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KRÁTKÉ NEKÓDUJÍCÍ RNA U NÁDORŮ MOZKU

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Abstrakt

Nádory mozku představují heterogenní skupinu onemocnění, a to jak z hlediska jejich původu, tak svým biologickým chováním. I v rámci stejných histopatologických jednotek pozorujeme u jednotlivých případů různý klinický průběh, přičemž současná klasifikace není schopna tuto skutečnost mnohdy reflektovat a stratifikovat pacienty podle jejich prognózy nebo odpovědi na léčbu. Diagnostika mozkových nádorů je někdy limitována jejich lokalizací a nedostupností reprezentativního vzorku tkáně. To může vést až k podhodnocení stupně sledovaného nádoru, nesprávné indikaci léčby a v konečném důsledku i ke kratšímu přežívání pacientů. Proto je pozornost odborníků čím dál více upírána k molekulárním biomarkerům, které jsou často přímo zodpovědné za biologické chování nádoru a mohou tak být významné nejen v diagnostice, ale také jako terapeutické cíle. Navíc bývají tyto molekuly buňkou cíleně secernovány nebo se z ní uvolňují v průběhu apoptózy či nekrózy a mohou tak být detekovatelné mimo jiné i v krvi a mozkomíšním moku. Intenzivně studovanou skupinu takových biomarkerů představují krátké nekódující RNA (small non-coding RNAs; sncRNA), které jsou zapojeny v kontrole mnoha buněčných procesů, včetně těch, které jsou zapojeny do patogeneze mozkových nádorů. Předkládaná habilitační práce proto shrnuje současné poznatky týkající se zapojení těchto molekul do patologie nejčastějších nádorů mozku s ohledem na jejich možné využití pro zpřesnění stávající diagnostiky, případně jako terapeutických cílů. Ve snaze přispět k uvedené problematice jsme ve spolupráci s klinickými pracovišti sestavili unikátní soubory pacientů a biologického materiálu, které nám umožnily identifikovat v nádorových tkáních a mozkomíšním moku sncRNA asociované s konkrétním typem nádoru a s prognózou onemocnění. U vybraných molekul jsme dále detailně prostudovali jejich biologickou funkci a navrhli jejich možné využití v terapii. Habilitační práce má charakter komentovaného souboru prací, přičemž příslušné části teoretického úvodu jsou vždy doplněny komentářem k publikovaným příspěvkům v rámci dané problematiky.

Abstract

Brain tumors represent a heterogeneous group of diseases both in terms of their origin and biological behavior. It is possible to observe different clinical courses in individual cases even within the same histopathological units, which the current classification is often unable to reflect and stratify patients according to their prognosis or response to treatment. The correct diagnosis of brain tumors is sometimes limited by their location and the unavailability of a representative tissue sample. This can lead to underestimating the grade of the tumor being monitored, incorrect indication of treatment, and, consequently, shorter patient survival. Therefore, experts' attention is increasingly focused on molecular biomarkers. An intensively studied group of such biomarkers are small non-coding RNAs (sncRNAs), which are involved in the control of many cellular processes, including those involved in brain tumors' pathogenesis. These molecules are often directly responsible for the tumor's biological behavior and, thus, can be important not only in diagnosis but also as therapeutic targets. In addition, these molecules are specifically secreted by the cell or are released during apoptosis or necrosis, and can be detectable in, inter alia, blood, and cerebrospinal fluid. The presented habilitation thesis summarizes the current knowledge of the involvement of these molecules in the pathology of the most common brain tumors, including their possible use to refine the existing diagnostics or as therapeutic targets. In an effort to contribute to this field, in collaboration with clinical institutions we collected unique patient cohorts and sets of biological material, that allowed us to identify sncRNAs in tumor tissues and cerebrospinal fluid associated with specific brain tumor types and disease prognosis. We further studied their specific biological function, and suggested their possible use in therapy. The proposed habilitation thesis is written as a collection of previously published scholarly works, while the relevant parts of the theoretical introduction are always supplemented by a commentary on published contributions within the given topic.

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Prohlášení

Prohlašuji, že jsem svoji habilitační práci vypracoval samostatně s využitím informačních zdrojů, které jsou v práci citovány.

Brno 15. prosince 2020

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Mgr. Jiří Šána, Ph.D.

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Úvod

Ačkoliv primární nádory mozku představují malé procento z celkového počtu nově diagnostikovaných neoplazií, ve více než třiceti procentech se jedná o maligní tumory s nepříznivou prognózou. Podíl intrakraniálních nádorů na celkové úmrtnosti způsobené nádorovými onemocněními navíc rapidně vzroste, započítáme-li i mozkové metastázy, které se dle některých zdrojů vyvinou až u čtyřiceti procent onkologických pacientů se solidními nádory (Kazda *et al.*, 2019). Incidence všech nádorů mozku navíc doposud mezi jednotlivými dekádami narůstala a tento nepříznivý trend lze předpokládat i v následujících letech. Obecně špatná prognóza maligních forem nádorů mozku je způsobená především jejich lokalizací znesnadňující často, při zvážení všech rizik, jejich efektivní terapii. Neméně významnou roli v krátkém přežívání pacientů však hraje v některých případech i nepřesná diagnostika, která je konfrontována jednak s již zmiňovanou lokalizací nádorů a dále s jejich značnou heterogenitou. V tomto ohledu se lze například setkat s odbornými pracemi uvádějícími až 49% diskrepanci mezi diagnózou stanovenou na základě stereotaktické biopsie a posléze z resekčního materiálu (Jackson *et al.*, 2001). Situaci navíc komplikuje i rozdílné biologické chování nádorů, které je často pozorováno i v rámci jednotlivých diagnóz, a které standardní histopatologická klasifikace nereflektuje. Zde proto nabývá na významu molekulární diagnostika.

Hlubší poznání molekulární patogeneze nádorů mozku bylo dosaženo až s vývojem nových sofistikovaných technologií umožňujících vysokokapacitní a velmi přesné molekulární analýzy. Kromě studia chromozomových aberací a variant v kódujících genech se stále více odborných prací zaměřuje na analýzu nekódující složky transkriptomu. Celogenomové analýzy odhalily, že lidský genom obsahuje asi 20 000 protein-kódujících genů, což reprezentuje méně než 2 % jeho celkové kapacity. Nicméně bylo rovněž zjištěno, že nejméně 90 % genomu je transkripčně aktivních. Lidský transkriptom tedy zdaleka neobsahuje pouze sekvence protein-kódujících genů, ale je v něm nepoměrně větší množství nekódujících RNA včetně RNA schopných cíleně na mnoha úrovních regulovat genovou expresi. Nejprve byly tyto nekódující transkripty považovány za evolučně nahromaděný genetický odpad vzniklý při časné sestavování genů nebo inzercí mobilních genetických elementů. Avšak další výzkumy prokázaly, že tyto nekódující RNA hrají zásadní role v buněčném vývoji, fyziologii, ale také patologii mnoha onemocnění. Právě jejich esenciální význam snad ve všech buněčných procesech a pozorování, že jejich deregulace souvisí s mnoha typy

onemocnění včetně nádorových, z nich činí velmi nadějně jak diagnostické, prognostické a prediktivní biomarkery, tak slibné terapeutické cíle. Na základě své délky se pak tyto RNA dělí do dvou hlavních skupin – krátké nekódující RNA a dlouhé nekódující RNA. Kromě délky se tyto dvě skupiny liší především mechanismem působení. Zatímco krátké RNA se uplatňují především v procesu posttranskripční regulace genové exprese, dlouhé RNA se navíc mohou účastnit regulace i na úrovni samotné transkripce, a dokonce mohou hrát úlohu i v některých posttranslačních procesech.

Následující komentář k souboru uveřejněných prací si klade za cíl seznámit s problematikou biologie krátkých nekódujících RNA včetně detailnější charakterizace jejich nejdůležitějších tříd, dále uvést problematiku nádorů mozku, a nakonec detailně shrnout význam krátkých nekódujících RNA u těchto nádorových onemocnění. Text habilitační práce je na příslušných místech doplněn o kapitoly dokládající a komentující příspěvek autora k dané problematice na základě odborných prací, které tvoří přílohy této práce.

Krátké nekódující RNA

Jak již bylo zmíněno v úvodu, skupinu krátkých nekódujících RNA (sncRNA; small non-coding RNAs) tvoří protein nekódující transkripty o délkách do 200 nukleotidů. V tomto smyslu se však nejedná o nedávno či dokonce nově definovanou skupinu RNA, a naopak lze říci, že objev některých krátkých RNA přímo souvisel s definováním centrálního dogmatu molekulární biologie Francisem Crickem publikovaným v roce 1958 (Crick, 1958; Cobb, 2017). Takovými molekulami jsou transferová a některé ribozomální RNA (tRNA, resp. rRNA) (Hoagland *et al.*, 1958). Na přelomu 60. a 70. let 20. století pak byly popsány rovněž malé jaderné RNA (snRNA; small nuclear RNA) hrající důležitou roli v sestřihu mRNA a malé jadéřkové RNA (snoRNA; small nucleolar RNA) zapojené v procesu modifikace rRNA. Uvedené RNA řadíme mezi tzv. „housekeepingové“ neboli provozní molekuly esenciální pro udržování bazálních funkcí buňky bez ohledu na její specifickou roli v tkáni nebo organismu, přičemž exprese těchto molekul přímo odpovídá metabolickým požadavkům buňky.

V pozdějších letech pak byly díky technologickým pokrokům v oblasti molekulární biologie a biochemie objeveny další sncRNA, jež v současné době řadíme do skupiny tzv. regulačních sncRNA, ačkoliv by některé z nich mohly být pro svoji funkci a význam řazeny rovněž mezi „housekeepingové“. Příkladem jsou krátké interagující RNA asociované s centromerovými repetitivními oblastmi (crasiRNA; centromere repeat associated small interacting RNAs), které se podílejí na rekrutaci heterochromatinu a centromerických proteinů v procesu segregace chromozomů a zdají se být tedy důležitým faktorem v udržování buněčné stability (Lindsay *et al.*, 2012; Sana *et al.*, 2012). Naopak již typickými zástupci skupiny regulačních sncRNA jsou krátké RNA asociované s promotorovými oblastmi (PASR; promoter-associated small RNAs), RNA iniciující transkripci (tiRNA, transcription initiation RNAs) a krátké RNA specifické pro telomery (tel-sRNA; telomere-specific small RNAs) podílející se pravděpodobně na epigenetickém umlčování protein-kódujících genů (Sana *et al.*, 2012). Studium těchto molekul je ovšem do značné míry omezené a hlubší znalosti o jejich biogenezi a mechanismech působení na úrovni buňky stále chybí, stejně tak jako není doposud jednoznačně objasněn jejich vliv na možné patofyziologické stavy organismu.

V rámci skupiny regulačních sncRNA se naopak značnému zájmu odborníků jak z řad vědeckovýzkumných pracovníků, tak lékařů těší krátké interferující RNA (siRNA; small interfering RNAs), mikroRNA (miRNA; microRNAs) a v posledních několika letech rovněž

PIWI-interagující RNA (piRNA; PIWI-interacting RNAs). Za hlavní milníky, které definitivně odstartovaly neustále se zvyšující zájem o regulační sncRNA, lze bezpochyby označit práce Victora Ambrose a Garyho Ruvkuna z roku 1993 zabývající se post-transkripční regulací protein-kódujícího genu *lin-14* prostřednictvím krátké RNA *lin-4* (Lee *et al.*, 1993; Wightman *et al.*, 1993) následované popisem mechanismu RNA interference v roce 1998 Andrew Firem a Craigem Melloem (Fire *et al.*, 1998).

Krátké interferující RNA – siRNA

Proces RNA interference je úzce spjat s přítomností siRNA. Tyto molekuly vznikají z dlouhých dvouřetězcových RNA (dsRNA; double stranded) formujících se ze dvou alespoň částečně vzájemně komplementárních jednořetězcových RNA (ssRNA; single stranded). Pomocí endonukleázy Dicer dojde následně k sestřihu za vzniku 19-23 nt dlouhých dsRNA, jejichž jedno vlákno (guide strand) je inkorporováno do enzymatického komplexu zvaného RISC (RNA-induced silencing complex), zatímco druhý řetězec (passenger strand) je degradován. Krátká ssRNA inkorporovaná v RISC se pak v buňce naváže na jinou komplementární sekvenci, čímž dochází k zahájení procesu degradace molekuly, která tuto sekvenci obsahovala (Leuschner *et al.*, 2006; Dana *et al.*, 2017). SiRNA jsou především exogenního původu vznikající v buňce v přítomnosti parazitických sekvencí RNA, které tam bývají nejčastěji vnášeny viry. Tyto parazitické sekvence jsou pak za pomoci procesu RNA interference degradovány. Analogicky k výše popsanému mechanismu pak reálně celý proces probíhá tak, že pokud se do buňky dostane RNA virus, v určitém okamžiku vytvoří dsRNA, buňka ji rozpozná a použije k výrobě siRNA. Ta se pak specificky váže na všechny virové RNA molekuly s identickou sekvencí a cíleně dojde k jejich degradaci (Carthew a Sontheimer, 2009; Šána *et al.*, 2018).

Pro svoji funkci se v laboratořích siRNA využívají při experimentálním cíleném transientním umlčování genů a ze stejného důvodu se zdají být slibnými terapeutickými cíli u mnoha onemocnění včetně nádorů. V roce 2008 byla zahájena první klinická studie fáze I (NCT00689065) s přípravkem CALAA-01 – cíleným terapeutikem určeným k inhibici růstu nádoru nebo ke zmenšení velikosti nádoru, kde aktivní složkou je siRNA inhibující buněčný růst prostřednictvím cílené regulace podjednotky RRM2 (M2 ribonukleotid reduktázy). Výsledky studie potvrdily, že systémové podávání preparátu vedlo u pacientů s melanomem k předpokládanému snížení hladin RRM2 v nádorové tkáni (Davis *et al.*, 2010). Během následujících deseti let pak proběhlo více než 50 klinických studií preparátů, jejichž

aktivní molekulou byla siRNA, přičemž dva z nich jsou již schváleny americkým Úřadem pro kontrolu potravin a léčiv (FDA; Food and Drug Administration) i Evropskou lékovou agenturou (EMA; European Medicines Agency) pro použití v klinické praxi. První z nich byl v roce 2018 přípravek Onpattro, jehož aktivní složku tvoří patisiran – siRNA, která specificky cílí mRNA genu pro transthyretin (TTR) v hepatocytech. Cílovou skupinou jsou pacienti s dědičnou transthyretinovou amyloidózou (hATTR; hereditary transthyretin-mediated amyloidosis), u kterých léčba významně zmírňuje polyneuropatii. Druhým schváleným preparátem je Givlaari s aktivní látkou givosiran – siRNA cílicí mRNA genu pro syntázu 1 kyseliny aminolevulinové (ALAS1; aminolevulinic acid synthase 1). Tento enzym se podílí na počátečním stupni produkce hemu v játrech a givosiran tak brání hromadění neurotoxických produktů kyseliny aminolevulinové (ALA; aminolevulinic acid) a porfobilinogenu (PBG; porfobilinogen), což jsou klíčové faktory vzniku ataků při akutní jaterní porfyrii (AHP; acute hepatic porphyria). V aktivních fázích klinického hodnocení je v současné době dalších 14 léčebných preparátů, z toho 3 se testují jako potenciální terapeutika u nádorových onemocnění. Jedná se o siRNA cílicí mutovaný gen KRAS G12D u pacientů s karcinomem pankreatu, siRNA homologní k části mRNA receptoru EphA2 obecně u pacientů s pokročilým solidním nádorovým onemocněním a siRNA specificky umlčující E3 ubikvitin-protein ligázu Cbl-b (Casitas B-lineage lymphoma proto-oncogene-b) u pacientů s karcinomem pankreatu, kolorektálním karcinomem a dalšími metastazujícími, recidivujícími nebo neresekovatelnými solidními tumory.

MikroRNA – miRNA

Další třídou sncRNA jsou miRNA, 18 až 25 nt dlouhé řetězce, které společně se siRNA patří mezi tzv. „Dicer dependent“ neboli na endonukleáze Dicer závislé RNA. Toto označení vychází z biogeneze obou molekul, během níž je aktivita zmiňované endonukleázy klíčová pro sestřih a vznik finálních krátkých dsRNA. Avšak na rozdíl od siRNA nevzniká prekurzorová dsRNA ze dvou na sobě nezávislých RNA molekul, ale párováním komplementárních sekvencí jedné molekuly RNA za vzniku vlásenky se smyčkou a je tedy vždy endogenního původu.

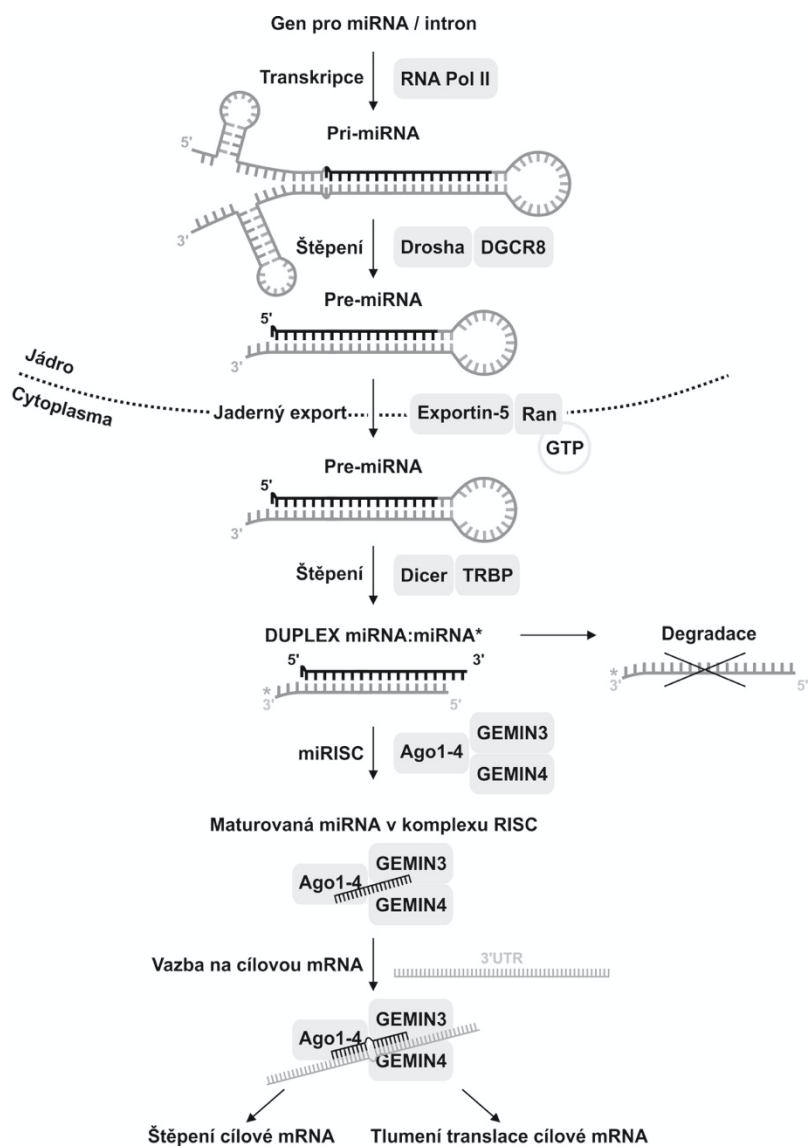
V detailnějším pohledu na biogenezi miRNA jsou v prvním kroku geny těchto molekul přepisovány z jejich vlastních promotorů pomocí RNA polymerázy II do primárních transkriptů, tzv. pri-miRNA. Tyto dlouhé molekuly jsou dále pomocí multiproteinového komplexu s ribonukleázovou funkcí sestřihovány na krátké, přibližně 70 nukleotidů dlouhé

vlásečkové struktury, označované jako pre-miRNA. Vlásoky jsou dále specificky za pomoci GTP-dependentního proteinu Exportin 5 transportovány z jádra do cytoplazmy, kde jsou endonukleázou Dicer v komplexu s dsRNA vazebným proteinem TRBP zpracovány do podoby maturovaných dvouřetězcových miRNA (Carthew a Sontheimer, 2009). Jeden z řetězců maturované miRNA označovaný jako vedoucí řetězec (guide strand) je inkorporován do multiproteinového komplexu miRISC (miRNA-induced silencing complex) zatímco druhý doprovodný řetězec (passenger strand) je uvolněn a degradován. O osudu řetězců rozhoduje stabilita párování na 5' konci, vedoucí („guide“) řetězec je ten, který je méně stabilní na 5' konci duplexu miRNA/miRNA (Winter a Diederichs, 2011). Zpracování prekurzorových struktur však není vždy totožné, což může vést ke vzniku izoform miRNA s rozdílnými zakončeními a následně i s rozdílným osudem jednotlivých řetězců (Winter *et al.*, 2009). Řetězec miRNA, který je začleněn v miRISC, realizuje ve většině případů svou funkci vazbou na místa v rámci 3' UTR cílových mRNA (Krol *et al.*, 2010). Jak již bylo uvedeno, miRNA inhibují expresi cílového genu post-transkripčně, přičemž komplex miRISC je tvořen především proteiny Argonautové rodiny, proteiny GEMIN3 a GEMIN4 a je analogický komplexu RISC. Úplná komplementarita miRNA s cílovou mRNA proto umožňuje její degradaci katalyzovanou proteinem AGO2, zatímco neúplná komplementarita je spojena s inhibicí translace (Carthew a Sontheimer, 2009). Text převzat a upraven z Šána, 2015. Kanonický model biogeneze miRNA je přehledně znázorněn na Obrázku 1.

MiRNA jsou tedy nepochybně významné posttranskripční regulátory genové exprese zapojené do většiny jak fyziologických, tak patologických procesů na úrovni buňky, a proto byla těmto molekulám věnována v posledních dvou desetiletích největší pozornost ze všech doposud známých nekódujících RNA. Dosavadní závěry aplikovaného výzkumu tedy jednoznačně potvrzují, že miRNA jsou velice slibné diagnostické biomarkery a potenciální terapeutické cíle u mnoha onemocnění včetně nádorů. V současné době je pod klíčovým slovem „microRNA“ registrováno více než 80 aktivních klinických studií, převážně biomarkerové povahy. Tato skutečnost odráží krom jiného fakt, že miRNA jsou vysoce stabilní molekuly velmi často aktivně vylučované buňkami do tělních tekutin, kde se stávají snadněji dostupnými a jejichž odběr v porovnání s tkáňovými biopsiemi významně snižuje rizika nežádoucích komplikací a je pro pacienta bezesporu komfortnější. Kromě zmiňovaných diagnostických studií proběhlo nebo stále probíhá i několik klinických hodnocení zaměřených na využití miRNA jako terapeutických molekul (Tabulka 1). V závislosti na způsobu deregulace těchto miRNA v patologicky pozměněných buňkách

anebo s ohledem na jejich role v patofyziologických procesech kauzálně souvisejících s daným onemocněním je přistoupeno buď k cílené inhibici nebo naopak suplování hladin konkrétních miRNA.

Obrázek 1 Kanonický model biogeneze miRNA (převzato a upraveno ze Slabý, 2012)



Inhibice bývá zprostředkována tzv. anti-miRNA oligonukleotidy, umělé navržené a syntetizované molekuly komplementární k cílové miRNA, které v sobě navíc obsahují specifické chemické modifikace jednak bránící anti-miRNA před působením exonukleáz a současně zvyšující jejich afinitu k cílové molekule. Takto upravená anti-miRNA je následně podána pacientovi buďto volně rozpuštěná ve fyziologickém roztoku (např. Miravirsen) nebo ve spojení se specifickým doručovacím systémem, který může jednak tuto anti-miRNA ještě více stabilizovat a poskytovat ji další ochranu před působení okolníhoho

prostředí nevyjímaje enzymatickou degradaci a současně může zvyšovat afinitu celého konstruktů ke konkrétním buněčným či tkáňovým strukturám. Nejčastěji používané takové systémy jsou modifikované liposomy, nanobuněčné vektory derivované z bakteriálních struktur (EnGeneIC Dream Vector, EDV) a organické (např. cholesterol nebo N-acetyl-D-galaktosamin) a anorganické nanočásticové konjugáty (např. zlato, silikáty nebo oxidy železa) (Fu *et al.*, 2019; Lee *et al.*, 2019; Bajan a Hutvagner, 2020). V experimentálních podmínkách se lze rovněž často setkat s využitím virových vektorů (adenoviry, retroviry a lentiviry) (Bajan a Hutvagner, 2020). Za účelem navyšování hladin konkrétních miRNA v buňkách se pak využívají buďto pre-miRNA anebo častěji tzv. miRNA mimic, což jsou uměle syntetizované a chemicky modifikované dvouřetězcové molekuly RNA napodobující maturované duplexy miRNA (Jin *et al.*, 2015).

Příspěvek k dané problematice

V roce 2019 jsme požádali Úřad průmyslového vlastnictví o zapsání užitého vzoru s názvem „Diagnostická sada pro neinvazivní diagnostiku mozkových nádorů“, který byl následně zapsán pod spisovým číslem 33336. V rámci předkládaného technického řešení byly identifikovány miRNA v mozkomíšním moku (CSF) asociované s přítomností vybraných mozkových nádorů. Předkládané technické řešení tedy poskytuje diagnostickou sadu pro detekci glioblastomu, meningeomu a/nebo mozkových metastáz na základě kvantifikace let-7i-5p, miR-151a-3p, miR-423-3p a alespoň jedné miRNA vybrané z let-7b-5p, miR-140-5p a miR-21-3p ve vzorku mozkomíšního moku metodou kvantitativní PCR v reálném čase (RT-qPCR) (Příloha 1).

Tabulka 1 Přehled ukončených a probíhajících klinických hodnocení mikroRNA jako terapeutických molekul

Výrobce	Název léčiva	Účinná látka	Cílové onemocnění	Klinická fáze	Status	ClinicalTrials.gov identifikátor
Santaris Pharma/Roche	Miravirsen	AntimiR-122	Hepatitida C (včetně chronických infekcí)	Fáze I	dokončeno	NCT01646489
				Fáze II	dokončeno	NCT01200420
				Fáze II	probíhá	NCT01872936
				Dlouhodobé prodloužení fáze II	dokončeno	NCT02031133
						NCT02508090
Regulus Therapeutics	RG-101	AntimiR-122	Chronická hepatitida C	Fáze II	dokončeno	700281685*
				Fáze II	probíhá	700262855*
				Fáze II	dokončeno	700252168*
	RG-125	AntimiR-103/107	Nealkoholická steatohepatitida	Fáze I	probíhá	700263697*
				Fáze I/II	dokončeno	700274303*
	RG-012 Lademirsen	AntimiR-21	Dědičná nefritida	Fáze I	dokončeno	700284590*
Fáze II				probíhá	700284665*	
RGLS4326	AntimiR-17	Autozomálně dominantní polycystické onemocnění ledvin	Fáze I	probíhá	NCT04536688	
miRagen Therapeutics	MRG-106	AntimiR-155	Kožní T-buněčný lymfom, mykóza fungoides	Fáze I	probíhá	NCT02580552
				Fáze II	probíhá	NCT03713320 NCT03837457
	MRG-110	AntimiR-92	Poranění	Fáze I	dokončeno	NCT03603431
	MRG-201	miR-29 mimic	Keloidní jizvy / Scleroderma	Fáze I	dokončeno	NCT02603224
Fáze II				probíhá	NCT03601052	
EnGeneIC	MesomiR-1	miR-16 mimic	Maligní mezoteliom pleury; nemalobuněčný karcinom plic	Fáze I	dokončeno	NCT02369198
Mirna Therapeutics Inc.	MRX-34	miR-34 mimic	Různé solidní nádory	Fáze I	ukončeno#	NCT01829971

* Adis Insight Database (<https://adisinsight.springer.com>); # Předčasně ukončeno – pět případů závažných nežádoucích imunitních reakcí

PIWI-interagující RNA – piRNA

V posledních deseti letech začalo skokově přibývat prací zabývajících se piRNA. Dnes tak již můžeme evidovat téměř 1 500 odborných publikací na toto téma, z toho téměř 200 v souvislosti s nádorovými onemocněními. Oproti předešlým dvěma sncRNA jsou piRNA charakteristické nepatrně delším řetězcem dosahujícím délky v rozmezí 24-32 nt s typickou 2-O-metylovou skupinou na 3' konci, která je společně s ordinárním O-monofosfátem na 5' konci zodpovědná za stabilitu molekuly (Iwasaki *et al.*, 2015; Romano *et al.*, 2017). PiRNA se vyznačují značnou sekvenční variabilitou a původně byly objeveny u mouchy *Drosophila melanogaster* jako molekuly asociované s PIWI (P-element Induced Wimpy Testis) proteiny (Romano *et al.*, 2017) – podrodinou Argonautových proteinů zodpovědných za vazbu siRNA a miRNA a tvorbu komplexů RISC, kde zprostředkovávají degradaci komplementárních transkriptů a mohou se také podílet na epigenetických regulačních procesech (Luteijn a Ketting, 2013). PIWI proteiny byly původně objeveny rovněž u mouchy *Drosophila melanogaster*, kde jsou zodpovědné za udržování stability a sebeobnovu zárodečných kmenových buněk (Ross *et al.*, 2014; Han *et al.*, 2017). Až později byly jejich homology identifikovány v dalších organismech včetně člověka, a to jak ve zmiňovaných pluripotentních kmenových, tak v menší míře i v plně diferencovaných buňkách (Ross *et al.*, 2014; Iwasaki *et al.*, 2015). Ačkoliv se objevují práce popisující dílčí role PIWI proteinů v somatických buňkách, jsou stále spojovány především s embryogenezí a jejich poškození je asociováno s poruchami mitózy a následnými chromozomálními aberacemi (Ross *et al.*, 2014). U člověka jsou popsány čtyři homology PIWI proteinů – HIWI (PIWIL1), HILI (PIWIL2), HIWI2 (PIWIL3) a HIWI3 (PIWIL4), přičemž jejich exprese byla kromě kmenových buněk pozorována i u různých nádorových onemocnění.

Analogicky i piRNA byly nejprve spojovány se zárodečnými buňkami a až posléze byly objeveny ve většině somatických buněk člověka včetně nádorově transformovaných (Rybecka *et al.*). Výzkumy prokázaly, že cílené snižování hladin piRNA vede v buňkách k mobilizaci transponovatelných elementů (TEs), poškození genomu, anomálnímu vývoji gonád a neplodnosti. Zdá se tedy, že piRNA chrání genom před nežádoucím působením zmiňovaných parazitických repetitivních sekvencí a hrají tak důležitou roli v udržování jeho stability (Toth *et al.*, 2016). Význam těchto sncRNA ještě roste, uvědomíme-li si, že sekvence pocházející z TEs tvoří přibližně 45 % lidského genomu. Zvýšená aktivita TEs je pak spojována s nádorovými onemocněními, s čímž pravděpodobně do značné míry souvisí

i narůstající nestabilita genomu v nádorových buňkách v procesu jejich maligní transformace (Lander *et al.*, 2001; Moyano a Stefani, 2015).

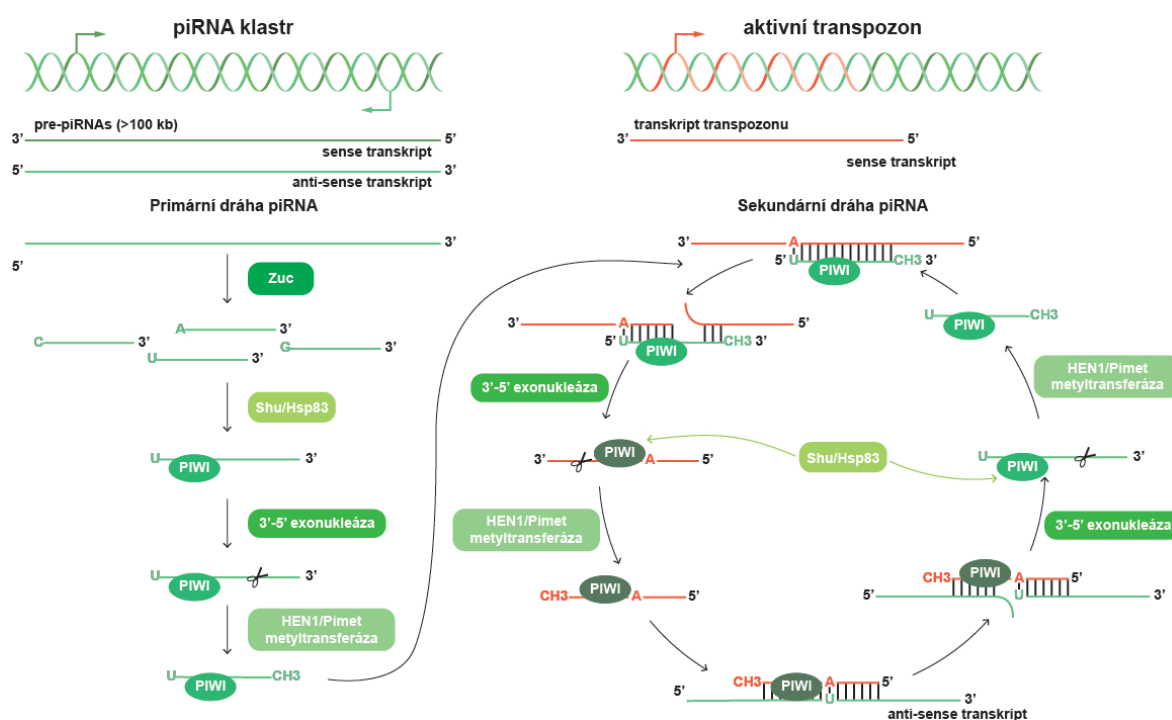
PiRNA mohou být děleny na základě jejich lokalizace v genomu do tří skupin: piRNA transkribované z oblastí bohatých na transpozony, které se typicky přepisují z obou řetězců DNA a produkují jak „sense“, tak „antisense“ piRNA; piRNA přepisované z protein-kódujících oblastí, které vždy odpovídají mRNA, ze které jsou odvozeny, a velmi často pocházejí z 3' UTR (untranslated region; nepřekládaná oblast), kde lze nalézt právě zbytky TEs; a piRNA odvozené z dlouhých nekódujících RNA, kde mohou být umístěny napříč celým transkriptem (Assumpcao *et al.*, 2015).

Nejlépe je v tuto chvíli prostudována funkce piRNA z první zmiňované skupiny, jejichž biogeneze probíhá ve dvou vzájemně oddělených drahách – primární a sekundární (Romano *et al.*, 2017). Kromě toho je biogeneze piRNA na rozdíl od siRNA a miRNA nezávislá na enzymu Dicer a na rozdíl od prekurzorů miRNA nedisponují pre-piRNA žádnými významnými sekundárními strukturami (Wei *et al.*, 2017). V primární dráze biogeneze piRNA jsou tedy nejprve více než 100 kb dlouhé pre-piRNA transkripty transportovány z jádra do cytoplazmy a štěpeny enzymem Zucchini (Zuc) na krátké řetězce, které jsou následně pomocí mediátorového komplexu Shu/Hsp93 navázány jednotlivě na proteiny z rodiny PIWI. Poté je 5' konec takového řetězce odštěpen a metylován metyltransferázou Hen1, čímž je dokončeno formování tzv. piRISC (Luteijn a Ketting, 2013; Ross *et al.*, 2014). Zralý piRISC může v tuto chvíli vstoupit do sekundárního cyklu biogeneze nazývaného rovněž jako „ping-pong“ cyklus, který je zodpovědný především za amplifikaci piRNA a posttranskripční umlčování TEs (Han *et al.*, 2017). Celý cyklus začíná vazbou piRISC na transpozon, jeho štěpení a vznikem piRISC podobného komplexu obsahujícího komplementární řetězec vlastní piRNA. Tento komplex se následně váže na RNA molekulu přepsanou z antisense vlákna již degradovaného transpozonu, dochází ke štěpení tohoto řetězce a opětovnému vzniku piRISC, přičemž se celý cyklus může opakovat. Mechanismus působení a lokalizace PIWI/piRNA komplexu jsou pak do značné míry závislé na konkrétní podskupině proteinů z rodiny PIWI, se kterou je piRNA v dané chvíli asociována (Luteijn a Ketting, 2013). Tímto procesem je tedy zajištěno umlčení nežádoucí aktivity transpozonů a současně jsou udržovány hladiny piRNA v buňce. Obě dráhy biogeneze piRNA jsou znázorněny na Obrázku 2.

Společně s narůstajícím počtem vědeckých prací popisujících významnou roli PIWI/piRNA regulačního mechanismu v procesu kancerogeneze včetně asociovaných molekulárních souvislostí přibývá rovněž studií aplikovaného a translačního výzkumu navrhujících piRNA

jako potenciálně využitelné diagnostické nádorové biomarkery a slibné terapeutické cíle, ačkoliv registrovaná intervenční klinická studie ověřující tuto skutečnost u jakéhokoliv nádorového onemocnění nebyla doposud iniciována (Weng *et al.*, 2018). Na druhou stranu některá vědecká pozorování jsou natolik zásadní, že přibývá mezinárodních patentových přihlášek tematicky se zabývajících ochranou duševního vlastnictví právě v oblastech využitelnosti piRNA v léčebně-diagnostické praxi. Příkladem je evropský patent EP3431609A1 - Method of diagnosis of colorectal cancer zapsaný týmem prof. Slabého, který se týká způsobu diagnostiky a stanovení prognózy kolorektálního karcinomu s využitím mimo jiné piRNA-5937.

Obrázek 2 Primární a sekundární dráha biogeneze PIWI-interagujících RNA (převzato a upraveno z Luteijn a Ketting, 2013)



Příspěvek k dané problematice

*Naše přehledová práce z roku 2012 přinesla jako první ucelený přehled známých, ale i nově popisovaných tříd nekódujících RNA a jejich významu u nádorových onemocnění. K dnešnímu dni eviduje časopis Journal of Translational Medicine více než 17 tisíc přístupů k této práci a ta se nachází na sedmém místě sledovanosti v rámci podobně starých článků publikovaných ve zmíněném časopisu. Dle databáze Web of Science byl tento článek již 160krát citován (Sana *et al.*, 2012; Příloha 2).*

Nádory mozku

Nádory mozku lze na základě jejich původu rozdělit na primární a sekundární. Zatímco primární nádory se derivují přímo z intrakraniálních struktur, sekundární malignity vznikají iniciálně v jiných částech těla a do mozku následně metastazují. Obě tyto skupiny postihují ročně celosvětově více než 40 pacientů na 100 tis. osob a toto číslo mezi jednotlivými dekádami neustále roste (Fox *et al.*, 2011; de Robles *et al.*, 2015). U mladých dospělých a dospělých pacientů jsou z primárních nádorů mozku nejvíce zastoupeny meningeomy. Ačkoliv se jedná většinou o benigní a chirurgicky dobře léčitelná onemocnění, díky své lokalizaci v omezeném prostoru lebky mohou i jinak tyto benigní nádory svým růstem a tlakem na okolní zdravou tkáň přímo ohrožovat pacienty na životech. To platí i pro další v porovnání s ostatními intrakraniálními neopláziemi relativně často diagnostikované nádory selární oblasti a mozkomíšních nervů reprezentované především adenomem hypofýzy, resp. schwannomy. Společně s ostatními spíše marginálně se vyskytujícími neopláziemi pak tvoří benigní nádory přibližně dvě třetiny všech primárních intrakraniálních nádorů. Největší podíl malignit primárně vznikajících v této anatomické oblasti reprezentují nádory derivované z neuroepiteliální tkáně – především nejvíce agresivní glioblastom (GBM), dále pak astrocytomy a vzácněji se vyskytující oligodendrogliomy. U pacientů středního a staršího věku lze rovněž pozorovat zvyšující se výskyt primárních intrakraniálních lymfomů s incidencí na úrovni difúzního a anaplastického astrocytomu. Společně s GBM jsou lymfomy nejagresivnějšími primárními malignitami centrální nervové soustavy a jejich prognóza je zpravidla fatální (Fadrus *et al.*, 2010; Porter *et al.*, 2010; Ostrom *et al.*, 2018; Brain a Other, 2019; Ostrom *et al.*, 2019).

Druhou výše zmiňovanou skupinu nádorů mozku tvoří sekundární nádory, jejichž primární ložisko je lokalizováno v jiné části těla a do mozku následně metastazovaly. Jejich přesnou incidenci je obtížné určit a údaje se napříč epidemiologickými studiemi mnohdy významně liší. Nicméně se předpokládá, že ročně dochází ve vyspělých zemích k diseminaci nějakého typu nádoru do mozku až u 15 pacientů na 100 tis. obyvatel, přičemž incidence společně s prevalencí neustále rostou ruku v ruce s pokroky v oblastech jak diagnostických metod, tak onkologické léčby (Fox *et al.*, 2011).

Gliomy

Gliomy jsou jedny z nejčastěji diagnostikovaných primárních nádorů mozku u dospělé populace. Jak název napovídá, jejich původ lze dohledat v neoplastické transformaci gliových buněk, které obklopují neurony a podporují jejich činnost. Podle konkrétního typu gliové buňky, ze které nádor vznikl, můžeme gliomy rozlišit na astrocytomy, oligodendrogliomy a ependymomy. V úplném výčtu jsou pak rovněž popisovány tzv. smíšené gliomy neboli oligoastrocytomy (Slabý *et al.*, 2013). Nejčastěji se vyskytující a současně nejvíce prostudovanou skupinou gliomů jsou nepochybně astrocytomy. Ty jsou tradičně na základě histopatologické klasifikace dále děleny na prognosticky příznivější gliomy nízkého stupně malignity (low-grade gliomas; LGG) a gliomy vysokého stupně malignity (high-grade gliomas; HGG), které se obecně vyznačují mnohem agresivnějším biologickým chováním. Hlavními hodnotícími kritérii jsou buněčnost, jaderné atypie, mitotická aktivita, angioproliferace a přítomnost nekróz. Podle aktuálně platné verze WHO klasifikace nádorů CNS z roku 2016, ve které je mimo jiné oproti předchozím vydáním významně akcentována molekulárně-genetická charakteristika nádorů, se mezi LGG řadí převážně v dětském věku se vyskytující pilocytární a subependymální obrovskobuněčné astrocytomy (WHO grade I) – od zdravé mozkové tkáně relativně dobře ohraničené benigní nádory přestože u druhého zmiňovaného mohou být v některých případech pozorovány vaskulární proliferace, nekrózy a mitotické figury (Zitterbart, 2014; Louis *et al.*, 2016); a dále do této skupiny astrocytárních tumorů spadají difúzní astrocytom, IDH-mutovaný/IDH-wildtype a pleomorfický xanthoastrocytom (WHO grade II). Difúzní astrocytom je středně diferencovaný nádor infiltrující často okolní tkáň a neřídka progredující do HGG; nejčastěji bývá diagnostikován mezi 20. a 50. rokem života. Oproti tomu pleomorfický xanthoastrocytom se vyskytuje spíše u mladších pacientů – nejvíce okolo 12. roku života. Obecně se tato skupina nádorů vyznačuje malou buněčností a nízkou mitotickou aktivitou (Louis *et al.*, 2016; Wesseling a Capper, 2018). Naopak HGG jsou vysoce invazivní malignity s četnými mitózami a silnou vaskularizací, bývají špatně ohraničeny a jsou náchylné k nekróze. V této skupině jsou zařazeny jednak anaplastický pleomorfní xanthoastrocytom a mnohem častěji se vyskytující anaplastický astrocytom, IDH-mutovaný/IDH-wildtype (WHO grade III), pro který jsou charakteristické jaderné atypie, atypické a četné mitózy a infiltrativní růst, a dále nejvíce agresivní formy gliomů (WHO grade IV) zahrnující difúzní středočarový gliom, H3 K27M-mutovaný a GBM, IDH-mutovaný/IDH-wildtype, který je vůbec nejčastěji diagnostikovaným intrakraniálním

nádorovým onemocněním astrocytárního původu. Jak anaplastický astrocytom, tak GBM mohou vznikat buď *de novo* nebo progresí z původně diagnostikovaného gliomu s nižším stupněm malignity. V takovýchto případech se pak hovoří o primární (často IDH-wildtype), resp. sekundární (často IDH-mutované) formě onemocnění (Louis *et al.*, 2007; Slabý *et al.*, 2013; Hendrych *et al.*, 2020). Text převzat a upraven z Šána, 2015.

Glioblastom

GBM reprezentuje nejagresivnější formu gliomů s infaustní prognózou, přičemž medián celkového přežívání takto postižených pacientů se podle různých zdrojů pohybuje v rozmezí 12 a 15 měsíců od stanovení diagnózy. Současný terapeutický standard se skládá v první řadě z maximálně možné chirurgické resekce, na kterou navazuje onkologická léčba – tzv. Stuppův protokol. Ten se skládá z radioterapie v celkové dávce 60 Gy a konkomitantně podávané chemoterapie s temozolomidem (TMZ) v délce 42 dnů. V případě, že nádor na léčbu reaguje a pacientův stav to umožňuje, je možné po dokončení této konkomitantní chemoradioterapie podávat adjuvantní TMZ v monoterapii (Ohgaki *et al.*, 2004; Stupp *et al.*, 2005; Stupp *et al.*, 2009; Lakomy *et al.*, 2011a). Nejvýznamněji se na celkovém přežívání pacientů s GBM ovšem podílí především radikalita chirurgického výkonu a radioterapeutická intervence. Samotný TMZ pak prodlužuje přežívání bez progresse onemocnění podle dostupných zdrojů o 13 až 21 týdnů (Wick *et al.*, 2004; Lakomy *et al.*, 2011a). Pokud pacient léčbu nepodstoupí, odhaduje se jeho přežití na pouhých 3 až 6 měsíců (Krex *et al.*, 2007).

GBM představuje s incidencí 3 až 5 nových případů na 100 tisíc obyvatel téměř 60 % všech diagnostikovaných gliomů, přičemž necelých 95 % tvoří *de novo* vznikající primární nádory a zbylých asi 5 % jsou sekundární formy onemocnění vznikající nejčastěji progresí z anaplastického astrocytomu. Primární GBM jsou velmi často popisovány u starších pacientů s nejvyšší incidencí okolo šedesátého roku života. Naopak mladší pacienti okolo čtyřiceti let bývají spíše postiženi sekundárním typem GBM (Watanabe *et al.*, 1996).

Symptomy typicky pozorované u GBM jsou asociovány, podobně jako u většiny nádorů mozku, jednak se zvětšující se velikostí primárního nádoru, a tedy zvyšujícím se tlakem na okolní struktury mozku, a jednak s infiltrací maligních buněk do zdravé tkáně a jejím poškozením. Nejčastější klinické příznaky GBM jsou tedy zvýšený nitrolebeční tlak, bolest hlavy, slabost, ztráta somatosenzorického a vizuálního vnímání, zvracení a parciální nebo generalizované epileptické záchvaty. U GBM neexistuje v současné době žádná možnost

prevence, plošného skríninku ani časné detekce, a stejně tak pro toto onemocnění neexistují ani žádné specifické diagnostické biomarkery. Diagnóza je tak určována na základě vyšetření pomocí magnetické rezonance a/nebo počítačové tomografie indikované obvykle z důvodů klinických příznaků a následně verifikována histologicky (Jenkinson *et al.*, 2007). Z molekulárně-genetického pohledu je GBM velice heterogenní onemocnění vyznačující se častými chromozomálními aberacemi a změnami na úrovni funkčně významných mutací nádorových supresorů a onkogenů. Zjevné rozdíly na této úrovni lze pak sledovat především mezi primárním a sekundárním GBM. Tato skutečnost je dána právě způsobem vzniku obou podtypů, kdy sekundární GBM nese nutně mutační vzor gliomu nižšího stupně, ze kterého se vyvinul. Z tohoto pohledu je klíčové vyšetření mutačního statusu genů pro IDH (isocitrátdehydrogenáza), konkrétně se pak jedná o nejčastější mutace v kodonu R132 genu IDH1 a v kodonu R172 genu IDH2, které se často vyskytují již u LGG a jsou tedy znakem sekundárních forem GBM (Louis *et al.*, 2016). IDH hraje významnou roli v Krebsově cyklu, kde katalyzuje přeměnu isocitrátu na alfa-ketoglutarát. Mutace IDH má za následek především změnu finálního produktu na onkometabolit 2-hydroxyglutarát, který kompetitivně inhibuje aktivitu α -ketoglutarát dependentních dioxygenáz, mezi které patří i histon demethylázy (Kramář *et al.*, 2016). Mutovaný gen pro IDH je tak nejen u gliomů velmi často asociován s rozsáhlými metylacemi v promotorových oblastech genů (cytosine-phosphate-guanine (CpG) island methylator phenotype; G-CIMP) postihujícími nezřídka i geny MGMT (O6-methylguanine-DNA methyltransferase); a dále RB1, CDKN2A-p14^{ARF} a CDKN2A-p16^{INK4a} představující krucální regulátory buněčného cyklu (Hughes *et al.*, 2013; Crespo *et al.*, 2015; Malta *et al.*, 2018). Dalším epigenetickým modulátorem, jehož mutantní forma je spojována s postupnou malignizací gliomů, je gen ATRX kódující chromatin-remodelující protein. U sekundárních GBM je oproti primární formě možno častěji pozorovat inaktivační mutaci nádorově supresorového genu TP53 mající vliv na apoptózu a stabilitu genomu. Ze strukturních aberací je pro sekundární GBM dále typická ztráta dlouhých ramének na chromozomech 19 a 13 a dvakrát častěji se u sekundárních GBM vyskytuje ztráta heterozygotnosti v oblasti dlouhého raménka chromozomu 22 (Huse *et al.*, 2011; Crespo *et al.*, 2015). Mezi klasické znaky primárního GBM patří především amplifikace anebo aktivační mutace EGFR, inaktivace nádorového supresoru PTEN nebo delece genu CDKN2A kódujícího nádorové supresory p14ARF a p16INK4a (Bleeker *et al.*, 2012). Rovněž zde byly oproti sekundární formě onemocnění pozorovány častější výskyt mutace v genu TERT a ztráta heterozygotnosti v oblasti krátkého raménka chromozomu 10 (Crespo *et al.*, 2015). U obou podtypů potom byly pozorovány zvýšené hladiny VEGF

a PDGFA, případně jejich receptorů, hrajících významnou úlohu v angiogenezi, nádorovém růstu a metastazování; mutace či amplifikace protoonkogenu MDM2 (mouse double minute 2), jenž kóduje negativní regulační faktor proteinu p53; a častá ztráta dlouhého raménka na chromozomu 10 (Biernat *et al.*, 1997; Stark *et al.*, 2003; Karcher *et al.*, 2006; Huse *et al.*, 2011; Popescu *et al.*, 2016; Hou *et al.*, 2019).

Obecně lze tedy u primárního GBM pozorovat častěji aktivovanou signální dráhu EGFR/PI3K/PTEN/Akt, zatímco se sekundárním GBM je typicky, nikoli však bezvýhradně, spojována signální dráha TP53/MDM2/CDKN2A/p14^{ARF} (Ohgaki a Kleihues, 2007). Dále lze u GBM často pozorovat aktivované signální dráhy RAS/MAPK a RB/CDKN2A-p16INK4a (Mao *et al.*, 2012; Slabý *et al.*, 2013; Crespo *et al.*, 2015).

Příspěvek k dané problematice

GBM je onemocnění s velice nízkou incidencí často diagnostikované v pokročilé formě nebo u pacientů, jejichž stav neumožňuje indikovat standardní léčebný protokol. Rovněž s ohledem na množství komplexních onkologických center v České republice a velikosti spádových oblastí je pro jednoho pracoviště značně problematické získat uniformní a dostatečně rozsáhlý soubor pacientů s tímto onemocněním. Právě to pak tvoří jednu z hlavních překážek translačního výzkumu. Našemu týmu se podařilo vytvořit relativně rozsáhlý kompletně histopatologicky a klinicky charakterizovaný soubor pacientů s GBM, kteří podstoupili jak dostatečně extenzivní chirurgický zákrok, tak intenzivní následnou onkologickou léčbu. Primárním cílem po vytvoření vlastního souboru bylo především zhodnotit vliv klinických faktorů a použité léčby ve vztahu k parametrům přežití, jako jsou čas do progresu onemocnění (PFS) a celkové přežívání (OS). Získané výsledky potom představovaly důležité informace, které jsme později využili při sestavování souboru pacientů pro sofistikovanější molekulární analýzy a byly u těchto prací také podkladem pro vstupní parametry multivariačních regresních analýz.

První práce na toto téma, publikovaná v roce 2011, zahrnovala soubor čítající 86 histopatologicky potvrzených primárních GBM s mediánem věku v době diagnózy 56 let (24-69), z toho 60 % zaujímali muži. Medián PFS byl 7 měsíců (2,0-5,5) a medián OS byl 13 měsíců (2,5-70,0), přičemž signifikantně nejdelšího přežívání dosahovali dle očekávání pacienti s pooperačním stavem tělesné výkonosti (performance status, PS; ECOG) rovnajícím se nule. Stejně tak prognosticky výrazně příznivěji se jeví pacienti, u kterých bylo dosaženo makroskopicky totálního odstranění nádoru, a pacienti, kteří absolvovali

konkomitantní chemoradioterapii bez výraznější redukce, tj. alespoň 54 Gy z celkových 60 Gy a 40 dní chemoterapie z plánovaných 42 dní. Věk, pohlaví a lokalizace nádoru nedosáhly ve vztahu k prognóze signifikantní významnosti (**Lakomý et al., 2011a; Příloha 3**).

V recentní práci na podobné téma z roku 2020 pak bylo cílem poskytnout reálné výsledky z léčby GBM a porovnat celkové přežití pacientů po léčbě Stuppovým režimem dnes a před deseti lety. Oproti předchozí studii bylo zařazeno celkem 155 nových pacientů, medián věku 60,9 let, 61 % mužů, 58 pacientů (37 %) podstoupilo totální chirurgickou resekci nádoru. Stuppův režim byl indikován u 90 pacientů (58 %), 65 pacientů (42 %) podstoupilo samotnou radioterapii. Medián PFS v kohortě pacientů, kteří podstoupili Stuppův režim, byl 6,7 měsíců, medián OS 16,0 měsíců a 2letý OS 30,7 %. OS byl delší, pokud byli pacienti schopni dokončit alespoň tři cykly adjuvantní chemoterapie (medián 23,3 měsíců a 43,9 % pacientů žilo 2 roky po operaci). Rychlá časná progresse před radioterapií byla negativním prognostickým faktorem s poměrem rizik – HR 1,87 ($p = 0,007$). Interval mezi operací a začátkem radioterapie (medián 6,7 týdnů) nebyl prognosticky významný. Medián OS v současné kohortě byl přibližně o 2 měsíce delší než v historické kontrolní skupině léčené před 10 lety (16 vs. 13,8 měsíců) s použitím stejného Stuppova režimu. S přihlédnutím k rozdílům v charakteristikách pacientů mezi současnými a historickými kohortami, věkem, rozsahem resekce a výkonnostním stavem pacienta podle ECOG byl HR (Stuppův režim vs. RT samotný) pro OS roven 0,45 ($p = 0,002$) (**Lakomý et al., 2020a; Příloha 4**).

Třetí velmi recentní práce se pak zabývala retrospektivním posouzením incidence a lokalizace nádorů a potenciálních prediktorů časné progresse u nově diagnostikovaných pacientů s GBM ještě před zahájením radioterapie; a dále byly porovnány výsledky dosahovaných přežití v kohortách pacientů s GBM vykazujících či nevykazujících rychlou progresi onemocnění, přičemž data byla vztažena k průběhu léčby. Celkem bylo analyzováno 90 pacientů s předoperační, pooperační a plánovací MRI: medián věku 59 let, 59 % mužů a 39 pacientů (43 %) podstoupilo celkovou chirurgickou resekci nádoru. Režim Stupp byl indikován 64 pacientům (71 %); 26 pacientů (29 %) podstoupilo samotnou radioterapii. Progrese již na plánovací MRI provedené krátce před radioterapií byla zjištěna u 46 (51 %) pacientů. Hlavním prediktorem této rychlé progresse onemocnění byl chirurgický výkon s nedostatečnou radikalitou ($p < 0,001$). Přítomnost rychlé progresse byla potvrzena jako silný negativní prognostický faktor; medián OS u pacientů s rychlou progresí byl 10,7 vs. 18,7 měsíců a dvouleté přežití bylo pozorováno pouze u 15,6 % pacientů vs. 37,7 % pacientů, u kterých rychlá progresse nebyla prokázána. Je zajímavé, že vliv rychlé progresse na OS se významně lišil u mladších (≤ 50 let) a starších pacientů (> 50 let) ($p = 0,047$);

v případě PFS tento rozdíl zaznamenán nebyl. Pacienti s rychlou progresí, kterým byla indikována léčba v režimu Stuppova protokolu, měli delší OS ve srovnání s pacienty, kteří podstoupili pouze radioterapii (medián OS 16,0 vs. 7,5; HR = 0,5, $p = 0,022$; dvouleté přežití 22,3 % vs. 5,6 %). Interval mezi chirurgickým zákrokem a zahájením radioterapie nebyl překvapivě prognostickým znakem ani u celé zkoumané kohorty pacientů, ani u pacientů s rychlou progresí (Lakomy *et al.*, 2020b; Příloha 5).

Glioblastomové kmenové buňky

Podle současných studií stojí za špatnou prognózou GBM, podobně jako u jiných nádorů, mimo jiné i malá populace nádorových buněk vyznačující se unikátními vlastnostmi jako jsou schopnost sebeobnovy, diferenciací a neomezené proliferace. Tyto buňky disponují rovněž zvýšenou rezistencí k podávané léčbě a jsou tak pravděpodobně zodpovědné za vznik časných recidiv. Pro svoji podobnost se zdravými kmenovými buňkami bývají označovány jako nádorové kmenové buňky (Cancer Stem Cells, CSC) nebo také tumor-iniciující buňky (Stopschinski *et al.*, 2013). Teorie nádorových kmenových buněk předpokládá, že buňky nádoru jsou stejně jako v případě normální zdravé tkáně hierarchicky uspořádány a na vrcholu pyramidy stojí právě CSC.

Přítomnost CSCs do značné míry vysvětluje nízkou efektivitu konvenční terapie u některých nádorových onemocnění včetně GBM. Tato terapie je totiž zaměřena především na rychle se dělící buňky nádoru. Avšak, jak již bylo zmíněno, CSCs se většinu času vyskytují v tzv. klidovém stádiu buněčného cyklu a rychlost jejich proliferace není tak rapidní. To poskytuje buňce dostatečný čas, aby opravila poškozenou DNA. V tom jí navíc významně pomáhají zvýšené hladiny DNA reparačních enzymů a transportérů lékové rezistence (ABC transportéry) (Pardal *et al.*, 2003; Foreman *et al.*, 2009). Dokonce bylo zjištěno, že CSCs, kterým se podařilo uniknout chemoterapii, byly více rezistentní a tuto vlastnost následně předávaly i svým dceřiným buňkám. Tato skutečnost koresponduje s faktem, že rekurentní tumory bývají často k léčbě méně citlivé než primární nádor (Lou a Dean, 2007).

Glioblastomové kmenové buňky (GSCs) pocházejí pravděpodobně z neurálních kmenových buněk. Nasvědčuje tomu i fakt, že poprvé byly z GBM tkáně vyizolovány na základě exprese povrchového glykoproteinu CD133, jehož přítomnost je typická pro normální neurální kmenové buňky (NSCs). GSCs stejně jako NSCs rostou v bezsérovém mediu jako suspenzní sférické buněčné kolonie neboli neurosféry a vyznačují se expresí stejných identifikujících markerů (Reynolds a Weiss, 1992; Singh *et al.*, 2003; Singh *et al.*, 2004). Historicky nejvíce

používaným markerem GSCs je již zmiňovaný CD133, který se však na základě posledních studií jeví jako nepříliš specifický (O'Brien *et al.*, 2007; Wright *et al.*, 2008). Mnohem větší specificita je v současné době připisována molekule CD15 (Son *et al.*, 2009) a cytoskeletálnímu proteinu nestinu, jehož exprese se mimo jiné u gliomů ukázala být negativním prognostickým faktorem. Jiný způsob identifikace GSCs je založen na schopnosti buněk aktivně se zbavovat škodlivých látek. Jde o metodu izolace tzv. „side population“, u které byla detekována zvýšená hladina ABC transportérů a jež rovněž disponuje kmenovými vlastnostmi (Fukaya *et al.*, 2010).

Pro GSCs je typická perivaskulární lokalizace a jejich výskyt byl také pozorován v okolí nekrotických ložisek, což dokládá fakt, že je „stem-like“ fenotyp těchto buněk značnou mírou závislý na okolním mikroprostředí. Často diskutován je vliv hypoxie na chování GSCs (Heddleston *et al.*, 2009). Hypoxie indukuje expresi HIF (hypoxia inducible factor), který zvyšuje schopnost sebeobnovy jak GSCs tak i non-GSCs. Zatímco HIF1 α je exprimován i neurálními kmenovými buňkami, HIF2 α je typický pouze pro GSCs, což z něj v podstatě činí ideální terapeutický cíl (Heddleston *et al.*, 2009). Text převzat a upraven z Kleinova *et al.*, 2015.

Molekulárně-genetické diagnostické, prognostické a prediktivní biomarkery u pacientů s difúzními gliomy

Stanovení diagnózy a prognózy onemocnění, případně predikce odpovědi na léčbu GBM vychází z integrované diagnostiky difúzních gliomů, v rámci níž dle platných doporučení WHO z roku 2016 nabyly na významu právě molekulárně-genetické biomarkery. Tento stav reflektuje poznatky dosavadního výzkumu a začleňuje molekulární charakteristiku společně s konvenčními histopatologickými nálezy a imunofenotypem mezi základní diagnostické přístupy klasifikace difúzních gliomů. Dle aktuálních doporučení by tedy nově diagnostikované difúzní astrocytární a oligodendroglíální gliomy měly být testovány na přítomnost mutace IDH1 pomocí imunohistochemického vyšetření a v případě negativního výsledku by u nich měla být provedena molekulární analýza verifikující tento výsledek a současně schopná odhalit případnou přítomnost vzácněji se vyskytujících mutací v genech IDH1/2. IDH mutované nádory by následně měly být vyšetřeny na přítomnost chromozomální kodelece 1p/19q vznikající nebalancovanou chromozomální translokací t(1;19)(q10;p10) (Griffin *et al.*, 2006). Tato kodelece se vyskytuje téměř výhradně u oligodendroglíálních nádorů, které zároveň vždy vykazují imunohistochemicky expresi

ATRX. Naopak alterace genu ATRX je často pozorovatelná, nikoliv však nutně, u IDH-mutovaných astrocytomů, kde je dále obvykle provázena i mutacemi v genu TP53.

Mimo standardní klasifikaci jsou mutační status genů IDH1/2 a integrita chromozomů 1 a 19 využívány rovněž jako prognostické biomarkery, kdy přítomnost mutací, resp. kodelece 1p/19q predikuje významně lepší prognózu u oligodendroglálních tumorů. Podobně přítomnost mutace v genu pro ATRX je pozitivním prognostickým znakem u pacientů s astrocytomy.

U pediatrických pacientů je pak při podezření na difúzní středočárový gliom doporučeno molekulární vyšetření dvacátého sedmého kodonu histonu H3, kde může vlivem mutace docházet k záměně aminokyseliny lysin na methionin – H3K27M. Přítomnost této mutace potvrzuje zmiňovaný podtyp difúzního gliomu a současně predikuje velmi špatnou prognózu (Gojo *et al.*, 2019). Na základě doporučení mezinárodního konsorcia neuropatologů a klinických neuroonkologů (The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy – not official WHO; cIMPACT-NOW), které prezentuje praktická doporučení pro tkáňovou diagnostiku mozkových nádorů na základě recentních zjištění, je vhodné doplnit molekulární vyšetření difúzních a anaplastických astrocytomů IDH-wt rovněž o analýzu numerických aberací chromozomů 7 a 10, a dále vyšetření amplifikace genu pro EGFR a mutačního statusu promotoru pro gen TERT, přičemž případy s alespoň jedním alterovaným znakem klasifikovat jako difúzní astrocytární gliomy, IDH-wt, s molekulárními znaky GBM, WHO IV (Louis *et al.*, 2019; Hendrych *et al.*, 2020).

Pacienti s HGG by měli být rovněž testováni na metylační status promotoru genu pro MGMT. Metylace promotoru pro MGMT byla opakovaně asociována nejen s prognózou, ale především s odpovědí na léčbu alkylačními činidly, mezi které patří i TMZ. Metylace promotoru MGMT vede na buněčné úrovni ke snížené kapacitě reparace DNA a s tím související zvýšené apoptóze, což v důsledku znamená lepší odpověď na léčbu TMZ indukující tato poškození (Criniere *et al.*, 2007; Lakomy *et al.*, 2011b; Sana *et al.*, 2014; Lakomy *et al.*, 2020).

Příspěvek k dané problematice

Metylace promotoru genu pro MGMT je v současné době široce využívaným molekulárním biomarkerem jak odpovědi na léčbu alkylačním činidlem TMZ, tak celkového přežívání pacientů s GBM. Z tohoto důvodu jsme v našich studiích zaměřených na identifikaci nových

prediktivních a prognostických biomarkerů u GBM metylační status promotoru rovněž analyzovali. Toto hodnocení bylo provedeno pomocí analýzy teploty tání s vysokým rozlišením, které předcházela bisulfitová konverze DNA. Tato metoda je vysoce citlivá a výrazně snižuje procento falešně pozitivních výsledků, které bývají pozorovány v případě využití technologie metylačně specifické PCR. Na základě dostupné literatury a získaných dat byla stanovena hraniční hodnota definující metylovaný promotor na alespoň 25% metylaci. Dle tohoto rozdělení jsme v první studii zjistili 32 % metylovaných oproti 68 % nemetylovaných vzorků, ačkoliv odborná literatura uvádí množství GBM s metylovaným promotorem pro MGMT v rozmezí 35 % až 47 % (Lakomy et al., 2011b; Příloha 6). Důvodem tohoto nižšího zastoupení metylovaných vzorků však mohlo být jednak použití technologie eliminující falešnou pozitivitu, ale také nedostatečně rozsáhlý soubor pacientů čítající 38 případů. V naší následné studii založené na analýze 58 pacientů s primárním GBM bylo procento případů s metylovaným promotorem MGMT 38 % (Sana et al., 2014; Příloha 7). V obou studiích jsme v souladu s literaturou potvrdili jak významnou asociaci metylačního stavu promotoru pro MGMT s PFS, tak s OS u pacientů s GBM.

Meningeomy

Meningeomy jsou nejčastějšími intrakraniálními nádory tvořícími dle různých zdrojů 20 až 30 % všech primárních neoplázií této anatomické oblasti. Vznikají z arachnoideálních buněk mozkových obalů a jedná se zpravidla o pomalu rostoucí, často kalcifikované tumory (Wiemels et al., 2010). Jejich incidence narůstá s věkem a maxima dosahuje kolem 50. roku života, přičemž jsou na rozdíl od gliomů častěji diagnostikovány u žen. Se stoupajícím věkem se obecně zhoršuje i prognóza pacientů, ačkoliv tato je primárně závislá na stupni malignity onemocnění. Biologické chování menigeomů je značně variabilní a kolísá od benigních (WHO stupeň malignity I; 94 % všech diagnostikovaných menigeomů) přes skupinu nádorů s rysy agresivního chování (WHO II; 5 %) až k menigeomům vysoce agresivním, které mají tendenci infiltrovat okolní tkáň (WHO III; 1 %) (Enam et al., 1996; Kane et al., 2011; Krejčí et al., 2013; Duba et al., 2015). Vyšší stupeň je spojován se statisticky vyšším rizikem recidivy, morbidity a mortality (Mawrin a Perry, 2010). Například atypické menigeomy recidivují osmkrát častěji než benigní, dobu přežití u atypických a maligních menigeomů udávají některé studie do dvou let (Mawrin a Perry, 2010; Krejčí et al., 2013). Za známku agresivity je také považována invaze do mozku či kosti, nebo výrazný perifokální edém v okolí léze. Dalším nepříznivým faktorem pro úspěšnou léčbu

může být lokalizace meningeomů, jež je může činit obtížně dosažitelnými k radikální chirurgické léčbě (Krejčí *et al.*, 2012; Krejčí *et al.*, 2013).

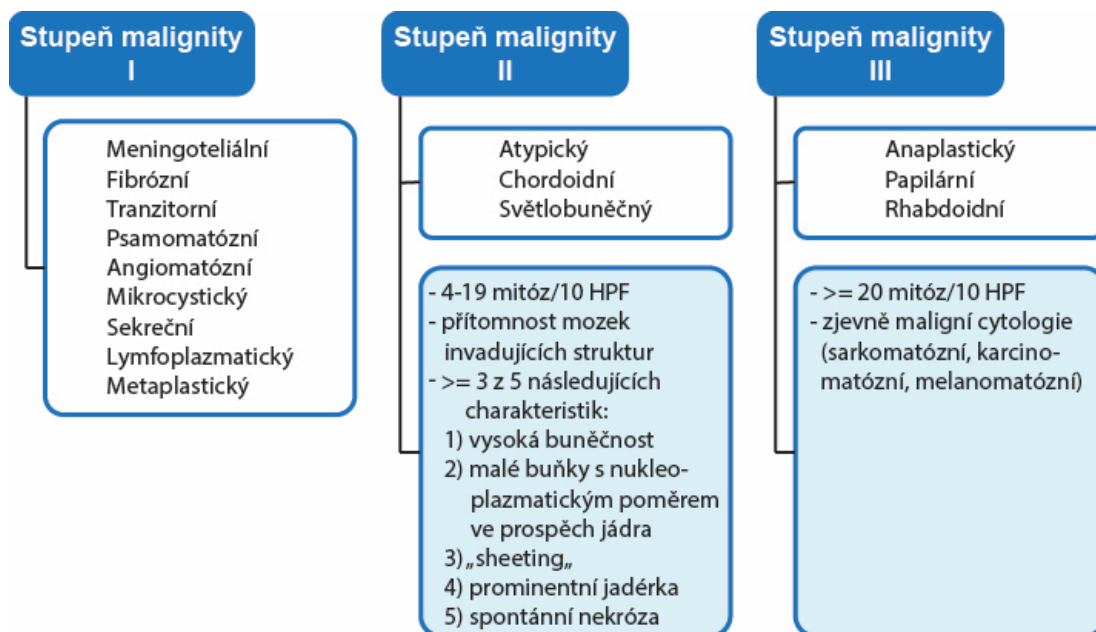
Právě chirurgická intervence je základním terapeutickým přístupem uplatňujícím se v managementu pacientů s meningeomem a její úspěšnost přímo souvisí s pravděpodobností recidivy a prognózou pacienta. Radikalita chirurgického zákroku je běžně hodnocena na základě kritérií podle Simpsona a klasifikována do pěti odpovídajících stupňů – prvními dvěma se hodnotí radikální výkon. Recidiva je tím pravděpodobnější, čím větší stupeň radikality byl dosažen; u stupně I je to asi 10 %, u stupně II 19 %, u stupně III se udává 29 % a u stupně IV 40 % během 10 let. Stupeň V pak odpovídá pouze biopsii tumoru a nelze tedy hovořit o následné recidivě onemocnění. Z nechirurgických možností léčby je nutné zmínit radiochirurgii, která může být zvažována jako doplňkový i alternativní přístup (Duba *et al.*, 2015).

Klinický obraz onemocnění tedy reflektuje především stupeň malignity a dále zejména u benigních forem velikost a lokalizaci tumoru, kdy lze pozorovat jak zcela asymptomatické případy, tak pacienty s časnou hemiparézou, epileptickými záchvaty anebo fatickou poruchou. Objemnější nádory mohou způsobovat nitrolební hypertenzi projevující se bolestmi hlavy, nevolností či zvracením.

Etiologie meningeomů, podobně jako většiny intrakraniálních nádorů u raně dospělých a dospělých pacientů, není jasná. Nicméně dle některých studií v ní může hrát kromě genetické predispozice roli radiace a působení virů a z důvodu častějšího výskytu onemocnění u žen je rovněž zmiňován hormonální vliv (Dumanski *et al.*, 1987; Seizinger *et al.*, 1987; Koehorst *et al.*, 1993; Duba *et al.*, 2015).

Revidovaná WHO klasifikace nádorů CNS z roku 2016 kategorizuje meningeomy do 15 různých variant (Obrázek 3), kdy každá z variant je diagnostikována na základě své charakteristické histologické morfologie. Mnoho meningeomů však vykazuje smíšenou histologii; v těchto případech se tedy kategorie stanoví s přihlédnutím k dominantnímu histologickému typu. Atypické meningiomy (WHO II) jsou diagnostikovány buďto na základě přítomnosti 4 – 19 mitóz na 10 HPF (high-power fields) nebo výskytem mozek invadujících struktur nebo přítomností alespoň tří z pěti dále uvedených znaků: vysoká buněčnost, malé buňky s nukleoplazmatickým poměrem posunutým ve prospěch jádra, tzv. „sheeting“, – nepřerušovaný růst bez vzoru, prominentní jadérka a spontánní nekróza. Anaplastické meningiomy (WHO III) jsou určeny buď výrazně zvýšenou mitotickou aktivitou (≥ 20 mitóz na 10 HPF), nebo zjevně maligní morfologií jako je karcinomatózní, sarkomatózní a melanomatózní cytologie (Lee a Lee, 2020).

Obrázek 3 Přehled klasifikačního systému Světové zdravotnické organizace z roku 2016 pro klasifikaci meningeomů (převzato a upraveno z Lee a Lee, 2020)



HPF, „high-power fields“; N / C, jaderné / cytoplazmatické

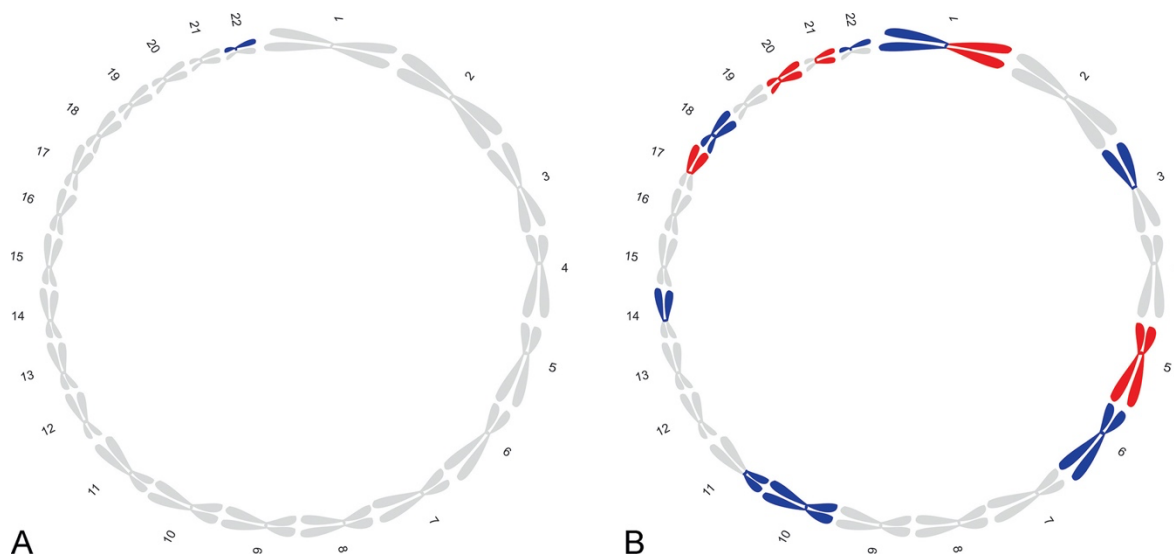
Přestože současný histologický stupeň malignity do značné míry odráží agresivitu a průběh onemocnění a je tedy cenným prognostickým ukazatelem, stále existuje na úrovni biologického chování meningeomů v rámci jednotlivých WHO skupin značná variabilita, což má nepochybný dopad na přežívání pacientů. Současný stav by mohla významně zlepšit integrace molekulárních charakteristik do stávající diagnostiky, podobně jako je tomu např. u gliomů. V souvislosti s genezí a biologií meningeomů je pravděpodobně nejdéle studovaným genem neurofibromin 2 (NF2), průvodce neurofibromatózy typu 2 a nádorový supresor lokalizovaný na dlouhém raménku chromozomu 22 v pozici 12.2 (Bi *et al.*, 2018). NF2, též nazývaný jako Merlin nebo Schwannomin, je membránový protein vázající vlákna aktinu k buněčné membráně a účastníci se tak tvorby cytoskeletu. Nachází se přednostně v nervové tkáni, konkrétně pak v adherentních mezibuněčných spojích, kde působí rovněž jako inhibitor buněčného růstu.

Kromě těchto genetických změn byla u meningeomů pozorována celá řada dalších běžně se opakujících alterací na úrovni jak genů (Tabulka 2), tak chromozomů (Obrázek 4), často asociovaných právě s konkrétním stupněm malignity. Jako nezávislé prognostické biomarkery se ukázaly rovněž mutace v genech TERT, BAP1, DMD a CDKN2A/B. Nicméně s výjimkou mutace TERT se jedná spíše o vzácně se vyskytující mutace a jejich implementace do klinicko-diagnostické praxe není v tuto chvíli reálná.

Tabulka 2 Hlavní molekulární alterace u meningeomů (převzato a upraveno z Birzu *et al.*, 2020)

Gen	Lokus	Frekvence	Dominantní WHO skupina	Dominantní histologie	Buněčný proces
<i>NF2</i>	22q12.2	40-60 %	1,2,3	Fibrózně tranzitorní	Hippo, PI3K/AKT/mTOR
<i>TRAF7</i>	16p13.3	15-26 %	1 > 2,3	Meningoteliálně sekreční	MAPK, IRF, NF-kB
<i>KLF4</i>	9p31	9-12 %	1	Sekreční	PI3K/AKT/mTOR
<i>AKT1</i>	14q32.33	7-12 %	1	Meningoteliální	PI3K/AKT/mTOR
<i>PIK3CA</i>	3q26.32	4-7 %	1 > 2,3	Meningoteliálně tranzitorní	PI3K/AKT/mTOR
<i>POLR2A</i>	17p13.1	6 %	1	Meningoteliální	Transkripce
<i>PTEN</i>	10q23.31	NA	2,3	NA	PI3K/AKT/mTOR
<i>SMO</i>	7p32.1	1-5 %	1	Meningoteliální	SHH dráha
<i>SUFU</i>	10q24.32	1 %	NA	NA	SHH dráha
<i>PTCH1/2</i>	9q22.32 1p34.1	Výjimečně	NA	NA	SHH dráha
<i>FAK</i>	8q24.3	Velmi raritně	NA	NA	Buněčná motilita
<i>BAP1</i>	3p21.1	< 1 %	2,3	Rhabdoidní, Papilární	DNA reparace
<i>CHEK2</i>	22q12.1	Výjimečně	1	NA	Regulace buněčného cyklu
<i>CDKN2A</i> <i>CDKN2B</i>	9p21.3	< 5 %	2,3	NA	Regulace buněčného cyklu
<i>DMD</i>	Yp21.1	NA	2,3	NA	Cytoskelet
<i>TERTp</i>	5p15.33	5-15 %	1,2,3	NA	Aktivita telomerázy
<i>SMARCB1</i>	22q11.23	5 %	1 < 2,3	NA	Remodelace chromatinu
<i>SMARCE1</i>	17q21.2	3-4 %	2	Světlobuněčný	Remodelace chromatinu
<i>ARID1A</i>	1p36.11	12 %	3	Anaplastický	Remodelace chromatinu
<i>PBRM1</i>	3p21.1	3 %	3	Papilárně rhabdoidní	Remodelace chromatinu

Obrázek 4 Chromozomální aberace často se vyskytující se u meningeomů (převzato z Bi *et al.*, 2018)



A – Meningeomy WHO I, B – Meningeomy s vyšším stupněm malignity; Modrá označuje ztrátu chromozomů; červená, chromozomální zisk.

Překvapivé údaje o epigenetické subklasifikaci meningeomů a klinickém vývoji onemocnění poskytlo rovněž studium metylomu. Výsledky neřízené shlukové analýzy odhalily dvě hlavní epigenetické skupiny, kdy první z nich obsahovala další čtyři (metylační klastr (MC) benigní 1, MC benigní 2, MC benigní 3 a MC středně rizikový A) a druhá dvě (MC středně rizikový B a MC maligní) podskupiny. Tumory v těchto podskupinách se vyznačovaly jak odlišným vývojem a genetickými změnami, tak různou prognózou (Tabulka 3) (Sahm *et al.*, 2017; Birzu *et al.*, 2020).

Tabulka 3 Identifikované metylační klastry meningeomů a jejich charakteristika (převzato a upraveno z Birzu *et al.*, 2020)

	MC benigní I	MC benigní II	MC benigní III	MC středně rizikový A	MC středně rizikový B	MC maligní
Histologie	Fibrózní Tranzitorní Atypický	Sekreční Tranzitorní Meningoteliální	Angiomatózní Tranzitorní Atypický	Fibrózní Tranzitorní Atypický	Atypický Anaplastický	Anaplastický
Cytogenetické alterace	22q ztráta		22q ztráta 5 zisk	22q ztráta 1p ztráta	22q, 1p, 10 ztráta CDKN2A delece	22q, 1p ztráta CDKN2A delece (70 %)
Mutace	NF2 (63 %)	TRAF7 (49 %), AKT1 (33 %), KLF4 (15 %), SMO (7 %), NF2 (7 %)	PIK3CA (11 %), NF2 (32 %)	NF2 (53 %)	NF2, TERT, SUFU (5 %)	NF2, TERT, SUFU (6 %)
Prognóza	Dobrá	Dobrá	Dobrá	Střední	Střední	Špatná

Mozkové metastázy

Sekundární mozkové nádory, mozkové metastázy, jsou nejčastějšími nitrolebními malignitami vyskytující se u 30–40 % onkologických pacientů s generalizovaným nádorovým onemocněním; v některých materiálech se udává podíl až 50 % (Pospíšková *et al.*, 2011; Kazda *et al.*, 2019). Metastatický rozsev probíhá nejčastěji hematogenně, méně často přímým prorůstáním nebo podél spinálních kořenů a likvorovými cestami. Vyšší tendence k metastazování do mozku je pozorována u melanomů, testikulárních nádorů a karcinomů plic. Z pohledu četnosti jsou zastoupeny nejvíce metastázy s primárním tumorem v plicích, prsou, ledvinách, gastrointestinálním traktu a již zmíněný melanom. U přibližně 10 % mozkových metastáz je pak origo neznámé. 80 % metastáz se nachází supratentoriálně. V 10 % se jedná o solitární metastatické ložisko v celém organismu a až 85 % metastáz v mozku je mnohočetných (Fadrus *et al.*, 2010; Pospíšková *et al.*, 2011). Lze předpokládat, že incidence bude nadále vzrůstat díky větší dostupnosti magnetické rezonance, a především díky rozšiřujícím se indikacím moderní systémové terapie vedoucím k obecně delšímu celkovému přežívání diseminovaných pacientů, u kterých stoupá riziko další diseminace právě do intrakraniální oblasti. Jelikož však moderní terapeutické agens

většinou jen obtížně pronikají do centrálního nervového systému, prognóza pacientů s mozkovými metastázami je i přes výše naznačené úspěchy moderní onkologické léčby nadále velice vážná s mediány celkového přežití udávanými v řádu měsíců. Existují však velké rozdíly v přežívání jednotlivých pacientů, kdy lze v některých případech dosáhnout kombinovanou onkologickou léčbou přežití až několika let (Shen *et al.*, 2016). Vzhledem k popisovaným rozdílům v přežívání je odhad prognózy u konkrétního pacienta s nově diagnostikovanou metastázou extrémně důležitý při rozhodování o nejvhodnějším diagnosticko-terapeutickém postupu. V denní rutinní praxi jsou tak neocenitelnou pomůckou nejrůznější prognostické skórovací systémy umožňující u daného pacienta validní odhad celkového přežití (Kazda *et al.*, 2015). Nejznámějším takovým skórovacím systémem je RPA (Recursive Partitioning Analysis), pomocí kterého lze zhodnocením celkového výkonnostního stavu (Karnofsky index), věku, kontroly primárního onemocnění a přítomnosti extrakraniálních metastáz rozdělit pacienty do tří skupin s predikovaným mediánem přežití v rozmezí 2,3 až 7,1 měsíců (Gaspar *et al.*, 1997). Novější skórovací systémy zohledňují i molekulárně-genetické charakteristiky nádoru. Nicméně právě molekulárně-genetická podstata onemocnění, ač je s ní nepochybně úzce spojena budoucnost onkologické léčby, je v případě mozkových metastáz jen velmi těžko uchopitelná a obtížně interpretovatelná tak, aby mohla sloužit jako samostatný diagnostický anebo prognostický nástroj. Důvodem je nesmírná molekulární heterogenita onemocnění logicky vyplývající jednak z různého původu metastáz a dále z nutnosti přizpůsobit se z pohledu metastazující buňky mnohdy extrémnímu prostředí. Text převzat a upraven z Kazda *et al.*, 2019.

Složitost a molekulární komplexnost celého procesu lze demonstrovat na metastatické kaskádě, která je iniciována invazí bazální membrány a blízké tkáně nádorovými buňkami a jejich intravazací do krevního řečiště nebo infiltrací lymfatických drah. Poté následuje aktivace mechanismů umožňujících přežití nádorových buněk ve stavu anoikis a jejich extravazace do vhodné tkáně organismu, kde mohou být schopné proliferace. Tento patogenní proces tedy vyžaduje přesnou koordinaci různých signálních drah, které mohou být aktivovány a řízeny v závislosti na buňkou získaných genetických alteracích vícero mechanismy (Hernández-Caballero, 2013). Nejznámější geny zapojené do jednotlivých kroků metastatického procesu jsou uvedeny v Tabulce 4. Kromě toho bylo popsáno vícero genů, které podporují nebo naopak potlačují schopnost invazivity a metastazování u konkrétních primárních nádorů (Tabulka 5) (Rahmathulla *et al.*, 2012).

Tabulka 4 Přehled genů asociovaných se zvýšeným metastatickým potenciálem (převzato a upraveno z Rahmathulla *et al.*, 2012)

Gen	Lokus	Význam
<i>RHoC</i>	1p21-p13	Remodelace cytoskeletu během morfogeneze a v procesech souvisejících motilitou; důležitá role v buněčné invazivitě
<i>LOX</i>	5q23.1-q23.2	Pozitivní regulace invazivního potenciálu nádorových buněk v hypoxických podmínkách
<i>VEGF</i>	6p21.1	Angiogenní růstový faktor Inhibuje tvorbu mozkových metastáz; reguluje angiogenezi a proliferaci buněk; zvyšuje apoptózu
<i>CSF1</i>	1p13.3	Stimuluje proliferaci makrofágů a následné uvolňování růstových faktorů
<i>ID1</i>	20q11.21	Podílí se na remodelaci matrix a angiogenezi
<i>TWIST1</i>	7p21.1	Způsobuje ztrátu buněčné adheze zprostředkovanou E-kadherinem a podporuje buněčnou motilitu skrze regulaci epiteliálně-mezenchymální tranzice
<i>MET</i>	7q31.2	Ovlivňuje širokou škálu buněčných procesů
<i>MMP-9</i>	20q13.12	Degradace extracelulární matrix, remodelace tkáně
<i>NEDD9</i>	6p24.2	Pozitivní regulace metastatického potenciálu
<i>LEF1</i>	4q25	Transkripční efektor - dráha WNT; sklon k mozkovým metastázám Knockdown genu vede k významnému snížení počtu mozkových metastáz, snižuje tvorbu kolonií a invazivitu
<i>HOXB9</i>	17q21.32	Patří do rodiny homeoboxových genů; klíčová role v embryonální segmentaci a vzorování Knockdown genu vede u buněk ke snížení jejich invazivity, schopnosti tvořit kolonie a schopnosti iniciovat mozkové metastázy
<i>BMP4</i>	14q22.2	Klíčová role v embryonálním vývoji a epiteliálně-mezenchymální tranzici
<i>STAT3</i>	17q21.2	Transkripční faktor Snížení vede k potlačení růstu mozkových metastáz; snižuje angiogenezi a buněčnou invazivitu

Tabulka 5 Přehled genů podporujících a tlumících metastazování a invazivitu nádoru (převzato a upraveno z Rahmathulla *et al.*, 2012)

Geny potlačující tvorbu metastáz			Geny podporující tvorbu metastáz		
Gen	Lokus	Primární nádor	Gen	Lokus	Primární nádor
<i>NM23</i>	17q21.3	Karcinom prsu, kolorektální karcinom, melanom	<i>ERBB2 (HER2)</i>	17q21.1	Karcinom prsu
<i>MKK4</i>	17p11.2	Karcinom prsu, ovária, prostaty	<i>TIAM1</i>	21q22.1	Karcinom ledvin, prostaty, prsu, kolorektální karcinom
<i>BRMS1</i>	11q13.1-q13.2	Karcinom prsu, melanom	<i>SRC</i>	20q12-q13	Karcinom prsu, plic, kolorektální karcinom, melanom
<i>KISS1</i>	1q32	Karcinom prsu, melanom	<i>S100A4</i>	1q21	Karcinom prsu, žaludku, kolorektální karcinom
<i>KAI1 (CD82)</i>	11p11.2	Karcinom močového měchýře, prsu, plic, slinivky, prostaty	<i>MTA1</i>	14q32.3	Karcinom prsu, vaječníku, plic, kolorektální karcinom
<i>CD44</i>	11p13	Kolorektální karcinom, karcinom prsu, plic, prostaty, melanom	<i>KRAS</i>	12p12.1	Karcinom slinivky, plic, kolorektální karcinom
<i>CRSP3</i>	6q23.2	Melanom	<i>HRAS</i>	11p15.5	Karcinom močového měchýře, ledvin, štítné žlázy
<i>RHOGDI2</i>	11p11.2	Karcinom močového měchýře, prsu, ledvin, plic, prostaty, kolorektální karcinom, hepatocelulární karcinom			
<i>VDUP1</i>	1q21	Melanom			
<i>PTEN/MMAC1</i>	10q23.31	Karcinom prsu, endometria, ledvin, plic, štítné žlázy, kolorektální karcinom			
<i>VHL</i>	3p25.3	Karcinom ledvin			
<i>TIMP2</i>	17q25.3	Melanom			
<i>SMAD4</i>	18q21.2	Karcinom slinivky, prostaty, kolorektální karcinom			
<i>RRM1</i>	11p15.4	Karcinom plic			
<i>PTPN11</i>	12q24.1	Karcinom plic, štítné žlázy, kolorektální karcinom, melanom			
<i>CDH1</i>	16q22.1	Karcinom prsu, žaludku			
<i>CASP8</i>	2q33	Karcinom prsu, žaludku, plic			

Příspěvek k dané problematice

V roce 2019 jsme publikovali přehledovou práci s názvem „Diagnostika, operační a systémová terapie metastáz solidních nádorů“, jejímž cílem bylo poskytnout základní a zároveň aktuální informace o současných diagnostických a léčebných možnostech pacientů s mozkovými metastázami včetně chirurgické léčby a radioterapie. Poznatky vycházely zejména z několika velkých randomizovaných studií potenciálně ovlivňujících denní praxi v léčbě pacientů s mozkovými metastázami, které byly publikovány v poslední době (Kazda et al., 2019b; Příloha 8).

Krátké nekódující RNA u nádorů mozku

Jak již bylo zmíněno v úvodní kapitole této práce, nejznámějšími a doposud nejvíce prostudovanými třídami regulačních sncRNA jsou siRNA, miRNA a piRNA. Z hlediska možné diagnostiky nádorových onemocnění, včetně predikce prognózy a odpovědi na léčbu onkologických pacientů, jsou relevantní pouze miRNA a piRNA, jelikož právě tyto RNA jsou kódovány v DNA samotné buňky. Na rozdíl od toho siRNA se v buňce většinou přirozeně nevyskytují a objevují se v ní převážně až v přítomnosti parazitických RNA sekvencí, které tam bývají nejčastěji vnášeny viry. Důvodem, proč je tedy tato třída nekódujících RNA často zmiňována v souvislosti s nádory, je jejich schopnost cíleně regulovat mRNA prostřednictvím mechanismu RNA interference, a tedy jejich terapeutický potenciál. V souvislosti s léčbou nádorů mozku však doposud nebyl schválen žádný terapeutický prostředek využívající siRNA. Z tohoto důvodu bude následující text zaměřen pouze na význam miRNA a piRNA u vybraných onkologických onemocnění mozku, které se zde jeví být jak slibné terapeutické cíle, tak především již zmiňované diagnostické, prognostické a prediktivní biomarkery.

Krátké nekódující RNA u gliomů

Gliomy jsou heterogenní skupinou onemocnění s velmi variabilním biologickým chováním, které úzce souvisí s prognózou a případně odpovědí na léčbu, nejen mezi histopatologicky klasifikovanými podskupinami, ale i v rámci nich. Důvodem je variabilita těchto nádorů na molekulární úrovni, kterou nelze reflektovat standardními histopatologickými vyšetřeními. Hlubšího poznání molekulární patogeneze gliomů bylo dosaženo až s vývojem nových sofistikovaných technologií umožňujících vysokokapacitní a velmi přesné molekulární analýzy nádorů především na úrovni jejich genomů a transkriptomů. Kromě studia chromozomových aberací a variant v kódujících genech se stále více odborných prací zaměřuje na studium nekódujících RNA včetně miRNA a piRNA, které již byly mnohokrát asociovány s patologií gliomů a hrají v jejich chování klíčovou roli.

Příspěvek k dané problematice

V roce 2011 jsme uveřejnili jednu z prvních přehledových prací na téma významu miRNA v biologii GBM, kde jsme sumarizovali tehdejší poznatky k dané problematice, včetně účasti miRNA v regulaci důležitých signálních drah GBM hrajících roli v esenciálních procesech jako jsou proliferace, apoptóza, regulace buněčného cyklu, invazivita a angiogeneze.

Rovněž zde byla vyzdvížena potenciální role miRNA jako diagnostických, prognostických a prediktivních biomarkerů u pacientů s GBM, což s ohledem na současné znalosti již můžeme považovat za pravdivá tvrzení (Sana et al., 2011; Příloha 9).

MikroRNA jako diagnostické biomarkery u gliomů

Studium expresních profilů miRNA asociovaných s gliomy o různém stupni malignity a nenádorovou mozkovou tkání není pouze prvním krokem vedoucím k odhalení miRNA zapojených do iniciace a následné progresu tohoto onemocnění, ale je také klíčové pro rozvoj nové molekulární taxonomie gliomů, jejímž cílem je přesněji odhadnout biologickou povahu onemocnění doposud predikovanou pouze na základě konvenčního histopatologického vyšetření. Odhalení expresních profilů miRNA charakteristických pro jednotlivé skupiny gliomů by rovněž mohlo vést k vytvoření efektivního diagnostického nástroje schopného reflektovat heterogenní povahu gliomů, která v některých případech může vést k podhodnocení skutečného rizika a následně k indikaci neodpovídající léčby.

První práci, ve které byly analyzovány expresní profily miRNA u GBM, publikoval v roce 2005 se svými kolegy Ciafré. Tento autorský kolektiv analyzoval konkrétně 254 miRNA u devíti párových vzorků GBM a nenádorových mozkových tkání. Z výsledků práce vyplývá, že devět miRNA (miR-10b, miR-130a, miR-221, miR-125b-1, miR-125b-2, miR-9-2, miR-21, miR-25 a miR-123) vykazovalo zvýšenou expresi a čtyři miRNA (miR-128a and miR-181a/b/c) naopak sníženou expresi v nádorové tkáni. Vůbec největší rozdíly v expresi mezi porovnávanými vzorky pak autoři pozorovali u miR-221, miR-128a a miR-181a/b/c (Ciafre et al., 2005). Velmi podobnou analýzu provedli později Chan a kol., kteří tímto způsobem porovnali tři vzorky primárních gliomů s vysokým stupněm malignity a celkem osm vzorků jak fetální, tak adultní mozkové tkáně. Výsledkem bylo odhalení pěti miRNA (miR-21, miR-138, miR-347, miR-135 a miR-291-5) s významně zvýšenou hladinou a tři miRNA (miR-198, miR-188 a miR-202) se sníženou hladinou v nádorové tkáni. Je vhodné rovněž zmínit, že průměrná hodnota exprese miR-21 ve sledovaných gliomech dosahovala téměř devítinásobku exprese pozorované v kontrolní zdravé mozkové tkáni (Chan et al., 2005). Jiná studie pod vedením dr. Silbera analyzovala expresní hladiny 192 maturovaných miRNA u čtyř různých vzorků anaplastických astrocytomů, čtyř GBM a rovněž čtyř vzorků nenádorové tkáně získané ze spánkových laloků v rámci chirurgicko-léčebného výkonu u pacientů s epilepsií. Statistická analýza dosažených dat následně odhalila třináct miRNA s deregulovanou expresí mezi GBM a nenádorovými vzorky a šest

miRNA s významně odlišnými expresními hladinami mezi vzorky anaplastických astrocytomů a GBM (Silber *et al.*, 2008). Podobná studie porovnávající třináct primárních a třináct sekundárních GBM se stejným počtem anaplastických astrocytomů a sedmi mozkovými tkáněmi bez histopatologicky prokázané neoplastické transformace popisuje 55 miRNA se zvýšenou expresí a 29 miRNA se sníženou expresí v gliálních tkáních. Současně tato práce identifikovala 67 rozdílně exprimovaných miRNA mezi gliomy WHO III a IV. Porovnání primárních a sekundárních GBM pak odhalilo sedm miRNA se statisticky významnou změnou v expresi mezi těmito skupinami. Dvacet jedna miRNA bylo též rozdílně exprimovaných mezi postupně progredujícími gliomy (anaplastický astrocytom a sekundární GBM) a *de novo* vzniklými GBM, 76 miRNA vykazovalo statisticky významný rozdíl v expresi mezi anaplastickým astrocytmem a primárním GBM a nakonec 68 miRNA bylo odlišně exprimováno anaplastickým astrocytmem a sekundárním GBM (Rao *et al.*, 2010). Podobně D'Urso a kol. našli soubor deseti miRNA schopných na základě své exprese správně klasifikovat primární a sekundární GBM (D'Urso *et al.*, 2012). Skalsky a Cullen studovali miRNA profily u šesti GBM a třech nenádorových vzorků mozkové tkáně pomocí vysokokapacitního sekvenování nové generace. Tato technologie umožnila mezi těmito dvěma skupinami odhalit devět rozdílně exprimovaných miRNA (miR-124, miR-10b*, miR-139-5p, miR-7, miR-10b, miR-132, miR-95, miR-543 a miR-7d) (Skalsky a Cullen, 2011). Sasayama a kol. analyzovali tři párové vzorky GBM a nenádorové mozkové tkáně a odhalili tak pět miRNA (miR-10b, miR-21, miR-183, miR-92b a miR-106b) s významně vyšší expresí a pět miRNA (miR-302c*, miR-379, miR-329, miR-134 a miR-369-3p) s nižší expresí ve vzorcích GBM (Sasayama *et al.*, 2009). Wang a kol. rovněž analyzovali expresní profily miRNA u párových vzorků GBM a nenádorové tkáně a identifikovali 91 miRNA, jejichž exprese byla přinejmenším dvojnásobně rozdílná mezi skupinami. Za zmínku stojí, že miR-483-5p byla téměř stonásobně snížena v nádorové tkáni (Wang *et al.*, 2012a). Další studie validující expresi osmi vybraných miRNA u deseti GBM a čtyř nenádorových vzorků mozku popsala miR-21 a miR-221 jako statisticky významně zvýšené a naopak miR-181b jako sníženou právě v GBM (Conti *et al.*, 2009). V neposlední řadě je nutno zmínit i naše studie, kde jsme v prvním případě porovnávali expresi osmi vybraných miRNA u GBM a nenádorových vzorků získaných chirurgickým odstraněním arteriovenózních malformací. Výsledky ukázaly, že zatímco miR-21 a miR-125b byly zvýšeny v nádorové tkáni, miR-128a, miR-181a/b/c a miR-221/222 vykazovaly opačný stav (Slaby *et al.*, 2010). Ve druhém případě jsme analyzovali pomocí vysokokapacitní technologie založené na platformě PCR globální expresní profil u 58 vzorků GBM a deseti

mozkových tkání odebraných stejně jako v případě studie dr. Silbera ze spánkových laloků od pacientů s diagnostikovanou epilepsií. Výsledky odhalily 28 miRNA s významně pozměněnou expresí v GBM tkáni, které byly následně schopny přesně klasifikovat všechny studované vzorky (Sana *et al.*, 2014). Další menší studie porovnávající pouze jednotlivé miRNA mezi nádorovými a nenádorovými vzorky potom popisují sníženou hladinu miR-31, miR-205, miR-124a a miR-34a v GBM tkáni (Fowler *et al.*, 2011; Li *et al.*, 2011; Hua *et al.*, 2012; Yue *et al.*, 2012).

Tabulka 6 MikroRNA významně deregulované ve tkáni gliomů, které byly pozorovány alespoň ve dvou na sobě nezávislých studiích (převzato z Šána *et al.*, 2018)

miRNA studie	Chan	Sasayama	Conti	Wang	Ciafrè	Slaby	Silber	Rao	Sana	Ostatní
miR-17								↑	↑	↑
miR-19a								↑		↑
miR-19b								↑		↑
miR-21	↑		↑	↑	↑	↑	↑	↑	↑	
miR-25					↑			↑	↑	
miR-92b		↑						↑		
miR-106b		↑						↑	↑	
miR-125b					↑	↑				
miR-130a					↑			↑		
miR-155							↑	↑	↑	
miR-182				↑				↑		
miR-335				↑						↑
miR-10b					↑			↓	↑	
miR-34a									↑	2x↓
miR-138	↑							↓	↓	
miR-221			↑		↑	↓			↓	
miR-483-5p				↓				↓	↑	
miR-128a					↓	↓	↓		↓	
miR-132							↓	↓	↓	
miR-181a					↓	↓				
miR-181b			↓		↓	↓				
miR-181c					↓	↓				
miR-198	↓							↓		
miR-219-5p				↓				↓	↓	
miR-329		↓						↓		
miR-338-3p				↓				↓	↓	

↑ zvýšená exprese u gliomů, ↓ snížená exprese u gliomů

Malzkron a kol. analyzovali expresní profily miRNA mezi gliomy s rozdílnými stupni malignity. Autoři tak konkrétně studovali expresní profily 157 miRNA u pacientů postižených low-grade astrocytomy (WHO II), které postupně progredovaly do sekundárních GBM. Následným porovnáním bylo nalezeno dvanáct miRNA (miR-9, miR-15a, miR-16, miR-17, miR-19a, miR-20a, miR-21, miR-25, miR-28, miR-130b, miR-140 a miR-210), které byly zvýšeny, a dvě miRNA (miR-184 a miR-328), jejichž exprese korelovala se zvyšující se malignitou gliomů (Malzkorn *et al.*, 2010). MiRNA, které byly pozorovány jako významně deregulované ve tkáni gliomů alespoň ve dvou na sobě nezávislých studiích, jsou uvedeny v Tabulce 6. Text převzat a upraven z Šána *et al.*, 2018.

Příspěvek k dané problematice

*Expresní profily miRNA u GBM jsme studovali a následně publikovali ve dvou originálních sděleních. V první publikaci jsme prezentovali výsledky naší pilotní studie, ve které jsme se zaměřili na analýzu exprese 8 miRNA (miR-21, miR-128a, miR-181c, miR-195, miR-196a, miR-196b, miR-221 a miR-222) vybraných na základě dostupné literatury jako asociovaných s molekulární patologií GBM. Hladiny exprese těchto miRNA ve tkáni primárních GBM jsme porovnávali s jejich hladinami ve vzorcích odebraných z okolí arteriovenózních malformací (AVM) jako nenádorovou kontrolou mozkové tkáně. Ve všech případech jsme pozorovali signifikantní změny v hladinách exprese mezi porovnávanými vzorky, přičemž miR-21 a miR-196a/b vykazovaly zvýšenou hladinu, zatímco miR-181c, miR-221, miR-222, miR-195 a miR-128a sníženou hladinu v tkáni GBM. Nejvíce významný rozdíl byl pozorován v případě miR-128a s hladinami 0,01krát nižšími v nádorové tkáni. Rozdíly v expresi pozorované u miR-21, miR-196a/b a miR-181c byly ve shodě s dříve publikovanými studiemi, avšak v případě miR-221 a miR-222 jsme pozorovali ve srovnání s dřívějšími pracemi opačný trend v jejich hladinách u nádorové a nenádorové tkáně. Důvodem však mohl být charakter nenádorové kontrolní tkáně použité v naší studii, která obsahuje vysoké množství arteriálních endoteliálních buněk charakteristických právě vysokou expresí miR-221/222 (Lakomy *et al.*, 2011b; Příloha 6). Z těchto důvodů jsme pro naši druhou studii jako kontrolní tkáň použili mozkovou tkáň z temporálních laloků resekovaných v rámci terapeutického chirurgického výkonu u pacientů s farmakorezistentní epilepsií. V této studii jsme na rozsáhleším souboru vzorků GBM ($n = 58$) a již zmíněných nenádorových tkáních ($n = 10$) analyzovali expresi miRNA pomocí technologie TaqMan LowDensity Array, která je založená na principu real-time PCR a u každého vzorku detekuje paralelně hladinu exprese 754 miRNA. Následná*

analýza odhalila 108 miRNA se signifikantně zvýšenou a 108 miRNA se sníženou hladinou v tkáni GBM. Mezi nimi 28 miRNA vykazovalo hodnotu P nižší než 10^{-9} a tyto byly schopny rozlišit nádorovou a nenádorovou tkáň se 100% senzitivitou a specificitou. Nejvíce zvýšené hladiny byly pozorovány u miR-21* a miR-155, zatímco miR-220 a miR-1247 byly nejvíce snižené v nádorové tkáni. Devět z identifikovaných miRNA byly již dříve u GBM popsány jako deregulované. Kromě nejznámější a nejvíce prostudované onkogenní miR-21 to byly ještě miR-10b*, 128a, miR-133b, miR-139-3p, miR-139-5p, miR-155, 196b a miR-328 (**Sana et al., 2014b; Příloha 7**).

Kromě tkáňových miRNA se zdají být významnými diagnostickými biomarkery rovněž cirkulující miRNA v tělních tekutinách. V případě mozkových nádorů se jeví být jako vhodný materiál k detekci cirkulujících miRNA mozkomíšní mok (cerebrospinal fluid, CSF), který přirozeně přichází do přímého kontaktu s centrální nervovou soustavou, a tedy i s případnou patologickou tkání. V roce 2018 jsme proto publikovali práci, ve které jsme shrnuli současné poznatky o potencionálním využití miRNA v CSF, jako diagnostických, prognostických a prediktivních molekul u nádorů mozku, včetně gliomů. Součástí práce byla i rozsáhlá rešerše a zamyšlení nad metodickými přístupy, které se využívají k analýze miRNA v CSF (**Kopkova et al., 2018a; Příloha 10**). Jelikož se tyto metodické postupy mezi jednotlivými pracemi značně lišily, včetně způsobu izolace RNA a následné kvantifikace miRNA, provedli jsme optimalizační studii, jejíž výsledky jsme opublikovali v práci „MicroRNA isolation and quantification in cerebrospinal fluid: A comparative methodical study“ v časopisu PLoS ONE (**Kopkova et al., 2018b; Příloha 11**). Získané poznatky jsme následně aplikovali v práci, kde jsme porovnávali profily miRNA ve vzorcích CSF odebraných od pacientů s GBM, nízkostupňovými gliomy (LGG), meningeomem a mozkovými metastázami a jako kontrolní skupina byli využiti pacienti s normotenzním hydrocefalem. Vysokokapacitní analýza hladin miRNA pomocí sekvenování nové generace odhalila celkem 25 miRNA významně deregulovaných mezi GBM a kontrolní skupinou a dále 14 miRNA deregulovaných mezi LGG a kontrolní skupinou. Následná validace potvrdila rozdílné hladiny mezi vzorky obou typů gliomů a kontrolní skupinou v případě let-7b, let-7c, miR-10a, miR-10b, miR-140 a miR-196b; mezi GBM a kontrolními vzorky byly navíc pozorovány signifikantně různé hladiny u miR-30e a miR-196a. Zajímavé bylo, že hladiny let-7c a miR-196a se lišily i mezi GBM a LGG. Toto zjištění tedy z obou miRNA dělá nadějně diagnostické biomarkery, jež by bylo možné využít k detekci skrytých ložisek vysokostupňových gliomů u LGG, což by umožnilo včas indikovat vhodný terapeutický přístup (**Kopkova et al., Příloha 12**).

Jedním z hlavních cílů výzkumu gliomů je objevit vysoce senzitivní prognostické a prediktivní markery umožňující stratifikovat pacienty podle rizika progresu a predikovat odpověď na indikovanou léčbu. Význam této výzvy pak ještě více zvyšuje příchod nových terapeutických možností v léčbě gliomů.

Výzkumný tým okolo Srinivasana analyzoval deset vybraných miRNA u 222 pacientů s histopatologicky potvrzeným GBM, přičemž cílem studie bylo najít rozdílně exprimované miRNA mezi krátce a déle přežívajícími pacienty. Závěrečné statistické zhodnocení získaných expresních dat odhalilo tři miRNA (miR-20a, miR-106a a miR-17-5p) s nádorově protektivními vlastnostmi, které byly vysoce exprimovány právě u déle přežívající skupiny pacientů. Naopak sedm miRNA (miR-31, miR-221, miR-222, miR-148a, miR-146b, miR-200b a miR-193a) bylo vysoce exprimovaných u pacientů s kratším celkovým přežíváním a lze tedy usuzovat na jejich spíše onkogenní charakter (Srinivasan *et al.*, 2011). Jiná studie pod vedením Niyazi již využila více sofistikovaný přístup analýzy exprese miRNA a studovala současně hladiny 1100 těchto molekul u souboru 35 vzorků nádorových tkání odebraných od pacientů s diagnostikovaným GBM, kteří shodně podstoupili adjuvantní konkomitantní chemoradioterapii s TMZ. Výsledky tohoto výzkumného kolektivu odhalily třicet nejvíce rozdílně exprimovaných miRNA mezi pacienty s lepší a horší prognózou, ačkoliv pouze pět z těchto nekódujících RNA dosahovalo při tomto porovnání statistické významnosti (miR-3163, násobná změna (FC) = 2,0, $p = 0,05$; miR-539, FC = 0,5, $p = 0,001$; miR-1305, FC = 0,5, $p = 0,05$; miR-1260, FC = 0,5, $p = 0,03$; let-7a, FC = 0,3, $p = 0,02$). Nicméně charakterizace studovaných vzorků na základě expresních profilů všech výše zmiňovaných třiceti miRNA umožnila tyto vzorky správně klasifikovat podle doby celkového přežívání pacientů, přičemž se tento test ukázal jako nezávislý na metylačním stavu promotoru pro MGMT, v současné době jediným rutinně užívaným molekulárním markerem odpovědi na léčbu TMZ. Nutno však uvést, že pokud byla zmíněná sada miRNA testována pomocí multivariační Coxovy regresní analýzy společně s metylačním statutem MGMT, informací o aplikaci adjuvantního TMZ v monoterapii, věkem a RPA (recursive partitioning analysis), pouze aplikace adjuvantního TMZ v monoterapii zůstala jediným významným prognostickým faktorem ($p = 0,01$). Jak sada miRNA, tak MGMT status zde svoji významnost ztratily ($p = 0,22$, resp. $p = 0,17$) (Niyazi *et al.*, 2011).

Z prací naší výzkumné skupiny bych chronologicky uvedl nejprve studii z roku 2010, ve které Slabý a kol. pozorovali negativní vliv zvýšených hladin miR-181c a miR-181b na

léčebnou odpověď u pacientů, kteří po chirurgickém odstranění nádoru podstoupili klasický Stuppův protokol (Slaby *et al.*, 2010). Ve druhé práci jsme pak u pacientů s primárním GBM analyzovali expresi osmi vybraných miRNA (miR-21, miR-128a, miR-181c, miR-195, miR-196a, miR-196b, miR-221 a miR-222), přičemž z dosažených výsledků jasně vyplývá, že miR-195 a miR-196b mají pozitivní vliv na OS pacientů ($p = 0,0124$, resp. $p = 0,0492$) a kombinace expresních profilů miR-181c a miR-21 umožnila identifikovat pacienty progredující do šesti měsíců od stanovení diagnózy (senzitivita 92 %, specificita 81 %, $p < 0,0001$) (Lakomy *et al.*, 2011b). V posledním případě jsme k analýze miRNA využili vysokokapacitní technologii TaqMan Low Density Array od společnosti ThermoFisher Scientific založenou na platformě real-time PCR. Tato technologie je schopna analyzovat ve dvou reakčních bězích více než 700 miRNA. Analýzu jsme provedli u 58 vzorků primárních GBM. Následná korelace s klinickými daty vedla k identifikaci šesti miRNA (miR-31, miR-224, miR-432*, miR-454, miR-672 a miR-885-5p), jejichž expresní vzor byl významně asociován jak s časem do progresu onemocnění (poměr rizik (HR) 1,98, 95% konfidenční interval (CI) 1,33–2,94, $p < 0,001$), tak celkovým přežíváním (HR 2,86, 95% CI 1,91–4,29, $p < 0,001$). Rizikové skóre vypočtené na základě výše uvedených šesti miRNA bylo navíc zcela nezávislé na metylačním stavu promotoru pro MGMT a na dalších parametrech včetně věku, pohlaví, KPS a adjuvantního podání TMZ v monoterapii (Sana *et al.*, 2014).

Výsledky Lakomého a kol. jsou však v částečném rozporu s publikovaným pozorováním Ujifukua a kol. Tito autoři identifikovali miR-195 jako jednu ze tří nejvíce zvýšených miRNA u TMZ rezistentních buněk, přičemž dalšími dvěma byly miR-455-3p a miR-10a*. Navíc prokázali, že cíleným umlčením miR-195 došlo u zkoumaných buněčných linií k navýšení jejich senzitivity k TMZ (Ujifuku *et al.*, 2010). Podobně Guan a kol. popsali významnou korelaci mezi vysokou expresí miR-196a/b a horší prognózou jak u pacientů s GBM, tak s anaplastickým astrocytmem (Guan *et al.*, 2010). Nicméně je zajímavé, že v případě miR-195 a miR-196b byl pozorován jejich totožný vztah s prognózou, který popisoval právě Lakomý u GBM, také u kolorektálního, hepatocelulárního a adrenokortikálního karcinomu (Soon *et al.*, 2009; Xu *et al.*, 2009; Liu *et al.*, 2010; Wang *et al.*, 2012c). Bude tedy nutné počkat na další práce, které se hlouběji zaměří právě na tyto diskutabilní miRNA a verifikují jejich biologický význam, vztah k prognóze a případně jejich schopnost predikovat odpověď na léčbu u pacientů s GBM.

Ve zcela jiné práci hodnotili Wang a kol. schopnost miR-214 predikovat celkové přežívání u 108 pacientů s diagnostickým gliomem (WHO I – 18, WHO II – 12, WHO III – 32 a WHO IV – 46). Z výsledků následně vyplynulo, že celkové přežívání pacientů, jejichž

nádory disponovaly nižší hladinou miR-214, bylo významně kratší než u pacientů, u kterých byla zjištěna vysoká hladina této miRNA ($p < 0,001$). Navíc byla miR-214 významně snížena v nádorové tkáni oproti nenádorovým vzorkům mozkové tkáně ($p = 0,001$) (Wang *et al.*, 2014). Podobně jiné studie prokázaly, že miR-34a, miR-203, miR-326 a miR-375 byly redukovány v gliomech a jejich nižší exprese byla významně asociována jak s kratším časem do progresse onemocnění, tak s kratším celkovým přežíváním pacientů (Chang *et al.*, 2012; Gao *et al.*, 2013; He *et al.*, 2013; Wang *et al.*, 2013).

Naopak zvýšená exprese v tkáních gliomů (WHO I – 18, WHO II – 14, WHO III – 38 a WHO IV – 58) byla pozorována v případě miR-9 a miR-128, přičemž vyšší exprese miR-9 byla častěji pozorována u gliomů s vyšším stupněm malignity ($p = 0,001$), u pacientů s nízkým KPS (Karnofsky performance status; $p = 0,008$) a u pacientů s kratším celkovým přežíváním ($p < 0,001$). Multivariační Coxova regresní analýza následně prokázala, že hladina miR-9 je u pacientů s gliomy zcela nezávislým prognostickým znakem ($p = 0,01$). Je ovšem nutné zmínit, že vliv na celkové přežívání byl prokázán pouze ve skupině gliomů s vysokým stupněm malignity. U gliomů s nízkým stupněm malignity byl tento efekt nevýznamný (Wu *et al.*, 2013). Jiná studie sledovala zvýšenou expresi v gliomech (WHO I – 35, WHO II – 40, WHO III – 41 a WHO IV – 52) v porovnání se zdravou mozkovou tkání u miR-650 a miR-168, přičemž miR-650 byla podobně jako miR-9 asociována se stupněm malignity a KPS. U pacientů s vysokou expresí miR-650 byla rovněž pozorována významně horší prognóza (Sun *et al.*, 2013).

MiR-21 je nejčastěji popisovanou a studovanou onkogenní miRNA u většiny nádorových onemocnění. K hlubšímu pochopení její role právě v biologii gliomů byla pomocí *in situ* hybridizace analyzována její exprese u 193 FFPE (formalin-fixed paraffin-embedded) nádorových vzorků odebraných od pacientů s gliomy o různém stupni malignity. Výsledky následně ukázaly, že miR-21 je lokalizována v nádorových buňkách a s nádorem asociovaných cévách, zatímco její exprese nebyla pozorována ve zdravém mozkovém parenchymu. Současně byla exprese miR-21 v nádorových buňkách asociována s horší prognózou (Hermansen *et al.*, 2013). Podobně jako v případě právě zmiňované miR-21 byla i hladina miR-335 významně vyšší v nádorových vzorcích oproti nenádorovým ($p < 0,001$) a její vysoká hladina přímo koreluje s kratším celkovým přežíváním pacientů ($p = 0,01$), přičemž multivariační analýza navíc prokázala, že se jedná o nezávislý prognostický faktor ($p = 0,02$) (Jiang *et al.*, 2012). V konečném výčtu byly jako onkogenní, s negativním vlivem na prognózu pacientů, popsány u gliomů rovněž miR-17, miR-224 a miR-372 (Lu *et al.*, 2012; Li *et al.*, 2013; Lu *et al.*, 2013). Text převzat a upraven z Šána *et al.*, 2018.

Příspěvek k dané problematice

Najít nové a silné prognostické a prediktivní biomarkery časné progresy nádoru u pacientů s GBM bylo našim hlavním cílem v obou již zmiňovaných studiích zabývajících se miRNA u tohoto onemocnění. V naší první práci jsme pomocí Kaplan-Meierovy analýzy našli významnou asociaci hladin miR-195 a miR-196b s celkovým přežíváním pacientů, přičemž obě miRNA měly snížené hladiny u pacientů s horší prognózou. Naopak hladiny miR-181c a miR-21 byly u pacientů s nejhorší prognózou zvýšené. Společná exprese těchto dvou miRNA pak byla schopná identifikovat pacienty s kratším než šestiměsíčním časem do progresy s 92% senzitivitou a 81% specificitou, a to na hladině významnosti $p < 0,0001$. Nezávislost všech studovaných miRNA pak potvrdila analýza, která nenalezla asociaci jejich exprese s metylačním stavem promotoru pro MGMT (Lakomy et al., 2011b; Příloha 6).

V naší poslední studii jsme provedli vysokokapacitní analýzy exprese miRNA u 58 histologicky potvrzených primárních GBM a získaná data statisticky korelovala s klinickými parametry pacientů. Výsledkem byla sada šesti prognostických miRNA (miR-224, miR-432, miR-672, miR-31, miR-885-5p a miR-454), na základě kterých lze stanovit individuální rizikové skóre (RS), umožňující predikovat jak celkové přežívání, tak čas do progresy onemocnění. Pacienti rozdělení do skupin dle RS vykazovali mnohem významnější rozdíly jak v celkovém přežívání, tak v čase do progresy onemocnění, než tomu bylo u skupin pacientů rozdělených pomocí metylačního stavu promotoru pro MGMT. Multivariační Coxova regresní analýza současně odhalila, že námi vytvořené RS je nezávislé na ostatních sledovaných faktorech, včetně zmiňovaného metylačního stavu promotoru pro MGMT, podání adjuvantního TMZ v monoterapii, performance statusu a rozsahu resekce. Pro nezávislou validaci získaných výsledků jsme využili data z databáze TCGA (The Cancer Genome Atlas), která však obsahovala pouze čtyři z našich šesti objevených prognostických miRNA (miR-31, miR-224, miR-432* a miR-454-3p). RS sestavené pouze na základě těchto čtyř miRNA i přesto umožnilo signifikantně odlišit pacienty dle délky přežívání. Toto statistické hodnocení bylo provedeno Kaplan-Meierovou analýzou expresních dat u souboru 485 primárních GBM deponovaných v TCGA (Sana et al., 2014b; Příloha 7).*

Zatímco zájem o studium miRNA ve vztahu k nádorovým onemocněním v posledních letech exponenciálně stoupal a v mnoha případech jsou již detailně charakterizovány jejich expresní vzorce nejen u konkrétních typů nádorů, ale i u jejich různě maligních podskupin, expresní profily piRNA nejsou ve většině případů stále známé, natož ověřené nezávislými studiemi. Nicméně se zvyšujícím se povědomím o biologickém významu piRNA zájem o tyto molekuly mezi odborníky na nádorová onemocnění postupně narůstá.

Deregulované hladiny piRNA u nádorových onemocnění a jejich možný vztah k prognóze či schopnost predikovat odpověď na léčbu byly doposud publikovány pouze v omezeném množství prací. A ačkoliv se ve většině případů nejednalo o gliální nádory, je zde vhodné některé z těchto studií zmínit. Jednou z nejvíce prostudovaných piRNA u pacientů s nádory je piR-823. Snížená hladina této piRNA byla pozorována u nádorů žaludku a u renálního karcinomu, přičemž její nádorově supresorový efekt byl v prvně jmenovaném případě potvrzen jak na úrovni *in vitro*, tak *in vivo* (Cheng *et al.*, 2012; Iliev *et al.*, 2016). Ke zcela opačnému výsledku ovšem dospěla studie zabývající se expresí piR-823 u mnohočetného myelomu. V tomto případě byla exprese piR-823 zvýšená jak u pacientů postižených tímto onemocněním, tak ve stabilních buněčných liniích odvozených od tohoto typu nádoru. Zvýšené hladiny zároveň pozitivně korelovaly s klinickým stádiem onemocnění. Umlčení piR-823 pomocí syntetických oligonukleotidů vedlo k zástavě buněčného cyklu a k expresi proteinů indukujících apoptózu (Yan *et al.*, 2015; Li *et al.*, 2019). Je velmi zajímavé, že jednou z hlavních příčin rozvoje mnohočetného myelomu je hypermetylace DNA vedoucí k potlačení transkripce nádorově supresorových genů. Význam metylace DNA je popisován i u GBM, a to v souvislosti s promotorem pro MGMT. Onkogenní charakter pak vykazovala piR-823 ještě u karcinomu jícnu (Su *et al.*, 2020).

Co se týče výzkumu piRNA přímo u gliomů, první piRNA popsaná v GBM byla piR-8041. Její hladiny v nádorové tkáni jsou oproti kontrole sníženy a *in vitro* studie naznačily, že má silný antiproliferativní účinek a je zodpovědná za zástavu buněčného cyklu v kontrolním bodě G1/S. Jejími cíli jsou ERK1/2, jejichž aktivace je nutným krokem pro postup buňky z G1 do S fáze buněčného cyklu (Jacobs *et al.*, 2018). Nižší expresi oproti nenádorové tkáni vykazuje u gliomů rovněž piR-30188. Uměle navýšené hladiny této piRNA společně s vyšší expresí proteinu PIWIL3, miR-367-3p a sníženou hladinou lncRNA OIP5-AS1 vede u gliomových buněk k inhibici jejich růstu (Liu *et al.*, 2018). Z pohledu mozkových nádorů obecně může být zajímavá rovněž piR-DQ590027, jelikož její nadměrná exprese ovlivňuje

expresi ZO-1, okludinu a claudinu-5, což dále vede k vyšší propustnosti hematoencefalické bariéry (Leng *et al.*, 2018).

Nakonec v roce 2016 Jacobs a kol. provedli metaanalýzu na souboru dat získaných z dbGaP (Database of Genotypes and Phenotypes), který po pročištění od duplikovaných dat obsahoval celkem 2401 kontrolních vzorků a 1840 pacientů s gliomy. Z těchto pacientů bylo asi 67 % s diagnostikovaným high-grade gliomem, přičemž 82 % z nich tvořily GBM. Nejednalo se ovšem o analýzu exprese, nýbrž o identifikaci variant piRNA odlišujících se vzájemně na úrovni DNA přítomností různých jednonukleotidových polymorfizmů (single-nucleotide polymorphisms; SNPs), které by mohly být asociovány s rozvojem gliomů u dospělých pacientů. Statistická analýza odhalila významný vztah mezi rizikem vzniku gliomu a vzácnou variantou rs149336947 lokalizovanou v blízkosti 3' konce genu pro piR-2799 na chromozomu 2q33.1. PiR-2799 je 30 nukleotidů dlouhá molekula mapovaná v intronové oblasti apoptotického inhibitoru CFLAR, který je značně exprimován v lidském těle včetně mozku. Dále byly nalezeny o něco méně významné asociace s rizikem vzniku gliomů v případě rs62435800 v oblasti pro piR-18913 na chromozomu 6q27, rs147061479 u piR-598 na chromozomu 8q13.1, rs142742690 u piR-11714 na chromozomu 9q22.1 a rs35712968 u piR-3266 na chromozomu 10q24.2. PiR-18913, piR-598, piR-11714, a piR-3266 byly následně pozorovány v transkribované podobě u gliomových buněčných linií U87, A172, a NHA. Avšak exprese piR-2799 nebyla v těchto buňkách překvapivě potvrzena. Finální funkční analýza jedné ze čtyř výše zmíněných v buňkách exprimovaných piRNA, piR-598, odhalila, že transfekce divokého typu této piRNA ovlivnila v buněčných liniích expresi genů zapojených do apoptózy a redukovala jak viabilitu buněk, tak formování kolonií v *in vitro* podmínkách. Avšak po doručení piR-598 s alelovou variantou obsahující rs147061479 proliferace testovaných linií výrazně vzrostla (Jacobs *et al.*, 2016).

Studiem piRNA se zabýváme rovněž v naší výzkumné skupině. S cílem identifikovat piRNA asociované s GBM jsme provedli globální expresní profilování krátkých RNA s využitím sekvenování nové generace, a to celkem u 19 zamražených vzorků GBM a 11 nenádorových vzorků mozku získaných od pacientů s farmakorezistentní epilepsií. Bioinformatická analýza odhalila celkem 38 různých piRNA s více než 50 čteními u alespoň 50 % zařazených vzorků, které byly mezi oběma sledovanými skupinami vzorků signifikantně rozdílně exprimovány – 15 piRNA vykazovalo sníženou expresi, zatímco 23 piRNA bylo více exprimováno u GBM. Hladin významnosti nižších než 0,0001 pak dosahovalo 17 piRNA. Vybrané piRNA pak byly pomocí TaqMan RT-qPCR validovány na nezávislém souboru vzorků získaných od 77 pacientů s GBM a 23 dárců bez potvrzeného

nádorového onemocnění, přičemž výsledky byly úspěšně potvrzeny u piR-1849, piR-9491, piR-12487 a piR-12488 ($p < 0,0001$). Kromě toho byla piR-23231 významně asociována s OS pacientů s GBM léčených v režimu Stuppa ($p = 0,007$) (Bartos *et al.*, 2020, práce odeslána k recenznímu řízení). Výše uvedená pozorování tedy společně podporují předpoklad, že piRNA hrají důležitou roli v molekulárních procesech vedoucích ke vzniku a následné progresi gliomů. Text převzat a doplněn z Šána *et al.*, 2018.

Význam mikroRNA v chemoradiorezistenci gliomů

Poškození DNA způsobené ionizujícím zářením aktivuje signální dráhy spojené s reparací DNA, jejichž hlavním úkolem je nejprve pozastavit buněčný cyklus, a tak poskytnout buňce dostatek času k opravě poškozené genetické informace. Tímto se buňka může úspěšně vyhnout apoptóze, kterou by musela nepochybně podstoupit, pokud by nedošlo k těmto opravám. Jako molekulární odpověď buněk na ionizující záření, a tedy i radioterapii, jsou nejčastěji popisovány signální dráhy PI3K/AKT (Lee *et al.*, 2011) a ATM (ataxia telangiectasia mutated)/Chk2 (checkpoint kinase 2)/p53 (Squatrito *et al.*, 2010).

Kromě radioterapie je důležitou součástí onkologické léčby rovněž chemoterapie. V případě léčby GBM je nejčastěji používaným cytostatikem alkylační činidlo TMZ. Nicméně buňky GBM velmi často disponují mechanismy, které tento efekt inhibují. V souvislosti s reparací poškození DNA způsobených TMZ je u GBM nejčastěji diskutován enzym MGMT. Jiným mechanismem chránícím buňky před působením cytostatik je jejich vylučování z intracelulárního prostoru pomocí ATPázových pump, které jsou zodpovědné za tzv. mnohočetnou lékovou rezistenci. Mnoho experimentálních prací ukázalo, že do regulace všech těchto procesů jsou zapojené i miRNA, které tak hrají významnou roli v chemoradiorezistenci GBM (Besse *et al.*, 2013).

Příspěvek k dané problematice

*Vliv miRNA na chemoradiorezistenci gliomů jsme přehledně zpracovali v publikaci „MicroRNAs involved in chemo- and radioresistance of high-grade gliomas“ uveřejněné v roce 2013 v časopise Tumor Biology (Besse *et al.*, 2013; Příloha 13). S přípravou této publikace byla v naší laboratoři současně zahájena studie, jejímž cílem bylo odhalit nové, doposud neopublikované miRNA zapojené v regulaci radiorezistence GBM a přispět tak k hlubším poznáním této, v léčbě GBM zcela klíčové problematiky. Výsledkem pak byly dvě*

původní práce opublikované v zahraničních časopisech s impakt faktorem zabývající se vlivem miRNA na radiorezistenci GBM buněk. Obě práce jsou uvedeny v následující podkapitole.

MikroRNA jsou zapojené do radiorezistence glioblastomu

Signální dráha PI3K/AKT hraje velmi významnou roli v buněčné rezistenci k ionizujícímu záření a bývá velmi často deregulována u GBM. Inaktivace této dráhy citelně narušuje mechanismus opravy DNA, což může v konečném důsledku vést ke zvýšené citlivosti k radioterapii. Několikrát již bylo demonstrováno, že miR-21 aktivuje tuto signalizaci prostřednictvím přímé suprese PI3K/AKT inhibitoru PTEN (Chakravarti *et al.*, 2004; Kao *et al.*, 2007; Gwak *et al.*, 2012). Snížení intracelulární hladiny miR-21 se manifestuje zvýšenou expresí PTEN, aktivací signální dráhy PI3K/AKT a v konečném důsledku blokadí γ -H2AX (H2A histone family, member X, gamma), který rozpoznává dvouřetězcové zlomy na DNA a aktivuje jejich reparaci. Zdá se tedy, že miR-21, která stojí na samém začátku signální dráhy a jejíž snížení vede ke zvýšené citlivosti k ionizujícímu záření, je také slibným terapeutickým cílem k překonání radiorezistence GBM (Gwak *et al.*, 2012). Další v tomto ohledu terapeuticky významnou molekulou je miR-7. Podobně jako miR-21 je i miR-7 zapojena do regulace signální dráhy PI3K/AKT, avšak její účinek je naopak nádorově supresivní. Zvýšené hladiny miR-7 u buněčných linií U251 a U87MG vedly v obou případech ke zvýšené citlivosti k radiaci, a to pravděpodobně skrze sníženou aktivitu EGFR, a tedy i AKT (Kefas *et al.*, 2008; Lee *et al.*, 2011). Další miRNA popisovaná v souvislosti s radioterapií GBM je miR-181. Hladina miR-181 bývá úměrně asociována s obdrženou dávkou radiace, přičemž její vysoká exprese vede, podobně jako u miR-7, ke zvýšené citlivosti k radiaci. Jako příčina tohoto efektu se zdá být přímý regulační vztah mezi miR-7 a anti-apoptotickým genem BCL-2 (Chen *et al.*, 2010).

Signální dráha ATM/Chk2/p53 je úzce spjata s buněčnými procesy jako jsou apoptóza a buněčný cyklus (Cao *et al.*, 2006). Význam ATM v radiorezistenci byl demonstrován na dvou buněčných liniích GBM, které se liší v jeho expresi. Bylo zjištěno, že miR-100 je odlišně exprimována oběma zmíněnými liniemi, což naznačuje její vztah s expresí ATM. Počítačové modelování navíc predikuje miR-100 jako přímý regulátor ATM (Ng *et al.*, 2010). Po ozáření buněčné linie s nižší hladinou ATM dochází k indukci miR-15a, miR-16, miR-21, miR-143 a miR-155, což naznačuje jejich zapojení do procesu buněčné odpovědi na ionizující záření (Chaudhry *et al.*, 2010b). Toto zapojení pak ve svých pracích na jiných

typech nádorových onemocnění potvrdily i další autoři (Chaudhry *et al.*, 2010a; Babar *et al.*, 2011; Lin *et al.*, 2011). Geny ATM a DNA-PK byly rovněž potvrzeny jako přímý cíl miR-101. Tato miRNA senzitivuje buňky U87MG k radiaci, což bylo potvrzeno jak v podmínkách *in vitro*, tak i na zvířecím modelu (Yan *et al.*, 2010).

Příspěvek k dané problematice

*Na základě porovnání tkáně GBM a tkáně temporálních laloků resekovaných u pacientů s epilepsií, které nevykazovaly žádné známky dysplastických změn, jsme odhalili signifikantní snížení exprese jak miR-338-5p, tak jejího vlásenkového partnera miR-338-3p v tkáni GBM. Tato skutečnost naznačovala, že by obě tyto miRNA, jež podléhají stejné regulaci transkripce pri-miRNA, mohly hrát roli v biologii GBM. Navíc miR-338-3p byla již dříve popsána jako miRNA specificky se exprimující v mozkové tkáni zapojená do procesů proliferace a diferenciac. Z těchto důvodů jsme se rozhodli provést v případě obou molekul *in vitro* navýšení jejich exprese a sledovat dopad této změny na jejich biologické a funkční vlastnosti. Zatímco navýšení hladin miR-338-3p se neprojevovalo žádným efektem, v případě miR-338-5p došlo k výraznému snížení proliferace u všech studovaných buněčných linií, tj. A172, T98G a U87MG. Další *in vitro* analýzy naznačily, že snížení proliferace je pravděpodobně způsobeno zástavou buněčného cyklu. Současně nebyl pozorován žádný efekt na apoptózu. Jelikož jedna z nejvíce účinných léčebných modalit GBM je radioterapie, rozhodli jsme se buňky jak s uměle navýšenou, tak endogenní hladinou miR-338-5p vystavit právě ionizujícímu záření. Po tomto ovlivnění jsme rovněž pozorovali rozdíly v proliferaci a zástavu buněčného cyklu u všech třech sledovaných linií s navýšenými hladinami miR-338-5p, avšak změna v těchto sledovaných parametrech byla mnohem významnější než v případě neozářených buněk. Navíc jsme u všech třech ozářených a současně miR-338-5p transfekovaných linií pozorovali signifikantní zvýšení počtu apoptotických buněk. Na závěr studie bylo pomocí celogenomového expresního profilování provedeno porovnání transkriptomu buněk s navýšenou a nenavýšenou hladinou miR-338-5p s cílem identifikovat mRNA regulované touto miRNA. Analýza transkriptomů získaných ze všech tří studovaných buněčných linií umožnila identifikovat deset genů s vysoce signifikantním rozdílem v expresi mezi skupinami. Tři z těchto genů, NDFIP1, RHEB a PPP2R5a, jsou úzce spojeny s genomovou nestabilitou a odpovědí buňky na poškození DNA. Pomocí těchto genů by vliv miR-338-5p na radiorezistenci GBM mohl být modulován skrze PTEN, který je známým inhibítorem dráhy PI3K/AKT, ale je zapojen také do transkripce genů E2F1 a RAD51. MiR-*

338-5p se proto jeví jako slibný terapeutický cíl umožňující zvýšit senzitivitu k radiaci a tak zlepšit celkovou prognózu pacientů s GBM (Besse *et al.*, 2016; Příloha 14).

V roce 2017 jsme pak uveřejnili výsledky druhé části studie, v rámci které jsme dlouhodobou expozicí ionizujícímu záření připravili radiorezistentní GBM buněčné linie T98G-R, U87MG-R a U251-R. Jak v radiorezistentních, tak v rodičovských buněčných liniích jsme provedli globální profilování exprese miRNA a identifikovaly tak 113 miRNA s významně odlišnou expresí mezi oběma skupinami; z toho 73 miRNA bylo zvýšeno a 40 miRNA naopak sníženo v radiorezistentních liniích. Správnost našeho pozorování podpořila skutečnost, že některé z těchto miRNA byly již dříve popsány ve vztahu právě k ionizujícímu záření. Věříme, že tyto miRNA představují potenciálně prediktivní biomarkery nebo terapeutické cíle v GBM (Ondracek *et al.* 2017; Příloha 15).

MikroRNA jsou zapojené do chemorezistence glioblastomu

TMZ je cytotoxické pro-léčivo, které se hydrolyzou stává účinným metylačním činidlem vedoucím k inhibici replikace DNA. Nicméně po dlouhodobějším působení TMZ dochází u GBM ke vzniku rezistence. Jedním z velmi diskutovaných mechanismů, pomocí kterého získává GBM rezistenci k TMZ, je zvýšená hladina reparačního proteinu MGMT. MGMT je DNA alkyltransferáza schopná opravovat mutagenní poškození na DNA způsobené TMZ metylujícím guanin na pozici O6. Tato poškození za normálních okolností zapříčiňují špatné párování guaninu s thyminem, a tedy změnu genetické informace, destabilizaci genomu a apoptózu. Nicméně vysoké hladiny MGMT vedou k demetylaci guaninu, a tak umožňují buňce vyhnout se apoptóze (Sharma *et al.*, 2009; Carmo *et al.*, 2011; Besse *et al.*, 2013).

MiRNA mají schopnost regulovat MGMT, ale také například anti-apoptotický protein BCL-2. V případě obou těchto proteinů byla pozorována negativní korelace jejich hladin s expresí miRNA rodiny miR-181. Navíc *in vitro* umlčení této rodiny vedlo k signifikantnímu navýšení expresní hladiny MGMT. Tato skutečnost naznačuje, že by rodina miR-181 mohla být jak prediktivním biomarkerem odpovědi na léčbu TMZ, tak potenciálním terapeutickým cílem zvyšujícím účinnost TMZ (Chen *et al.*, 2010; Zhang *et al.*, 2012).

Opakovaně byl prokázán vztah mezi rezistencí k TMZ a signálními drahami PI3K/AKT a RAS/MAPK. Tato pozorování byla provedena například na buněčné linii U118 derivované z tkáně gliomu. Zmíněná buněčná linie disponuje značnou rezistencí k TMZ, nicméně po inhibici výše uvedených signálních drah došlo k významnému poklesu rezistence (Carmo *et al.*, 2011). Podobně jako v případě radiorezistence, i rezistence GBM buněk k TMZ může

být částečně zprostředkována skrze přímou inhibici PTEN pomocí miR-21 a tedy aktivací signální dráhy PI3K/AKT. Umělé snížení hladiny miR-21 se projevilo inhibicí proliferace, zvýšenou apoptózou a zástavou buněčného cyklu, což bylo způsobeno snížením BAX (BCL2-associated X protein) / BCL-2 poměru a snížením aktivity kaspázy 3 (Ren et al., 2010a; Shi et al., 2010). Význam miR-21 byl rovněž zkoumán u buněk vystavených působení cytostatika 5-fluorouracilu, u kterých tato miRNA potencovala apoptózu a snižovala migrační potenciál (Ren *et al.*, 2010).

Mezi miRNA regulující apoptotické faktory jako jsou BAX, cytochrom c a kaspáza 3 patří také miR-221/222. Jejich snížení zvyšovalo nezávisle na proteinu p53 senzitivitu k TMZ (Chen *et al.*, 2012). V jiné studii zase bylo zjištěno, že miR-195, miR-455-3p a miR-10a* vykazují shodně zvýšenou hladinu u buněk rezistentních k TMZ. Zvýšená hladina miR-195 v kombinaci s TMZ navíc silně potencovala indukci apoptózy (Ujifuku *et al.*, 2010).

Za chemorezistenci není ovšem zodpovědná pouze signalizace PI3K/AKT, ale velice důležité jsou v tomto směru rovněž proteiny patřící do rodiny ABC transportérů, které přispívají k mnohočetné lékové rezistenci. Jedna z mnoha prací zabývajících se možnostmi překlenutí této rezistence ukázala, že zvýšená hladina miR-328 vede ke snížené expresi ABCG2 a může být tedy, jak se zdá, rovněž zapojená v chemorezistenci GBM (Li *et al.*, 2010b).

Význam mikroRNA u glioblastomových kmenových buněk

Regulační efekt miRNA se uplatňuje ve většině důležitých buněčných procesech jako je proliferace, diferenciace, apoptóza, buněčný cyklus a stejně tak udržování kmenových vlastností buněk (Jansson a Lund, 2012). Tyto molekuly jsou tedy zapojeny i do biologie GSCs, což z nich činí ohnisko zájmu mnoha výzkumných kolektivů. Cílená regulace miRNA, které jsou zapojeny do řízení kmenových vlastností GSCs, by totiž mohla být využita jako nový efektní přístup léčby nádorových chorob, včetně GBM (Liu a Tang, 2011). Tato strategie však v sobě skrývá mnohá úskalí. GSCs jsou velmi blízké normálním neurálním kmenovým buňkám (NSCs) a mechanismy, udržující jejich kmenovost, jsou často stejné či podobné u obou těchto buněčných populací. Důležité tedy je, aby léčba zaměřující se na GSCs cílila opravdu jenom tyto buňky. Lang a kol. proto za použití metody hlubokého sekvenování identifikovali miRNA, které jsou rozdílně exprimované mezi těmito buněčnými populacemi, a tím získali set potenciálních terapeutických cílů pro bezpečnou cílenou léčbu, která by mířila pouze na GSCs a nikoli na NSCs. Signifikantně zvýšenou

hladinu v GSCs vykazovaly miR-10a, miR-10b a miR-140-5p. Naopak snížená exprese byla detekována u miR-874 a miR-124. Jako přímý cíl miR-10a a miR-10b byl identifikován nádorový supresor CSMD1 (CUB and SUSHI multiple domain protein 1), jehož ztráta nebo snížená hladina byla zaznamenána i u jiných typů nádorů (Kamal *et al.*, 2010; Lang *et al.*, 2012). Tento transmembránový protein je podle některých studií zapojen do signalizace TGF- β , jež patří mezi hlavní regulátory kmenovosti a rovněž se podílí na mechanismech buněčného růstu a apoptózy (Tang *et al.*, 2012; Sakaki-Yumoto *et al.*, 2013). Regulační vliv této miRNA byl objeven i v případě dalších genů zapojených v nádorové transformaci jako je PTEN, STAT3, SDC-1, TIAM1, NF-1. Inhibice miR-10b v GSC a GBM buňkách měla za následek výrazné snížení proliferace, migrace, invazivity a buněčného růstu, a to zejména v případě GSC (Guessous *et al.*, 2013).

Mezi přímé cíle nádorově supresorové miR-124 pak patří jednak známý onkogen NRAS, jenž je zapojen do procesu proliferace, diferenciaci a celkového přežívání buněk, a dále serin/treonin kináza PIM3 modulující buněčný cyklus (Lang *et al.*, 2012). MiR-124 byla rovněž identifikována jako cíl transkripčního faktoru REST, jednoho z hlavních represorů neurální diferenciaci. Mezi její další cílové molekuly totiž patří fosfatáza SCP-1, která výrazně potlačuje neurální diferenciaci (Visvanathan *et al.*, 2007; Conti *et al.*, 2012). Silber a kol. rovněž potvrdili zapojení miR-124 spolu s miR-137 do procesu diferenciaci, a to jak v NSCs, tak i v mozkových nádorových kmenových buňkách. Obě tyto miRNA ovlivňují proliferaci GBM buněčných linií prostřednictvím přímé inhibice cyklin dependentní kinázy 6 (CDK6), která se skrze fosforylaci proteinu Rb podílí na řízení buněčného cyklu (Silber *et al.*, 2008). Bylo rovněž zjištěno, že supresorová miR-137 vykazuje v GBM z důvodu hypermetylace svého promotoru sníženou hladinu exprese. Exprese této molekuly narůstá spolu s mírou diferenciaci NSCs i GSCs. Po transfekci pre-miR-137 do GSCs klesá schopnost sebeobnovy, tvoření neurosfér a rovněž exprese markerů kmenových buněk Oct4, Nanog, Sox2 a Shh. MiR-137 reguluje kmenové vlastnosti GSCs skrze svůj přímý cíl RTVP-1, jehož exprese přímo koreluje se stupněm malignity astrocytomů. RTVP-1 zvyšuje expresi CXCR-4, který je zapojen v signalizaci SHH-GLI-Nanog, a tím přispívá ke zvýšení schopnosti sebeobnovy buněk (Bier *et al.*, 2013). Tumorigenní a invazivní marker CXCR-4 je přímým cílem klastru miR-302-367, proto se i tato skupina miRNA podílí na inhibici sebeobnovy, invazivity a infiltrace glioblastom iniciujících buněk, a to rovněž přes signalizaci SHH-GLI-Nanog, ve které, jak už bylo uvedeno, je CXCR-4 zapojen. Po zvýšení hladiny miR-302-367 v GSCs, došlo k poklesu schopnosti tvořit neurosféry a naopak ke zvýšení exprese astrocytárního markeru GFAP, což dokládá zapojení tohoto klastru i do

procesu diferenciaci (Fareh *et al.*, 2012). Další miRNA zapojená do procesu diferenciaci je miR-128. Po jejím zvýšení dochází k indukci diferenciaci, a tudíž poklesu markerů kmenovosti jako je Nestin nebo Sox2. Jejimi přímými cíli jsou známé mitogenní tyrozinkinázy EGFR a PDGFR α , u nichž byl už v předešlých studiích prokázán inhibiční vliv na neurální diferenciaci (Boockvar *et al.*, 2003; Jackson *et al.*, 2006; Papagiannakopoulos *et al.*, 2012). Výzkumný tým Aldaz a kol. se zaměřil na identifikaci molekul miRNA, jejichž exprese se během diferenciaci GSCs mění, tudíž se dá předpokládat jejich přímé zapojení do tohoto procesu. Míra exprese miR-93 a miR-106 se během diferenciaci snižovala, naopak u miR-21, miR-29a, miR-29b, miR-221 a miR-222 se zvyšovala. Inhibice miR-221/222 v diferencujících GSCs měla za následek nárůst exprese nestinu, ale pokles astrocytárních (GFAP) a neurálních markerů (TUJ1). Tento trend nebyl pozorován v případě miR-29a/b. Jejich zvýšení v GSCs nevyvolalo žádné změny v expresi markerů kmenovosti ani v diferenciaci, ale vedlo k prokazatelnému snížení viability a zvýšení apoptózy. MiR-29a/b jsou do procesu apoptózy zapojeny skrze svůj přímý cíl, anti-apoptotický protein MCL1, patřící do rodiny apoptotických regulátorů BCL2. Překvapením studie bylo zjištění výrazné prodiferenční role miR-21, mnohými studiemi postulovanou jako miRNA se silně onkogenními vlastnostmi. Po zvýšení této miRNA v GSCs opět došlo ke snížení exprese nestinu a nárůstu TUJ1 a GFAP. Jejím přímým cílem byl určen SPRY1, který byl již dříve popsán jako inhibitor neurální diferenciaci v myších embryonálních kmenových buňkách. (Aldaz *et al.*, 2013). MiR-221/222 i miR-21 jsou všeobecně známé pro své onkogenní vlastnosti (Schramedei *et al.*, 2011; Li *et al.*, 2014). Ve výše popsané studii však autoři polemizují, že pokud by léčba cílila na tyto miRNA, neměla by požadovaný efekt zabraňující vzniku recidiv právě kvůli zapojení těchto miRNA do procesu diferenciaci (Aldaz *et al.*, 2013). Schraivogel a kol. se zaměřili na rozdíly v expresním profilu miRNA mezi CD133 pozitivními a negativními buňkami. CD133, jak již bylo uvedeno, je jedním z nejpoužívanějších markerů pro identifikaci a izolaci GSCs. MiR-9/9*, miR-15b, miR-17-5p, miR-106 vykazovaly nejvyšší expresi u CD133 pozitivních buněk, miR-221/222, miR-27, miR-21 zase u CD133 negativních buněk. Cílena inhibice miR-9/9* a miR-17-5p vedla k redukci CD133 pozitivních buněk a jejich schopnosti tvořit neurosféry. Jako možný cíl těchto dvou miRNA byl identifikován transkripční faktor CAMTA1, který indukuje expresi krátkého secernovaného proteinu NPPA a jeho receptoru, jež se podílejí na inhibici proliferace (Schraivogel *et al.*, 2011). Jiná výzkumná skupina zase uvedla, že k nejvíce sníženým miRNA v CD133 pozitivních oproti CD133 negativním buňkám patří miR-125b, jež je zapojena do řízení proliferace. Jejím přímým cílem je totiž

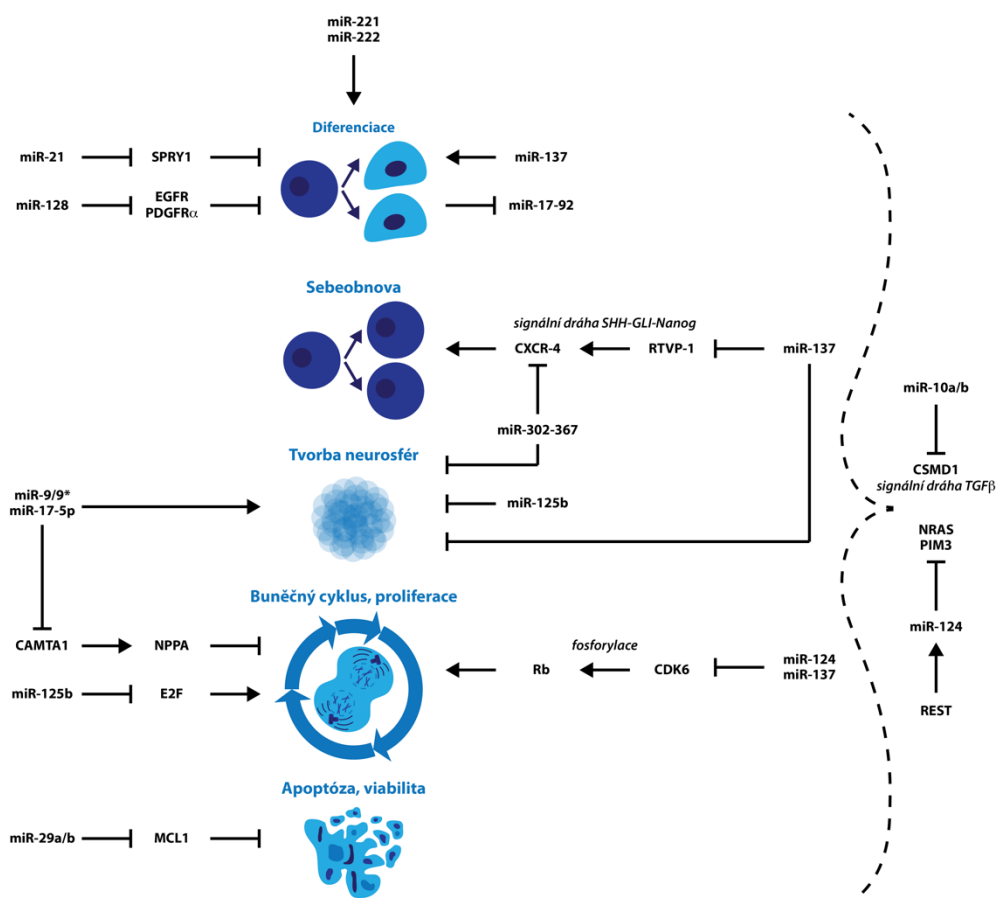
člen rodiny E2F, což jsou významní regulátoři buněčného cyklu. Po zvýšení její hladiny v CD133 pozitivních buňkách došlo k represi exprese Nestinu a CD133 a schopnosti těchto buněk tvořit neurosféry (Wu *et al.*, 2012). Exprese miR-17-92 se během diferenciaci snižuje, což dokládá jejich význam v udržování kmenových vlastností GSCs. Tento klastr je zapojen v řízení proliferace a apoptózy skrze své cíle CDKN1A, E2F1 a PTEN. Jako přímý cíl této molekuly byl rovněž určen růstový faktor pojivové tkáně (CTFG), který váže VEGFA (vaskulární endoteliální růstový faktor) a tím přispívá k inhibici nádorové angiogeneze (Ernst *et al.*, 2010). Zapojení miRNA v regulaci biologických vlastností u GSCs je shrnuto na Obrázku 5. Text převzat a upraven z Kleinova *et al.*, 2015.

Příspěvek k dané problematice

*Problematiku významu miRNA u GSC jsme v roce 2015 shrnuli v přehledovém článku v českém recenzovaném časopise, kde jsme současně postulovali, že na základě dostupných poznatku se GSC zdají být skutečně jednou z příčin biologicky nepříznivého chování tumoru a jsou tedy nadějným terapeutickým cílem. Jejich cílené ovlivnění prostřednictvím miRNA, přirozených regulátorů genové exprese, by pak mohlo vést ke zvýšení senzitivity GBM k adjuvantní terapii, významnému prodloužení času do progresu onemocnění, a tedy i zlepšení celkové prognózy pacientů (Kleinová *et al.*, 2015; Příloha 16).*

*V roce 2018 jsme pak uveřejnili výsledky studie, která měla za cíl především odhalit panel miRNA specificky deregulovaných právě u GSC. Pomocí globální analýzy exprese miRNA u deseti párovaných in vitro i in vivo charakterizovaných primárních kultur GSC a GBM buněk postrádajících kmenové vlastnosti (non-GSC) jsme identifikovali miRNA spojené s kmenovým fenotypem. 51 nejvíce deregulovaných miRNA bylo schopno klasifikovat buněčné kultury do dvou hlavních klastrů – GSC a non-GSC a zároveň identifikovalo podskupinu GSC kultur s výraznějšími charakteristikami kmenových buněk. Rizikové skóre založené na expresi 7 vybraných v GSC zvýšených miRNA pak bylo schopno predikovat OS u pacientů s GBM z databáze TCGA, a to nezávisle na mutačním statusu genu IDH1. Tato práce tak potvrdila významnou úlohu miRNA v biologii GSC a vybrané miRNA mohou představovat užitečné prognostické markery a potenciální terapeutické cíle (Sana *et al.*, 2018; Příloha 17).*

Obrázek 5 Zapojení mikroRNA v regulaci biologických vlastností glioblastomových kmenových buněk (převzato z Kleinova *et al.*, 2015)



MikroRNA u meningeomů

V porovnání s gliomy není význam miRNA u meningeomů zdaleka prozkoumán. Přesto se však v posledních letech ruku v ruce s masivním rozšířením pokročilých vysokokapacitních technologií pro studium transkriptomu objevilo několik prací zabývajících se studiem globálních expresních profilů miRNA právě u tohoto onemocnění. Zmíněné studie přinesly především řadu důležitých poznatků o zapojení miRNA v biologii nádorů a jejich potenciální využití v diagnostické praxi, případně jako terapeutických cílů (Wang *et al.*, 2020). Právě identifikace rozdílné exprese miRNA mezi nádorovými vzorky a normální kontrolní tkání je běžným přístupem ke studiu mechanismů tumorigeneze. El-Gewely a kol. identifikovali 6 rozdílně exprimovaných miRNA mezi meningeomy stupně malignity WHO I a II a durální kontrolou z nichž miR-218 a miR-24a vykazovaly zvýšenou expresi, zatímco miR-143, miR-193b, miR-451 a miR-21 byly sníženy ve tkáni nádoru (El-Gewely *et al.*, 2016). Porovnáním exprese miRNA u meningeomů s rozdílnými stupni malignity byla

odhalena sada 4 miRNA (miR-222, miR-34a-3p, miR-136 a miR-497) vzájemně odlišující stupně WHO I a WHO II (Ludwig *et al.*, 2015). Tyto rozdílně exprimované miRNA byly již dříve spojeny s proliferací a přežíváním u mnoha různých nádorů, což naznačuje, že by mohly být slibnými biomarkery procesů vzniku a progresu meningeomů.

Zhi a kol. naznačili, že efektivními a současně lehce dostupnými molekulami pro diagnostiku a monitorování meningeomů by mohly být podobně jako u jiných nádorových onemocnění cirkulující miRNA v krevním séru nebo plazmě pacientů. Tito autoři konkrétně analyzovali miRNA v krevním séru 210 pacientů s meningeomem a současně 210 zdravých dárců bez histopatologicky potvrzeného jakéhokoliv nádorového onemocnění a odhalili panel 6 miRNA, ve kterém 219-5p, miR-409-3p, miR-106a-5p a miR-409-3p byly signifikantně zvýšené u pacientů s meningeomem, zatímco miR-224 a miR-197 vykazovaly vyšší hladiny u zdravých dárců. Stojí za zmínku, že čtyři u pacientů s meningeomem zvýšené miRNA byly významně sníženy, zatímco zbývající dvě miRNA byly významně zvýšeny po chirurgickém odstranění nádoru (Zhi *et al.*, 2016).

V procesech vzniku a postupného vývoje meningeomů bylo popsáno několik deregulovaných miRNA jak onkogenního, tak nádorově supresorového charakteru. Vůbec nejznámější onkogenní miRNA napříč nádory je miR-21, která inhibuje expresi mnoha nádorových supresorů. Její význam v biologii meningeomů naznačilo i několik prací, ve kterých figurovala jako nejčastěji deregulovaná miRNA jednak mezi tkání nádoru a normální tkání a současně mezi meningeomy s různými stupni malignity (Galani *et al.*, 2015; Ludwig *et al.*, 2015; El-Gewely *et al.*, 2016; Katar *et al.*, 2017). MiR-21 je exprimována v nízkých hladinách u meningeomů WHO I, a naopak jsou její hladiny signifikantně zvýšeny u meningeomů WHO II a III stejně jako např. u glioblastomu (Wang *et al.*, 2012b; Galani *et al.*, 2015; Ludwig *et al.*, 2015; Katar *et al.*, 2017). Navíc již bylo u různých nádorů potvrzeno mnoho přímých cílů miR-21 zapojených značnou měrou do onkogeneze a vývoje maligních forem onemocnění, a to včetně PDCD4 (programmed cell death factor 4), PTEN (phosphatase and tensin homolog) a BTG2 (B-cell translocation gene 2) (Murugaiyan *et al.*, 2015). Ve vztahu k meningeomu bylo prokázáno, že miR-21 podporovala jeho růst represí aktivačních inhibitorů AKT včetně BTG2 (B-cell translocation gene 2) a PTEN (Wang *et al.*, 2020). PTEN je nádorový supresor, který inhibuje signální dráhu AKT prostřednictvím antagonizace PI3K redukcí PIP3 na PIP2 (Jiang a Liu, 2008). Exprese jak miR-21, tak jeho přímého cíle PTEN byla významně rozdílná mezi benigními a maligními meningeomy; vysoká exprese miR-21 negativně korelovala s hladinou PTEN, což se projevilo ve stimulaci proliferace (Zhi *et al.*, 2013; Galani *et al.*, 2017).

Další onkogenní miRNA hrající významnou roli u meningeomu je miR-224, přičemž vyšší exprese miR-224 byla pozorována jak v nádorové tkáni oproti normálním vzorkům mozku, tak u maligních meningeomů WHO III v porovnání se stupněm malignity WHO I (Ludwig *et al.*, 2015; Wang *et al.*, 2015). Funkční studie současně popsaly pozitivní korelaci mezi nízkou expresí miR-224 a pomalou buněčnou proliferací, a naopak zvýšenou apoptotickou aktivitou (Wang *et al.*, 2015). Navíc meningeomy s nízkou expresí miR-224 byly asociovány se signifikantně lepší prognózou a pacienti vykazovali jak delší celkové přežívání, tak delší čas bez rekurence tumoru po chirurgické intervenci (Wang *et al.*, 2015). Stojí rovněž za zmínku, že hladiny miR-224 v krevním séru vykazují zcela opačný trend v porovnání s expresí miR-224 v nádorové tkáni; u pacientů s meningeomy byly pozorovány nižší hladiny miR-224 a po odstranění nádoru jejich hladiny v krevním séru významně vzrostly (Zhi *et al.*, 2016). Zvýšená exprese ve tkáni meningeomů všech stupňů malignity byla pozorována ještě v případě miR-335 a její vysoká exprese vedla ke zvýšení buněčné proliferace a inhibici G0 kontrolního bodu buněčného cyklu skrze přímou inaktivaci signální dráhy Rb1 (Shi *et al.*, 2012).

Oproti zmiňovaným onkogenním miRNA byly miR-34a a miR-218, u jiných nádorů známé nádorově supresorové miRNA, signifikantně zvýšené v meningeomech WHO I a II a naopak snižené v meningeomech WHO III v porovnání s normální durální tkání (Zhi *et al.*, 2013; Ludwig *et al.*, 2015). Zvýšená exprese miR-34a-3p vedla v podmínkách *in vitro* u buněk derivovaných z meningeomu k inhibici buněčné proliferace a indukci apoptózy skrze snížení proteinů SMAD4, FRAT1 a BCL2 (Hu *et al.*, 2019; Wang *et al.*, 2020). Podobně zvýšená exprese miR-218 vedla ke zpomalení buněčné proliferace, invazivity, schopnosti tvořit kolonie a formovat nádorové sféry skrze přímé cílení transkriptu receptoru pro IL-6 a JAK3 (Yang *et al.*, 2017). Někteří autoři proto naznačují, že miR-218 může hrát důležitou roli ve zvratu benigního meningeomu na maligní formu (El-Gewely *et al.*, 2016).

Jiná studie dále prokázala, že miR-200a, člen rodiny miR-200 a známý regulátor EMT, inhibuje růst buněk meningeomu skrze cílenou regulaci mRNA transkriptu pro β -katenin a blokuje tak Wnt/ β -kateninovou signalizaci (Saydam *et al.*, 2009). Navíc zvýšená exprese miR-200a vedla k inhibici invazivity maligních buněk meningeomu cíleným umlčováním transkriptu NMMHC-B (non-muscle myosin heavy chain IIb) (Senol *et al.*, 2015). Poslední miRNA s nádorově supresorovou funkcí popsána doposud u meningeomu je miR-145, jejíž nízká exprese je popsána i mnoha dalších nádorů. U meningeomů je její exprese signifikantně snižená u maligních forem onemocnění WHOII/III a její umělé navýšení v *in vitro* podmínkách vede k redukci buněčné proliferace a migrace (Kliese *et al.*, 2013).

Příspěvek k dané problematice

V práci „Cerebrospinal Fluid MicroRNA Signatures as Diagnostic Biomarkers in Brain Tumors“ publikované v roce 2019 v časopise Cancers jsme mimo jiné analyzovali hladiny miRNA v CSF odebraného od pacientů s meningeomem a dále od pacientů s gliomy, mozkovými metastázami a kontrolními vzorky CSF získanými v rámci standardní terapeutické péče od pacientů s normotenzním hydrocefalem bez potvrzeného onkologického onemocnění. Globální kvantifikace miRNA pomocí technologie sekvenování nové generace u vzorků CSF od pacientů s meningeomem (n = 11) a kontrolních vzorků (n = 19) odhalila celkem 12 miRNA s odlišnými hladinami (miR-196a-5p, miR-10a-5p, miR-549a, miR-196b-5p, miR-199b-3p, miR-101-3p, miR-152-3p, miR-10a-3p, miR-148a-3p, miR-140-5p, miR-1247-5p a miR-205-5p; $p_{adj} < 0,01$). Následná validace na nezávislém souboru CSF vzorků od pacientů s meningeomem (n = 44) a kontrolních vzorků (n = 21) potvrdila předešlé výsledky pouze u miR-140-5p a miR-196b-5p. Porovnání s ostatními nádory navíc ukázalo, že let-7b, miR-10a-5p a miR-10b-5p jsou rozdílně exprimovány v CSF mezi skupinou meningeomů a GBM a LGG, ale neliší se od mozkových metastáz. Na druhou stranu rozdíly hladin mezi meningeomy a mozkovými metastázami byly pozorovány u miR-21-3p a miR-196b. Výsledky tedy jasně naznačují, že cirkulující miRNA v CSF jsou nadějnými biomarkery pro diagnostiku meningeomů (Kopkova et al., 2019; Příloha 12).

MikroRNA u mozkových metastáz

Význam sncRNA u mozkových metastáz je obecně studován ze dvou základních úhlů pohledu. Prvním z nich je role těchto molekul ve vícestupňovém procesu diseminace primárního nádoru do mozku a druhým je pak jejich přítomnost a úloha v biologii vlastní metastázy. Zaměříme-li se nyní na proces metastazování, významnou roli zde bezesporu hraje epitelo-mezenchymální tranzice (EMT). Tento buněčný program vede ke změně epiteliálního fenotypu buňky v mezenchymální typ. Nádorové buňky tak získávají větší mechanickou odolnost, nezávislost na bazální membráně, motilitu a v neposlední řadě i odolnost k protinádorové léčbě. Takto pozměněné buňky aktivně vycestovávají do krevního oběhu, kterým jsou pak bez nebezpečí mechanického poškození unášeny do místa nidace. Následně se mechanismem opačným, zvaným mezenchymo-epiteliální tranzice (MET), vracejí ke svému původnímu epiteliálnímu fenotypu a zakládají dceřiná ložiska, mikrometastázy, z nichž se při příznivých podmínkách může vyvinout plnohodnotné

metastatické ložisko (Matejka *et al.*, 2017). Ačkoliv se EMT může na molekulární úrovni u jednotlivých typů nádorů částečně lišit, obecně se jedná o vysoce konzervovaný proces s dobře popsányými mechanismy. Jsou například známy transkripční faktory TWIST1, SNAIL1 a SLUG, které významně zvyšují metastatický potenciál a jsou spojeny se špatnou prognózou (Alsidawi *et al.*, 2014; Imani *et al.*, 2016; Kanchan *et al.*, 2020). Bylo potvrzeno, že TWIST1 může indukovat disintegrin a metaloproteinázu ADAM12, což vede k podpoře invazivity skrze regulaci tvorby invadopodií a fokálních adhezí (Eckert *et al.*, 2017; Kanchan *et al.*, 2020). Jako supresorová molekula se zde významně uplatňuje miR-34a, která potlačuje vznik metastáz regulací jak transkripčních faktorů TWIST1 a SLUG, tak obecně signální dráhy NOTCH1 (Imani *et al.*, 2016). Mimo to je ADAM12 přímým cílem miRNA rodin miR-29 a miR-200, které se tak rovněž podílejí na regulaci metastazování (Duhachek-Muggy a Zolkiewska, 2015). MiRNA z těchto dvou rodin mohou navíc ovlivňovat přeskupování cytoskeletu cílením klíčových molekul zapojených do regulace buněčné adheze. Dále byly u karcinomu prsu popsány miR-8084, miR-708-3p, klastr miR-96-182-183, miR-484, miR-210 a miR-142-3p, které modulovaly invazivitu nádorových buněk skrze regulaci EMT; a miR-124, miR-199a/214, miR-3178, miR-30a, miR-508-3p a miR-212-5p, které ovlivňují hladinu EMT markerů a transkripčních faktorů regulujících expresi E-cadherinu u triple negativního karcinomu prsu, běžně metastazujícího do mozku (Kanchan *et al.*, 2020).

Jakmile buňky primárního nádoru změni svůj fenotyp prostřednictvím mechanismu EMT, zahájí proces intravazace do blízkých kapilár, ve kterých začnou prostřednictvím adhezních molekul a proteinových receptorů kontaktovat endotelové buňky s cílem přichytit se ke stěně kapilár a extravazovat zpět do vhodné tkáně ve vzdálených místech organismu (Dua *et al.*, 2005). Bylo prokázáno, že některé extracelulární miRNA mohou regulovat integritu endotelu a tím i procesy intravazace, případně extravazace. Například miR-105, která je vylučována nádorovými buňkami, narušuje endotel cílením proteinu těsného spoje ZO-1 (Zonula occludens protein-1) (Zhou *et al.*, 2014). Rovněž angiogenní a růstové faktory uvolňované nádorovými nebo stromálními buňkami přispívají k intravazaci (Dua *et al.*, 2005). Nedávná studie objevila novou roli TGF- β produkovaného fibroblasty asociovanými s nádorem v organizaci kapilár, kdy přítomnost fibroblastů vedla ke zvýšení počtu pericytů na povrchu cév (Zonneville *et al.*, 2018). Hlubší analýza naznačila, že zvýšená exprese miR-520/373 u metastatických buněk karcinomu prsu vedla k významnému potlačení signalizace řízené právě molekulou TGF- β , což dále vedlo ke snížení hladin angiogenních faktorů PAI-1 (plasminogen activator inhibitor-1), PTHrP (parathyroid hormone-related protein)

a ANGPTL4 (angiopoietin-like 4), a tedy v konečném důsledku u zkoumaných buněk ke snížené schopnosti metastazovat (Keklikoglou *et al.*, 2012). V jiné studii bylo dále prokázáno, že miR-204 potlačuje vaskularizaci a angiogenezi inhibicí pro-angiogenních molekul ANGPT1 a TGF β R2 (Flores-Perez *et al.*, 2016). Rodina miR-200 by také mohla hrát roli v regulaci angiogeneze přímým cílením pro-angiogenních cytokinů IL-8 a CXCL1 v endoteliálních buňkách (Pecot *et al.*, 2013).

Aby nádorové buňky cirkulující v krevním oběhu přežily, musejí překonat jednak anoikis a současně se vyhnout nástrojům imunitního dohledu, přičemž většina takovýchto buněk je fagocytována nebo podstoupí apoptózu. Jedním z nástrojů využívaných cirkulujícími nádorovými buňkami po vstupu do krevního oběhu je aktivace krevních destiček, kdy indukci agregace krevních destiček jsou tyto buňky chráněny před imunitním dozorem. Současně pak u těchto buněk dojde k dočasné zástavě buněčného cyklu, čímž významně zvýší pravděpodobnost přežití ve vaskulatuře (Strilic a Offermanns, 2017; Ponert *et al.*, 2018). Debeb a kol. prokázali, že nadměrná exprese miR-141 zvyšuje u nádorových buněk mozkový tropismus a současně snížené hladiny miR-141 inhibují schopnost těchto buněk metastazovat do mozku, což naznačuje, že miR-141 chrání buňky v krevním oběhu a pomáhá při kolonizaci v mozku (Debeb *et al.*, 2016). Krevní destičky agregované na povrchu cirkulujících nádorových buněk rovněž secernují TGF β a PDGF (platelet-derived growth factor), které inhibují aktivitu NK (natural killer) buněk, a tím přispívají k imunitnímu úniku anoikis (Gersuk *et al.*, 1991). Mikročástice derivované z krevních destiček jsou významnými rezervoáry miRNA, které krevní destičky mohou transportovat a modulovat jimi genovou expresi v cirkulujících nádorových buňkách. Bylo prokázáno, že miR-183 enkapsulovaná právě v mikročásticích derivovaných z krevních destiček potlačuje signifikantním způsobem aktivitu NK buněk, a to skrze cílené umlčení DAP12, proteinu klíčového pro stabilizaci povrchových NK receptorů, a následnou transdukcí signálu (Donatelli *et al.*, 2014).

Jakmile jsou nádorové buňky schopné přežít v krevním oběhu, přichytí se na stěny kapilár a zahájí proces extravazace, který je koordinován mnoha selektiny, integriny, kadheriny, CD44 a imunoglobulinovými receptory. Extravazace značně zpomaluje celý proces metastazování, jelikož nádorové buňky musí překonat obranné mechanismy jednak astrocytů, ale i dalších ochranných mechanismů stimulovaných mikroprostředím mozku (Wasilewski *et al.*, 2017). Právě astrocyty jsou mobilizovány po invazi cizích buněk do mozku jedny z prvních a indukují u těchto buněk vnější apoptotickou dráhu skrze povrchový receptor Fas (Valiente *et al.*, 2014), přičemž nedávné studie prokázaly, že některé miRNA

včetně miR-7, miR-21 a let-7c regulují expresi ligandu FasL a jiná současně jiná miRNA – miR-200c cílí mRNA transkriptu genu FAP-1, čímž reguluje vnější apoptotickou dráhu spouštěnou skrze CD95 (Sayed *et al.*, 2010; Schickel *et al.*, 2010). Ve snaze potlačit apoptotické účinky astrocytů, uvolňují nádorové buňky proteázové inhibitory známé jako serpiny, přičemž bylo popsáno, že např. miR-21 inhibuje Serpin1 a může tak hrát důležitou roli v procesu metastazování (Yamanaka *et al.*, 2012). Pokud se metastatické buňky vyhnou těmto signálům buněčné smrti, vyžadují pro svoji nidaci aktivní podporu opět astrocytů, které stabilizují mezerovitá spojení konexinů, a umožní tak vznik mikrometastázy (Chen *et al.*, 2016). V tomto ohledu bylo popsáno pozorování, kdy exprese miR-206 nepřímo koreluje s expresí konexinového genu Cx43 a je na úrovni fenotypu buněk asociována se sníženou proliferací a migrací (Kanchan *et al.*, 2020). Ke stabilizaci mezerovitých spojení mezi astrocyty a nádorovými buňkami je dále nutná exprese interleukinů IL-6 a IL-8, které ovlivňují oba buněčné typy – indukují expresi endoteliového ligandu ET-1 na astrocytech a endoteliových receptorů ETAR a ETBR na nádorových buňkách (Kim *et al.*, 2014; Wasilewski *et al.*, 2017), přičemž exprese ET-1 je posttranskripčně regulována pomocí miR125a/b-5p (Li *et al.*, 2010a). Je nepochybné, že v procesu extravazace hrají důležitou roli rovněž molekuly buněčné adheze (CAM) a jejich receptory, které jsou rovněž posttranskripčně regulovány pomocí miRNA. Konkrétně pak bylo ověřeno, že miR-17 reguluje ICAM-1 a E-selektin (Suarez *et al.*, 2010); miR-126 a miR-1185 reguluje endoteliální expresi VCAM1 (Harris *et al.*, 2008; Deng *et al.*, 2017); a miR-483-5p přímo cílí transkript genu ALCAM (Lu *et al.*, 2017).

Kromě výše uvedeného přispívají reaktivní astrocyty sekrecí vybraných molekul také k tvorbě protumorigenního „niche“. U myši byla v tomto ohledu identifikována skupina 17 genů, jejichž exprese specificky korelovala s geny již dříve popsanými ve spojitosti s metastázami karcinomu prsu. Mezi těmito 17 geny pak byly identifikovány čtyři konkrétní (COX2, EGFR ligand HBEGF, ANGPTL4 a 2,6-sialyltransferase ST6GALNAC5), které jsou zodpovědné za nasměrování buněk karcinomu prsu do mozku (Bos *et al.*, 2009). Z těchto genů se COX2 aktivní podílí na regulaci exprese MMP-1 a jeho exprese je asociována s propustností hematoencefalické bariéry. COX2 rovněž v buňkách indukuje fenotyp kmenových buněk, a to zvýšením exprese miR-655 a miR-526b, díky čemuž nabývají tyto buňky na schopnosti metastazovat (Majumder *et al.*, 2015; Majumder *et al.*, 2018). MiR-212 pak přímo cílí molekulu HBEGF a suprimuje buněčný růst, migraci a invazivitu (Wei *et al.*, 2014). Transkript ST6GALNAC5, specifický mediátor mozkových metastáz karcinomu prsu, je přímým cílem miR-200c a miR-200b a může také regulovat

EMT (Bos *et al.*, 2009; Kurcon *et al.*, 2015). MikroRNA zapojené v regulaci jednotlivých kroků procesu metastazování jsou přehledně uvedeny v Tabulce 7.

Příspěvek k dané problematice

*Pomocí globální analýzy hladin miRNA v CSF odebraného od pacientů s mozkovými metastázami (n = 13) a od pacientů s normotenzním hydrocefalem bez potvrzeného onkologického onemocnění (n = 19) jako kontrolních vzorků jsme identifikovali 14 miRNA s rozdílnými hladinami (miR-5100, miR-92a-3p, miR-143-3p, miR-196a-5p, miR-196b-5p, miR-490-3p, miR-1247-5p, miR-199b-3p, miR-21-3p, miR-3607-3p, miR-205-5p, miR-532-5p, miR-381-3p a miR-10a-5p; $p_{adj} < 0,001$). Následná validace na nezávislém souboru CSF vzorků od pacientů s mozkovými metastázami (n = 12) a kontrolních vzorků (n = 21) potvrdila předešlé výsledky pouze u miR-21-3p. Porovnání s ostatními nádory navíc ukázalo, že miR-10a-5p je signifikantně snížena v CSF u pacientů s mozkovými metastázami v porovnání s jak GBM, tak LGG, ale neliší se od meningeomu ani od kontrolních vzorků. V případě miR-196b-5p pak vzorky CSF od pacientů s mozkovými metastázami vykazovaly nejnižší hladiny napříč všemi sledovanými pacienty s mozkovými nádory, zatímco miR-31-3p dosahovala naopak nejvyšších hladin u pacientů s mozkovými metastázami v porovnání se všemi ostatními zkoumanými vzorky. Nakonec miR-10b-5p a miR-30e-5p byly významně zvýšené v CSF u LGG v porovnání s mozkovými metastázami, ačkoliv tento trend nebyl pozorován u GBM. Výsledky tedy jasně naznačují, že cirkulující miRNA v CSF jsou nadějnými biomarkery pro diagnostiku pacientů s mozkovými metastázami (Kopkova *et al.*, 2019; Příloha 12).*

Tabulka 7 MikroRNA zapojené v regulaci procesu metastazování (převzato a upraveno z Kanchan *et al.*, 2020)

mikroRNA	molekulární cíl	mikroRNA	molekulární cíl
EMT		Prostupnost hematoencefalickou bariérou	
miR-8084	ING2, p53-BAX	miR-181c	PDPK1
miR-484	PAX-5	miR-143	PUMA
miR-708-3p	ZEB1, CDH2 a vimentin	miR-125a-5p	ICAM-1
miR-210	E-cadherin (ORF), PAX-5	miR-1258	HPSE
miR-142-3p	Bach-1, CXCR4, MMP9 a VEGFR	miR-210	Occludin, β -catenin
miR-199a/214	Slug	Mezibuněčná komunikace a formování "niche"	
miR-3178	Notch1	miR-26a	PTEN
miR-212-5p	Prrx2		ATM
miR-29,miR-30	ADAM12-L	miR-19a	PTEN
miR-200 family		miR-345	KISS1
Intravazace		miR-124, miR-155, miR-689	Souvisí s fenotypem M1 mikroglií
miR-126	VEGF/PI3K/AKT axis, MAPK	miR-711 and miR-145	Souvisí s fenotypem M2 mikroglií
miR-520/373	ANGPTL4, PTHrP, PAI-1		L1CAM
miR-204	ANGPT1 a TGF β R2	miR-503	Spouští M1 – M2 polarizaci mikroglií
miR-200 family	IL-8 a CXCL1	Reprogramování metabolismu	
miR-105	ZO-1	miR-122	PKM2, GLUT-1
Intravaskulární mikroprostředí		miR-155	PIK3R1-PDK/AKT-FOXO3a-cMYC axis
miR-141	Ochrana v krevním oběhu	miR-7	RelA
miR-183	DAP12/NK cells	Kolonizace	
Extravazace a mikroprostředí mozku		miR-200 family (miR-200a,200b, 200c, miR-141, and miR-429)	ZEB1 a ZEB2
miR-7, let-7c, miR-21	FasL, SERPIN1	miR-147	ZEB1
miR-200c	FAP-1	miR-126	IGFBP2, PITPNC1 a MERTK
miR-206	Cx43		
miR-19a, miR-32,miR-124a, miR-130b, miR-148a, and miR-583	PCDH7		
miR-125a/b-5p	ET-1		
miR-1266, miR-185 and miR-30c	BCL2L1		
miR-151-3p	TWIST1		
miR-17	ICAM-1and E-Selectin		

miR-126 and miR-1185	VCAM1
miR-483-5p	ALCAM
miR-21-3p	L1CAM
miR-212	HBEGF
miR-655	COX2
miR-200b, 200c	ST6GALNAC5

Závěrečný komentář

Přestože současná WHO klasifikace některých nádorů mozku, zejména pak gliomů, již bere v úvahu kromě histopatologických znaků částečně i jejich molekulární charakteristiky, stále neumožňuje dostatečně citlivě stanovit prognózu onemocnění, případně predikovat léčebnou odpověď na konvenční terapii. Navíc u dalších diagnóz včetně meningeomu nejsou molekulární znaky doposud reflektovány vůbec, ačkoliv třeba právě meningeomy stupně malignity WHO II vykazují velmi nejisté biologické chování, které na základě standardní histopatologie nelze odhadnout. V kontextu současného trendu personalizované či precizní medicíny je v porovnání s jinými diagnózami právě nedostatek dostatečně citlivých a zároveň robustních prognostických a prediktivních biomarkerů odpovědi na léčbu možná jeden z důvodů pomalého zařazování cílených léčiv do terapeutických standardů mozkových malignit. Navíc s ohledem na heterogenitu některých typů mozkových nádorů a jejich lokalizaci je mnohdy limitováno i samotné stanovení správné diagnózy. Zde by mohly být užitečnými nástroji diagnostiky biomarkery cirkulující v periferní krvi nebo s ohledem na přítomnost hematoencefalické bariéry lépe v mozkomíšním moku. Ten se jako tekutina omývající všechny struktury centrální nervové soustavy a rezervoár jak odpadních produktů, tak molekul zprostředkovávajících mezibuněčnou komunikaci jeví být v případě nádorů mozku vhodným zdrojem biomarkerů včetně krátkých nekódujících RNA. Právě krátké nekódující RNA disponují všemi vlastnostmi, aby mohly být v tomto ohledu považovány za vhodné kandidátní molekuly. Nejenže jsou zapojeny do řízení všech klíčových buněčných procesů a jejich specifické expresní profily již byly asociovány s většinou nádorových onemocnění, jsou rovněž vysoce stabilními molekulami ať už v tělních tekutinách nebo v běžných laboratorních podmínkách. Kromě toho jsou tyto molekuly v nádoru relativně snadno regulovatelné a představují tak nadějně terapeutické cíle. Mnohé z uvedených skutečností jsme u nádorů mozku, zejména pak u glioblastomu, v posledních deseti letech několikrát sami potvrdili a pozorované výsledky prezentovali v řadě odborných publikacích, které jsou komentované v této habilitační práci. Deset let výzkumu v tomto oboru zároveň představuje období, během kterého znalosti a poznatky o významu krátkých nekódujících RNA u nádorových onemocnění obecně přibývaly exponenciálním tempem a dosáhly úrovně, kdy snaha o zavedení těchto molekul do rutinní diagnostické a onkologické praxe je dalším logickým krokem, který by mohl zefektivnit management a prodloužit celkové přežívání pacientů nejen s nádory mozku.

Commentary

Although the current WHO classification of some brain tumors, especially gliomas, already considers not only histopathological features but also their molecular characteristics, it still does not allow sufficient sensitivity for determining the prognosis of the disease or prediction of therapeutic response to conventional therapy. In other diagnoses, molecular features have not yet been reflected at all. Although meningiomas WHO II, for example, show very uncertain biological behavior, which cannot be estimated based on standard histopathology. In the context of the current trend of personalized and precision medicine, the lack of sufficiently sensitive and at the same time robust prognostic and predictive biomarkers is one of the reasons for the slow inclusion of targeted drugs in the therapeutic standards for brain malignancies.

In addition, due to the heterogeneity of some brain tumors and their location, the correct diagnosis determination is often limited. Biomarkers circulating in the peripheral blood or, better yet, in the cerebrospinal fluid because of the blood-brain barrier, could be useful diagnostic tools. Cerebrospinal fluid is a body fluid that washes all the structures of the central nervous system and is a reservoir of both waste products and molecules mediating intercellular communication; therefore, it appears to be a suitable source of brain tumors biomarkers, including small non-coding RNAs. Small non-coding RNAs have all the properties to be considered as suitable candidate molecules in this regard. They are not only involved in the control of key cellular processes, and their specific expression profiles have already been associated with most cancers, but they are also highly stable molecules both in body fluids and under normal laboratory conditions. Besides, these molecules are relatively easily regulatable in the tumor and, thus, represent promising therapeutic targets. We have confirmed many of these facts in glioblastoma and other brain tumors, and presented the observed results in many scholarly publications in the last ten years; these works are commented in this habilitation thesis. Ten years of research in this field also represents a period during which the knowledge about the importance of small non-coding RNAs in cancer in general has increased exponentially. It has reached a level where efforts to introduce these molecules into routine diagnostic and oncological practice are the next logical step to make the disease management more effective and to prolong the overall survival of cancer patients.

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Seznam použitých zkratek

AHP	Acute hepatic porphyria (Akutní jaterní porfyrie)
cIMPACT-NOW	The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy – not official WHO (Mezinárodní konsorcium neuropatologů a klinických neuroonkologů)
CNS	Central nervous system (Centrální nervová soustava)
CpG	Cytosine-phosphate-guanine (Cytosin-fosfát-guanin)
crasiRNA	Centromere repeat associated small interacting ribonucleic acid (Krátká interagující ribonukleová kyselina asociovaná s centromerovými repetitivními oblastmi)
CSC	Cancer stem cells (Nádorové kmenové buňky)
CSF	Cerebrospinal fluid (Mozkomíšní mok)
dbGaP	Database of Genotypes and Phenotypes (Databáze genotypů a fenotypů)
DNA	Deoxyribonucleic acid (Deoxyribonukleová kyselina)
dsRNA	Double stranded ribonucleic acid (Dvouřetězcová ribonukleová kyselina)
ECOG	Eastern cooperative oncology group (Veřejně financovaná odborná skupina zabývající se multicentrickými klinickými studiemi v oblasti výzkumu nádorových onemocnění)
EDV	EnGeneIC Dream Vector (vektor derivovaný z bakteriálních struktur)
EMA	European Medicines Agency (Evropská léková agentura)
EMT	Epithelial–mesenchymal transition (Epitelo-mezenchymální tranzice)
FC	Fold change (Násobná změna)
FDA	Food and Drug Administration (Úřad pro kontrolu potravin a léčiv v USA)
FFPE	Formalin-fixed paraffin-embedded (Fixovaný ve formalínu a zalitý v parafínu)
G-CIMP	Cytosine-phosphate-guanine (CpG) island methylator phenotype (Fenotyp ovlivněný rozsáhlými metylacemi cytosin-fosfát-guaninových (CpG) oblastí)

GBM	Glioblastoma (Glioblastom)
GSCs	Glioblastoma stem cells (Glioblastomové kmenové buňky)
GTP	Guanosine triphosphate (Guanosin trifosfát)
hATTR	Hereditary transthyretin-mediated amyloidosis (Dědičná transthyretinová amyloidóza)
HGG	High-grade gliomas (Gliomy vysokého stupně malignity)
HPF	High-power fields (V mikroskopii zorné pole pod maximálním zvětšením použitého objektivu – často 400násobné zvětšení)
HR	Hazard ratio (Poměr rizik)
KPS	Karnofsky Performance Status (Celkový stav pacienta hodnocený dle stupnice D.A. Karnofského)
LGG	Low-grade gliomas (Gliomy nízkého stupně malignity)
MC	Methylation cluster (Metylační klastr)
MET	Mesenchymal–epithelial transition (Mezenchymo-epiteliální tranzice)
miRISC	MiRNA-induced silencing complex (Umlčovací komplex indukovaný mikroRNA)
miRNA	MicroRNA - micro ribonucleic acid (mikroRNA - mikro ribonukleová kyselina)
MRI	Magnetic resonance imaging (Magnetická rezonance)
mRNA	Messenger ribonucleic acid(Protein-kódující ribonukleová kyselina)
NA	Not available (Údaje nejsou dostupné)
NK	Natural killer (Přírození zabíječi)
NSCs	Neural stem cells (Neurální kmenové buňky)
nt	Nucleotide (Nukleotid)
OS	Overall survival (Celkové přežívání)

PASR	Promoter-associated small ribonucleic acid (Krátká ribonukleová kyselina asociovaná s promotorovými oblastmi)
PCR	Polymerase chain reaction (Polymerázová řetězová reakce)
PFS	Progression-free survival (Čas do progrese onemocnění)
PIP	Phosphatidylinositol (3,4,5)-trisphosphate (Fosfatidylinositol (3,4,5)-trifosfát)
piRNA	PIWI-interacting ribonucleic acid (PIWI-interagující ribonukleová kyselina)
pre-miRNA	Precursor of microRNA (Prekurzor mikroRNA)
pri-miRNA	Primary transcript of microRNA (Primární transkript mikroRNA)
RISC	RNA-induced silencing complex (Multiproteinový umlčovací komplex)
RNA	Ribonucleic acid (Ribonuklová kyselina)
RPA	Recursive Partitioning Analysis (Analýza umožňující predikci prognózy u pacientů s mozkovými metastázami)
rRNA	Ribosomal ribonucleic acid (Ribozomální ribonukleová kyselina)
RS	Risk Score (Rizikové skóre)
RT	Radiotherapy (Radioterapie)
RT-qPCR	Quantitative reverse transcription polymerase chain reaction (Kvantitativní polymerázová řetězová reakce, které předchází reverzní transkripce)
siRNA	Small interfering ribonucleic acid (Krátká interferující ribonukleová kyselina)
sncRNA	Small non-coding ribonucleic acid (Krátká nekódující ribonukleová kyselina)
snoRNA	Small nucleolar ribonucleic acid (Malá jadéřková ribonukleová kyselina)
SNPs	Single-nucleotide polymorphisms (Jednonukleotidové polymorfizmy)
snRNA	Small nuclear ribonucleic acid (Malá jaderná ribonukleová kyselina)

ssRNA	Single stranded ribonucleic acid (Jednořetězcová ribonukleová kyselina)
TCGA	The Cancer Genome Atlas (Databáze nádorových genomů)
tel-sRNA	Telomere-specific small ribonucleic acid (Krátká ribonukleová kyselina specifická pro oblasti telomer)
TEs	Transposable elements (Transponovatelné elementy)
tiRNA	Transcription initiation ribonucleic acid (Ribonukleová kyselina iniciující transkripci)
TMZ	Temozolomide (Temozolomid)
tRNA	Transfer ribonucleic acid (Transferová ribonukleová kyselina)
UTR	Untranslated region (Nekódující oblast)
WHO	World health organization (Světová zdravotnická organizace)

Seznam uvedených genů

ABCG2	ATP binding cassette subfamily G member 2
ADAM12	ADAM metallopeptidase domain 12
AGO	Argonaute
AKT	AKT serine/threonine kinase 1
ALA	Aminolevulinic acid
ALAS1	Aminolevulinic acid synthase 1
ALCAM	Activated leukocyte cell adhesion molecule
ANGPT1	Angiopoietin 1
ANGPTL4	Angiopoietin-like 4
ARID1A	AT-rich interaction domain 1A
ATM	Ataxia telangiectasia mutated
ATRX	ATRX chromatin remodeler
BACH1	BTB domain and CNC homolog 1
BAP1	BRCA1 associated protein 1
BAX	BCL2-associated X protein

BCL-2	BCL2 apoptosis regulator
BMP4	Bone morphogenetic protein 4
BRMS1	BRMS1 transcriptional repressor and anoikis regulator
BTG2	B-cell translocation gene 2
CAM	Calmodulin
CAMTA1	Calmodulin binding transcription activator 1
CASP8	Caspase 8
Cbl-b	Casitas B-lineage lymphoma proto-oncogene-b
CDH	Cadherin
CDKN2A	Cyclin dependent kinase inhibitor 2A
p14ARF	Alternate reading frame protein product of the CDKN2A
p16INK4a	Alternate reading frame protein product of the CDKN2A
CFLAR	CASP8 and FADD like apoptosis regulator
CHEK2	Checkpoint kinase 2
COX2	Cytochrome c oxidase subunit II
CRSP3	Mediator complex subunit 23
CSF1	Colony stimulating factor 1
CSMD1	CUB and SUSHI multiple domain protein 1
CTFG	Cellular communication network factor 2
Cx43	Connexin 43
CXCL1	C-X-C motif chemokine ligand 1
CXCR4	C-X-C motif chemokine receptor 4
DAP12	Transmembrane immune signaling adaptor TYROBP
DMD	Dystrophin
DNA-PK	Protein kinase, DNA-activated, catalytic subunit
E2F1	E2F transcription factor 1
EGFR	Epidermal growth factor receptor
EphA2	EPH receptor A2
ERBB2	Erb-b2 receptor tyrosine kinase 2
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin 1
ETAR	Endothelin receptor type A
ETBR	Endothelin receptor type B
FAK	Protein tyrosine kinase 2
FAP-1	Protein tyrosine phosphatase non-receptor type 13

FasL	Fas ligand (TNF superfamily, member 6)
GEMIN	Gem nuclear organelle associated protein
GFAP	Glial fibrillary acidic protein
GLUT-1	Glucose transporter 1
HBEGF	Heparin binding EGF like growth factor
Hen1	Hen1 methyltransferase
HER2	Erb-b2 receptor tyrosine kinase 2
HIF	Hypoxia inducible factor
HOXB9	Homeobox B9
HPSE	Heparanase
HRAS	HRas proto-oncogene, GTPase
ICAM-1	Intercellular adhesion molecule 1
ID1	Inhibitor of DNA binding 1, HLH protein
IDH	Isocitrate dehydrogenase
IGFBP2	Insulin like growth factor binding protein 2
IL	Interleukin
ING2	Inhibitor of growth family member 2
KAI1	CD82 molecule
KISS1	KiSS-1 metastasis suppressor
KLF4	Kruppel like factor 4
KRAS	KRAS proto-oncogene, GTPase
L1CAM	L1 cell adhesion molecule
LEF1	Lymphoid enhancer binding factor 1
lin-14	Protein lin-14
lin-4	Non-coding ribonucleic acid lin-4
LOX	Lysyl oxidase
MAPK	Mitogen activated kinase-like protein
MCL1	MCL1 apoptosis regulator, BCL2 family member
MDM2	Mouse double minute 2
MERTK	MER proto-oncogene, tyrosine kinase
MET	MET proto-oncogene, receptor tyrosine kinase
MGMT	O6-methylguanine-DNA methyltransferase
MKK4	Mitogen-activated protein kinase kinase 4
MMAC1	Phosphatase and tensin homolog
MMP-9	Matrix metalloproteinase 9

MTA1	Metastasis associated 1
mTOR	Mechanistic target of rapamycin kinase
Nanog	Nanog homeobox
NDFIP1	Nedd4 family interacting protein 1
NEDD9	Neural precursor cell expressed, developmentally down-regulated 9
NF	Neurofibromin
NM23	NME/NM23 nucleoside diphosphate kinase 1
NMMHC-B	Non-muscle myosin heavy chain Iib
NOTCH	Notch receptor
NPPA	Natriuretic peptide A
NRAS	RAS proto-oncogene, GTPase
PAI-1	Plasminogen activator inhibitor-1
PAX-5	Paired box 5
PBG	Porfobilinogen
PBRM1	Polybromo 1
PCDH7	Protocadherin 7
PDCD4	Programmed cell death factor 4
PDGFA	Platelet derived growth factor subunit A
PDGFR α	Platelet derived growth factor receptor alpha
PDPK1	3-phosphoinositide dependent protein kinase 1
PI3K	Phosphatidylinositol 3-kinase
PIM3	Pim-3 proto-oncogene, serine/threonine kinase
PITPNC1	Phosphatidylinositol transfer protein cytoplasmic 1
PIWI	P-element Induced Wimpy Testis
PKM2	Pyruvate kinase M 2
POLR2A	RNA polymerase II subunit A
PPP2R5a	Protein phosphatase 2 regulatory subunit B'alpha
Prrx2	Paired related homeobox 2
PTCH1/2	Patched 1/2
PTEN	Phosphatase and tensin homolog
PTHrP	Parathyroid hormone-related protein
PTPN11	Protein tyrosine phosphatase non-receptor type 11
PUMA	p53 upregulated modulator of apoptosis
RAD51	RAD51 recombinase
RB1	RB transcriptional corepressor 1

RelA	RELA proto-oncogene, NF-kB subunit
REST	RE1 silencing transcription factor
RHEB	Ras homolog, mTORC1 binding
RHoC	Ras homolog family member C
RHOGDI2	Rho GDP dissociation inhibitor beta
RRM1/2	Ribonucleotide reductase catalytic subunit M1/2
RTVP-1	GLI pathogenesis related 1
S100A4	S100 calcium binding protein A4
SCP-1	Synaptonemal complex protein 1
SERPIN1	Serine protease inhibitor 1
Shh	Sonic hedgehog signaling molecule
Shu/Hsp93	Shutdown protein/Hsp93 chaperone
SLUG	Snail family transcriptional repressor 2
SMAD4	SMAD family member 4
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
SMO	Smoothened, frizzled class receptor
SNAIL1	Snail family transcriptional repressor 1
Sox2	SRY-box transcription factor 2
SPRY1	Sprouty RTK signaling antagonist 1
SRC	SRC proto-oncogene, non-receptor tyrosine kinase
ST6GALNAC5	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5
STAT3	Signal transducer and activator of transcription 3
SUFU	SUFU negative regulator of hedgehog signaling
TERT	Telomerase reverse transcriptase
TGF- β	Transforming growth factor beta
TGF β R2	Transforming growth factor beta receptor 2
TIAM1	TIAM Rac1 associated GEF 1
TIMP2	TIMP metalloproteinase inhibitor 2
TP53	Tumor protein p53
TRAF7	TNF receptor associated factor 7
TRBP	TARBP2 subunit of RISC loading complex
TTR	Transthyretin
TUJ1	Neuron-specific class III beta-tubulin

TWIST1	Twist family bHLH transcription factor 1
VCAM1	Vascular cell adhesion molecule 1
Vdup1	Vitamin D[[3]] up-regulated protein 1/TXNIP thioredoxin interacting protei
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau tumor suppressor
Wnt	Wingless-type MMTV integration site family
ZEB1	Zinc finger E-box binding homeobox 1
ZO-1	TJP1 tight junction protein 1
Zuc	Zucchini
γ -H2AX	H2A histone family, member X, gamma

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Diagnostická sada pro neinvazivní diagnostiku mozkových nádorů

Oblast techniky

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Předkládané technické řešení se týká diagnostické sady pro neinvazivní diagnostiku mozkových nádorů. Sada je určena pro stanovení mikroRNA v mozkomíšním moku umožňující detekci různých typů mozkových nádorů.

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Dosavadní stav techniky

Primární nádory mozku a mozkové metastázy postihnou ročně v celosvětovém měřítku téměř 40 pacientů na 100 tisíc obyvatel a jejich incidence neustále stoupá. Přibližně 33 % primárních nádorů mozku pak tvoří maligní formy s pětiletým přežíváním v průměru u 34,4 % pacientů, přičemž prognóza úzce souvisí s konkrétním typem nádoru. Nejlepší prognózu mají nízkostupňové gliomy společně s meningeomy, naopak nejkratší přežívání je asociováno s multifonním glioblastomem (GBM). Velmi špatná prognóza bývá rovněž spojována s mozkovými metastázami, které se vyskytují u 20 % až 40 % dospělých pacientů s nádorovým onemocněním. Určení správné diagnózy hraje rozhodující roli i při výběru nejvhodnější terapie. Navzdory významným pokrokům v diagnostice nádorů mozku jako jsou různé modifikace zobrazovacích metod a následné histopatologické vyšetření tkáně, je diagnostika stále omezena lokalizací nádoru a často jeho značnou heterogenitou. Zavedení dostatečně senzitivních a specifických metod pro detekci a stanovení diagnózy u pacientů s mozkovými nádory je proto velice žádoucí. Vhodným diagnostickým nástrojem u mozkových nádorů je analýza mikroRNA, krátkých nekódujících RNA, v mozkomíšním moku (cerebrospinal fluid; CSF), který omývá centrální nervovou soustavu a je tak v přímém kontaktu s případnou patologickou tkání.

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Podstata technického řešení

V rámci předkládaného technického řešení byly identifikovány mikroRNA v mozkomíšním moku asociované s přítomností vybraných mozkových nádorů. Předkládané technické řešení tedy poskytuje diagnostickou sadu pro detekci multifonního glioblastomu (GBM), meningeomu a/nebo mozkových metastáz na základě kvantifikace let-7i-5p, miR-151a-3p, miR-423-3p, a alespoň jedné miRNA vybrané z let-7b-5p, miR-140-5p, miR-21-3p, ve vzorku CSF metodou kvantitativní PCR v reálném čase (qRT-PCR).

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Nukleotidové sekvence předmětných mikroRNA jsou následující:

let-7b-5p:	5' UGAGGUAGUAGGUUGUGUGGUU 3'
miR-140-5p:	5' CAGUGGUUUUACCCUAUGGUAG 3'
miR-21-3p:	5' CAACACCAGUCGAUGGGCUGU 3'
let-7i-5p:	5' UGAGGUAGUAGUUUGUGCUGUU 3'
45 miR-151a-3p:	5' CUAGACUGAAGCUCCUUGAGG 3'
miR-423-3p:	5' AGCUCGGUCUGAGGCCCCUCAGU 3'

Uvedené sady diagnostického setu mají následující složení:

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- stanovení hladiny exprese let-7b-5p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu pro qPCR sestávající ze sekvencí:

stem-loop primer:

5'- GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC AACCAC - 3'

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univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'

forward primer: 5' - GGGTGAGGTAGTAGGTTGT - 3'

TaqMan sonda: 5' - AGCCAACAACCACACAACC - 3'

- 5 - stanovení hladiny exprese miR-140-5p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu pro qPCR sestávající ze sekvencí:

stem-loop primer:

5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC CTACCA - 3'

univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'

10 forward primer: 5' - GGGCAGTGGTTTTACCCTA - 3'

TaqMan sonda: 5' - AGCCAACCTACCATAGGGT - 3'

- 15 - stanovení hladiny exprese miR-21-3p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu pro qPCR sestávající ze sekvencí:

stem-loop primer:

5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC ACAGCC - 3'

univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'

20 forward primer: 5' - GTTGCAACACCAGTCGATG - 3'

TaqMan sonda: 5' - AGCCAACACAGCCCATCGA - 3'

- 25 - stanovení hladiny exprese let-7i-5p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu pro qPCR sestávající ze sekvencí:

stem-loop primer:

5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC AACAGC - 3'

univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'

forward primer: 5' - GTTTGGTGAGGTAGTAGTTTGT - 3'

30 TaqMan sonda: 5' - AGCCAACAACAGCACAAAC - 3'

- 35 - stanovení hladiny exprese miR-151a-3p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu pro qPCR sestávající ze sekvencí:

stem-loop primer:

5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC CCTCAA - 3'

univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'

forward primer: 5' - GTTGCTAGACTGAAGCTCC - 3'

TaqMan sonda: 5' - AGCCAACCCTCAAGGAGCT - 3'

- 40 - stanovení hladiny exprese miR-423-3p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu pro qPCR sestávající ze sekvencí:

stem-loop primer:

5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC ACTGAG - 3'

45 univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'

forward primer: 5' - TTGAGCTCGGTCTGAGGC - 3'

TaqMan sonda: 5' - AGCCAACACTGAGGGGCCT - 3'

- 50 Sada může také s výhodou obsahovat deionizovanou vodu sloužící jako negativní kontrola a qRT-PCR standard odpovídající expresi let-7b-5p ve vzorku pacienta s GBM, expresi miR-140-5p ve vzorku pacienta s meningeomem, expresi miR-21-3p ve vzorku pacienta s mozkovými metastázami a expresi let-7i-5p, miR-151a-3p a miR-423-3p ve vzorcích pacientů s glioblastomem, meningeomem a mozkovými metastázami a jedinců bez jakéhokoliv prokázaného nádorového onemocnění.

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Diagnostika multifornního glioblastomu

5 Kvantitativní analýza hladiny let-7b-5p normalizované na průměr hladin let-7i-5p, miR-151a-3p a miR-423-3p, jejichž nukleotidové sekvence jsou specifikovány výše, pomocí qRT-PCR umožňuje s dostatečnou specificitou a senzitivitou odlišit pacienty s multifornním glioblastomem (GBM) od jedinců bez jakéhokoliv histopatologicky prokázaného nádorového onemocnění. Hladiny let-7b-5p, let-7i-5p, miR-151a-3p a miR-423-3p se s pomocí předmětné sady stanovují v RNA izolované ze supernatantu CSF.

10 Bylo zjištěno, že normalizovaná hladina let-7b-5p v supernatantu CSF je významně změněna (zvýšena) u pacientů s GBM ve srovnání s dárci bez jakéhokoliv prokázaného nádorového onemocnění.

Diagnostika meningeomu

15 Kvantitativní analýza hladiny miR-140-5p normalizované na průměr hladin let-7i-5p, miR-151a-3p a miR-423-3p, jejichž nukleotidové sekvence jsou specifikovány výše, pomocí qRT-PCR umožňuje s dostatečnou specificitou a senzitivitou odlišit pacienty s meningeomem od jedinců bez jakéhokoliv histopatologicky prokázaného nádorového onemocnění. Hladiny
20 miR-140-5p, let-7i-5p, miR-151a-3p a miR-423-3p se s pomocí předmětné sady stanovují v RNA vyzolované ze supernatantu CSF.

25 Bylo zjištěno, že normalizovaná hladina miR-140-5p v supernatantu CSF je významně změněna (zvýšena) u pacientů s meningeomem ve srovnání s dárci bez jakéhokoliv prokázaného nádorového onemocnění.

Diagnostika mozkových metastáz

30 Kvantitativní analýza hladiny miR-21-3p normalizované na průměr hladin let-7i-5p, miR-151a-3p a miR-423-3p, jejichž nukleotidové sekvence jsou specifikovány výše, pomocí qRT-PCR umožňuje s dostatečnou specificitou a senzitivitou odlišit pacienty s mozkovými metastázami od jedinců bez jakéhokoliv histopatologicky prokázaného nádorového onemocnění. Hladiny miR-21-3p, let-7i-5p, miR-151a-3p a miR-423-3p se s pomocí předmětné sady stanovují
35 v RNA vyzolované ze supernatantu CSF.

Bylo zjištěno, že normalizovaná hladina miR-21-3p v supernatantu CSF je významně změněna (zvýšena) u pacientů s mozkovými metastázami ve srovnání s dárci bez jakéhokoliv prokázaného nádorového onemocnění.

Objasnění výkresů

40 Obrázek 1 zobrazuje rozdíl v normalizovaných hladinách let-7b-5p v mozkomíšním moku mezi pacienty s multifornním glioblastomem (GBM) a jedinci bez jakéhokoliv prokázaného nádorového onemocnění pro ($p < 0,0001$). Rozdíl exprese byl analyzován pomocí Mann-Whitneyho testu.

50 Obrázek 2 zobrazuje ROC křivku, která udává, s jakou senzitivitou a specificitou je mozkomíšní let-7b-5p schopna rozlišit pacienty s GBM a jedince bez jakéhokoliv prokázaného nádorového onemocnění (AUC 0,9091; specificita 81 %; senzitivita 83 %).

Obrázek 3 zobrazuje rozdíl v normalizovaných hladinách miR-140-5p v mozkomíšním moku mezi pacienty s meningeomem a jedinci bez jakéhokoliv prokázaného nádorového onemocnění pro ($p < 0,001$). Rozdíl exprese byl analyzován pomocí Mann-Whitneyho testu.

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Obrázek 4 zobrazuje ROC křivku, která udává, s jakou senzitivitou a specificitou je mozkomíšní cirkulující miR-140-5p schopna rozlišit pacienty s meningeomem a jedince bez jakéhokoliv prokázaného nádorového onemocnění (AUC 0,8073; specificita 83 %; senzitivita 75 %).

- 5 Obrázek 5 zobrazuje rozdíl v normalizovaných hladinách miR-21-3p v mozkomíšním moku mezi pacienty s mozkovými metastázami a jedinci bez jakéhokoliv prokázaného nádorového onemocnění pro ($p < 0,01$). Rozdíl exprese byl analyzován pomocí Mann-Whitneyho testu.

- 10 Obrázek 6 zobrazuje ROC křivku, která udává, s jakou senzitivitou a specificitou je mozkomíšní cirkulující miR-21-3p schopna rozlišit pacienty s mozkovými metastázami a jedince bez jakéhokoliv prokázaného nádorového onemocnění (AUC 0,7969; specificita 75 %; senzitivita 100 %).

15 Příklad uskutečnění technického řešení

Pro testování diagnostické schopnosti miRNA podle předkládaného technického řešení byly připraveny qRT-PCR sety o následujícím složení:

Komponenta	Množství	Účel
Mix RT – reverzní transkripce ¹	1 x 4,67 μ l (1 rxn)	Přepis miRNA do cDNA
Mix PCR ²	1 x 17,4 μ l (1 rxn)	Amplifikace miRNA
Detekční sada pro miR-140-5p (RT primer; qPCR primery; TaqMan sonda)	2 μ l; 1 μ l (1 rxn)	Reverzní transkripce a amplifikace miR-140-5p
Detekční sada pro miR-21-3p (RT primer; qPCR primery; TaqMan sonda)	2 μ l; 1 μ l (1 rxn)	Reverzní transkripce a amplifikace miR-21-3p
Detekční sada pro let-7i-5p (RT primer; qPCR primery; TaqMan sonda)	2 μ l; 1 μ l (1 rxn)	Reverzní transkripce a amplifikace let-7i-5p
Detekční sada pro miR-151a-3p (RT primer; qPCR primery; TaqMan sonda)	2 μ l; 1 μ l (1 rxn)	Reverzní transkripce a amplifikace miR-151a-3p
Detekční sada pro miR-423-3p (RT primer; qPCR primery; TaqMan sonda)	2 μ l; 1 μ l (1 rxn)	Reverzní transkripce a amplifikace miR-423-3p
Deionizovaná voda RT, qPCR	3,33 μ l; 1,6 μ l (1 rxn)	Negativní kontrola
Pozitivní kontrola RT, qPCR	3,33 μ l; 1,6 μ l (1 rxn)	Pozitivní kontrola pro let-7b-5p, miR-140-5p, miR-21-3p, let-7i-5p, miR-151a-3p a miR-423-3p

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Pozn. ¹Mix RT obsahuje 10X RT pufr, dNTP mix w/dTTP (100M total), inhibitor RNáz (20U/ μ L), and MultiScribe™ RT enzym (50U/ μ L). ²Mix PCR obsahuje AmpliTaq Gold® DNA Polymerase, dNTPs s dUTP/dTTP směsí, ROX Passive Reference a optimalizovaný pufr. Uvedené objemy komponent platí pro jednu miRNA v rámci RT reakce v objemu 10 μ l a qPCR reakce v objemu 20 μ l. Pozitivní kontrola obsahuje qRT-PCR standard odpovídající hladinám let-7b-5p ve vzorku pacienta s multifornním glioblastomem, hladinám miR-140-5p ve vzorku pacienta s meningeomem, hladinám miR-21-3p ve vzorku pacienta s mozkovými metastázami a hladinám let-7i-5p, miR-151a-3p a miR-423-3p ve vzorcích pacientů s glioblastomem,

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meningeomem a mozkovými metastázami a jedinců bez jakéhokoliv prokázaného nádorového onemocnění.

5 Set podle předkládaného technického řešení může obsahovat všech šest sad pro stanovení hladin všech šest miRNA, může však také obsahovat detekční sady pro let-7i-5p, miR-151a-3p a miR-423-3p, a dále jednu nebo dvě sady vybrané z detekčních sad pro let-7b-5p, miR-140-5p, miR-21-3p.

10 Stem-loop primer (RT primer), qPCR primery (forward, reverse), a TaqMan sonda měly sekvence uvedené v nároku 1.

15 K izolaci RNA byl použit CSF pacientů odebraný pomocí lumbální punkce v objemu 3 až 5 ml do sterilní zkumavky. Poté byl CSF centrifugován 10 minut při RCF 500 g a 4 °C s následným oddělením a uschováním supernatantu. Celková RNA obohacená o frakci krátkých RNA byla izolována z 1 ml CSF supernatantu za použití kitu Urine microRNA Purification Kit od Norgen Biotek.

20 K detekci hladin let-7b-5p, miR-140-5p, miR-21-3p, let-7i-5p, miR-151a-3p a miR-423-3p byly použity výše uvedené sady obsahující jak specifické stem-loop primery pro reverzní transkripci (RT), pomocí níž je RNA přepsána do cDNA, tak primery a fluorescenčně značené sondy pro qPCR. Finální amplifikace a kvantifikace konkrétních miRNA byla provedena za použití přístroje QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). Kvantita stanovovaných miRNA je vyjádřena tzv. Cq (quantitation cycle), tedy číslem cyklu, při kterém fluorescenční signál dosáhl nastavené prahové hodnoty. Detekované hladiny let-7b-5p, miR-140-5p a miR-21-3p byly normalizovány na průměr hladin referenčních let-7i-5p, miR-151a-3p a miR-423-3p.

30 Relativní exprese let-7b-5p, miR-140-5p a miR-21-3p byly stanoveny metodou 2^{-dCq} , kde hodnota dCq představuje rozdíl mezi Cq jednotlivých stanovovaných miRNA a průměrem hladin tří referenčních miRNA.

35 Všichni pacienti, jejichž RNA byla použita k analýze, byli předem diagnostikováni pomocí histologického vyšetření odebrané tkáně. Do studie byli zařazeni pouze pacienti, u nichž byl histopatologicky potvrzen GBM, meningiom nebo mozkové metastázy. Kontrolní skupinu tvořili jedinci bez jakéhokoliv prokázaného nádorového onemocnění.

40 U pacientů s GBM byla v CSF detekována významně zvýšená hladina let-7b-5p ve srovnání s jedinci bez jakéhokoliv histopatologicky prokázaného nádorového onemocnění ($p < 0,0001$) (Obrázek 1). Rozdíl hladin mezi sledovanými skupinami byl testován pomocí Mann-Whitneyho testu.

45 Pomocí ROC (receiver operating characteristic) analýzy byla získána hodnotící, prahová hodnota normalizované exprese let-7b-5p v CSF (cut-off = 0,6418), podle které lze rozlišit pacienty s GBM od jedinců bez jakéhokoliv prokázaného nádorového onemocnění se specificitou 81 % a senzitivitou 83 % (AUC [area under curve] = 0,9091) (Obrázek 2).

50 Vzorky s vyšší normalizovanou hladinou let-7b-5p, než je prahová hodnota, jsou považovány za vzorky pacientů s GBM, naopak vzorky s nižší normalizovanou hladinou let-7b-5p, než je prahová hodnota, jsou považovány za vzorky jedinců bez jakéhokoliv prokázaného nádorového onemocnění.

55 U pacientů s meningeomem byla v CSF detekována významně zvýšená hladina miR-140-5p ve srovnání s jedinci bez jakéhokoliv prokázaného nádorového onemocnění ($p < 0,001$) (Obrázek 3). Rozdíl hladin mezi sledovanými skupinami byl testován pomocí Mann-Whitneyho testu.

Pomocí ROC (receiver operating characteristic) analýzy byla získána hodnotící, prahová hodnota normalizované exprese miR-140-5p v CSF (cut-off = 0,2248), podle které lze rozlišit pacienty s meningeomem od jedinců bez jakéhokoliv prokázaného nádorového onemocnění se specificitou 83 % a senzitivitou 75 % (AUC [area under curve] = 0,8073) (Obrázek 4).

Vzorky s vyšší normalizovanou hladinou miR-140-5p, než je prahová hodnota, jsou považovány za vzorky pacientů s meningeomem, naopak vzorky s nižší normalizovanou hladinou miR-140-5p, než je prahová hodnota, jsou považovány za vzorky jedinců bez jakéhokoliv prokázaného nádorového onemocnění.

U pacientů s mozgovými metastázami byla v CSF detekována významně zvýšená hladina miR-21-3p ve srovnání s jedinci bez jakéhokoliv histopatologicky prokázaného nádorového onemocnění ($p < 0,01$). Rozdíl hladin mezi sledovanými skupinami byl testován pomocí Mann-Whitneyho testu.

Pomocí ROC (receiver operating characteristic) analýzy byla získána hodnotící, prahová hodnota normalizované exprese miR-21-3p v CSF (cut-off = 0,0661), podle které lze rozlišit pacienty s mozgovými metastázami od jedinců bez jakéhokoliv prokázaného nádorového onemocnění se specificitou 75 % a senzitivitou 100 % (AUC [area under curve] = 0,7969) (Obrázek 6).

Vzorky s vyšší normalizovanou hladinou miR-21-3p, než je prahová hodnota, jsou považovány za vzorky pacientů s mozgovými metastázami, naopak vzorky s nižší normalizovanou hladinou miR-21-3p, než je prahová hodnota, jsou považovány za vzorky jedinců bez jakéhokoliv prokázaného nádorového onemocnění.

NÁROKY NA OCHRANU

1. Diagnostický set pro detekci multiformního glioblastomu, meningeomu a/nebo mozgových metastáz ze vzorku mozkomíšního moku metodou kvantitativní PCR v reálném čase, **vyznačený tím**, že obsahuje sady pro stanovení hladin následujících mikroRNA: let-7i-5p, miR-151a-3p, miR-423-3p a alespoň jedné mikroRNA vybrané z let-7b-5p, miR-140-5p a miR-21-3p, přičemž uvedené sady jsou:

- sada pro stanovení hladiny let-7b-5p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu typu TaqMan sestávající ze sekvencí:

stem-loop primer:

5'- GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC AACCAC - 3'

univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'

forward primer: 5' - GGGTGAGGTAGTAGGTTGT - 3'

TaqMan sonda: 5' - AGCCAACAACCACACAACC - 3'

- sada pro stanovení hladiny miR-140-5p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu typu TaqMan sestávající ze sekvencí:

stem-loop primer:

5'- GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCTACCA - 3'

univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'

forward primer: 5' - GGGCAGTGGTTTTACCCTA - 3'

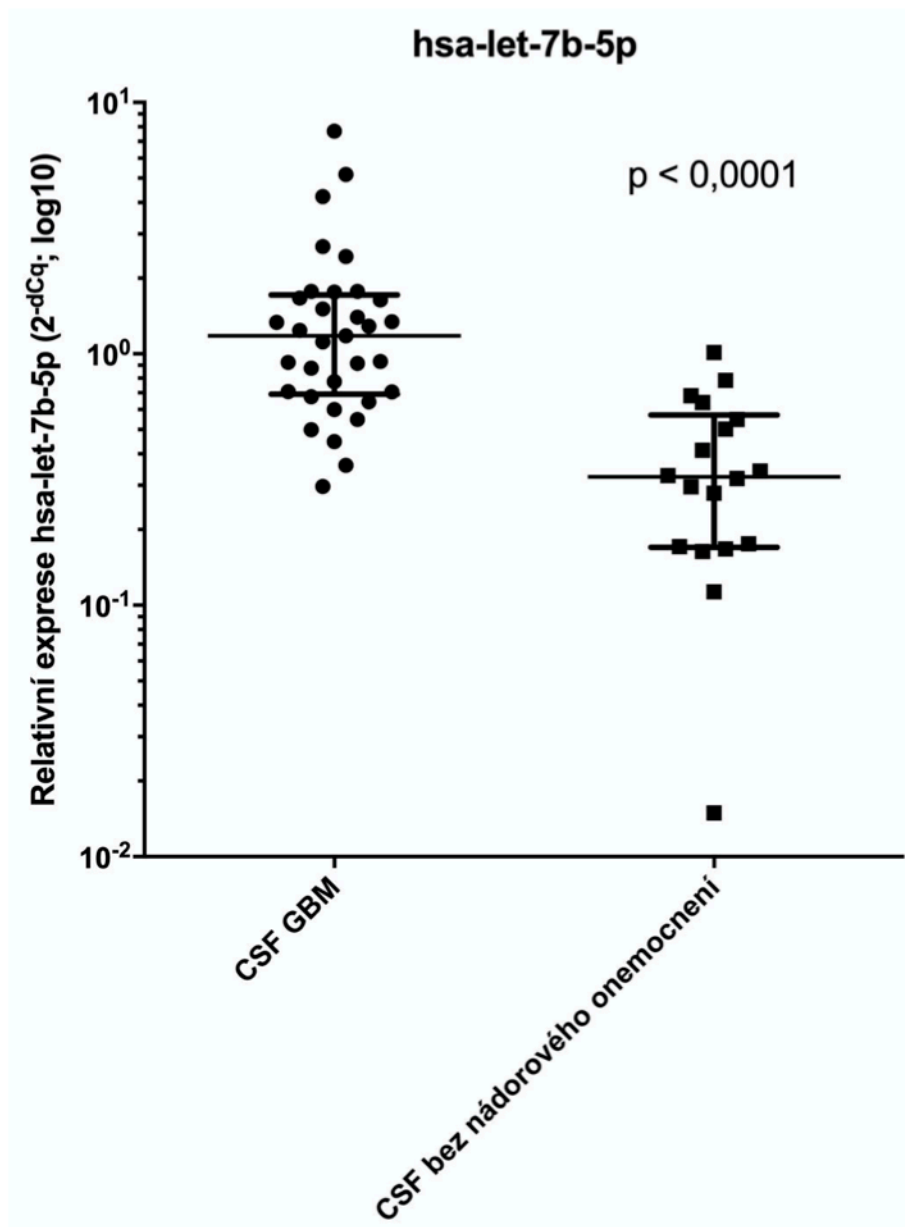
TaqMan sonda: 5' - AGCCAACCTACCATAGGGT - 3'

- sada pro stanovení hladiny miR-21-3p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu typu TaqMan sestávající ze sekvencí:
- stem-loop primer:
 5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACAGCC - 3'
 univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'
 forward primer: 5' - GTTGCAACACCAGTCGATG - 3'
 TaqMan sonda: 5' - AGCCAACACAGCCCATCGA - 3'
- sada pro stanovení hladiny let-7i-5p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu typu TaqMan sestávající ze sekvencí:
- stem-loop primer:
 5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC AACAGC - 3'
 univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'
 forward primer: 5' - GTTTGGTGAGGTAGTAGTTTGT - 3'
 TaqMan sonda: 5' - AGCCAACAACAGCACAAAC - 3'
- sada pro stanovení hladiny miR-151a-3p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu typu TaqMan sestávající ze sekvencí:
- stem-loop primer:
 5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCCTCAA - 3'
 univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'
 forward primer: 5' - GTTGCTAGACTGAAGCTCC - 3'
 TaqMan sonda: 5' - AGCCAACCCTCAAGGAGCT - 3'
- sada pro stanovení hladiny miR-423-3p, obsahující stem-loop primer pro reverzní transkripci a primery a fluorescenčně značenou sondu typu TaqMan sestávající ze sekvencí:
- stem-loop primer:
 5'– GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC ACTGAG - 3'
 univerzální reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'
 forward primer: 5' - TTGAGCTCGGTCTGAGGC - 3'
 TaqMan sonda: 5' - AGCCAACACTGAGGGGCCT - 3'
2. Diagnostický set pro detekci multiformního glioblastomu podle nároku 1, **vyznačený tým**, že obsahuje sady stem-loop primerů pro reverzní transkripci, primerů a fluorescenčně značených sond pro stanovení hladin mikroRNA let-7b-5p, let-7i-5p, miR-151a-3p a miR-423-3p.
3. Diagnostický set pro detekci meningeomu podle nároku 1, **vyznačený tým**, že obsahuje sady stem-loop primerů pro reverzní transkripci, primerů a fluorescenčně značených sond pro stanovení hladin mikroRNA miR-140-5p, let-7i-5p, miR-151a-3p a miR-423-3p.
4. Diagnostický set pro detekci mozkových metastáz podle nároku 1, **vyznačený tým**, že obsahuje sady stem-loop primerů pro reverzní transkripci, primerů a fluorescenčně značených sond pro stanovení hladin mikroRNA miR-21-3p, let-7i-5p, miR-151a-3p a miR-423-3p.
5. Diagnostický set podle kteréhokoliv z předcházejících nároků, **vyznačený tým**, že dále obsahuje deionizovanou vodu jako negativní kontrolu.
6. Diagnostický set podle kteréhokoliv z předcházejících nároků, **vyznačený tým**, že dále obsahuje směsný qRT-PCR standard odpovídající hladinám let-7b-5p ve vzorku pacienta s multiformním glioblastomem, hladinám miR-140-5p ve vzorku pacienta s meningeomem, a/nebo hladinám miR-21-3p ve vzorku pacienta s mozkovými metastázami; a směsný qRT-PCR

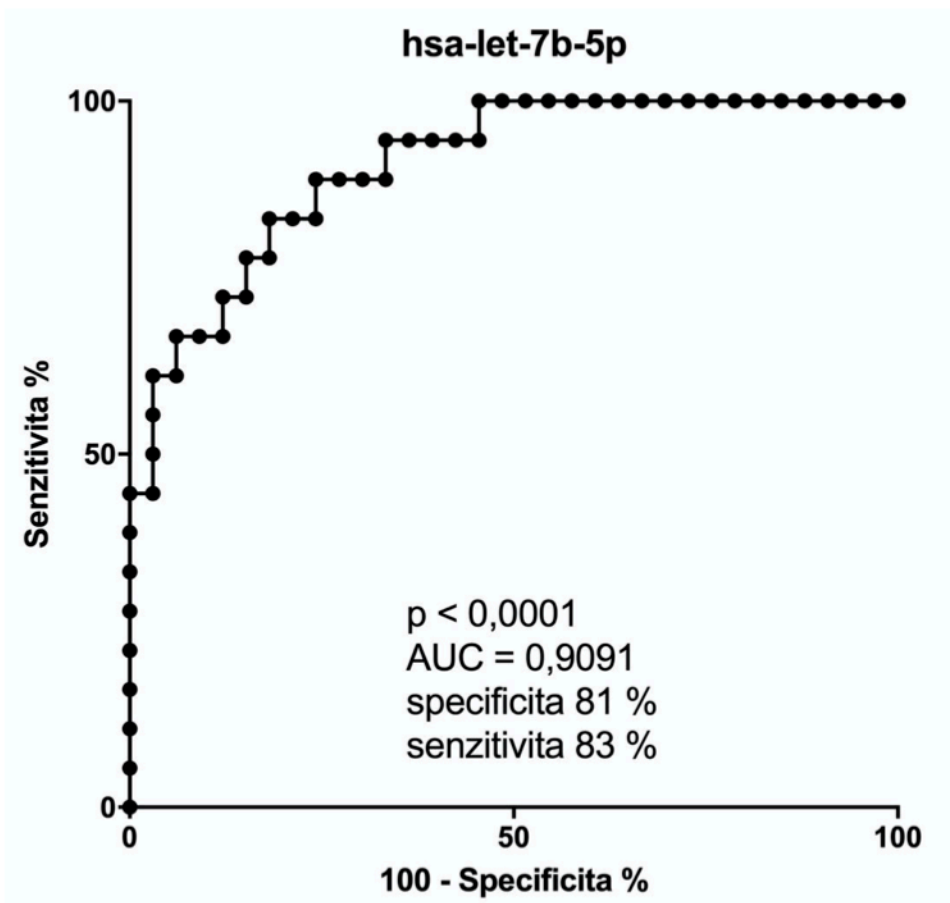
standard odpovídající hladinám let-7i-5p, miR-151a-3p a miR-423-3p ve vzorcích pacientů s glioblastomem, meningeomem a/nebo mozkovými metastázami; a směsný qRT-PCR standard odpovídající hladinám uvedených miRNA u jedinců bez jakéhokoliv prokázaného nádorového onemocnění.

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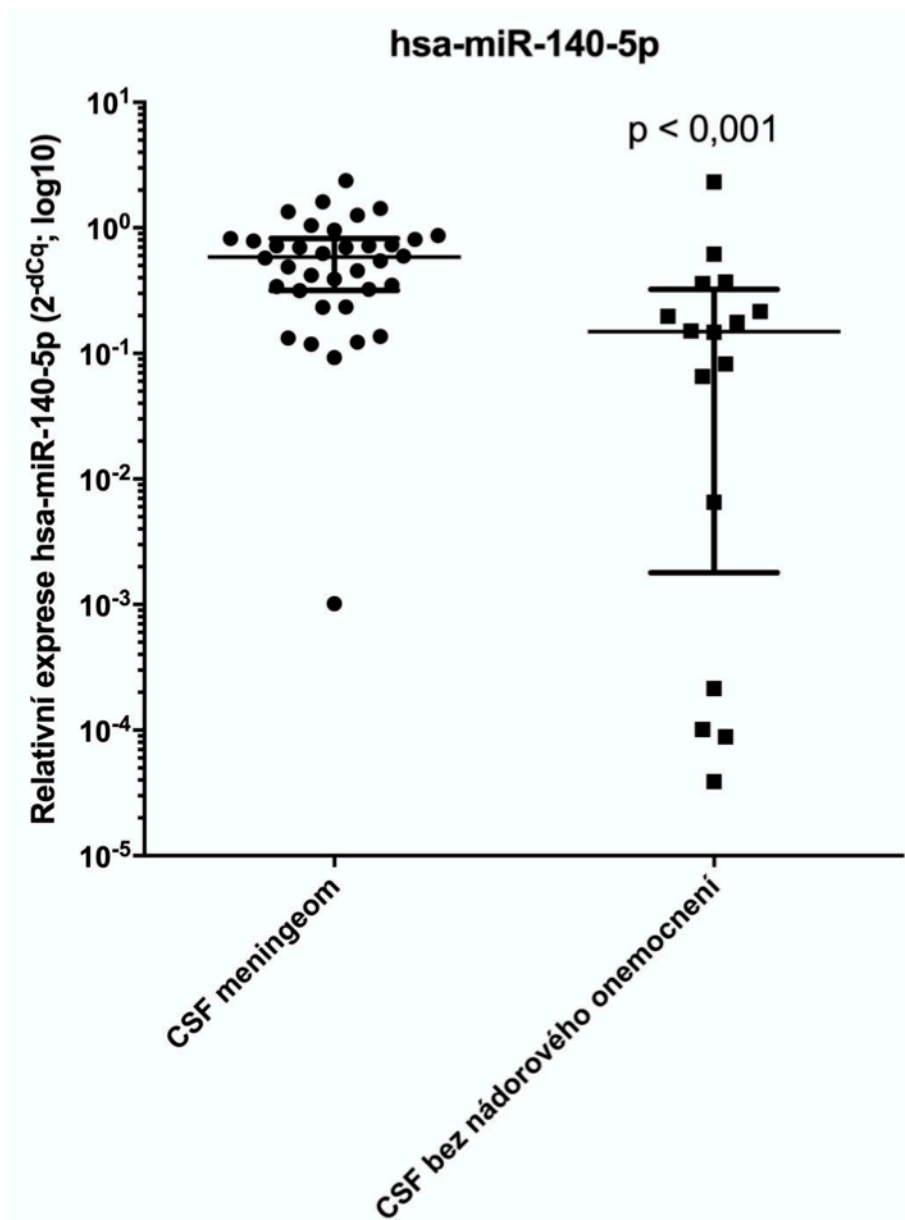
6 výkresů



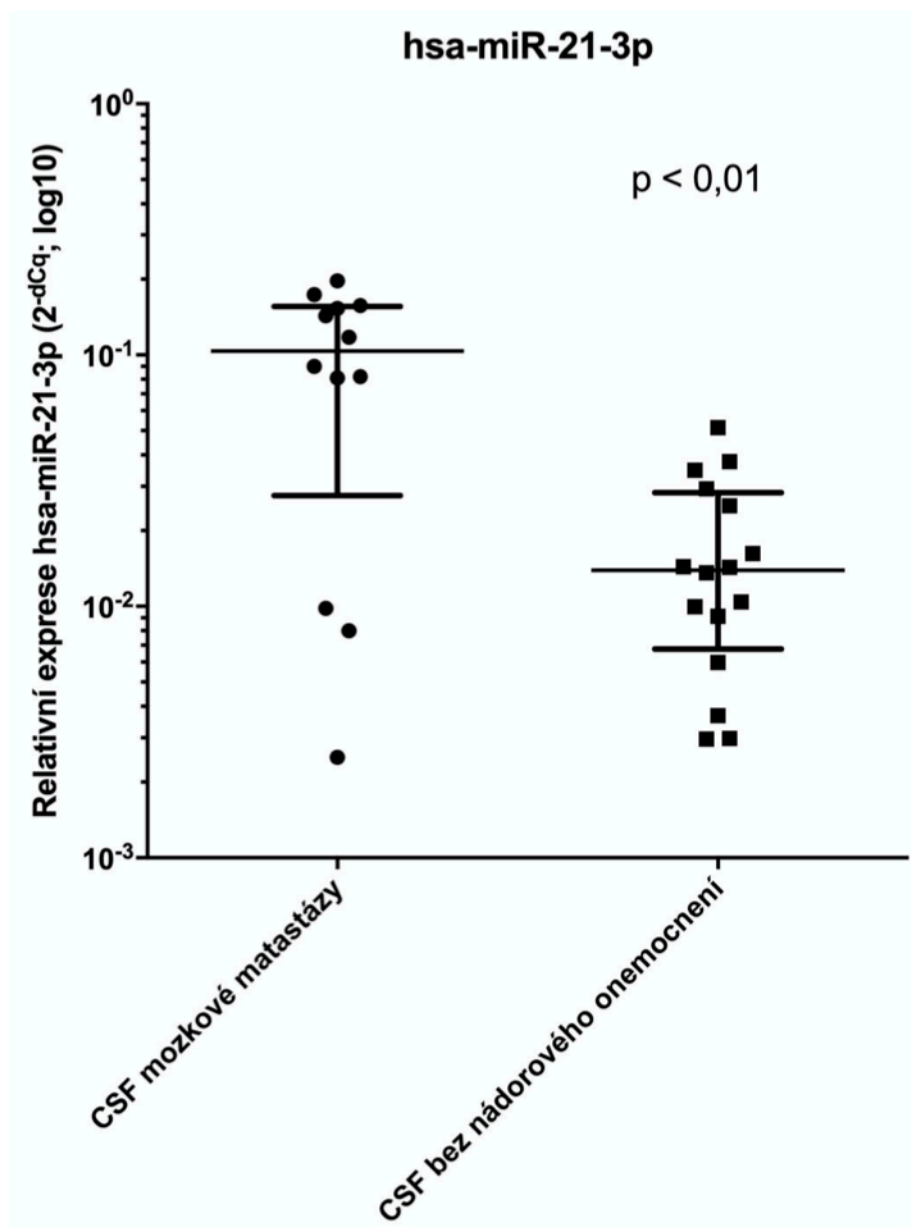
Obr. 1



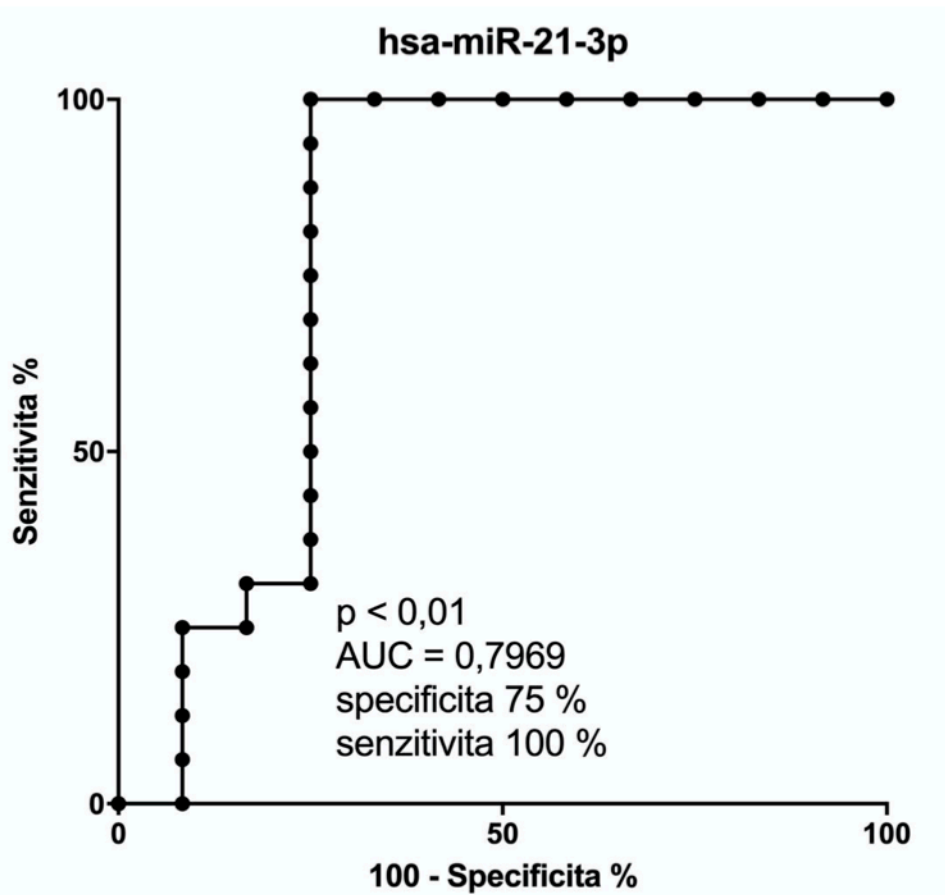
Obr. 2



Obr. 3



Obr. 5



Obr. 6

PŘÍLOHA 2

REVIEW

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Novel classes of non-coding RNAs and cancer

Jiri Sana^{1,2}, Petra Faltejskova^{1,2}, Marek Svoboda¹ and Ondrej Slaby^{1,2,3*}

Abstract

For the many years, the central dogma of molecular biology has been that RNA functions mainly as an informational intermediate between a DNA sequence and its encoded protein. But one of the great surprises of modern biology was the discovery that protein-coding genes represent less than 2% of the total genome sequence, and subsequently the fact that at least 90% of the human genome is actively transcribed. Thus, the human transcriptome was found to be more complex than a collection of protein-coding genes and their splice variants. Although initially argued to be spurious transcriptional noise or accumulated evolutionary debris arising from the early assembly of genes and/or the insertion of mobile genetic elements, recent evidence suggests that the non-coding RNAs (ncRNAs) may play major biological roles in cellular development, physiology and pathologies. ncRNAs could be grouped into two major classes based on the transcript size; small ncRNAs and long ncRNAs. Each of these classes can be further divided, whereas novel subclasses are still being discovered and characterized. Although, in the last years, small ncRNAs called microRNAs were studied most frequently with more than ten thousand hits at PubMed database, recently, evidence has begun to accumulate describing the molecular mechanisms by which a wide range of novel RNA species function, providing insight into their functional roles in cellular biology and in human disease. In this review, we summarize newly discovered classes of ncRNAs, and highlight their functioning in cancer biology and potential usage as biomarkers or therapeutic targets.

Keywords: Non-coding RNAs, microRNAs, siRNAs, piRNAs, lncRNAs, Cancer

Introduction

The abundance of non-translated functional RNAs in the cell has been a textbook truth for decades. Most of these non-coding RNAs (ncRNAs) fulfil essential functions, such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) involved in mRNA translation, small nuclear RNAs (snRNAs) involved in splicing and small nucleolar RNAs (snoRNAs) involved in the modification of rRNAs. The central dogma of molecular biology, developed from the study of simple organisms like *Escherichia coli*, has been that RNA functions mainly as an informational intermediate between a DNA sequence ('gene') and its encoded protein. The presumption was that most genetic information that specifies biological form and phenotype is expressed as proteins, which have not only diverse catalytic and structural functions, but also regulate the activity of the system in various ways.

This is largely true in prokaryotes and presumed also to be true in eukaryotes [1]. But one of the great surprises of modern biology was definitely the discovery that the human genome encodes only ~20,000 protein-coding genes, representing less than 2% of the total genome sequence (see Figure 1). Subsequently, with the advent of tiling resolution genomic microarrays and whole genome and transcriptome sequencing technologies (ENCODE project) it was determined that at least 90% of the genome is actively transcribed. The human transcriptome was found to be more complex than a collection of protein-coding genes and their splice variants; showing extensive antisense, overlapping and ncRNA expression [1,2]. Although initially argued to be spurious transcriptional noise or accumulated evolutionary debris arising from the early assembly of genes and/or the insertion of mobile genetic elements, recent evidence suggests that the proverbial "dark matter" of the genome may play a major biological role in cellular development, physiology and pathologies. In general, the more complex an organism, the greater is its number of ncRNAs. The enticing possibility that although the number of protein-coding

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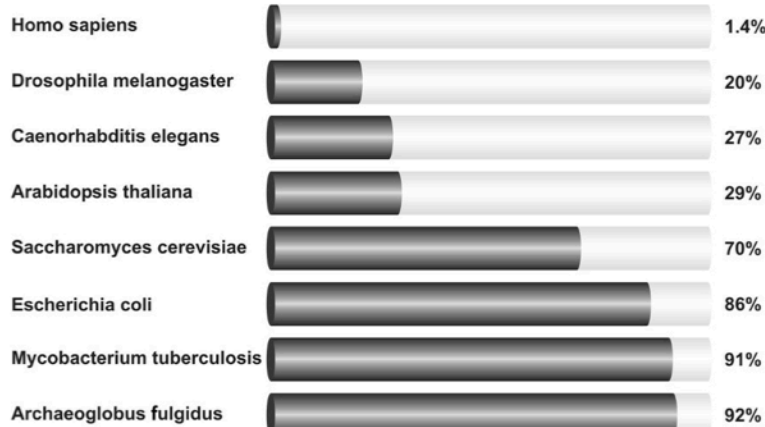


Figure 1 The percentage of protein-coding genes sequences in several eukaryotic and bacterial genomes.

transcripts between organisms is similar, the ultimate control of cellular function may be through interactions between proteins and ncRNA, is corroborated by the fact that the majority of chromatin-modifying complexes do not have DNA binding capacity and therefore, must utilize a third party in binding to DNA. It has been largely demonstrated that this third party may be represented by transcription factors as well as by ncRNAs [2,3].

The beginnings of the present-day understanding on regulatory non-coding RNAs were inspired mainly by the pioneering ideas of John S. Mattick, who has long argued that proteins comprise only a minority of the eukaryotic genome's information output. Considering unique ability of RNA to both fold in three-dimensional space and hybridize in a sequence-specific manner to other nucleic acids, ncRNAs are proposed to behave as a digital-to-analogue processing network, allowing the expansion of complexity in biological systems, well beyond purely protein-based regulatory networks [4].

Non-coding RNAs are grouped into two major classes based on transcript size; small ncRNAs and long ncRNAs (lncRNAs) (classification of recently discovered non-coding RNAs is summarized in Table 1). Small ncRNAs are represented by a broad range of known and newly discovered RNA species, with many being associated with 5' or 3' regions of protein-coding genes. This class includes the well-documented miRNAs, siRNAs, piRNAs, etc. Most of them significantly extended our view of molecular carcinogenesis, and at present they are subject of intensive translational research in this field. In contrast to miRNAs, lncRNAs are mRNA-like transcripts ranging in length from 200 nt to ~100 kilobases (kb) and lacking significant open reading frames. LncRNAs' expression levels appear to be lower than protein-coding genes, and some lncRNAs are preferentially expressed in specific tissues. The small number of

characterized human lncRNAs have been associated with a spectrum of biological processes including alternative splicing or nuclear import. Moreover they can serve as structural components, precursors to small RNAs and even as regulators of mRNA decay. Furthermore, accumulating reports of misregulated lncRNA (HOTAIR, MALAT1, HULC, T-UCRs, etc.) expressions across numerous cancer types suggest that aberrant lncRNA expression may be an important contributor to tumorigenesis. In this review, we summarize recent knowledge of novel classes of ncRNAs, their biology and function, with special focus on their significance in cancer biology and oncology translational research, which is the field where the number of publications focusing this topic is rapidly growing [5-7].

Small non-coding RNAs

Post-transcriptional RNA silencing or RNA interference (RNAi) is a naturally conserved mechanism of regulation of gene expression described in almost all eukaryotic species including humans [8,9]. It is mostly triggered by dsRNA precursors that vary in length and origin. These dsRNAs are rapidly processed into short RNA duplexes subsequently generating small ncRNAs (small ncRNAs), which are associated with Argonaute family proteins and guide the recognition and ultimately the cleavage or translational repression of complementary single-stranded RNAs, such as messenger RNAs or viral genomic/antigenomic RNAs. Moreover, the small ncRNAs have also been implicated in guiding chromatin modifications [9,10]. Since the discovery of the first small ncRNA, various classes of small ncRNAs have been identified. Based on whether their biogenesis is dependent on Dicer, the dsRNA specific RNA III ribonuclease, all the known eukaryotic small ncRNAs can be classified into two groups: Dicer-dependent, such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and in some cases small nucleolar RNAs (snoRNAs); and

Table 1 Types of recently discovered human non-coding RNAs

	Class	Symbol	Characteristic	Disease / biological function associations
Small non-coding RNAs	MicroRNAs	miRNAs	18–25 nt; account 1–2% of the human genome; control the 50% of protein-coding genes; guide suppression of translation; Drosha and Dicer dependent small ncRNAs	initiation of various disorders including many, if not all, cancers / regulation of proliferation, differentiation, and apoptosis involved in human development
	Small interfering RNAs	siRNAs	19–23 nt; made by Dicer processing; guide sequence specific degradation of target mRNA	great potential in diseases treatment / posttranscriptional gene silencing mainly through RISC degradation mechanism; defence against pathogenic nucleic acids
	Piwi-interacting RNAs	piRNAs	26–30 nt; bind Piwi proteins; Dicer independent; exist in genome clusters; principally restricted to the germline and somatic cells bordering the germline	relationship between piRNAs and diseases has not yet been discovered / involved in germ cell development, stem self-renewal, and retrotransposon silencing
	Small nucleolar RNAs	snoRNAs	60–300 nt; enriched in the nucleolus; in vertebrate are excised from pre-mRNA introns; bind snoRNP proteins	association with development of some cancers / important function in the maturation of other non-coding RNAs, above all, rRNAs and snRNAs; miRNA-like snoRNAs regulate mRNAs
	Promoter-associated small RNAs	PASRs	20–200 nt; modified 5' (capped) ends; coincide with the transcriptional start sites of protein- and non-coding genes; made from transcription of short capped transcripts	relationship with diseases has not yet been discovered / involved in the regulation of the transcription of protein-coding genes by targeting epigenetic silencing complexes
	Transcription initiation RNAs	tiRNAs	~ 18 nt ; have the highest density just downstream of transcriptional start sites; show patterns of positional conservation; preferentially located in GC-rich promoters	
	Centromere repeat associated small interacting RNAs	crasiRNAs	34–42 nt; processed from long dsRNAs	relationship between crasiRNAs and diseases has not yet been discovered / involved in the recruitment of heterochromatin and/or centromeric proteins
	Telomere-specific small RNAs	tel-sRNAs	~ 24 nt; Dicer independent; 2'-O-methylated at the 3' terminus; evolutionarily conserved from protozoa to mammals; have not been described in human up to now	relationship between tel-sRNAs and diseases has not yet been discovered / epigenetic regulation
	Pyknons		subset of patterns of variable length; form mosaics in untranslated and protein-coding regions; more frequently in 3' UTR	expected association with cancer biology / possible link with posttranscriptional silencing of genes, mainly involved in cell communication, regulation of transcription, signaling, transport, etc.
Long non-coding RNAs	Long intergenic non-coding RNAs	lincRNAs	ranging from several hundreds to tens of thousands nts; lie within the genomic intervals between two genes; transcriptional cis-regulation of neighbouring genes	involved in tumorigenesis and cancer metastasis / involved in diverse biological processes such as dosage compensation and/or imprinting
	Long intronic non-coding RNAs		lie within the introns; evolutionary conserved; tissue and subcellular expression specified	aberrantly expressed in human cancers / possible link with posttranscriptional gene silencing
	Telomere-associated ncRNAs	TERRAs	100 bp - >9 kb; conserved among eukaryotes; synthesized from C-rich strand; polyadenylated; form inter-molecular G-quadruplex structure with single-stranded telomeric DNA	possible impact on telomere-associated diseases including many cancers / negative regulation of telomere length and activity through inhibition of telomerase
	Long non-coding RNAs with dual functions		both protein-coding and functionally regulatory RNA capacity	deregulation has been described in breast and ovarian tumors / modulate gene expression through diverse mechanisms
	Pseudogene RNAs		gene copies that have lost the ability to code for a protein; potential to regulate their protein-coding cousin; made through retrotrans-position; tissue specific	often deregulated during tumorigenesis and cancer progression / regulation of tumor suppressors and oncogenes by acting as microRNA decoys
	Transcribed-ultraconserved regions	T-UCRs	longer than 200 bp; absolutely conserved between orthologous regions of human, rat, and mouse; located in both intra- and intergenic regions	expression is often altered in some cancers; possible involvement in tumorigenesis / antisense inhibitors for protein-coding genes or other ncRNAs

Dicer-independent small ncRNAs, such as PIWI-interacting RNAs (piRNAs) [11] (Figure 2). Moreover, phylogenetic analysis indicates that known Argonaute family proteins can be divided into two subgroups namely AGO based on AGO1 and PIWI based on PIWI. Interestingly, Ago proteins interact with miRNAs and siRNAs while Piwi subgroup is characterized by interaction with piRNAs [12]. Biogenesis of other small non-coding RNAs is less or completely undescribed yet. These RNAs are generally classified according to their genome and function localization. Among them belong promoter-associated small RNAs (PASRs), transcription initiation RNAs (tiRNAs), centromere repeat associated small interacting RNAs (crasiRNAs), and telomere-specific small RNAs (tel-sRNAs). To the class of small non-coding RNAs also belong the recently discovered pyknons that, as suggested by current findings, are involved in many biological functions. It was many times described that some of above mentioned small non-coding RNAs play important roles in pathogenesis of various diseases including tumors. In this respect, the most studied ncRNAs are miRNAs, which have been described in many, if not all, cancers [13-16].

MicroRNAs

The most frequently studied subclass of small ncRNAs are microRNAs (miRNAs), originally discovered by Victor Ambros in *Caenorhabditis elegans*. They are 18–25 nucleotides long, evolutionary conserved, single-stranded

RNA molecules involved in specific regulation of gene expression in eukaryotes [17]. It is predicted that miRNA genes account for 1–2% of the human genome and control the activity of ~50% of all protein-coding genes [18,19]. Early annotation for the genomic position of miRNAs indicated that most miRNAs are located in intergenic regions (>1 kb away from annotated or predicted genes), although a sizeable minority was found in the intronic regions of known genes in the sense or antisense orientation. This led to the postulation that most miRNA genes are transcribed as autonomous transcription units [19]. A detailed analysis of miRNA gene expression showed that miRNA genes can be transcribed from their own promoters and that miRNAs are generated by RNA polymerase II (RNAPII) as primary transcripts (pri-miRNAs). These are processed to short 70-nucleotide stem-loop structures known as pre-miRNAs by the ribonuclease called Drosha and the double-stranded-RNA-binding protein known as Pasha (or DGCR8 – DiGeorge critical region 8), which together compose a multiprotein complex termed a microprocessor. The pre-miRNAs are transported to cytoplasm by the RAN GTP-dependent transporter exportin 5 (XPO5). In the cytoplasm, the pre-miRNAs are processed to mature miRNA duplexes by their interaction with the endonuclease enzyme Dicer in complex with dsRNA binding protein TRBP [19,20]. One strand (“guide strand”) of the resulting 18–25-nucleotide mature miRNA duplex ultimately gets integrated into the miRNA-induced silencing complex (miRISC) with the

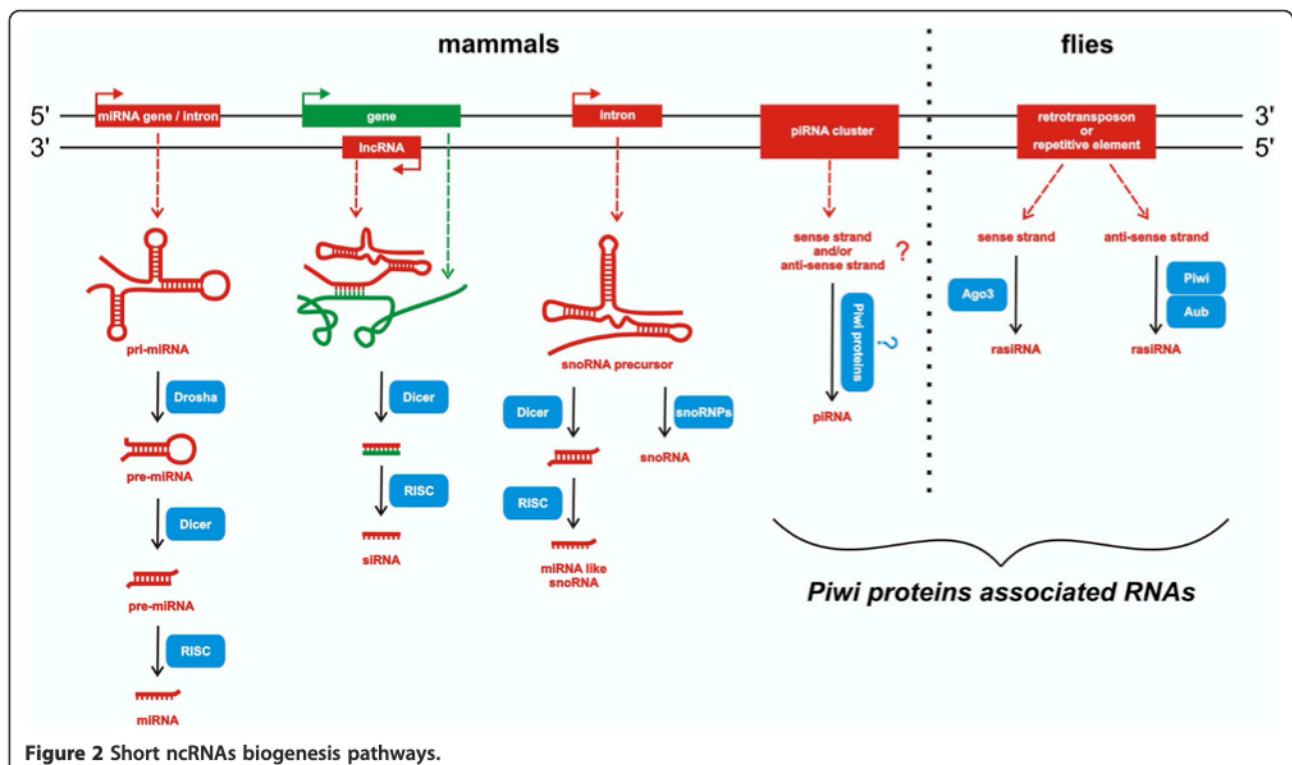


Figure 2 Short ncRNAs biogenesis pathways.

central part formed by proteins of the Argonaute family, whereas the other strand (passenger or miRNA*) is released and degraded. The retained ("guide") strand is the one that has the less stably base-paired 5' end in the miRNA/miRNA* duplex. Generally, most miRNA genes produce one dominant miRNA species. However, the ratio of miRNA to miRNA* can vary in different tissues or developmental stages, which probably depends on specific properties of the pre-miRNA or miRNA duplex, or on the activity of different accessory processing factors [19]. Moreover, the ratio might be modulated by the availability of mRNA targets as a result of enhanced destabilization of either miRNA or miRNA* occurring in the absence of respective complementary mRNAs [20]. Mature miRNAs in miRISC exert their regulatory effects by binding to imperfect complementary sites. MiRNAs repress target-gene expression post-transcriptionally, apparently at the level of translation, through a miRISC complex that is similar to, or possibly identical with, that used for the RNAi pathway discussed later. Perfect complementarity of mRNA-miRNA allows Ago-catalyzed cleavage of the mRNA strand, whereas central mismatches exclude cleavage and promote repression of mRNA translation. Consistent with translational control, miRNAs that use this mechanism reduce the protein levels of their target genes, but the mRNA levels of these genes are barely affected [21-23]. Current studies indicate that miRNA targeting in mammalian cells occurs predominantly through binding to sequences within 3'UTRs [24,25], however inhibition of gene expression through targeting the 5'UTR has been also demonstrated [26]. Nevertheless, statistical analyses of conserved miRNA target sequences proved that mammalian miRNA target sites rarely occur within 5'UTRs [24,25,27]. Moreover, it was found out that miR-10a induces, rather than inhibits, protein expression through binding to 5'UTRs of cellular transcripts [23]. It is therefore supposed that binding to 5'UTR results in mechanistic effects divergent from 3'UTR binding.

Most of the miRNAs described to date regulate crucial cell processes such as proliferation, differentiation, and apoptosis. Therefore, these RNAs are involved in human development as well as in initiation of various disorders including many, if not all, cancers where miRNAs have been found to be also significant prognostic and predictive markers [13,28-35]. Examples of miRNAs with significant functional effects in cancer are mentioned below.

Bloomston *et al.* [36] identified 6 miRNAs linked to long-term survival in pancreatic adenocarcinoma. They found also that expression level of miR-196a-2 was able to predict patients' survival, since higher miRNA levels marked the poor survivors group. In HCC, up-regulation of miR-221 and down-regulation of

miR-122 were associated with shorter time to recurrence [37,38]. MiR-21 is up-regulated in many solid tumors, including CRC. Slabý *et al.* [39] proved that miR-21 over-expression shows a strong correlation with the established prognostic factors as nodal stage, metastatic disease and UICC stage. Moreover, Kulda *et al.* [40] correlated miR-21 expression to disease-free interval (DFI). There was shorter DFI in patients with a higher expression of miR-21. Several studies proved that down-regulated expression of miR-221, miR-137, miR-372, miR-182*, let-7 and miR-34a is associated with shorter survival in patients with lung cancer [41-43]. Breast cancer metastatic process has been connected with up-regulation of miR-10b [44] and with loss of expression of miR-126 and miR-335 [45]. Finally, higher levels of miR-15b were associated with poor survival and recurrence in melanoma [46]. Another important question for management of cancer patients is the possibility of predicting therapy response. Nakajima *et al.* [47] identified let-7g and miR-181b as significant indicators for chemoresponse to S-1-based chemotherapy. The same year, Markou *et al.* [48] demonstrated that inhibition of miR-21 and miR-200b increases the sensitivity of cholangiocarcinoma cells to gemcitabine. Yang *et al.* [49] identified miR-214, a miRNA up-regulated in ovarian cancer, as responsible of cisplatin resistance through its action on PTEN/AKT pathway. Subsequently, there is a large number of publications which confirmed many 3'UTRs of oncogenes and tumor suppressor genes to be direct targets of selected miRNAs. According to a recent study by Nagel *et al.* [50], miR-135a and miR-135b decrease translation of the APC transcript *in vitro*. Concerning CRC, KRAS oncogene has been reported to be a direct target of the let-7 miRNA [51]. Another miRNA associated with KRAS regulation is miR-143 [52]. MiRNAs arrays-based studies revealed the p85 β regulatory subunit of PI3K as a direct target of miR-126 [53]. Moreover, another important regulatory component of PI3K pathway, the tumor suppressor gene *PTEN*, is strongly repressed by miR-21 in hepatocellular carcinoma [54]. MiR-17-5p belongs to a highly conserved, polycistronic miRNA cluster miR-17-92. Yu *et al.* [55] described the function of this cluster as a negative regulator of cell cycle and proliferation of human breast cancer cells, which directly regulates cyclin D1 (CCND1). The same cluster is also involved in malignancies of B cell origin [56] and a direct regulation by c-MYC has been reported [57,58]. Some of the most often deregulated miRNAs with their experimentally proved mRNA targets are summarized in the Table 2, however, the number of described miRNAs and putative targets is much more higher and it is not possible to mention all of them.

Table 2 Gene targets of the most common described human cancer-associated miRNAs

MiRNA	Associated cancers	<i>In vitro</i> confirmed gene targets
MiR-21	CRC, PC, RCC, GBM, BrC, NSCLC, BCL, PTC, HCC, HNSCC, ESCC, GC, CML, CCC, MM, OC, M, LC, PDA	PDCD4, TIMP3, RhoB, Spry1, PTEN, TM1, CDK2AP1, ANP32A, SMARCA4, ANKRD46, THRB, Cdc25A, BMPRII, LRRFIP1, BTG2, MARCKS, TPM1
MiR-155	NSCLC, SCLC, HCC, BrC, M, CCC, HL, PDA, RCC, GBM, PTC, CML, CRC, SPA, AML, NPC, CLL	FOXO3A, SOX6, SATB1, SKI, Wee1, SOCS1, SHIP1, S/EBP β , IFN- γ R α , AGTR1, FGF7, ZNF537, ZIC3, IKK β , RhoA, BACH1, ZIC3, HIVEP2, CEBPB, ZNF652, ARID2, SMAD5, TP53INP1
MiR-145	BrC, CRC, ESCC, NSCLC, PC, BCL, OC, GC, BIC, NPC, HCC	c-Myc, ERK5, FSCN1, SMAD2/3, IGF-1R, FLI1, DFF45, mucin 1, MYO6, CBF β , PPP3CA, CLINT1, ICP4, RTKN
MiR-221	BrC, PC, CRC, M, GBM, ALL, HCC, PTC, PDA, GC, CML, NSCLC, AML, OC	DVL2, KIT, CDKN1B, Bmf, p27, HOXB5, CDKN1C/p57, CDKN1B/p27, MMP1, SOD2, TIMP3, Dicer1, ER α , ARHI, PUMA, p27Kip1, p57
Let-7a	M, HL, nHL, CRC, SLC, NSCLC, GC, HNSCC, ESCC, OC, CLL, HCC	PRDM-1, STAT3, Caspase-3, Integrin β 3, PRDM1/blimp-1
MiR-16	LC, OC, NPC, GC, PC, BrC, HCC, MM, CLL, HL	VEGFR2, FGFR1, Zyxin, Cyclin E1, Bmi-1, BRCA-1, BCL2
MiR-200	BrC, PDA, GC, HNSCC, M, OC, PC	FN1, MSN, NTRK2, LEPR, ARHGAP19, ZEB1/2, Fli1/VEGFR1, FAP-1, FOG2, ERRFI-1
MiR-205	M, BrC, PC, ESCC, HNSCC	Runx2, E2F1, ErbB3, Zeb1
MiR-31	PTC, CRC, BrC, LC, GC, HCC	LATS2, WAVE3, SATB2, ITGA5, RDX, RhoA, FIH
MiR-126	CRC, GC, BrC, SCLC, AML, NSCLC, HCC	SLC7A5, SOX2, PLAC1, VEGFA, PIK3R2, Crk, EGFL7, p85 β
MiR-210	PDA, RCC, BrC, PC, GBM, NSCLC, OC, GC, HNSCC	FGFRL1, SDHD, MNT
MiR-9	GBM, PC, nHL, EC, OC	CAMTA1, PDGFR- β , CDX2, PRDM-1, E-cadherin, NF-kappaB1
MiR-141	PC, EC, CRC, HNSCC, LC, BrC, ESCC, OC, RCC	SIP1, YAP1
MiR-122	HCC, RCC	Bcl-w, ADAM17

CRC colorectal cancer, PC prostate cancer, RCC renal cell carcinoma, GBM glioblastoma multiforme, BrC breast cancer, LC lung cancer, NSCLC non-small cell lung cancer, SCLC small cell lung cancer, BCL B-cell lymphoma, PTC papillary thyroid carcinoma, HCC hepatocellular carcinoma, HNSCC head and neck squamous cell carcinoma, ESCC esophagus squamous cell carcinoma, GC gastric cancer, CLL chronic lymphocytic leukemia, CML chronic myelogenous leukemia, ALL acute lymphocytic leukemia, AML acute myeloid leukemia, CCC cervical cell carcinoma, MM multiple myeloma, OC ovarian cancer, M melanoma, LC laryngeal carcinoma, PDA pancreatic ductal adenocarcinoma, HL Hodgkin lymphoma, nHL Non-Hodgkin lymphoma, SPA sporadic pituitary adenomas, NPC nasopharyngeal carcinoma, BIC bladder cancer, EC endometrial cancer.

Small interfering RNAs

Another class of small ncRNAs involved in post-transcriptional RNA silencing are so-called small interfering RNAs (siRNAs). They are produced from long dsRNAs of exogenous or endogenous origin [59]. These short helical RNA molecules are formed by two at least partially complementary RNA single strands, namely the passenger strand and the guide strand. Typical strand lengths of these dsRNAs are 19–23 nucleotides and they are made by Dicer processing as miRNAs [60]. One of the arisen single strands is subsequently incorporated into RISC (RNA-induced silencing complex) where guides sequence-specific degradation of complementary target mRNAs unlike miRNA that rather suppresses translation and does not lead to degradation of the mRNA target [9,61,62]. SiRNAs are worldwide used in gene silencing experiments and have become a specific and powerful tool to turn off the expression of target genes, and also turned into a promising experimental tool in molecular oncology. SiRNAs could be used in cancer therapy by several strategies. These include the suppression of overexpressed oncogenes, retarding cell division by interfering with cyclins and related genes or enhancing apoptosis by inhibiting anti-apoptotic genes. For example, Vassilev *et al.* [63] developed new siRNA-based inhibitors of the p53-MDM2 protein interaction. A year later, Wu *et al.* [30] demonstrated that down-regulation of RPL6 (ribosomal protein L6) in gastric cancer SGC7901 and AGS cell lines by siRNA reduced colony forming ability and cell growth. Moreover, the cell cycle of these cells was suppressed in G1 phase. Similarly, CDK8 specific siRNA transfection down-regulated the expression of CDK8 in colon cancer cells, which was also associated with a decrease in the expression of β -catenin, inhibition of proliferation, increased apoptosis and G0/G1 cell cycle arrest [64]. Dufort *et al.* [65] described that cell transfection of IGF-IR siRNAs decreased proliferation, diminished phosphorylation of downstream signaling pathway proteins, AKT and ERK, and caused a G0/G1 cell cycle block in two murine breast cancer cell lines, EMT6 and C4HD. The IGF-IR silencing also induced secretion of two proinflammatory cytokines, TNF- α and IFN- γ . Another study showed that mTOR-siRNA transfection significantly inhibits cell proliferation, increases the level of apoptosis and decreases migration of NSCLC cells, and could be used as an alternative therapy targeting mTOR with fewer side effects [66]. RNAi against multidrug resistance genes or chemoradioresistance and angiogenesis targets may also provide beneficial cancer treatments. He *et al.* [67] proved that silencing of MDR1 by siRNA led to decreased P-glycoprotein activity and lower drug resistance of L2-RAC cells, which could be used as a novel approach of combined gene and chemotherapy for yolk

sac carcinoma. Another study showed that combination of proteasome inhibitors with Mcl-1 siRNA enhances the ultimate anticancer effect in DLD-1, LOVO, SW620, HCT-116, SKOV3 and H1299 cell lines [68]. Bansal *et al.* [69] states that selective siRNA depletion of CDK1 increases sensitivity of patients with ovarian cancer to cisplatin-induced apoptosis. The number of publications dealing with siRNAs is rapidly growing and successful cancer therapy by siRNA *in vitro* and *in vivo* provides the enthusiasm for potential therapeutic applications of this technique [70]. Some examples of siRNA cancer therapies in clinical trials are summarized in Table 3.

Piwi proteins associated RNAs

Extensive research in the past few years has revealed that members of the Argonaute protein family are key players in gene-silencing pathways guided by small RNAs. This family is further divided into AGO and PIWI subfamilies [72]. It was proved that the AGO proteins are present in diverse tissues and bind to miRNAs and siRNAs, whereas PIWI proteins are especially present in germline, and associate with a new class of small ncRNAs termed PIWI-interaction RNAs (piRNAs). PiRNAs are typically 24–32 nucleotides long RNAs that are generated by a Dicer-independent mechanism. It was thought that they are derived only from transposons and other repeated sequence elements [73] and therefore, they were alternatively designated as repeat-associated small interfering RNAs (rasiRNAs) [74]. But it is now clear that piRNAs can be also derived from complex DNA

sequence elements [75] and that rasiRNAs are a subset of piRNAs.

The precise mechanism of piRNAs biogenesis is not clear, but in 2007 Brennecke *et al.* [73] described a new mechanism similar to secondary siRNA generation, called as ping-pong model. He observed that antisense piRNAs associate with PIWI/AUB complex while sense piRNAs associate with AGO3 protein. This information led to the suggestion that PIWI and AUB proteins bind to maternally deposited piRNAs (primary piRNA) and this complex is subsequently bound to the transcripts produced by retrotransposons and cleaves a transcript generating a sense piRNAs (secondary piRNAs) that bind to AGO3. Finally, piRNA-AGO3 complex binds to the retrotransposon transcript, creating another set of anti-sense piRNAs. However, the model of piRNAs biogenesis is still incomplete and precise mechanisms of action remain poorly characterized (for a review, see [76-78]).

The PIWI subfamily as well as piRNAs have been implicated in germ cell development, stem cell self-renewal, and retrotransposon silencing. Recently, several studies were published describing the association between HIWI (the human ortholog of PIWI) expression and diverse group of cancers including pancreatic [79] and gastric [80] adenocarcinomas, sarcomas [81], hepatocellular carcinomas [82], colorectal cancer [83], gliomas [84] and esophageal squamous cell carcinomas [85]. It was proved that higher levels of HIWI mRNA are connected with worse clinical outcome. Moreover, the expression pattern of HIWI in gastric cancer tissues was similar to that of Ki67 and suppression of HIWI induced cell cycle arrest in G2/M phase [80]. Lee *et al.* [86] described that PIWIL2 (PIWI-like 2) protein is widely expressed in tumors and inhibits apoptosis through activation of STAT3/BCL-X(L) signalling pathway. Similarly, the newest study of Lu *et al.* [87] shows that this protein forms a PIWIL2/STAT3/c-Src complex, where STAT3 is phosphorylated by c-Src and translocated to nucleus. Subsequently, STAT3 binds to *P53* promoter and represses its transcription. These findings indicate that PIWI proteins may be involved in the development of different types of cancer and could be a potential target for cancer therapy. Recently, it was also proved, that not only PIWI proteins, but also piRNAs can play an important role in carcinogenesis. It was discovered that expression of piR-823 in gastric cancer tissues was significantly lower than in non-cancerous tissues. Artificial increase of the piR-823 levels in gastric cancer cells inhibited their growth. Moreover, the observations from the xenograft nude mice model confirmed its tumor suppressive properties [88]. On the contrary, levels of the piR-651 were upregulated in gastric, colon, lung, and breast cancer tissues compared to the paired

Table 3 Small RNA-based therapeutics in clinical trials (adapted from [71])

Gene target	Drug type	Drug name	Clinical phase	Notes
Bcl-2	LNA-oligo	SPC2996	I/II	CLL
Immunoproteasome β -subunits LMP2, LMP7 and MECL1	siRNA	Proteasome siRNA	I	Metastatic lymphoma
PLK1	siRNA	PLK SNALP	pre-clinical	
M2 subunit of ribonucleotide reductase	siRNA	CALAA-01	I	Solid tumors
PKN3	siRNA	Atu027	I	Solid tumors
KSP and VEGF	siRNA	ALN-VSP	I	Solid tumors
Survivin	LNA-oligo	EZN3042	I/II	Solid tumors
HIF-1 α	LNA-oligo	EZN2968	I/II	Solid tumors
Furin	shRNA	FANG vaccine	I	Solid tumors
eIF-4E	LNA-oligo	eIF-4E ASO	I	Solid tumors
Survivin	LNA-oligo	Survivin ASO	II	Solid tumors

non-cancerous tissues. The growth of gastric cancer cells was efficiently inhibited by a piR-651 inhibitor and the cells were arrested at the G2/M phase [89]. Interestingly, the peripheral blood levels of piR-651 and piR-823 in the patients with gastric cancer were significantly lower than those from controls. Thus, piRNAs may be valuable biomarkers for detecting circulating gastric cancer cells [90]. Resolving the function of PIWI proteins and piRNAs has broad implications not only in understanding their essential role in fertility, germline, stem cell development, and basic control and evolution of animal genomes, but also in the biology of cancers [12].

Small nucleolar RNAs

Small nucleolar RNAs (snoRNAs), 60 – 300 nucleotides long, represent one of the abundant groups of small ncRNAs characterized in eukaryotes. SnoRNAs are enriched in the nucleolus, which is the most prominent organelle in the interphase nucleus providing the cellular locale for the synthesis and processing of cytoplasmic ribosomal RNAs (rRNAs) [91]. Most of the snoRNAs are located within introns of protein-coding genes and are transcribed by RNA polymerase II, however, they can also be processed from introns of longer ncRNA precursors [92]. Nevertheless, while vertebrate snoRNAs are prevalently excised from pre-mRNA introns, in plant and yeast these RNAs are mainly generated from independent transcription units, as either monocistronic or (especially in plants) polycistronic snoRNA transcripts [93].

All snoRNAs fall into two major classes based on the presence of short consensus sequence motifs. First group contains the box C (RUGAUGA) and D (CUGA) motifs, whereas members of the second group are characterized by the box H (ANANNA) and ACA elements [94]. In both classes of snoRNAs, short stems bring the conserved boxes close to one another to constitute the structural core motifs of the snoRNAs, which coordinate the binding of specific proteins to form small nucleolar RNPs (snoRNPs) distinct for both groups [91,95]. SnoRNAs have important functions in the maturation of other non-coding RNAs. Above all, they manage post-transcriptional modification of rRNA and snRNA by 2'-O-methylation and pseudouridylation (for a review, see [91]). Interestingly, it was identified number of human snoRNAs with miRNA-like function. These snoRNAs are processed to small 20–25 nucleotides long RNAs that stably associate with Ago proteins. Processing is independent of the Drosha, but requires Dicer. Moreover, cellular target mRNA, whose activity is regulated by snoRNA, was identified [96].

Several studies have indicated that alterations of snoRNAs play important functions in cancer development and progression. The first report linking snoRNAs to

cancer was published in 2002 by Chang *et al.* [97]. He proved that h5sn2, a box H/ACA snoRNA, was significantly downregulated in human meningiomas compared with normal brain tissues. Subsequently, Dong *et al.* [98] identified snoRNA U50 as a reasonable candidate for the 6q tumor-suppressor gene in prostate cancer and this statement was confirmed in another study describing involvement of snoRNAs U50 in the development and/or progression of breast cancer [99]. Interestingly, chromosome 6q14-15 is a breakpoint of chromosomal translocation t(3;6)(q27;q15) for human B-cell lymphoma [100]. The same year, the GAS5 (growth arrest-specific transcript 5) was identified to control mammalian apoptosis and cell growth. GAS5 transcript levels were found to be significantly lower in breast cancer samples relative to adjacent unaffected normal breast epithelial tissues and despite the fact that this gene has no significant protein-coding potential, it was proved that several snoRNAs are encoded in its introns [101]. By profiling ncRNAs signatures in NSCLC tissues and matched noncancerous lung tissues, four snoRNAs (snoRD33, snoRD66, snoRD76 [102] and snoRA42 [103]) were found to be overexpressed in lung tumor tissues and it is supposed that they could be used as potential markers for early detection of non-small cell lung cancer [102]. Moreover, snoRD33 is located at chromosome 19q13.3 that contains oncogenes involved in different malignances including lung cancer, whereas snoRD66 and snoRD76 are located at chromosomal regions 3q27.1 and 1q25.1, respectively. These two chromosomal segments are the most frequently amplified in human solid tumors [28,104,105]. Recently, low levels of four snoRNAs (RNU44, RNU48, RNU43, RNU6B), commonly used for normalization of miRNA expression, were associated with a poor prognosis of the cancer patients [106]. Martens-Uzunova *et al.* [107] analyzed the composition of the entire small transcriptome by Illumina/Solexa deep sequencing and he revealed several snoRNAs with deregulated expression in samples of patients with prostate cancer. The newest publication concerning snoRNAs proved that snoRD112-114 located at the DLK1-DIO3 locus are ectopically expressed in acute promyelotic leukemia (APL), which shows that a relationship exists between a chromosomal translocation and expression of snoRNA loci. Moreover, *in vitro* experiments revealed that the snoRD114-1 [14q (II-1)] variant promotes cell growth through G0/G1 to S phase transition mediated by the Rb/p16 pathways [108]. Finally, it was also published that snoRNAs are present in stable form in plasma and serum samples [102,106] and therefore could be used as fluid-based biomarkers for cancers. These facts indicate that snoRNAs are critically associated with the development and progression of cancer, however further research for

comprehensive understanding their role in carcinogenesis is required.

Promoter-associated RNAs

Recently, a new class of ncRNAs known as promoter-associated RNAs (paRNAs) (sometimes termed as promoter-upstream transcripts – PROMPTs [109], transcription start site-associated RNAs [110] or promoter-proximal transcription start site RNAs [111]), were discovered. These ncRNAs are derived from eukaryotic promoters and have the potential to regulate the transcription of protein-coding genes by targeting epigenetic silencing complexes [71,112,113]. Their size ranged from 18 to 200 nucleotides and they include long, small and tiny RNAs.

The short paRNAs (PASRs) were identified in 2007 [114] using RNA maps. They are located near the promoter or transcription start site (TSS), but they are not associated with a known protein-coding genes. These transcripts are 20–90 nt long and it was proved that they are not Dicer product [110]. Human PASRs are expressed at low levels and their number per gene is positively correlated with promoter activity and mRNA level [109]. The tiny paRNAs or transcription initiation RNAs (tiRNAs) are shorter than 23 nt and they are transcribed in both sense and antisense directions around the promoter [115]. Furthermore, they are closely associated with highly expressed promoters and are preferentially located in GC-rich promoters [71,115]. It is still unclear how these two classes of small RNAs are related to one another, or if they share common biogenesis pathways [115]. Recently, a long paRNAs (PALRs, 100–200 nt) has been identified at a single-gene level and they were associated with regulatory functions (for a review, see [112,113,116,117]), especially with modification of DNA methylation [118].

It is supposed, that because of potential of paRNAs to regulate transcription, their deregulation could be associated with different types of diseases, including cancer. It was proved, that transfection of mimetic paRNAs into HeLa and HepG2 cells resulted in the transcriptional repression of human *C-MYC* and connective tissue growth factor (CTGF) [119]. Hawkins *et al.* [120] described that targeting of the human ubiquitin C gene (*UbC*) with a small paRNA led to long-term silencing which correlated with an early increase in histone methylation and a later increase in DNA methylation at the targeted locus. Furthermore, it was shown that PASRs play an important role in maintaining accessible chromatin architecture for transcription and releasing negative supercoils during transcription [110]. Concerning tiRNAs, they may have similar functions like PASRs, moreover they are usually found at CTCF-binding sites. Taft *et al.* [121] proved, that overexpression of tiRNAs decreased CTCF binding and associated gene expression, whereas inhibition of

tiRNAs resulted in increased CTCF localization and associated gene expression. Wang *et al.* [122] described, that an RNA-binding protein TLS (for translocated in liposarcoma) can specifically bind to CREB-binding protein (CBP) and p300 histone acetyltransferase depending on its allosteric modulation by PALRs, and so repress gene target *CCND1* in human cell lines. Finally, it was shown that paRNAs have the potential to form double-stranded RNAs and to be processed into endogenous siRNAs [123]. These facts indicate, that this novel class of ncRNAs has a great potential to regulate expression of various tumor suppressors and oncogenes on transcriptional level and therefore be involved in human cancerogenesis.

Centromere repeat associated small interacting RNAs

Cell stresses can induce incorrect centromere function manifesting in loss of sister chromatid cohesion, abnormal chromosome segregation, and aneuploidy, which have been observed in many human diseases including cancers [124]. These defects are often correlated with the aberrant accumulation of centromere satellite transcripts [125]. Moreover, it was observed that human cells under stress accumulate large transcripts of SatIII satellites [126]. The accumulation of similar transcripts in vertebrate cells is thought to result from defective RNA processing of larger transcripts that leads to a reduction of the small RNAs that participate in the recruitment of specific histones critical for centromere function [125,127]. The research on mammalian model uncovered the strong bidirectional promoter capability of the kangaroo endogenous retrovirus (KERV-1) LTR to produce long double-stranded RNAs for both KERV-1 and surrounding sequences, including sat23. These long dsRNAs are then processed into centromere repeat associated small interacting RNAs (crasiRNAs), 34 - 42 nucleotides in length. Unfortunately, the mechanism by which full-length KERV-1 and sat23 transcripts are processed into crasiRNAs remains unknown. The crasiRNAs are involved in the recruitment of heterochromatin and/or centromeric proteins. These findings have profound implications for understanding of centromere function and epigenetic identity by suggesting that a retrovirus, KERV-1, may participate in the organization of centromere chromatin structures indispensable to chromosome segregation in vertebrates [124]. These small centromere-associated ncRNAs occur conserved among eukaryotes suggesting their impact also in human.

Telomere-specific small RNAs

Another group of recently described short ncRNAs are telomere-specific small RNAs (tel-sRNAs). Tel-sRNAs are ~ 24 nt long, Dicer-independent, and 2'-O-methylated at the 3' terminus. They are asymmetric with specificity

toward telomere G-rich strand, and evolutionarily conserved from protozoan to mammalian cells. Interestingly, tel-sRNAs are up-regulated in cells that carry null mutation of H3K4 methyltransferase MLL and down-regulated in cells that carry null mutations of histone H3K9 methyltransferase SUV39H, suggesting that they are subject to epigenetic regulation. These results support that tel-sRNAs are heterochromatin associated pi-like small RNAs [128]. Recently, it was also reported that an 18-mer RNA oligo of (UUAGGG)₃ has potential to inhibit telomerase TERT activity *in vitro* by RNA duplex formation in the template region of the telomerase RNA component [129]. Therefore, it is supposed that tel-sRNAs containing UUAGGG repeats could act as sensors of chromatin status and create a feedback loop between the telomeric heterochromatic regulation and telomere length control. Although tel-sRNAs have not been described in human until to date, they could play an important role in carcinogenesis and contribute to unlimited replicative potential of cancer cells.

Pyknons

Pyknons are a subset of 127998 patterns of variable length, which form mosaics in untranslated as well as protein-coding regions of human genes. Nevertheless, they are found more frequently in the 3'UTR of genes than in other regions of the human genome [130,131]. Pyknons are present in statistically significant manner in genes that are involved in specific processes such as cell communication, transcription, regulation of transcription, signaling, transport, etc. Pyknons involve ~40% of the known miRNA sequences, thus suggesting possible link with posttranscriptional gene silencing and RNA interference [131]. Different sets of pyknons are connected to allele-specific sequence variations of disease-associated SNPs and miRNAs, suggesting that increased susceptibility to multiple common human disorders is associated with global alterations in genome-wide regulatory templates affecting the biogenesis and functions of non-coding RNAs [132].

In the time since their discovery, evidence has been slowly accumulating that these pyknon motifs mark transcribed, non-coding RNA sequences with potential functional relevance in human disease. Tsirigos *et al.* [133] described two GO terms (GO:0006281/DNA repair, GO:0006298/mismatch repair) that were significantly enriched in pyknons-containing regions of the human introns. He pointed out that these two terms are uniquely associated with pyknons and a search of the ENSEMBL database [134] for human genes labeled with these two GO terms identified a *MLH1* gene, that has been associated with hereditary non-polyposis colorectal cancer and other types of carcinomas and microsatellite instabilities. The human *MLH1* transcript has 17 introns

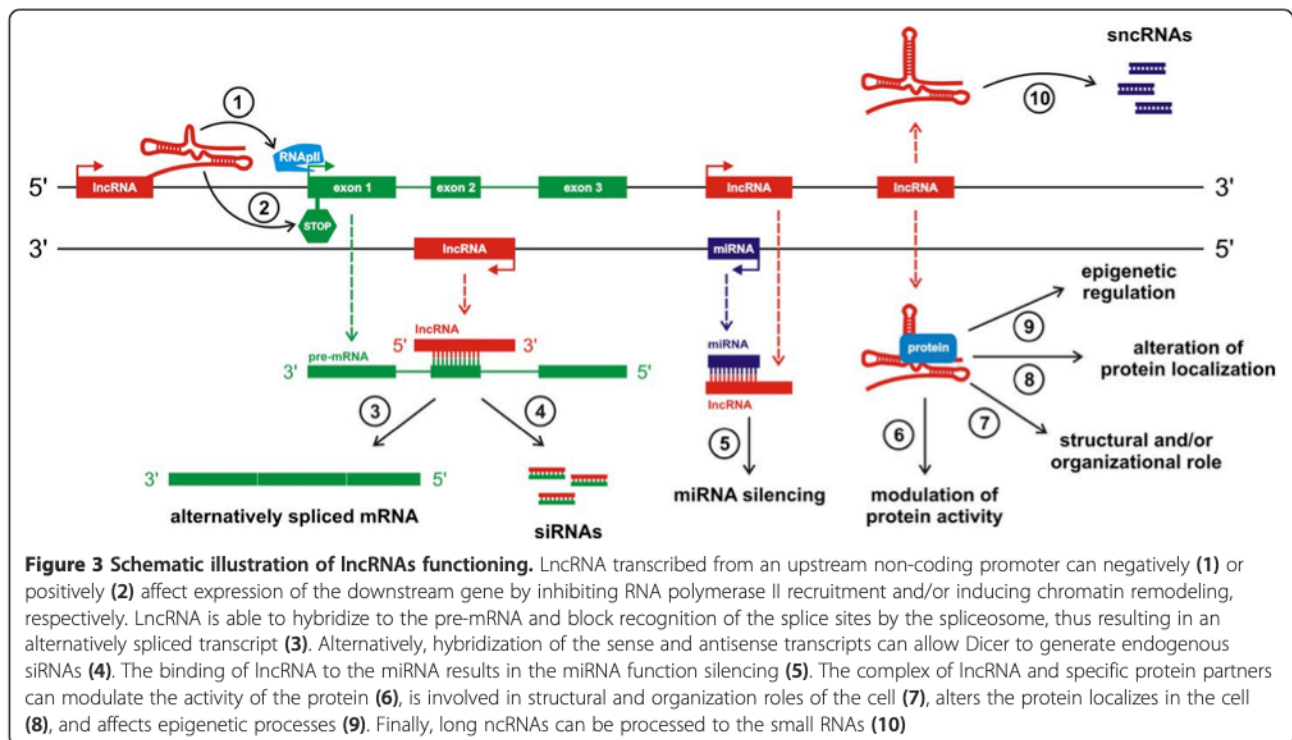
and the authors proved that these introns contain more than 10 different pyknons. Nevertheless, further research for comprehensive understanding their role in carcinogenesis is necessary.

Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are the broadest class encompassed all non-protein-coding RNA species with length more than 200 nucleotides, however, frequently ranging up to 100 kb. Many identified lncRNA are transcribed by RNA polymerase II (RNAPII), spliced, and usually contain canonical polyadenylation signals, but this is not a fast rule [2]. On the other hand, Pagano *et al.* [135] found out that some of these lncRNAs are due to their promoter structure likely to be transcribed by polymerase III (RNAPIII) and he marked them as cogenes since they could specifically coact with a protein-coding pol II gene. There is substantial evidence to suggest that lncRNAs mirror protein coding genes. Additionally, lncRNAs' promoters are bound and regulated by transcriptional factors and epigenetically marked with specific histone modifications [136]. LncRNAs are developmentally and tissue specific, and have been associated with a spectrum of biological processes, for example, alternative splicing, modulation of protein activity, alternation of protein localization, and epigenetic regulation. LncRNAs can be also precursors of small RNAs and even tools for miRNAs silencing [71,137-141]. However, one of their primary tasks appears to be regulators of protein-coding gene expression (Figure 3) [142]. Recently, Wang *et al.* [143] described four different mechanisms of lncRNAs action. He supposes that these molecules can function as signals, decoys, guides or as scaffolds (Figure 4). It is not surprising, then, that dysregulation of lncRNAs seems to be an important feature of many complex human diseases, including cancer (Table 4), ischaemic heart disease [144] and Alzheimer's disease [145]. Also dysregulation of lncRNAs that function as regulators of the expression of tumor suppressors or oncogenes, and not the protein-coding sequence itself, may be one of the 'hits' that leads to oncogenesis [2]. That is why they might be suitable as potential biomarkers and targets for novel therapeutic approaches in the future.

Long intergenic non-coding RNAs

Long intergenic non-coding RNAs (lincRNAs) are newly discovered ncRNAs belonging to lncRNAs. RNAs of this subclass ranging in length from several hundred to tens of thousands of bases and they lie within the genomic intervals between two genes. More than 3000 human lincRNAs have been identified, but less than 1% has been characterized [136,186]. It was shown that distinct lincRNAs are involved in diverse biological processes

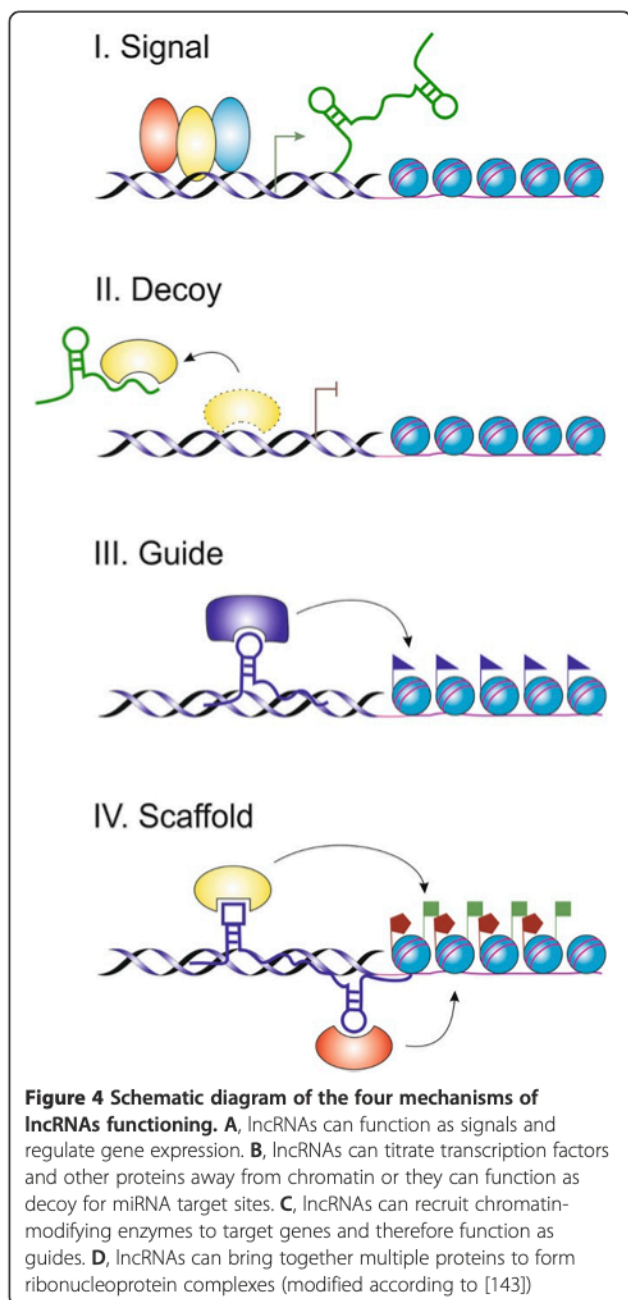


such as imprinting or cancer metastasis [7,140,186]. Moreover, recent studies proved that lincRNAs are exquisitely regulated during development and in response to diverse signaling cues, and exhibit distinct gene expression patterns in primary tumors and metastases [136]. Therefore, these lincRNAs could be utilized for cancer diagnosis, prognosis, and serve as potential therapeutic targets.

Recently it has been demonstrated that lincRNAs can act as natural 'miRNA sponges' to reduce miRNA levels [155]. The most highly upregulated transcript found in a microarray-based study of gene expression in hepatocellular carcinoma was determined to be the ncRNA HULC, or Highly Upregulated in Liver Cancer. Transcribed from chromosome 6p24.3, this lincRNA demonstrates the hallmarks of a typical mRNA molecule, including a single spliced GT-AG intron, canonical polyadenylation signals upstream of the poly(A) tail and nuclear export demonstrating strong localization to the cytoplasm. Although HULC was found to co-purify with ribosomes, no translation product for this lincRNA has been detected, supporting its classification as a non-coding transcript [156]. In addition to liver cancer, HULC was found to be highly upregulated in hepatic colorectal cancer metastasis and in hepatocellular carcinoma cell lines (HCC) producing hepatitis B virus (HBV) [157]. HULC exists as part of an intricate auto-regulatory network, which when perturbed, resulted in increased HULC expression (Figure 5a). The HULC

RNA appeared to function as a 'molecular decoy' or 'miRNA sponge' sequestering miR-372, of which one function is the translational repression of PRKACB, a kinase targeting cAMP response element binding protein (CREB). Once activated, the CREB protein was able to promote HULC transcription by maintaining an open chromatin structure at the HULC promoter resulting in increased HULC transcription [158].

Another well known RNA that belongs to lincRNA subclass described in previous paragraph is HOXA antisense intergenic RNA (HOTAIR) (see Figure 5b). HOTAIR is 2.2 kb gene localized within the human HOXC gene cluster on the long arm of chromosome 2. It has been shown that this lincRNA has a potential to regulate HOXD genes in trans via the recruitment of polycomb repressive complex 2 (PRC2), followed by the trimethylation of lysine 27 of histone H3 [7]. In general, the 5' region of the RNA binds the PRC2 complex responsible for H3K27 methylation, while the 3' region of HOTAIR binds LSD1 (flavin-dependent monoamine oxidase), a histone lysine demethylase that mediates enzymatic demethylation of H3K4Me2. HOTAIR exists in mammals, has poorly conserved sequences and considerably conserved structures, and has evolved faster than nearby HOXC genes [187]. HOTAIR was one of the first metastasis-associated lincRNAs, described to have a fundamental role in cancer. This lincRNA was found to be highly upregulated in both primary and metastatic breast tumors, showing up to 2000-fold increased transcription



over normal breast tissue. This phenotype seems to be closely linked with PRC2-dependent gene repression induced by HOTAIR. High levels of HOTAIR expression correlate with both metastasis and poor survival rate, connecting lincRNAs with tumor invasiveness and patient prognosis [140]. In addition, it was observed that the high expression level of HOTAIR in hepatocellular carcinoma could be a candidate biomarker for predicting tumor recurrence in hepatocellular carcinoma patients who have undergone liver transplant therapy and might be a potential therapeutic target [188]. Huarte *et al.* [189] identified several lincRNAs that are regulated by

p53. Furthermore, he proved that lincRNAs-p21 serves as a repressor in p53-dependent transcriptional responses, since inhibition of this lincRNA affected the expression of hundreds of gene targets enriched for genes normally repressed by p53.

While targeting cancer-specific miRNAs has proven to be successful, it will be necessary to design molecules with potential to inhibit lincRNAs. Gupta *et al.* [140] proved that these molecules can be depleted by siRNAs, but this possibility is quite complicated because of extensive secondary structures in lincRNAs [187]. Nevertheless, it is evident that cancer-associated lincRNAs may provide new approaches to the diagnosis and treatment of cancer.

Long intronic non-coding RNAs

The biogenesis of long intronic ncRNAs is poorly understood at this time. Nevertheless, there are some indirect evidences that indicate an involvement of RNA polymerase II (RNAPII). Among such evidences belong a concordant and co-regulated expression profiles of many intronic ncRNAs and their corresponding protein-coding genes, the broad contribution of RNAPII associated transcription factors and physiological stimuli in the transcription of intronic ncRNAs as well the presence of poly(A+) tail [190-194]. Nonetheless, it is described that over 10% of long intronic poly(A+) ncRNAs are up-regulated compared to only 4% of protein-coding transcripts after treatment with the RNAPII specific inhibitor α -amanitin [190,193,195]. These findings suggest that some intronic ncRNA and peculiar protein-coding RNAs could be transcribed by another RNA polymerase such as the recently described spRNAP-IV, whose transcriptional output seems to be enhanced by α -amanitin, or also could be transcribed by RNAP III [190,195-199].

Similarly to lincRNAs, there are also described evolutionary conserved long intronic ncRNAs sequences from mouse and human [200,201]. When the introns of a larger selection of vertebrates were aligned, the length of the conserved region became only 100 bp, while in the alignment of a smaller group of closely related species (human-mouse-cow-dog) the evolutionary conservation of the region extended to as much as 750 bp [201].

The widespread occurrence, tissue and subcellular expression specificity, evolutionary conservation, environment alteration responsiveness and aberrant expression in human cancers are features that accredit intronic ncRNAs to be mediators of gene expression regulation. A few sets of intronic ncRNAs have the same tissue expression pattern as the corresponding protein-coding genes, whereas others are inversely correlated. These findings point to complex regulatory relationships between intronic ncRNAs and their host loci [190,193,202,203]. Some small ncRNAs are encoded within intronic regions; moreover, intronic

Table 4 Human cancer associated lncRNAs (adapted from [4])

lncRNA	Size	Cytoband	Cancer types	References
HOTAIR	2158 nt	12q13.13	breast	[7,140]
MALAT1/α/NEAT2	7.5 kb	11q13.1	breast, lung, uterus, pancreas, colon, prostate, liver, osteosarcoma, neuroblastoma, cervix	[146-151]
HULC	500 nt	6p24.3	liver	[152,153]
BC200	200 nt	2p21	breast, cervix, esophagus, lung, ovary, parotid, tongue	[154,155]
H19	2.3 kb	11p15.5	bladder, lung, liver, breast, endometrial, cervix esophagus, ovary, prostate, colorectal	[156-159]
BIC/MIRHG155/MIRHG2	1.6 kb	21q11.2	B-cell lymphoma	[160]
PRNCR1	13 kb	8q24.2	prostate	[161]
LOC285194	2105 nt	3q13.31	osteosarcoma	[162]
PCGEM1	1643 nt	2 g32.2	prostate	[163-165]
UCA1/CUDR	1.4–2.7 kb	19p13.12	bladder, colon, cervix, lung, thyroid, liver, breast, esophagus, stomach	[166]
DD3/PCA3	0.6–4 kb	9q21.22	prostate	[167,168]
anti-NOS2A	1.9 kb	17q23.2	brain	[169]
uc.73A	201 nt	2q22.3	colon	[170]
uc.338	590 nt	12q13.13	liver	[171]
ANRIL/p15AS/CDK2BAS	34.8 kb	9p21.3	prostate, leukemia	[172-175]
MEG3	1.6 kb	14q32.2	brain	[176-178]
GAS5/SNHG2	isoforms	1q25.1	breast	[101]
SRA-1/SRA	1965 nt	5q31.3	breast, uterus, ovary	[179,180]
PTENP1	3.9 kb	9p13.3	prostate	[181,182]
ncRAN	2186 nt 2087 nt	17q25.1	bladder, neuroblastoma	[183,184]
LSINCT5	2.6 kb	5p15.33	breast, ovary	[185]

miRNAs tend to be present in large introns with 5'-biased position distribution, what correlates with the previous observation that most long intronic transcripts are expressed within first introns of the host genes. Thus, it is expected that a number of long intronic ncRNAs are processed into smaller ncRNAs [68,190,204,205]. Similar to lincRNAs HOTAIR, Heo *et al.* [206] described a long intronic non-coding RNA termed as cold assisted intronic non-coding RNA – COLDAIR, which is required for the vernalization-mediated epigenetic repression of FLC mediated by PRC2. Interestingly, the newest study of Tahira *et al.* [207] shows that long intronic non-coding RNAs are differentially expressed in primary and metastatic pancreatic cancer. Moreover, loci harbouring intronic lincRNAs differentially expressed in pancreatic ductal carcinoma metastases were enriched in genes associated to the MAPK pathway. These findings indicate potential relevance of this class of transcripts in biological processes related to malignant transformation and metastasis.

Telomere-associated ncRNAs

Telomeres protect linear chromosome ends from being recognized and processed as double-strand breaks by

DNA repair activities. This protective function of telomeres is essential for chromosome stability. Until recently, the heavily methylated state of subtelomeric regions, the gene-less nature of telomeres, and the observed telomere position effect led to the notion that telomeres are transcriptionally silent [208]. This hypothesis was recently challenged when several groups independently demonstrated that subtelomeric and telomeric regions, although devoid of genes, have the potential to be transcribed into telomeric UUAGGG-repeat containing ncRNAs (TERRA) [209-211]. TERRA molecules are conserved among eukaryotes and have been identified also in human. TERRA transcripts are synthesized from the C-rich strand and polyadenylated, and their synthesis is α -amanitin-sensitive, suggesting that they are transcripts of RNAPII [208,212]. TERRA molecules range between 100 bp and >9 kb in length and were reported to form intermolecular G-quadruplex structure with single-stranded telomeric DNA, but can also fold into a compact repeated structure containing G-quartets [211]. TERRA transcripts can be found throughout the different stages of the cell cycle, and their levels are affected by several factors that include telomere length, tumor

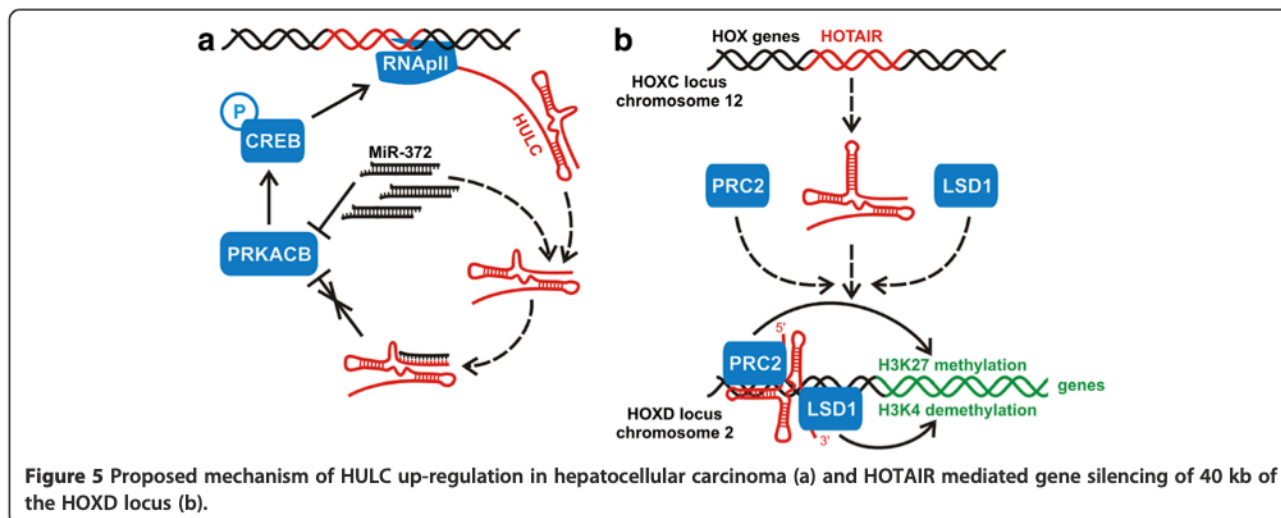


Figure 5 Proposed mechanism of HULC up-regulation in hepatocellular carcinoma (a) and HOTAIR mediated gene silencing of 40 kb of the HOXD locus (b).

stage, cellular stress, developmental stage, and telomeric chromatin structure [208].

TERRA most likely negatively regulates telomere length [211]. Increased TERRA levels by interfering with TERRA decay, such as the impairment of non-sense-mediated RNA decay in human cells or by deletion of the 5'–3' exonuclease Rat1p in *Saccharomyces cerevisiae*, are associated with a loss of telomere reserve [209,212]. Current models propose a role for TERRA in controlling telomerase activity. In yeast, the formation of a DNA/RNA hybrid between TERRA and telomeres is thought to inhibit elongation by telomerase, whereas in mammals, TERRA was shown to efficiently inhibit telomerase activity *in vitro*, presumably by base pairing with the template region of the RNA component of telomerase [208,210,212]. Caslini *et al.* [213] described that telomere uncapping through either TRF2 shelterin protein knockdown or exposure to telomere G-strand DNA oligonucleotides significantly increases the transcription of TERRA, an effect mediated by the functional cooperation between transcriptional regulator MLL and the tumor suppressor p53. Sampl *et al.* [214] found out that the expression of TERRA in patients with glioblastoma multiforme negatively correlates with the grade. Moreover, this finding of a diagnostic value of TERRA levels in astrocytoma WHO grade 2 to 4 corresponded with preliminary data in advanced stages of human tumors of larynx, colon, and lymph node [210]. Unfortunately, it is largely unclear how the expression of TERRA and the amount of TERRA transcripts are regulated in the cell [208]. Nevertheless, TERRA opens new avenues for telomere research that will impact on telomere-associated diseases including many cancers [215].

Long ncRNAs with dual functions

Until not long ago, ncRNAs were strictly considered as RNA molecules with regulatory functions but not

associated with the protein coding capacity typical of messenger RNAs. However, the recent identification and characterization of bifunctional RNAs, i.e. RNAs for which coding capacity and activity as functional regulatory RNAs have been reported, suggests that a definite categorization of some RNA molecules is far from being straightforward [216]. The steroid receptor RNA activator (SRA) is a unique co-regulator that functions as a non-coding RNA, although incorporation of an additional 5' region can result in translation of an SRA protein (SRAP) that also has co-activator activity [180,217,218]. SRA was initially shown to enhance gene expression through a ribonucleoprotein complex with steroid receptors and SRC-1 [217]. Currently, SRA is known as an RNA co-activator for many other nuclear receptors. In addition, SRA may act as an RNA scaffold for co-repressor complexes [216,219]. SRA transcripts have been identified in normal human tissues, with a higher expression in liver, skeletal muscle, adrenal and pituitary glands, whereas intermediate expression levels were observed in the placenta, lung, kidney and pancreas [217]. In some pathological cases, increased RNA levels of SRA were reported like in breast and ovarian tumors [179,220,221]. Interestingly, levels of SRA expression could be characteristic of tumor grade or particular subtypes of lesions among different tumors. Indeed, serous ovarian tumors showed higher levels of SRA than granulosa tumor cells [216,220].

Pseudogene RNAs

Pseudogenes are gene copies that have lost the ability to code for a protein; they are typically identified through annotation of disabled, decayed or incomplete protein-coding sequences. These molecules have long been labeled as “junk” DNA, failed copies of genes that arise during the evolution of genomes. However,

recent results showed that some pseudogenes appear to harbor the potential to regulate their protein-coding cousins [222,223]. Processed pseudogenes are made through retrotransposition of mRNAs, especially as a possible by-product of LINE-1 (Long INterspersed Elements) retrotransposition. Thus, these mRNAs are reverse transcribed and re-integrated into the genomic DNA [224,225]. The parent gene of the mRNA need not to be on the same chromosome as the retrotransposed copy. Retrotransposed mRNAs have three possible fates in the genome: formation of processed genes, formation of non-transcribed pseudogenes, or formation of pseudogenes transcribed into RNAs [222]. Interestingly, some of these RNAs exhibit a tissue-specific pattern of activation. Pseudogene transcripts can be processed into short interfering RNAs that regulate coding genes through the RNAi pathway. In another remarkable discovery, it has been shown that pseudogene RNAs are capable of regulating tumor suppressors and oncogenes by acting as microRNA decoys [223,225]. Moreover, Devor *et al.* [226] found out that primate-specific miRNAs, miR-220 and miR-492, each lie within a processed pseudogene. Several studies also show deregulated expression of these molecules during cancer progression, which provides evidence for the functional involvement of pseudogene RNAs in carcinogenesis and suggests these molecules as a potential novel diagnostic or therapeutic target in human cancers. One of these pseudogenes is myosin light chain kinase pseudogene (MYLK). MYLK_{P1} is partially duplicated from the original MYLK gene that encodes nonmuscle and smooth muscle myosin light chain kinase (smMLCK) isoforms and regulates cell contractility and cytokinesis. Despite strong homology with the smMLCK promoter (~ 90%), the MYLK_{P1} promoter is minimally active in normal bronchial epithelial cells, but highly active in lung adenocarcinoma cells. Moreover, MYLK_{P1} and smMLCK exhibit negatively correlated transcriptional patterns in normal and cancer cells with MYLK_{P1} strongly expressed in cancer cells and smMLCK highly expressed in non-neoplastic cells. For instance, expression of smMLCK decreased in colon carcinoma tissues compared to normal colon tissues. Mechanistically, MYLK_{P1} overexpression inhibits smMLCK expression in cancer cells by decreasing RNA stability, leading to increased cell proliferation. These findings provide strong evidence for the functional involvement of pseudogenes in carcinogenesis and suggest MYLK_{P1} as a potential novel diagnostic or therapeutic target in human cancers [227]. Using massively parallel signature sequencing (MPSS) technology, RT-PCR, and 5' rapid amplification of cDNA ends (RACE) a novel androgen regulated and transcribed pseudogene of kallikreins termed as KLK31P

was discovered. It was further proved that this pseudogene may play an important role in prostate carcinogenesis [228]. He *et al.* [229] found out that pseudogene RNAs are also able to regulate a dosage of PTEN tumor suppressor during tumor development. Pseudogene RNAs however, warrant further investigation into the true extent of their function [223,227].

Transcribed-ultraconserved regions

Ultraconserved regions (UCRs) are a subset of conserved sequences that are located in both intra- and intergenic regions. They are 481 sequences, longer than 200 bp that are absolutely conserved between orthologous regions of human, rat, and mouse genomes [230]. Calin *et al.* [170] have proved in cancer systems that differentially expressed UCR could alter the functional characteristics of malignant cells. The link between genomic location of UCRs and analyzed cancer-related genomic elements is highly statistically significant and comparable to that reported for miRNAs. UCRs are frequently located at fragile sites and genomic regions involved in cancers. Using northern blot, qRT-PCR and microarray analysis, it was revealed that UCRs have distinct signatures in human leukemias and carcinomas [170].

Majority of UCRs are transcribed (T-UCRs) in normal human tissues, both ubiquitously and tissue specifically. From the molecular point of view, untranscribed UCRs might have regulatory functions as enhancers [231], while many functions can be assigned for T-UCRs, such as antisense inhibitors for protein-coding genes or other ncRNAs, including miRNAs. On the other hand, instead of T-UCRs interacting with protein-coding genes and miRNAs, it is possible that miRNAs control T-UCRs. Evidence supporting this predication is that many T-UCRs have significant antisense complementarity with particular miRNAs and negative correlation between expression of specific T-UCRs and predicted interactor miRNAs [170,232].

The expression of many T-UCRs is significantly altered in cancer, especially in adult chronic lymphocytic leukemias, colorectal and hepatocellular carcinomas and neuroblastomas [170]. Their aberrant transcription profiles can be used to distinguish types of human cancers and have been linked to patient outcome [233]. Especially in neuroblastoma, functional T-UCR annotations, inferred through a functional genomics approach and validated using cellular models, reveal associations with several cancer-related cellular processes such as apoptosis and differentiation [234]. Further, DNA hypomethylation induces release of T-UCR silencing in cancer cells. Studies of primary human tumors have shown that hypermethylation of T-UCR CpG islands is common event

among the various tumor types. Thus in addition to miRNAs, another class of ncRNAs (T-UCRs) undergoes DNA methylation-associated inactivation in transformed cells, and so supports model that both epigenetic and genetic alterations in coding and noncoding sequences cooperate in human tumorigenesis. Most importantly, restoration of T-UCR expression was observed upon treatment with the DNA-demethylating agent [232]. Another study proved, that SNPs (single nucleotide polymorphisms) rs9572903 and rs2056116 in ultraconserved regions were associated with increased familial breast cancer risk [235]. Because of increasing number of studies concerning T-UCRs is published, it is supposed that the more specific roles of these molecules in cancer will be known in a short time.

Conclusions and future perspectives

For a long time, the central dogma of molecular biology proposed RNA molecules primarily to be informational “messenger” between DNA and protein. But, surprisingly, only 2% of the human genome sequence encodes proteins, while a large part of it is devoted to the expression of ncRNAs, which are divided into two main groups according to their nucleotide length – small and long ncRNAs. These molecules are suggested to be important regulators of gene expression. Nevertheless, the two groups of ncRNAs are distinct in their biological functions and mechanisms of gene regulations. Small ncRNAs are involved mainly in the post-transcriptional gene regulation using translational repression or RNAi pathway, while long ncRNAs are much more involved in epigenetic regulation. In many cases, differential expression of ncRNAs is becoming recognized as a one of the hallmarks of cancer cell, indicating their potential usage as the novel diagnostic, prognostic, or predictive biomarkers. Growing evidence also suggests that ncRNAs have the promising potential in targeted regulation of gene expression and, therefore, in cancer targeted therapy. However, the function of many ncRNAs remains unknown and it will be necessary to discover the precise mechanisms by which are these molecules involved in carcinogenesis.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

SJ and FP drafted the manuscript, SM and SO revised the manuscript critically for important and intellectual content. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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PŘÍLOHA 3

Výsledky multimodální léčby glioblastoma multiforme: Konsektivní série 86 pacientů diagnostikovaných v letech 2003–2009

Multimodal Treatment of Glioblastoma Multiforme: Results of 86 Consecutive Patients Diagnosed in Period 2003–2009

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Souhrn

Východiska: Glioblastoma multiforme patří k nejčastějším primárním nádorům mozku u dospělých. Standardní léčba spočívá v maximální resekci nádoru, adjuvantní konkomitantní chemoradioterapii a následné chemoterapii s temozolomidem. Tento postup zlepšuje medián celkového přežití ve srovnání se samotnou radioterapií. **Soubor pacientů a metody:** Retrospektivně jsme vyhodnotili konsektivní soubor pacientů s histologicky potvrzeným glioblastomem, kteří v období od ledna 2003 do prosince 2009 podstoupili po primárním chirurgickém zákroku konkomitantní radioterapii (1,8–2,0 Gy/den, plánováno celkem 60 Gy) s chemoterapií (temozolomid 75 mg/m²/den) s následným záměrem podání 6 cyklů adjuvantní chemoterapie (temozolomid 150–200 mg/m² D1–5, interval 28 dní). Primárním cílem bylo zhodnotit vliv klinických faktorů a použité léčby na základní parametry přežití, jako je čas bez progresu onemocnění (PFS) a celkové přežití (OS). Dále jsme se zaměřili na toxicitu léčby a vyhodnocení její bezpečnosti. **Výsledky:** Do souboru bylo zařazeno celkem 86 pacientů. Medián věku byl 56 let (rozmezí 24–69), převažovali muži (60%). Většina pacientů byla v době zahájení chemoradioterapie v dobrém fyzickém stavu, u více než 80 % byl performance status (PS) 0–1. U 20 % pacientů byla iniciálně provedena makroskopicky totální resekce nádoru, v 65 % subtotální resekce, v 9 % parciální resekce a v 6 % se jednalo pouze o biopsii. Medián PFS byl 7,0 měsíců (2,0–35,5), medián OS byl 13,0 měsíců (2,5–70,0). Pooperační PS, rozsah resekce a absolvování plánované konkomitantní léčby bez nutnosti její redukce měly statisticky signifikantní vliv na PFS i OS. Medián PFS a OS byl u pacientů s PS 0, 1 a 2 22,0, 7,0 a 6,0 měsíců v případě PFS ($p = 0,0018$) a 32,0, 13,0 a 9,0 měsíců v případě OS ($p = 0,0023$). Pacienti, u kterých bylo dosaženo totálního odstranění tumoru, měli delší PFS (14,0 vs 6,0 měsíců, HR = 0,5688, $p = 0,0301$) i OS (23,0 vs 12,0 měsíců, HR 0,4977, $p = 0,0093$), stejně jako pacienti, kteří absolvovali konkomitantní chemoradioterapii bez výraznější redukce. Pokud celková dávka radioterapie přesáhla 54 Gy, byl PFS 8,0 vs 3,0 měsíce (HR = 0,3313, $p = 0,0001$) a OS 15,0 vs 5,0 měsíce (HR = 0,1730, $p < 0,0001$). Podobně pokud počet dnů chemoterapie přesáhl 40, byl PFS 8,0 vs 5,0 měsíců (HR = 0,5300, $p = 0,0023$) a OS 17,0 vs 9,5 měsíců (HR = 0,5943, $p = 0,0175$). Věk, pohlaví a lokalizace nádoru nedosáhly statistické významnosti. U hematologické toxicity hodnocené 3. nebo 4. stupněm závažnosti (grade 3 nebo 4) byla relativně často zaznamenána trombocytopenie (9%), leukopenie (6%), neutropenie (6%) a selektivní lymfopenie (25%). U nehematologické toxicity jednoznačně dominovaly tromboembolické příhody (12%). Toxicita byla častější především u pacientů s horším PS (PS 2). V léčbě recidivy nebo progresu onemocnění přinášel pacientovi benefit zejména neurochirurgický výkon (OS 24,0 vs 12,5 měsíce, HR = 0,5325, $p = 0,0111$). **Závěr:** Performance status, rozsah resekce, úspěšné podání většiny plánované dávky konkomitantní chemoradioterapie a možnost chirurgického řešení případné recidivy/progresu onemocnění významně ovlivnily prognózu našich pacientů s glioblastomy. Dle našich zkušeností má být hlavním faktorem pro rozhodování o typu použité léčby především celkový stav nemocného. Léčba maligních gliomů vyžaduje multidisciplinární přístup.

Klíčová slova

glioblastoma multiforme – chemoterapie – radioterapie – přežití – toxicita

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Summary

Backgrounds: Glioblastoma multiforme is the most common malignant primary tumor of the brain in adults. Standard therapy consists in maximal surgical resection and adjuvant concurrent chemoradiotherapy and adjuvant therapy with temozolomid. This approach improves survival in comparison with postsurgical radiotherapy alone. **Patients and Methods:** Consecutive patients with histologically confirmed glioblastoma multiforme in the period from January 2003 to December 2009 underwent postoperative radiotherapy (1.8–2.0 Gy/d, total of 60 Gy) plus concurrent daily chemotherapy (temozolomide 75 mg/m²/d), followed by 6 cycles of temozolomide (150 to 200 mg/m² for 5 days, every 28 days) and were analyzed retrospectively. The primary end point was to describe the correlation between known clinical factors, treatment and progression free survival (PFS) and overall survival (OS). We assessed the toxicity and safety of the chemoradiotherapy. **Results:** Eighty-six patients (median age, 56 years; 60% male) were included. Most of them (> 80%) were of performance status (PS) 0-1 at the beginning of chemoradiotherapy. Total macroscopic resection was performed in 20% of the patients, subtotal in 65%, partial in 9%, and just biopsy in 6%. Median PFS was 7.0 months (2.0–35.5), median OS was 13.0 months (2.5–70). Postoperative performance status (PS), the extent of resection, and administration of planned treatment without reduction had statistically significant influences on PFS and OS. Median PFS and OS were 22.0, 7.0 and 6.0 months for PFS ($p = 0.0018$) in patients with PS 0, 1 and 2 respectively and 32.0, 13.0 and 9.0 months for OS ($p = 0.0023$). Patients with total removal of tumor had longer PFS (14.0 vs 6.0 months, HR = 0.5688; $p = 0.0301$) and OS (23.0 vs 12.0 months, HR 0.4977; $p = 0.0093$), as did patients without dose reduction of radiotherapy and/or chemotherapy. Patients with radiotherapy dose of over 54 Gy had PFS 8.0 vs 3.0 months (HR = 0.3313; $p = 0.0001$) and OS 15.0 vs 5.0 months (HR = 0.1730; $p < 0.0001$). Similarly, treatment with concurrent chemotherapy for more than 40 days was also important: PFS 8.0 vs 5.0 months (HR = 0.5300; $p = 0.0023$) and OS 17.0 vs 9.5 months (HR = 0.5943; $p = 0.0175$). Age, gender and position of tumor had no significant influence. Treatment-related hematology toxicity grades 3 and 4 occurred relatively often: thrombocytopenia (9%), leukopenia (6%), neutropenia (6%) and lymphopenia (25%). Thrombo-embolic events were dominant in non-hematology toxicity. Serious toxicity occurred mainly in the subgroup of patients with PS 2. Treatment of progression was useful in selected patients. Second surgery was of the most benefit (OS 24.0 vs 12.5 months, HR = 0.5325; $p = 0.0111$). **Conclusion:** Postoperative performance status, extent of resection, successful administration of the majority of planned concurrent chemoradiotherapy and possibility of surgical treatment at the time of recurrence correlate with better prognosis for our patients with glioblastoma. Our experience indicates that performance status should be the main factor in decisions about treatment intensity. Treatment of malignant glioma requires a multidisciplinary team.

Key words

glioblastoma multiforme – chemotherapy – radiotherapy – survival – toxicity

Východiska

Glioblastoma multiforme patří k nejčastějším primárním nádorům mozku u dospělých. Incidence se pohybuje mezi 3 a 4 případy na 100 000 obyvatel za rok. Postihuje převážně dospělé mezi 45 a 75 lety, více než 80 % pacientů je v době diagnózy starší 50 let. Radikální operačního výkonu je důležitým prognostickým faktorem [1,2]. Maximální resekční výkon a pooperační radioterapie byla po léta léčebným standardem. Indikace pooperační chemoterapie nejčastěji s deriváty nitrosourey byla sporná, její vliv na prodloužení přežití nebyl významný, léčba byla navíc zatížena vyšší toxicitou [3–6].

V roce 2005 byly publikovány výsledky Stuppovy studie fáze III, kde byla jasně potvrzena účinnost chemoterapie (temozolomid) v konkomitantním podání s frakcionovanou radioterapií a s její následnou adjuvantní aplikací [7]. Pooperační chemoradioterapie s temozolomidem v této studii prodloužila medián celkového přežití z 12,1 měsíce na 14,6 měsíce a dle poslední aktualizace dat sledovaných pacientů zvyšuje i prav-

děpodobnost pětiletého přežití, a to bez ohledu na věk či rozsah výkonu [7,8]. Dle retrospektivního hodnocení této průlomové studie mají největší benefit z léčby pacienti po makroskopicky totální resekci, mladší 50 let, s performance statusem (PS) 0–1 a přítomností metylace promotoru genu reparačního enzymu O⁶-Methylguanin-DNA-methyltransferázy (MGMT) [9].

Léčba primárních nádorů mozku má v Masarykově onkologickém ústavu (MOU) dlouholetou tradici. Důležitým faktorem je úzká spolupráce s Neurochirurgickou klinikou Fakultní nemocnice Brno (FN Brno) a dalšími pracovišti. Za zásadní považujeme komisionální řešení každého pacienta.

V následující retrospektivní analýze bude vyhodnocen soubor pacientů s glioblastomy, kteří byli diagnostikováni a léčeni naším multioborovým týmem v letech 2003–2009.

Soubor pacientů a metody

Do retrospektivního hodnocení byli zařazeni všichni pacienti starší 18 let s histologicky potvrzeným glioblastomem, kteří v období od ledna 2003 do pro-

since 2009 zahájili po chirurgickém zákroku konkomitantní chemoradioterapii s temozolomidem s následným záměrem podání 6 cyklů adjuvantní chemoterapie. Více než 95 % pacientů bylo primárně operováno na Neurochirurgické klinice FN Brno, následná léčba probíhala v Masarykově onkologickém ústavu. Resekce primárního nádoru byla považována za makroskopicky totální (bez přítomnosti evidentního rezidua), pokud bylo dosaženo shody neurochirurga i pooperační zobrazovací metody. V ostatních případech byla resekce vyhodnocena buď jako subtotalní (reziduum do 20 %), nebo parciální (reziduum nad 20 %).

Schéma konkomitantní chemoradioterapie a adjuvantní chemoterapie

Temozolomid byl podáván v dávce 75 mg/m², den 1.–42., p.o., po celou dobu radioterapie, včetně víkendů.

Konformní radioterapie byla prováděna standardní frakcionací (5 × 1,8–2,0 Gy / týden, celková dávka 60 Gy za 6 týdnů). Nejčastěji byla použita technika dvou la-

terolaterálních nebo konvergentních polí brzdného záření lineárního urychlovače o energii 6 MV a 18 MV. Plánovací cílový objem (PTV 1) zahrnoval lůžko nádoru a reziduum (GTV) s bezpečnostním lemem 2–3 cm. Po 4 týdnech léčby (aplikovaná dávka $20 \times 1,8$ – $2,0$ Gy) byl bezpečnostní lem zmenšen na 1–2 cm (PTV 2) a bylo pokračováno v radioterapii ($10 \times 1,8$ – $2,0$ Gy) do celkové dávky 60 Gy. Adjuvantní léčba temozolomidem v monoterapii byla indikována po ukončení konkomitantní chemoradioterapie (ve 4. týdnu po ukončení ozařování) v dávce 150 – 200 mg/m², p.o., den 1.–5., interval 28 dní, celkem 6 cyklů nebo do progresu onemocnění. Toto léčebné schéma bylo totožné s postupem, který ve své studii uplatnili Stupp et al [7].

Schéma sledování pacientů

v průběhu léčby a po jejím ukončení

K hodnocení velikosti pooperačního rezidua bylo prováděno časné CT nebo MRI vyšetření (do 72 hod po výkonu). Další CT nebo MRI vyšetření k hodnocení efektu proběhlé konkomitantní chemoradioterapie bylo standardně indikováno za 4 týdny po jejím ukončení. V průběhu adjuvantní chemoterapie s temozolomidem a/nebo následného sledování bylo CT nebo MRI prováděno každé 3 měsíce, pokud aktuální stav pacienta nevyžadoval kontrolu dříve. Ze zobrazovacích metod bylo vždy jednoznačně preferováno MRI vyšetření, CT bylo použito v případech, kdy nebylo MRI dostupné nebo nebylo z medicínských důvodů možné. Odborné neurologické vyšetření atestovaným neurologem bylo standardně prováděno před zahájením konkomitantní chemoradioterapie, dále za 4 týdny po jejím ukončení a poté každé 3 měsíce. V případě klinických potíží byl neurolog konzultován kdykoliv mimo původní plán.

V případě progresu onemocnění (progrese tumoru o 25 % a více, nové satelitní léze, klinické zhoršení s nutností navýšení kortikosteroidů) byl další postup posouzen multidisciplinární komisí pro mozkové nádory. Zde byly zváženy alternativy následné léčby: operace, reiradiace (včetně stereotaktické radioterapie a radiochirurgie), paliativní chemoterapie, symptomatická léčba.

Tab. 1. Charakteristika souboru a orientační srovnání se Stuppovým souborem.

Sledované parametry		CHT/RT n = 86	CHT/RT(Stupp) n = 287
věk (roky) n (%)	< 50	27 (31 %)	90 (31 %)
	> 50	59 (69 %)	197 (69 %)
	50–60	37 (43 %)	
	> 60	22 (26 %)	
věk – medián (roky)		56 (24–69) m 56 (28–68) ž 51 (24–69)	56
pohlaví n (%)	muži	51 (60 %)	185 (64 %)
	ženy	35 (40 %)	102 (36 %)
performance status (PS) dle WHO a Karnofsky index (KI) n (%)	PS 0 (KI 100 %)	11 (13 %)	113 (39 %)
	PS 1 (KI 90 %)	64 (74 %)	136 (47 %)
	(KI 80 %)	27 (31 %)	
	(KI 70 %)	37 (43 %)	
	(KI 60 %)	11 (13 %)	38 (13 %)
rozsah resekčního výkonu n (%)	totální resekce	17 (20 %)	113 (39 %)
	subtotální a parciální resekce	56 (65 %) 8 (9 %)	126 (44 %)
	biopsie	5 (6 %)	48 (17 %)
adjuvantní CHT n (%)		34/86 (40 %)	223/287 (78 %)
medián cyklů adjuvantní CHT		4 (1–7)	3 (0–7)
ukončeno 6 cyklů CHT		32 % (11/34)	47 %

CHT/RT – chemoradioterapie, CHT – chemoterapie, n – počet

Primárním cílem studie bylo zhodnotit vliv klinických faktorů (rozsah resekce, celkový stav pacienta, věk, pohlaví, lokalizace nádoru) a použité primární a následné léčby na základní parametry přežití, jako je čas bez progresu onemocnění (PFS), celkové přežití (OS) a čas přežití od zjištění recidivy/progrese (EFS). Parametr PFS je definován jako doba od operace do recidivy/progrese nádoru nebo úmrtí. Parametr OS je definován jako doba od operace do úmrtí pacienta. Parametr EFS je definován jako doba od recidivy/progrese do úmrtí.

Druhotným cílem bylo vyhodnocení bezpečnosti/toxicity léčby. Toxicita léčby byla stanovena na základě klasifikace dle National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 3.0.

K základní charakteristice dat byly použity běžné statistické funkce (např. medián, procentuální vyjádření výsledku).

V analýzách přežití byl při porovnání přežívání jednotlivých skupin pacientů využit Gehanův-Wilcoxonův test, případně Ln-pořadový test. Křivky přežití byly sestaveny klasickou Kaplan-Meierovou metodou. Za statisticky signifikantní byly považovány hodnoty $p \leq 0,05$. Statistické vyhodnocení dat bylo provedeno pomocí programu MedCalc, verze 9.3.9.0. Práce byla zpracována programovými produkty společnosti Microsoft (Microsoft Word, Microsoft Excel).

Charakteristiku souboru pacientů a jeho srovnání se souborem Stuppovy studie uvádí tab. 1.

Výsledky

Výsledky léčby

V době od ledna 2003 do prosince 2009 bylo ke konkomitantní chemoradioterapii s temozolomidem a následně adjuvantní léčbě indikováno

Tab. 2. Celkové přežití (OS) a čas bez progresu (PFS), orientační srovnání se Stuppovým souborem.

	CHT/RT (MOU) n = 86	CHT/RT (Stupp) n = 287
medián OS (měsíce)	13,0	14,6
overall survival v (%)		
6 měsících	84,0	86,3
12 měsících	57,0	61,1
18 měsících	39,0	39,4
24 měsících	26,0	27,2
3 letech	7,0	16,0
4 letech	3,0	12,1
5 letech	3,0	9,8
medián PFS (měsíce)	7,0	6,9
progression-free survival v (%)		
6 měsících	62,0	53,9
12 měsících	29,0	26,9
18 měsících	15,0	18,4
24 měsících	8,0	10,7

CHT/RT – chemoradioterapie, n – počet

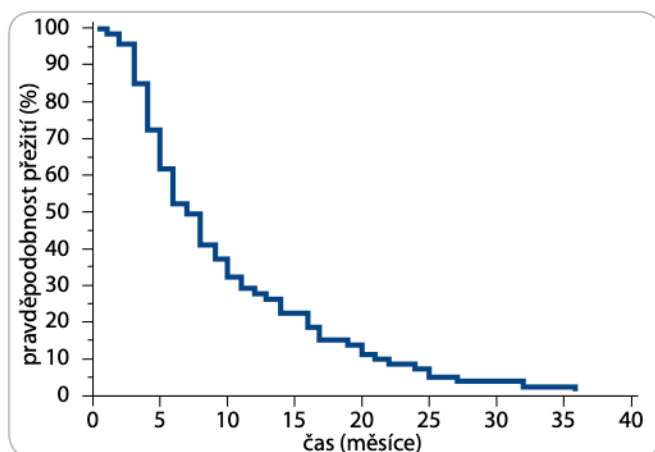
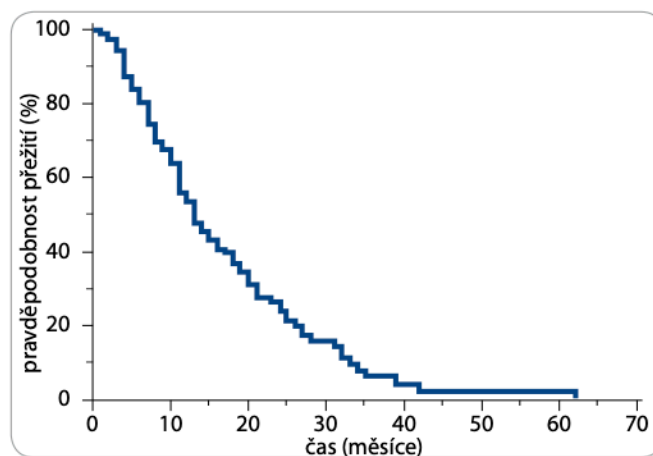
a chemoterapie. Dle očekávání pooperační PS, rozsah resekce a absolvování plánované konkomitantní léčby bez nutnosti její redukce měly statisticky signifikantní vliv na PFS i OS. Medián PFS a OS byl u pacientů s PS 0, 1 a 2 22,0, 7,0 a 6,0 měsíců v případě PFS ($p = 0,0018$) a 32,0, 13,0 a 9,0 měsíců v případě OS ($p = 0,0023$), viz graf 3. Pacienti, u kterých bylo dosaženo makroskopicky totálního odstranění tumoru, měli ve srovnání s pacienty s jakýmkoliv pooperačním reziduem delší PFS (14,0 vs 6,0 měsíců, HR = 0,5688, $p = 0,0301$) i OS (23,0 vs 12,0 měsíců, HR 0,4977, $p = 0,0093$), viz graf 4. V případě hodnocení vlivu dávek konkomitantně aplikované radioterapie a chemoterapie jsme zjistili, že lepší výsledky léčby zaznamenali pacienti, u nichž celková dávka radioterapie přesáhla 54 Gy a počet dnů chemoterapie přesáhl 40. V prvním případě byl PFS 8,0 vs 3,0 měsíce (HR = 0,3313, $p = 0,0001$) a OS 15,0 vs 5,0 měsíců (HR = 0,1730, $p < 0,0001$), ve druhém případě byl PFS 8,0 vs 5,0 měsíců (HR = 0,5359, $p = 0,0023$) a OS 17,0 vs 9,5 měsíce (HR = 0,5943, $p = 0,0175$), viz tab. 3. Naopak věk (tab. 3, graf 5) a pohlaví pacienta nebo lokalizace tumoru neměly v naší studii signifikantní vliv ani na PFS, ani na OS.

Podobně jako v případě primární léčby i možnost pokračovat v adjuvantním podávání temozolomidu měla vliv na přežití pacientů. Dle statistického hodnocení byl PFS u pacientů, kteří pokračovali v adjuvantním temozolomidu, signifikantně delší (10,0 vs 5,0 mě-

celkem 86 pacientů s nově diagnostikovaným glioblastoma multiforme. Medián věku pacientů byl 56 let, převažovali muži (60%). Většina pacientů byla v době zahájení chemoradioterapie v dobrém fyzickém stavu, více než 80% mělo PS 0–1. U 20% pacientů byla iniciálně provedena makroskopicky totální resekce nádoru, v 65% subtotální resekce, v 9% parciální resekce a v 6% se jednalo pouze o biopsii (tab. 1). Medián PFS v našem souboru byl 7,0 měsíců

(2,0–35,5) a medián OS byl 13,0 měsíců (2,5–70,0), viz křivky přežití (graf 1 a 2). V prvním a druhém roce od diagnózy choroby přežilo 57% a 26% pacientů, ve stejném období bylo bez progresu onemocnění 29% a 8% pacientů (podrobně viz tab. 2).

Data přežití byla podrobena analýze z pohledu PS (stavu fyzické výkonnosti), věku a pohlaví pacienta, lokalizace nádoru, radikality neurochirurgického výkonu, aplikované dávky radioterapie

**Graf 1. Kaplan-Meierova analýza – přežití bez progresu onemocnění (PFS).****Graf 2. Kaplan-Meierova analýza – celkové přežití (OS).**

Tab. 3. Parametry přežití (medián OS a medián PFS) ve vztahu k věku, rozsahu resekce, stavu výkonnosti po operaci (PS), dávce podané radioterapie a počtu dní chemoterapie při konkomitantní fázi léčby.

	Věk (roky)		Rozsah resekce		Performance status (PS)			Radioterapie (Gy)		Chemoterapie (počet dní)	
	< 50	> 50	radikální	neradikální	0	1	2	< 54	> 54	< 40	> 40
OS (měsíce)	16,0	11,0	23,0	12,0	32,0	13,0	9,0	5,0	15,0	9,5	17,0
p	p = 0,6159		p = 0,0093		p = 0,0023			p < 0,0001		p = 0,0175	
HR	0,8933		0,4977		-			0,1730		0,5943	
PFS (měsíce)	8,0	6,0	14,0	6,0	22,0	7,0	6,0	3,0	8,0	5,0	8,0
p	p = 0,5217		p = 0,0301		p = 0,0018			p = 0,0001		p = 0,0023	
HR	0,8674		0,5688		-			0,3313		0,5300	

síců, HR = 0,6480, p = 0,0393) než ve skupině, která adjuvantní léčbu z různých důvodů neabsolvovala. V případě OS byl sice rovněž patrný rozdíl mezi oběma skupinami, nicméně nebyl statisticky signifikantní (18,0 vs 11,0 měsíců, HR = 0,6874, p = 0,0896). Výsledky však mohou být ovlivněny relativně nízkým počtem pacientů, kteří pokračovali v adjuvantním temozolomidu (40%). K hlavním příčinám přerušení léčby již po konkomitantní části patřila progresse onemocnění a především toxicita léčby, která byla vyšší než ve Stuppově studii. K adjuvantnímu temozolomidu byli indikováni jen pacienti, kteří absolvovali konkomitantní chemoradioterapii bez závažnější G3/4 toxicity, bez jasné progresse nádoru na kontrolním CT nebo MRI vyšetření a bez významného zhoršení stavu fyzické výkonnosti.

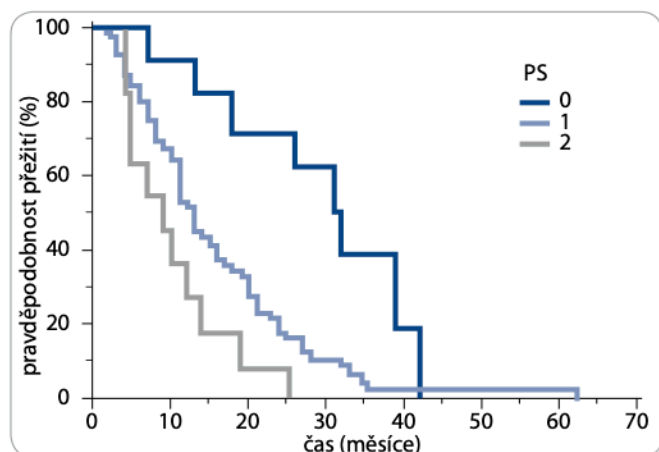
Tab. 4. Léčba recidivy/progrese po chemoradioterapii a adjuvantní chemoterapii.

Léčebná metoda	MOU – n (%)	Stupp – n (%)
operace	21/86 (24 %)	64/272 (24 %)
reiradiace	8/86 (9 %)	13/272 (5 %)
paliativní chemoterapie	39/86 (45 %)	148/272 (54 %)

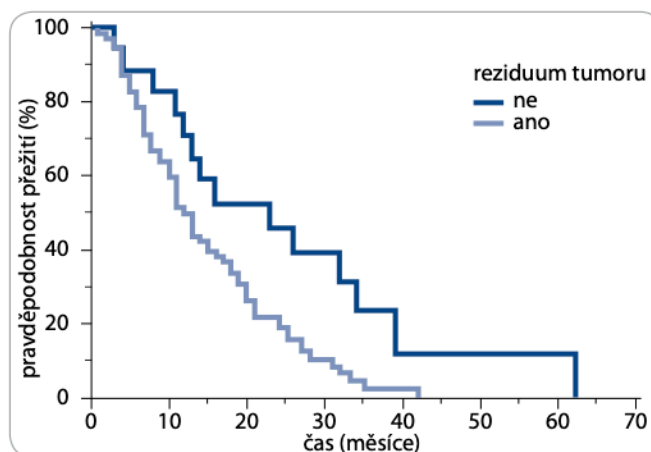
n – počet

Na podskupině 67 pacientů, u kterých byla zobrazovacími metodami (CT nebo MRI) potvrzena recidiva nebo progresse onemocnění, jsme hodnotili vliv dalšího postupu na celkové přežití a na čas přežití od zjištění recidivy/progrese (EFS). V našem souboru byla reoperace provedena u 24% pacientů, v ostatních případech pacienti podstoupili buď paliativní chemoterapii, radioterapii, nebo symptomatickou léčbu. Využití jednotlivých modalit ukazuje tab. 4. Přestože je hod-

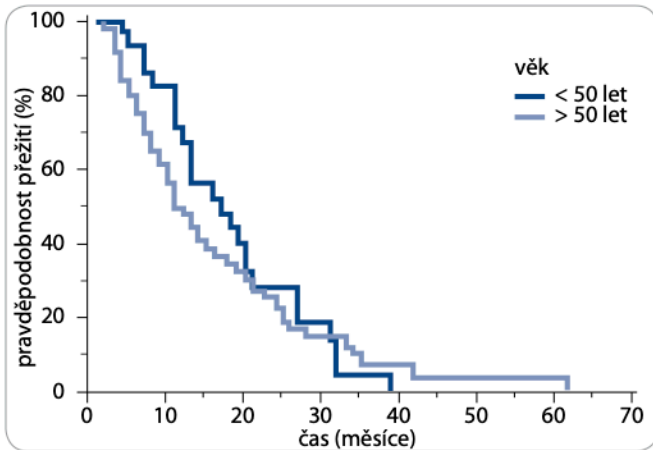
nocení výsledků léčby recidivy/progrese značně problematické, neboť charakter progresse významně ovlivňuje celkový stav pacienta a možnost použít protinádorovou léčbu, naše výsledky potvrzují její pozitivní vliv na další vývoj nemoci. Skupina léčených pacientů, bez ohledu na použitou modalitu léčby, znamenala signifikantně delší přežití od recidivy/progrese než pacienti na symptomatické terapii (7,0 vs 3,0 měsíce, HR = 0,5675, p = 0,0187), viz graf 6.



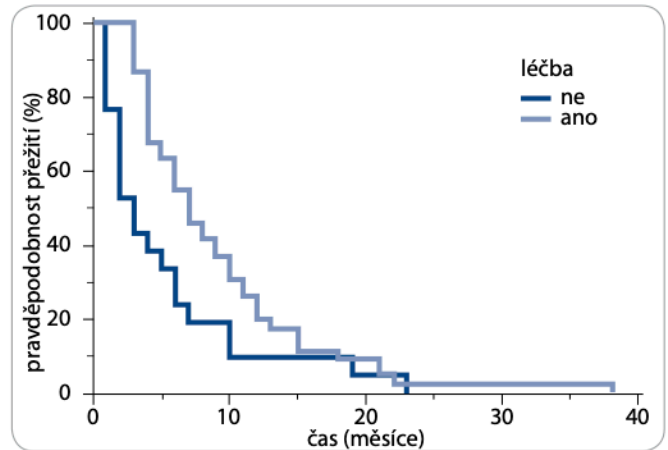
Graf 3. Kaplan-Meierova analýza – celkové přežití (OS) v závislosti na celkovém stavu výkonnosti (PS).



Graf 4. Kaplan-Meierova analýza – celkové přežití (OS) v závislosti na přítomnosti pooperačního rezidua tumoru.



Graf 5. Kaplan-Meierova analýza – celkové přežití (OS) v závislosti na věku.



Graf 6. Kaplan-Meierova analýza – čas přežití od recidivy/progrese (EFS) v závislosti na léčbě.

Tab. 5. Použitá léčba v době recidivy/progrese a ovlivnění délky života (EFS).

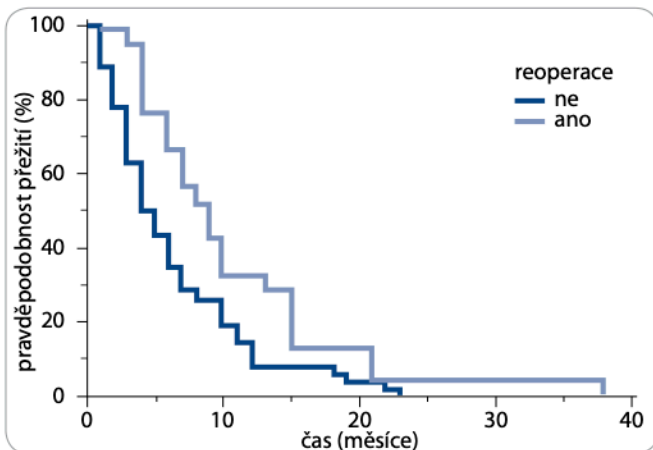
	Léčba relapsu/progrese		Reoperace		Reiradiace		Paliativní chemoterapie	
	ano	ne	ano	ne	ano	ne	ano	ne
n	46	21	21	46	8	59	39	28
přežití od progrese do úmrtí (měsíce)	7,0	3,0	9,0	4,0	11,0	5,0	6,0	4,5
p	p = 0,0187		p = 0,0247		p = 0,1207		p = 0,4008	
HR	0,5675		0,5855		0,5883		0,8286	
n – počet								

Toxicita léčby

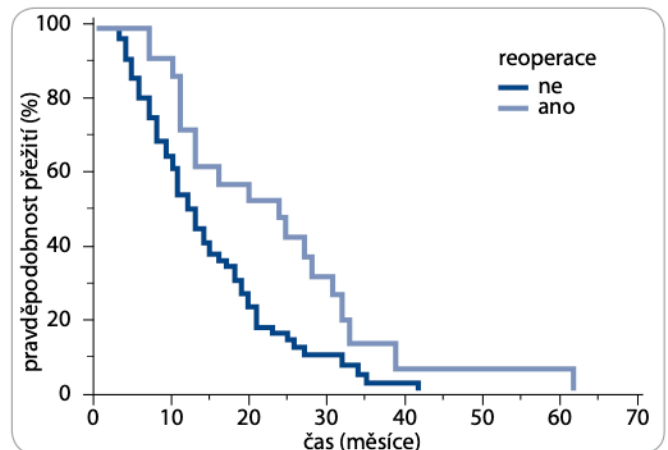
Kromě výsledků léčby jsme se rovněž zajímali o její toxicitu. Výskyt závažné hematologické a nehematologické toxicity, hodnocené stupněm 3 a 4 (G3/4), je uveden v tab. 6. U hematologické toxicity byla ve srovnání se Stuppovým souborem častěji zaznamenána trombocytopenie (celkem 8 pacientů, 9%), ve třech případech s krvácivými projevy s nutností aplikací trombonáplavů. Leukopenie a neutropenie G3/4 byla také častější (celkem 5 pacientů, 6%), ve dvou případech kvůli probíhajícímu infektu s nutnou podporou myelopoézy (G-CSF). Důležité je také poukázat na často podceňovanou G3/4 lymfopenii. V našem souboru se vyskytla u celkem 22 pacientů (25%), ve 4 případech mohla mít podíl na rozvoji infekce (bez dopro-

Z pohledu významnosti použité metody měl největší benefit operační výkon. Pacienti, kteří podstoupili reoperaci, měli ve srovnání s ostatními pacienty EFS 9,0 vs 4,0 měsíce (HR = 0,5855, p = 0,0247)

a OS 24,0 vs 12,5 měsíce (HR = 0,5325, p = 0,0111), viz graf 7 a 8. Samostatný vliv paliativní chemoterapie nebo reiradiace na prodloužení délky života od progrese nedosáhl statistické významnosti (tab. 5).



Graf 7. Kaplan-Meierova analýza – čas přežití od recidivy/progrese (EFS) v závislosti na operabilitě.



Graf 8. Kaplan-Meierova analýza – celkové přežití (OS) v závislosti na možnosti chirurgické léčby progrese nemoci.

Tab. 6. Závažná toxicita (G3/4) dle NCI-CTC version 3.0 a komplikace konkomitantní chemoradioterapie (v průběhu a do 1 měsíce od ukončení).

Toxicita	G3	G4	G3+4 (MOU)	G3+4 (Stupp)
Hematologická				
anémie	1	0	1/86 (1 %)	1 (< 1 %)
leukopenie	1	4	5/86 (6 %)	7/284 (2 %)
neutropenie	1	4	5/86 (6 %)	12/284 (4 %)
lymfopenie	16	6	22/86 (25 %)	neuveďeno
trombocytopenie	4	4	8/86 (9 %)	9/284 (3 %)
Nehematologická				
hepatopatie	3	0	3/86 (3 %)	neuveďeno
pneumonie	6/86 (7 %), 4x nekomplikovaná, 2x atypická			3/284 (1 %)
plicní embolizace + flebotrombózy DKK		4/86 (5 %)		12/284 (4 %)
		6/86 (7 %)		
		3/86 (3 %)		
úmrtí	* 2x atypická pneumonie (<i>Pneumocystis carinii</i>) * 1x plicní embolizace			2/284 (1 %) * krvácení do mozku

vodné G3/4 neutropenie) i přes profylaxi sumetrolimem u většiny pacientů. U nehematologické toxicity jednoznačně dominovaly tromboembolické příhody, z nichž jedna byla fatální. Zanedbatelná nejsou ani dvě úmrtí na pneumocystovou pneumonii, z nichž jedna vznikla v terénu lymfopenie G4. Tento relativně vysoký výskyt toxicity měl zcela jistě vliv na indikaci adjuvantní léčby a tím pravděpodobně ovlivnil i medián PFS a OS a zřejmě i snížil procento pacientů žijících déle než 2 roky. Na základě známých vstupních klinických faktorů jsme se proto snažili blíže popsat skupinu pacientů, která je vystavena vyššímu riziku vážných nežádoucích účinků léčby. Dle našich zkušeností je evidentní, že především pacienti s PS 2 mají vyšší riziko komplikací při probíhající chemoradioterapii a tím i vyšší pravděpodobnost jejího předčasného ukončení.

Opačná situace nastala v případě adjuvantní léčby temozolomidem, tj. po ukončení chemoradioterapie, kde jsme závažnou toxicitu (G3/4) nepozorovali. Důvodem byla jistě selekce pacientů pro pokračování v adjuvantní léčbě.

Diskuze

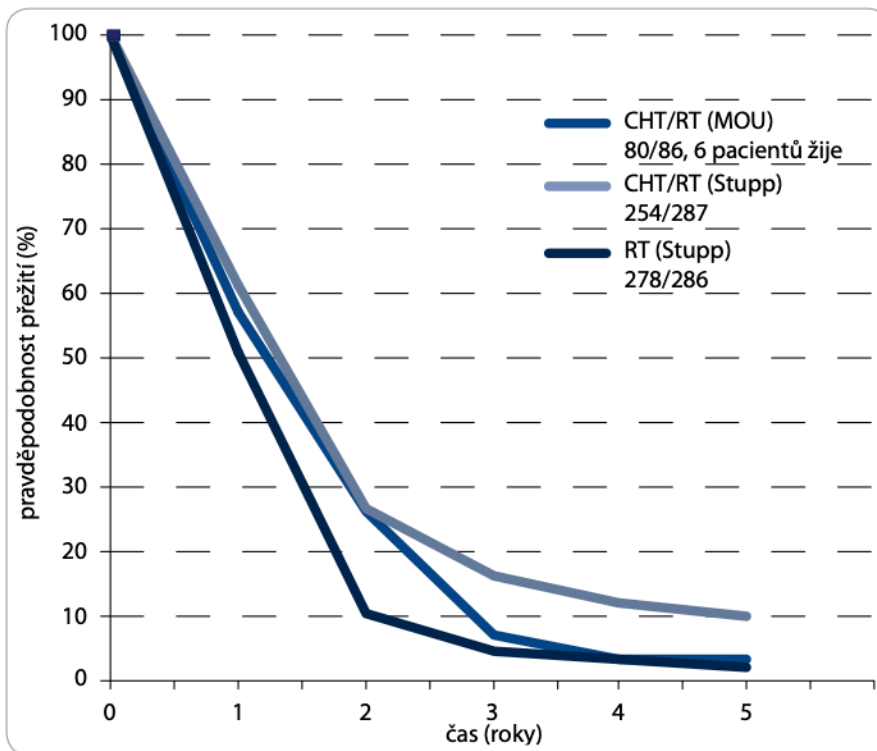
I v případě protinádorové léčby glioblastomů došlo v průběhu posledních 20 let k postupnému vývoji v otázce zařazení chemoterapie a typu cytostatik. Na počátku byly klinické studie zamě-

řeny zejména na hodnocení významu chemoterapie na bázi derivátů nitrosourey aplikovaných po chirurgickém zákroku a radioterapii u pacientů s high-grade gliomy. Dle metaanalýzy 12 klinických studií (GMT Group, 2002) zahrnujících přes 3 000 pacientů léčebných pooperačně samotnou radioterapii nebo současným podáním radioterapie a chemoterapie bylo zjištěno, že díky chemoterapii dochází k absolutnímu navýšení jednoletého přežití ze 40 % na 46 % a hraničnímu prodloužení mediánu přežití o 2 měsíce [6].

Od konce 90. let se začaly objevovat výsledky ze studií fáze II s konkomitantní chemoradioterapií s temozolomidem. V roce 2005 byly Stuppem publikovány výsledky průlomové studie fáze III, které potvrdily významný vliv konkomitantní chemoradioterapie a následné adjuvantní chemoterapie s temozolomidem na prodloužení času bez progresu i mediánu celkového přežití ve srovnání s pacienty léčenými jen operací a radioterapií [7]. Režim použitý v této studii se stal novým standardem léčby pacientů s glioblastomy a je i základem srovnávacím režimem v klinických studiích s novými léky, především z oblasti cílené léčby. Diskuze, která stále probíhá, se týká zejména počtu cyklů adjuvantní chemoterapie po ukončení konkomitantní léčby a také dávkového schématu temozolomidu. Iničiální Stup-

pova studie byla opakovaně podrobena retrospektivním analýzám, jejichž cílem bylo popsat jednotlivé podskupiny pacientů a nalézt zásadní prognostické a prediktivní faktory. Poslední hodnocení s výsledky pětiletého sledování bylo publikováno v březnu roku 2009 [8]. Signifikantní vliv chemoradioterapie na délku přežití byl zaznamenán ve všech analyzovaných podskupinách, bez ohledu na věk či rozsah výkonu. Největší benefit z léčby z pohledu klinických faktorů měli pacienti po makroskopicky totální resekci, mladší 50 let, s performance statusem (PS) 0–1. V případě resekčního výkonu a následné kombinované chemoradioterapie se 5 let dožívalo cca 10 % pacientů versus 1–2 % pacientů, u kterých byla pooperačně aplikována pouze samotná radioterapie. Pokud pacient podstoupil pouze biopsii, byly jeho šance na pětileté přežití při použití konkomitantní chemoradioterapie cca 5 %, kdežto s pouhou radioterapií téměř nulové [8].

V našem souboru jsme rovněž zaznamenali významný vliv radikality resekce a PS na celkové přežití. Věk byl jako prognostický faktor nesignifikantní, což mohlo být ovlivněno skladbou pacientů, kdy v souboru výrazně převažovali pacienti s PS 1 (74 %). Při srovnávání s výsledky Stuppova souboru jsme u našich pacientů dosáhli podobného dvouletého přežití, nicméně v pětiletém přežití



Graf 9. Křivky celkového přežití a orientační srovnání se Stuppovou studií.

jsou naše výsledky horší (podrobně viz tab. 2 a graf 9). Příčinou může být menší podíl pacientů po totálních resekcích (20% vs 39%) a nižší procento pacientů adjuvantně léčených temozolomidem po ukončené konkomitantní chemoradioterapii (40% vs 78%, viz tab. 1).

Naše studie má rovněž určité limity v možnosti přesného hodnocení léčebné odpovědi bezprostředně po primární chirurgické léčbě a po chemoradioterapii. V období od ledna 2003 až června 2005 nebylo vždy možné provádět pooperační MRI vyšetření k posouzení rezidua nádoru (bylo pouze CT vyšetření) a u části těchto pacientů nebylo k dispozici ani MRI za měsíc po ukončení konkomitantní chemoradioterapie. K hodnocení léčebné odpovědi bylo proto nejčastěji používáno CT vyšetření s i.v. kontrastem, u některých nejasných nálezů v kombinaci s PET vyšetřením, což nelze pokládat za optimální algoritmus poléčebného sledování. Dle dnešních doporučení má být pooperační MRI vyšetření provedeno do 24–72 hod po výkonu, další MRI vyšetření s odstupem 2–6 týdnů po ukončení konkomitantní chemoradioterapie a následné MRI kontroly v intervalu 2–4 měsíců

v průběhu adjuvantního temozolomidu a sledování.

Zajímavé bylo i srovnání výskytu závažné hematologické a nehematologické toxicity. V průběhu konkomitantní chemoradioterapie byla zaznamenána častější hematologická toxicita stupně G3/4, a to především trombocytopenie (9% vs 3%) a lymfopenie (25%), což opodstatňuje doporučení profylaktického podávání sumetrolimu. U nehematologické toxicity dominovaly tromboembolické příhody, viz tab. 6. Pacienti s PS 2 častěji konkomitantní léčbu nedokončili a jejich přežití bylo v řadě případů kratší než u podobných pacientů indikovaných k samotné radioterapii. Z toho vyplývá, že indikace konkomitantní chemoradioterapie u pacientů, kteří jsou po operaci v celkově horším klinickém stavu, musí být dobře zvážena. V případě, že kromě PS 2 jsou přítomny další negativní prognostické faktory, např. četné a závažné interkurence nebo inoperabilní nádor, je vhodnější volit pouze samotnou radioterapii.

Kromě klasických klinických prognostických faktorů je v současnosti diskutován i význam molekulárních prognostických a prediktivních faktorů. Za nadějný

prediktivní molekulární faktor se považuje stav metylace promotoru genu reparačního enzymu O⁶-Methylguanin-DNA-methyltransferázy (MGMT) [9–12]. Bohužel problémy se standardizací vyšetřovací metody komplikují zavedení vyšetřování tohoto faktoru do rutinní klinické praxe. Stav metylace MGMT u části našich pacientů je právě testován a výsledky budou publikovány později.

Další velmi významnou problematikou v této oblasti je léčba recidivy/progrese glioblastomu po předchozí primární léčbě. Pro určité pacienty může být metodou volby další neurochirurgický výkon, reiradiace nebo chemoterapie. Výsledky paliativní chemoterapie založené na derivátech nitrosourey v našem souboru nebyly nijak přesvědčivé. Četnost léčebných odpovědí se pohybovala do 10%, trvání bylo krátkodobé, medián času do progresu se pohyboval kolem 3 měsíců. Podobné výsledky byly dosaženy i v případě opakovaní radioterapie, jejíž aplikace je omezena předchozím ozařováním. Naopak i naše zkušenosti jednoznačně potvrzují, že u vybraných pacientů může být významným přínosem reoperace.

Určité zlepšení léčebných výsledků u rekurentních glioblastomů by mohly přinést dose-dense a metronomické režimy s temozolomidem [13,14]. Jejich potenciální účinnost i po selhání režimů se standardně dávkovaným temozolomidem je vysvětlována odlišným patofyziologickým mechanismem působení metronomické protinádorové léčby. Metronomické režimy převyšují dávkovou intenzitou standardní režimy, mohou působit antiangiogenně a díky kontinuálnímu podávání mohou způsobovat chronickou depleci reparačního enzymu MGMT. K dnešnímu datu však neproběhla žádná klinická studie fáze III, která by tyto hypotézy potvrdila. V současné době probíhá řada klinických studií s cílenou léčbou a v této souvislosti jsou i hledány nové molekulární prognostické faktory [15]. Nejčastěji se jedná o léčiva cílená proti mechanismům angiogeneze [16,17], signálním drahám receptorů pro růstové faktory [18–20], m-TOR [21], integrinům [22]. V tomto směru nejnadějnějších výsledků dosáhl bevacizumab [23,24], který je v USA již doporučen

k léčbě rekurentních high-grade gliomů jak v monoterapii, tak v kombinaci s chemoterapií. Začlenění bevacizumabu do první linie léčby ke konkomitantní chemoradioterapii s temozolomidem je nyní předmětem probíhajících klinických studií fáze III (AVAglio, RTOG 0825) [25]. Z dalších testovaných léčiv měl nadějný výsledek ve studiích fáze II orální inhibitor receptorů pro VEGF – cediranib [26]. Bohužel studie fáze III tato očekávání nepotvrdila [27]. Další studie fáze III probíhá také s inhibitorem integrinů – cilengitidem. Tento preparát je zkoušen v rámci první linie léčby se Stuppovým režimem u pacientů s přítomnou metylací promotoru genu pro MGMT.

Závěr

K dosažení optimálních výsledků léčby pacientů s glioblastoma multiforme je nutné, aby léčebná strategie byla vedena multidisciplinárním týmem. Standardní komplexní léčba je v současnosti založena na chirurgickém výkonu s maximální radikalitou při současném zachování kvality života, následováním konkomitantní aplikací radioterapie a chemoterapie s temozolomidem, který dále pokračuje i v adjuvantní léčbě. Větší benefit z uvedené léčby lze očekávat u pacientů po makroskopicky totální resekci, mladších 50 let, s performance statusem 0–1 a přítomností metylace promotoru genu reparačního enzymu MGMT. Důležitým faktorem pro rozhodování o indikaci konkomitantní chemoradioterapie je i potenciální toxicita léčby vedoucí k jejímu předčasnému ukončení a v konečném důsledku kratšímu přežití než při radioterapii samotné. Proto je nezbytné každého pacienta posuzovat individuálně. Dle našich zkušeností musíme být zvláště opatrní s indikací chemoradioterapie u pacientů s PS 2. Dalším důležitým faktorem je také léčba recidivy/progrese onemocnění po předchozí primární léčbě. Na zá-

kladě našich výsledků je evidentní, že u vybraných pacientů může být významným přínosem reoperace. Ve snaze zlepšit léčebné výsledky probíhá v posledních letech řada klinických studií s cílenou léčbou. Nejdále je výzkum v oblasti inhibice angiogeneze, především s bevacizumabem. Výsledky těchto studií mohou zlepšit obecně špatnou prognózu pacientů s glioblastomy.

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PŘÍLOHA 4



Real-World Evidence in Glioblastoma: Stupp's Regimen After a Decade

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The aim of this retrospective study is to provide real-world evidence in glioblastoma treatment and to compare overall survival after Stupp's regimen treatment today and a decade ago. A current consecutive cohort of histologically confirmed glioblastoma irradiated from 1/2014 to 12/2017 in our cancer center was compared with an already published historical control of patients treated in 1/2003–12/2009. A total of new 155 patients was analyzed, median age 60.9 years, 61% men, 58 patients (37%) underwent gross total tumor resection. Stupp's regimen was indicated in 90 patients (58%), 65 patients (42%) underwent radiotherapy alone. Median progression-free survival in Stupp's regimen cohort was 6.7 months, median OS 16.0 months, and 2-year OS 30.7%. OS was longer if patients were able to finish at least three cycles of adjuvant chemotherapy (median 23.3 months and 43.9% of patients lived at 2 years after surgery). Rapid early progression prior to radiotherapy was a negative prognostic factor with HR 1.87 ($p = 0.007$). The interval between surgery and the start of radiotherapy (median 6.7 weeks) was not prognostically significant ($p = 0.825$). The median OS in the current cohort was about 2 months longer than in the historical control group treated 10 years ago (16 vs. 13.8 months) using the same Stupp's regimen. Taking into account differences in patient's characteristics between current and historical cohorts, age, extent of resection, and ECOG patient performance status adjusted HR (Stupp's regimen vs. RT alone) for OS was determined as 0.45 ($p = 0.002$).

Keywords: glioblastoma, chemotherapy, radiotherapy, rapid early progression, overall survival, real-world evidence

INTRODUCTION

Despite intensive multimodal treatment of glioblastoma consisting of maximal safe resection followed by combined chemoradiotherapy (well-known Stupp's regimen), ultimately all patients develop tumor recurrence and subsequently die for further glioblastoma progression (1, 2). The greatest benefit from multimodal treatment has been demonstrated in patients after macroscopic gross total resection (GTR), those under 50 years of age, with ECOG (Eastern Cooperative Oncology Group) performance status of 0–1 and the presence of promoter methylation of O6-methylguanine-DNA-methyltransferase (MGMT) gene (3–5). Currently, more approaches are considered standard of care in older people who are less tolerant to the standard Stupp's regimen and are treated by Perry's modification (3 weeks chemoradiotherapy) or by chemotherapy alone, for example (6–8).

Real-world evidence data are an increasingly important supplement to clinical and translational research. These analyses of current real-world patients treated outside controlled clinical trials may identify hidden needs as well as provide survival data for proper powering in future clinical trials. This is especially relevant in glioblastoma where no positive practice changing trial, focused on the treatment of the best prognostic glioblastoma subcohort, was published during the last 15 years despite huge advances in the understanding of glioblastoma in general (9, 10).

This single institutional retrospective study unbiased by inter-center variability aims to analyze the outcomes of consecutive glioblastoma patients irradiated in our cancer center from 1/2014 to 12/2017 and to compare their outcomes with a historical control of patients treated in 1/2003–12/2009. This control cohort with a median survival of 13 months (2-year overall survival 26%) was published in 2011 and was treated by the same Stupp's regimen as most patients from the present cohort (11, 12). Comparison of survival data in respective control arms in recently published global clinical trials (Stupp's regimen used as the standard of care in control arm) with those published in original trial by Stupp et al. (patients enrollment from 8/2000 to 3/2002) reveals remarkable improvement in survival around 5 months after the same treatment regimen (1, 10, 13, 14). We aim to describe this possible improvement also in patients treated in real-world care outside of clinical trials in single institutional report unbiased by variability associated with the inclusion of patients in many countries and institutions.

MATERIALS AND METHODS

Consecutive patients over 18 years of age with histologically proven newly diagnosed glioblastoma irradiated from 1/2014 to 12/2017 in our cancer center were eligible for this analysis approved by our institutional review board. All patients signed informed consent with the usage of their data for research purposes. All patients after glioma surgery were discussed in the multidisciplinary neurooncology tumor board and those eligible for postsurgery oncology treatment were referred to radiotherapy consultation. A subgroup of patients indicated to concurrent

chemoradiation with subsequent adjuvant chemotherapy was further analyzed in detail.

Radiotherapy (RT) was performed in all patients within study cohorts. A planning CT scan for 3-dimensional RT dose calculation was utilized in all patients. Some of them underwent also planning MRI (including postcontrast T1 weighted scan with submillimeter slices) which was rigidly registered to CT scan for proper RT target definition. Individual prescription of RT dose and scheduling was guided mainly by patient's performance status and by volume, size, shape, and location of the target volume. Both standards of care approaches in target volume definitions were employed in patients eligible for treatment by Stupp's regimen—the Radiation Therapy Oncology Group (RTOG) contouring approach that defines two clinical target volumes accommodating hyperintensity at T2/FLAIR MRI in addition to T1 contrast-enhanced MRI (15) and the European Organization for Research and Treatment of Cancer (EORTC) single-phase contouring approach that defines one target utilizing mainly T1 postcontrast MRI (16). The total dose of normofractionated 60 Gy was prescribed irrespective of the used target volumes definition approach. RT was prepared employing planning system EclipseTM (Varian medical systems, Palo Alto, CA, USA) and performed on linear accelerator Varian Clinac iX or TrueBeam (Varian medical systems, Palo Alto, CA, USA).

Concurrent chemoradiotherapy and adjuvant chemotherapy were prescribed according to the original Stupp et al. (1) protocol. Temozolomide (75 mg/m²) was administered on days 1 through 42 with concomitant RT (60 Gy). After 4 weeks, treatment follows by the administration of temozolomide alone (150–200 mg/m²) on days 1–5 in six consecutive 4-week cycles or to progression. The prophylaxis against *Pneumocystis jirovecii* pneumonia was at the discretion of the treating physician.

Response to treatment was evaluated based on regular follow up MRI scanning. Progression presented already on planning MRI was considered only in patients who had available early postsurgery (within 72 h) control MRI enabling a clear definition of eventual postsurgery residuum. The first post (chemo)radiotherapy MRI was usually ordered 4–6 weeks after the last RT session, followed by regular MRI every 3 months unless clinically indicated for earlier examination. No routine RANO criteria (17) usage in daily clinical practice was employed and MRI were visually evaluated by servicing radiologist. Unclear findings were reviewed by a multidisciplinary neurooncology tumor board, mostly with a recommendation for an earlier control exam. Treatment at progression was highly individualized with options for resurgery, reirradiation, temozolomide rechallenge, palliative chemotherapy (mostly lomustine), or symptomatic treatment.

The primary objective is to evaluate the impact of clinical and laboratory factors (gender, age, extent of resection, ECOG patient status, tumor location, early tumor progression on planning MRI, MGMT methylation) and used treatment on survival parameters such as progression-free survival (PFS) and overall survival (OS). PFS was defined as the time from the date of initiation of RT to the date of relapse. Considering retrospective nature of this analysis, no strong measures according to differential diagnosis

TABLE 1 | Basic patients' characteristics of current cohort (GBM 2014–2017) and historical group (GBM 2003–2009).

Study cohort	GBM 2014–2017 (n = 155)		GBM 2003–2009 (n = 145)		Current vs. historical group Stupp's regimen p-value*
	Stupp's regimen n = 90	RT alone n = 65	Stupp's regimen n = 86	RT alone n = 59	
Age (years)					
Median (IQR)	56 (30–76)	66 (20–86)	56 (24–69)	67 (41–82)	0.034
≤50	22 (24%)	10 (15%)	30 (35%)	5 (8%)	0.140
Mens	61 (68%)	34 (52%)	51 (59%)	33 (56%)	0.274
Performance status (ECOG) and Karnofsky index (KI)					0.450
ECOG 0 (KI 90–100%)	45 (50%)	11 (17%)	38 (44%)	6 (10%)	
ECOG 1 (KI 70–80%)	44 (49%)	38 (58%)	48 (56%)	35 (59%)	
ECOG 2 (KI 50–60%)	1 (1%)	16 (25%)	0 (0%)	18 (31%)	
Tumor location					
Deep brain location	23 (26%)	26 (40%)	NA	NA	
Extent of resection					<0.001
GTR	44 (49%)	14 (22%)	17 (20%)	8 (13%)	
STR	36 (40%)	24 (37%)	56 (65%)	21 (36%)	
Partial resection or biopsy	10 (11%)	27 (41%)	13 (15%)	30 (51%)	
IDH status					
Mutated/evaluated	5/57 (9%)	1/22 (5%)	NA	NA	
MGMT status					
Methylated/evaluated	11/48 (23%)	8/25 (32%)	12/38 (32%)	NA	

GBM, glioblastoma; CHT/RT, chemoradiotherapy; CHT, chemotherapy; RT, radiotherapy; NA, Not Available; ECOG, Eastern Cooperative Oncology Group; GTR, gross total resection; STR, subtotal resection; IDH, Isocitrate dehydrogenase; MGMT, O6-methylguanine-DNA-methyltransferase. *p-values <0.05 are marked in bold.

of pseudoprogression were possible to be utilized. In the cases, where progression was described by the radiologist and there was subsequent change in the treatment, we recorded date of that MRI as a date of progression. On the other hand, in the cases where there was no change in the treatment after radiologist call of possible progression and subsequent MRI did not confirm progression, we did not record the previous MRI as that with progression and the subsequent MRI were evaluated in PFS analysis. OS was defined as the time from the date of diagnosis to the date of death (from tumor cause). The last control date was considered when relapse/death was not presented. The secondary goal is to compare the current treatment results using the Stupp's regimen with the results of patients treated 10 years ago adjusted for age, extent of resection, and ECOG patient status.

Patients' characteristics of both current and historical cohorts were described using standard summary statistics i.e., median and interquartile range (IQR) for continuous variables and frequency distributions for categorical variables. The following comparison of both groups was examined with Fisher's exact test, chi-squared test, or Mann–Whitney test, as appropriate. Survival probabilities were estimated using the Kaplan–Meier method. The log-rank test was performed to compare OS and PFS between the groups. Characteristics associated with the time-to-event outcomes were evaluated using Cox models where hazard ratios

(HR) and their 95% confidence interval (CI) were calculated. The proportional hazard assumption was verified based on scaled Schoenfeld residuals. The multivariable model was fitted using stepwise backward selection. All statistical analyses were performed employing R version 3.6.2 (18) and the significance level of 0.05 was considered.

RESULTS

A total of 155 patients was indicated to postsurgery RT. The median age was 61 years, 21% were younger 50 years, slightly higher number of men (61%). Gross total resection was achieved in 58 (37%) patients and more than 80% were in good general condition (ECOG 0–1). The other basic patients and tumor characteristics are summarized in **Table 1** including corresponding data from the historical cohort (11, 12). Patients treated with the Stupp's regimen in 2014–2017 were older than the historical cohort ($p = 0.034$) but underwent more often radical resection ($p < 0.001$). Postsurgery MRI exam was performed in 97 (63%) patients and was more common in patients after GTR or subtotal resection (STR).

The median time to first RT session was 6.7 weeks (range 2.1–11.7 weeks). The majority of patients (91%) were irradiated by intensity-modulated radiotherapy technique (including arc

TABLE 2 | Patients' treatment.

Study cohort	GBM 2014–2017 (n = 155)		GBM 2003–2009 (n = 145)		Current vs. historical group Stupp's regimen p-value*
	Stupp's regimen n = 90	RT alone n = 65	Stupp's regimen n = 86	RT alone n = 59	
Time to RT initiation					
Median (weeks)	6.7	6.9	5.1	5.3	<0.001
>6 weeks	57 (63%)	43 (66%)	27 (31%)	22 (39%)	<0.001
Radiotherapy					
Median dose	60	40	60	50	0.430
Abbreviated RT 15 × 2.67 Gy	0	11/65 (17%)	0	0	
Abbreviated RT 20 × 2.5 Gy	0	17/65 (26%)	0	9/59 (15%)	
Contouring approach EORTC	32 (36%)	53 (82%)	NA	NA	
Contouring approach RTOG	58 (64%)	5 (8%)	NA	NA	
Chemoradiotherapy					
Duration (days; IQR)	42 (37–44)	0	42	0	
Corticosteroids use	53/86 (62%)	57/62 (92%)	NA	NA	
Adjuvant chemotherapy					
No. of patients	65/90 (72%)	0	34/86 (40%)	0	<0.001
No. of cycles: median (range)	4 (1–15)	0	4 (1–12)	0	
No. of cycles: ≥3	47/90 (52%)		26/86 (30%)	0	
No. of cycles: ≥6	31/90 (34%)	0	11/86 (13%)	0	
Treatment after progression					
No. of patients	47/79 (59%)	9/32 (28%)	46/67 (69%)	NA	
Surgery	20/47 (43%)	1/9 (11%)	21/46 (46%)	NA	
Chemotherapy	33/47 (70%)	8/9 (89%)	39/46 (85%)	NA	
Reirradiation	20/47 (43%)	1/9 (11%)	8/46 (17%)	NA	

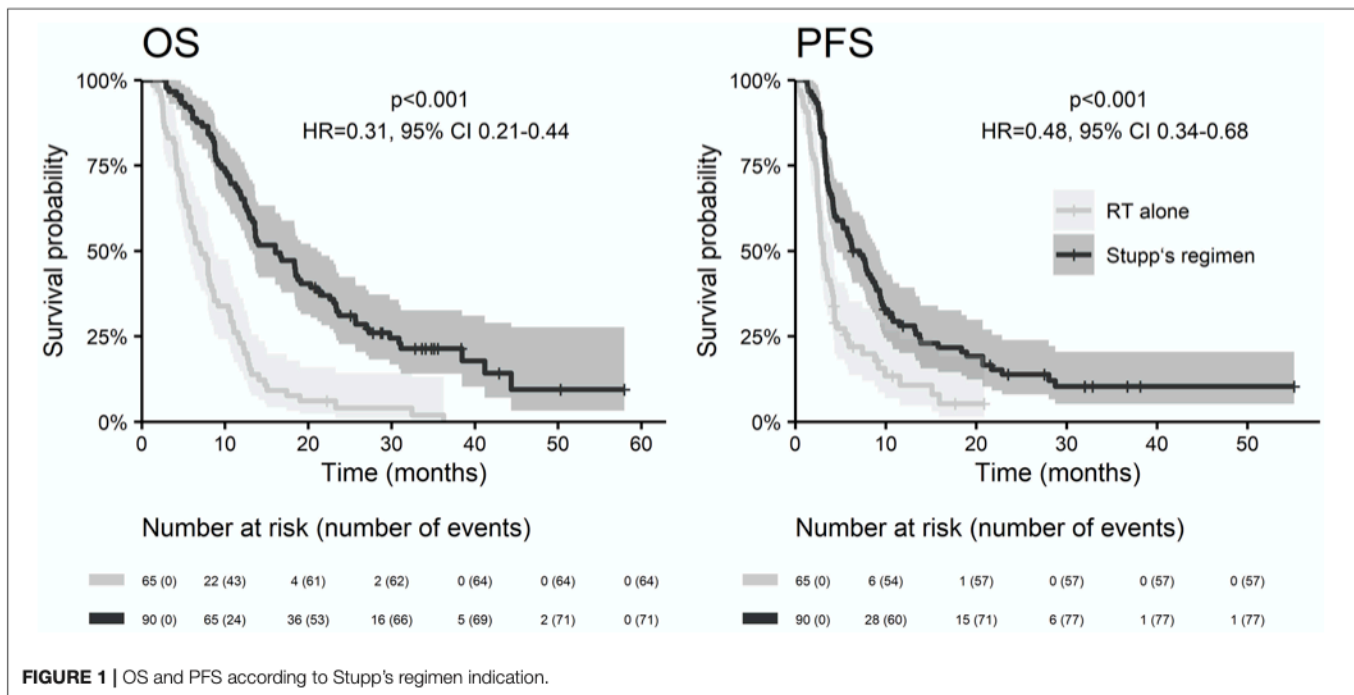
GBM, glioblastoma; CHT/RT, chemoradiotherapy; CHT, chemotherapy; RT, radiotherapy; NA, Not Available. *p-values < 0.05 are marked in bold.

therapy—volumetric modulated RT). Among patients who were not indicated to Stupp's regimen, the most common abbreviated schedule was 15 × 2.67 Gy (11/65; 17%) and 20 × 2.5 Gy (17/65; 26%). Stupp's regimen was indicated in 90/155 patients (58%). Only 31/90 (34%) patients finished the whole Stupp's protocol with all six cycles of adjuvant chemotherapy, 47/90 (52%) finished at least three cycles. More details about patients' treatment are summarized in **Table 2**.

With a median follow up of 34.8 months, the median PFS for the whole study cohort was 4.2 months and 2-year PFS 10%. Corresponding values for OS were 11.6 months and 19.8%. Treatment with Stupp's regimen was a strong positive prognostic factor with HR 0.31 ($p < 0.001$, median 16.0 vs. 7.1 months) and HR 0.48 ($p < 0.001$, median 6.7 vs. 3.1 months) for OS and PFS, respectively (**Figure 1**). Univariable analysis of prognostic factors for OS and PFS in the whole study cohort, Stupp's regimen cohort, and radiotherapy alone cohort is summarized in **Figure 2** and **Table 3**. In the whole cohort, the median OS of patients over 50 years was significantly shorter than that of younger patients (10.7 vs. 20.2 months; HR 2.31; $p < 0.001$). Better OS was observed in patients after GTR (median 15.4 vs. 11.8 months; HR 0.54; $p = 0.003$), those with better ECOG

score (median 13.6 vs. 10.3 vs. 5.8 months for ECOG 0, 1, 2 respectively; $p < 0.001$), patients with contouring based on RTOG approach (median 14.0 vs. 10.7 months; HR 0.60; $p = 0.005$) and patients without corticosteroids, as well as without deep brain tumor location (related to possibility to achieve GTR). No difference in OS and PFS was observed in our considered cohorts with respect to MGMT methylation status. The interval between surgery and the start of radiotherapy (median 6.7 weeks) was not prognostically significant ($p = 0.825$ and 0.603 for OS and PFS, respectively). On the other hand, the presence of rapid early progression on planning MRI (in 46 patients out of 90 evaluable patients who had postsurgery MRI) was associated with significantly worse survival (median 10.7 vs. 18.7 months; HR 1.87; $p = 0.007$, 2-year OS 15.6 vs. 37.7%), **Figure 3**.

The best outcomes had patients, who were able to continue in adjuvant chemotherapy after chemoradiotherapy (median OS 23.3 months and 2-year survival of 43.9% in those who finished at least three cycles of adjuvant chemotherapy), **Figure 4**. Survival outcome was associated with adjuvant chemotherapy also after adjusting for age, extent of resection, and ECOG patient status (**Figure 5**).



In the subgroup of patients treated by Stupp's protocol, the age, deep brain tumor location, contouring approach, corticosteroids, and adjuvant chemotherapy were independently associated with OS and age, deep brain tumor location, and adjuvant chemotherapy were independently associated with PFS (Figure 6).

In comparison with 86 patients from historical control treated by "the same" Stupp's regimen, the positive trend in the increase of overall survival was observed (median OS 13.8 vs. 16.0 months), Table 4 (11). The hazard ratios (Stupp's regimen vs. RT alone) were adjusted for age, extent of resection, and ECOG patient status due to comparability with historical results (historical cohort and original Stupp's trial) (1, 11). Adjusted HRs in current cohort were 0.45 (95% CI: 0.27–0.75, $p = 0.002$) and 0.55 (95% CI: 0.32–0.93, $p = 0.025$) for OS and PFS, respectively.

DISCUSSION

Remarkable improvement in overall survival after Stupp's regimen treatment was observed in recent years in comparison to the historical cohort treated a decade ago in our cancer center. Using the same treatment protocol in daily real-world practice, we observed improvement in median OS more than 2 months, with similar median PFS. Absence in PFS improvement may probably be due to the frequent unavailability of postoperative MRI examination 10 years ago. In the cohort of subsequently treated patients according to Stupp's regimen, postoperative MRI was performed in only 20% (17/86) of patients. The evaluation of the finding on the first MRI examination after completed radiotherapy was then very problematic. Progression was often closed according to the second MRI examination. Similar improvement in OS through decade was also observed

in reports from recent prospective randomized clinical trials where treatment in control study arm usually consisted of Stupp's regimen. However, care must be taken in comparison of survival data with respect to time of randomization (some of the recent clinical trials randomized patients after the end of chemoradiotherapy phase) (10, 13, 14). In the ACT IV study with rindopepimut, the median OS with Stupp's regimen alone (control arm) was 20.2 months (median 2.8 months from diagnosis to randomization + median 17.4 months from randomization to death) and in EF-14 with Optune median OS 19.8 months as discussed below (13, 14). Randomization in the EF-14 study (Stupp's regimen + Optune vs. Stupp's regimen alone) occurred after chemoradiotherapy was finished and only patients without progression of the disease were enrolled (92%), the remaining 8% of patients were excluded. Hypothetically, if the same proportion of patients with the worst prognosis (8%) were excluded from the Stupp's study EORTC 26981–22981/NCIC CE3, then the median OS of patients treated with Stupp's regimen would increase to around 16.5 months from randomization and to around 17.7 months from diagnosis. The difference in overall survival of 2–3 months is probably due to advances in diagnostic and treatment methods (20.2 months in ACT IV and 19.8 months in EF-14 vs. hypothetical 17.7 months in the Stupp's study) (10, 13, 14). The same improvement was also observed in our real-world cohort of patients treated outside of clinical trials.

The original Stupp's regimen (EORTC 26981–22981/NCIC CE3) was published already in 2005 and represents one of the most influencing prospective clinical trial in general (1). Indeed, the referred paper is unequivocally the most cited one (Publication Year 2005) in premium *the New England Journal of Medicine* journal. Only a few subsequent reports indicate so far possible improvements, mainly based on

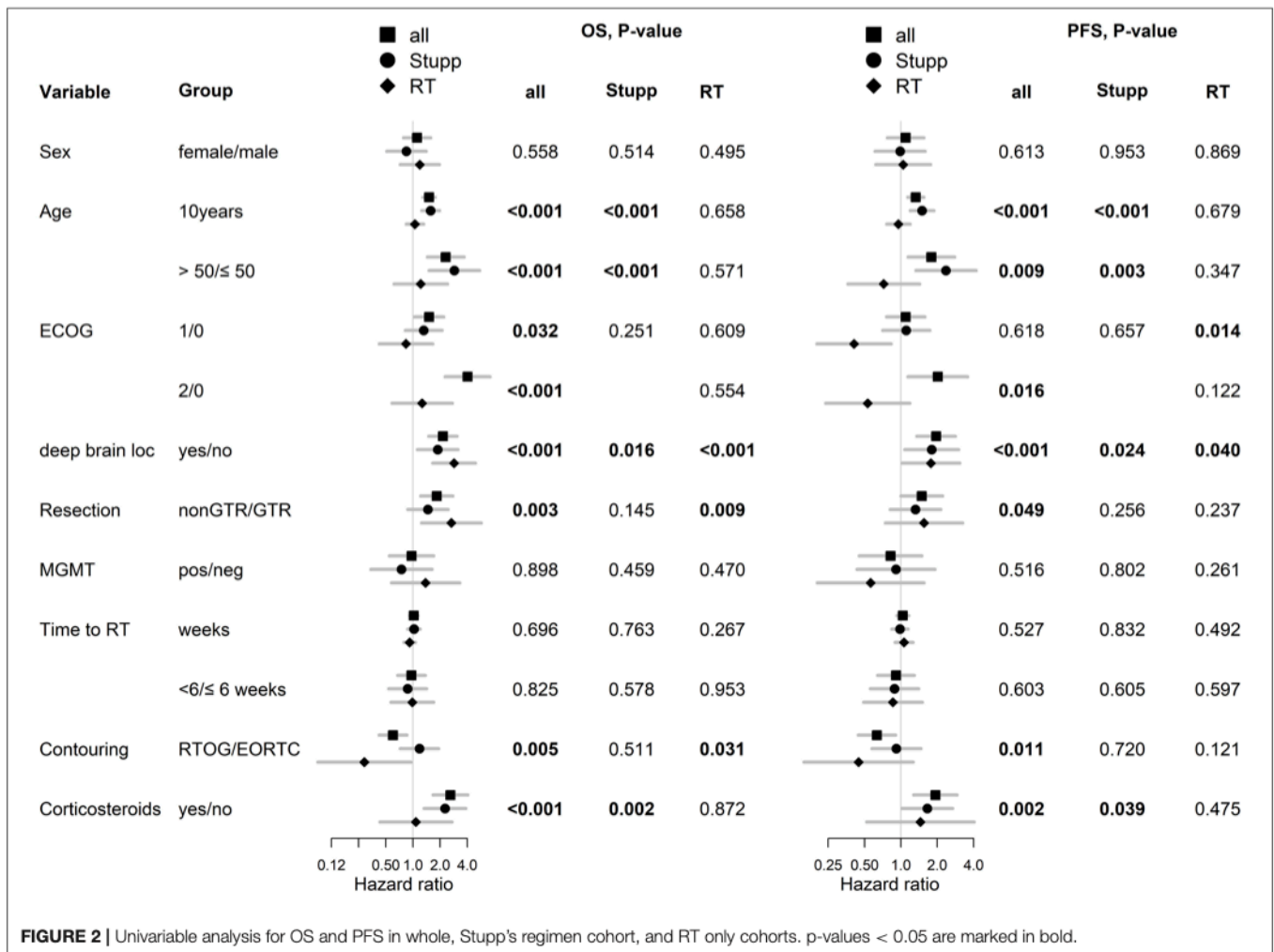


FIGURE 2 | Univariable analysis for OS and PFS in whole, Stupp's regimen cohort, and RT only cohorts. p-values < 0.05 are marked in bold.

trials focused on alternative temozolomide scheduling, as is prolonged administration to 12 or even more months (19). However, based on a meta-analysis of 4 randomized clinical trials comparing outcomes after six vs. more cycles adjuvant chemotherapy, no difference in OS was observed next to only slight improvement in PFS (19). Moreover, not every patient is actually able to finish in daily clinical practice the predefined 6 months of adjuvant treatment due to worsening clinical status or already progressing tumor. In our recent cohort, 52% (47/90) of patients were able to finish three and more cycles, and only 34% (31/90) completed six cycles of temozolomide (in original Stupp's cohort 47% of patients finished six cycles).

Neither other modern targeted therapies (bevacizumab, anti-EGFR inhibitors and antibodies, integrin inhibitors, anti-EGFR antibody conjugate, and depatuzumab mafodotin cytostatics) have been able to improve the Stupp regimen (20–26). Similarly, immunotherapy with rindopepimut or the anti-PD-1 antibody nivolumab, which has been currently successful in a number of poorly treatable diagnoses, has not been successful (13).

The only positive phase 3 clinical trial since 2005 that partially changed the glimpse of the treatment standard of newly diagnosed glioblastomas is the EF-14 study with Optune. The principle of treatment is based on the application of alternating current via Tumor Treating Fields to the tumor by means of electrodes adhered to the scalp, which prevent tumor cell mitosis (14, 27). Since treatment with TTF is not appropriate/accepted by and also available to every patient with glioblastoma, the Stupp's regimen should still be considered the gold standard (28). Similarly, intensification of chemotherapy in patients with MGMT methylation (lomustine + temozolomide combination) will probably not be a major breakthrough. The scheme is accepted with embarrassment, mainly because of concerns about a potential increase in toxicity (29).

The essential prerequisite for further optimization of treatment in daily clinical practice is the auto evaluation of own cohorts with comparison to published guidelines defining clinical trials. We performed the first major analysis of glioblastoma patients treated by the Stupp's regimen in 2011. At that time, patients treated in our cancer center achieved a similar median overall survival (13.8 vs. 14.6 months), the same 2-year survival

TABLE 3 | Univariable analysis for OS and PFS in whole, Stupp's regimen, and RT only cohorts.

Variable	Group	OS					
		All		Stupp's regimen		RT alone	
		HR (95% CI)	P-value*	HR (95% CI)	P-value*	HR (95% CI)	P-value*
Sex	Female/male	1.11 (0.78–1.57)	0.558	0.85 (0.51–1.40)	0.514	1.19 (0.72–1.97)	0.495
Age	10 years	1.51 (1.28–1.76)	<0.001	1.57 (1.25–1.97)	<0.001	1.05 (0.84–1.31)	0.658
	>50/≤50	2.31 (1.44–3.69)	<0.001	2.87 (1.50–5.48)	<0.001	1.22 (0.61–2.42)	0.571
ECOG	1/0	1.51 (1.04–2.19)	0.032	1.32 (0.82–2.11)	0.251	0.84 (0.42–1.66)	0.609
	2/0	4.02 (2.27–7.14)	<0.001	NA	NA	1.26 (0.58–2.73)	0.554
Deep brain loc	Yes/no	2.15 (1.49–3.08)	<0.001	1.88 (1.12–3.15)	0.016	2.85 (1.65–4.92)	<0.001
Resection	Non-GTR/GTR	1.84 (1.22–2.77)	0.003	1.46 (0.87–2.45)	0.145	2.66 (1.24–5.72)	0.009
MGMT	Pos/neg	0.96 (0.55–1.70)	0.898	0.74 (0.34–1.63)	0.459	1.38 (0.58–3.30)	0.470
Time to RT	Weeks	1.02 (0.92–1.14)	0.696	1.02 (0.87–1.20)	0.763	0.92 (0.79–1.07)	0.267
	<6/≤6 weeks	0.96 (0.67–1.37)	0.825	0.87 (0.54–1.41)	0.578	0.98 (0.57–1.70)	0.953
Contouring	RTOG/EORTC	0.60 (0.42–0.86)	0.005	1.18 (0.72–1.93)	0.511	0.29 (0.09–0.95)	0.031
Corticosteroids	Yes/no	2.60 (1.67–4.05)	<0.001	2.26 (1.33–3.85)	0.002	1.08 (0.43–2.71)	0.872
PFS							
Sex	Female/male	1.09 (0.77–1.55)	0.613	0.99 (0.61–1.59)	0.953	1.04 (0.62–1.76)	0.869
Age	10 years	1.33 (1.14–1.55)	<0.001	1.50 (1.19–1.88)	<0.001	0.95 (0.76–1.19)	0.679
	>50/≤50	1.79 (1.15–2.79)	0.009	2.36 (1.33–4.20)	0.003	0.72 (0.36–1.43)	0.347
ECOG	1/0	1.10 (0.76–1.58)	0.618	1.11 (0.71–1.74)	0.657	0.41 (0.20–0.83)	0.014
	2/0	2.02 (1.14–3.57)	0.016	NA	NA	0.53 (0.24–1.18)	0.122
Deep brain loc	Yes/no	1.96 (1.35–2.83)	<0.001	1.80 (1.07–3.01)	0.024	1.77 (1.02–3.08)	0.040
Resection	Non-GTR/GTR	1.49 (1.00–2.22)	0.049	1.32 (0.82–2.14)	0.256	1.56 (0.74–3.25)	0.237
MGMT	Pos/neg	0.82 (0.45–1.49)	0.516	0.91 (0.43–1.91)	0.802	0.56 (0.20–1.55)	0.261
Time to RT	Weeks	1.04 (0.92–1.17)	0.527	0.98 (0.84–1.15)	0.832	1.06 (0.89–1.26)	0.492
	<6/≤6 weeks	0.91 (0.64–1.30)	0.603	0.88 (0.56–1.41)	0.605	0.86 (0.49–1.51)	0.597
Contouring	RTOG/EORTC	0.63 (0.44–0.90)	0.011	0.92 (0.57–1.47)	0.720	0.45 (0.16–1.27)	0.121
Corticosteroids	Yes/no	1.93 (1.28–2.92)	0.002	1.66 (1.02–2.68)	0.039	1.45 (0.52–4.05)	0.475

OS, overall survival; PFS, progression free survival; RT, radiotherapy; NA, Not applicable; ECOG, Eastern Cooperative Oncology Group; GTR, gross total resection; MGMT, O6-methylguanine-DNA-methyltransferase. *p-values < 0.05 are marked in bold.

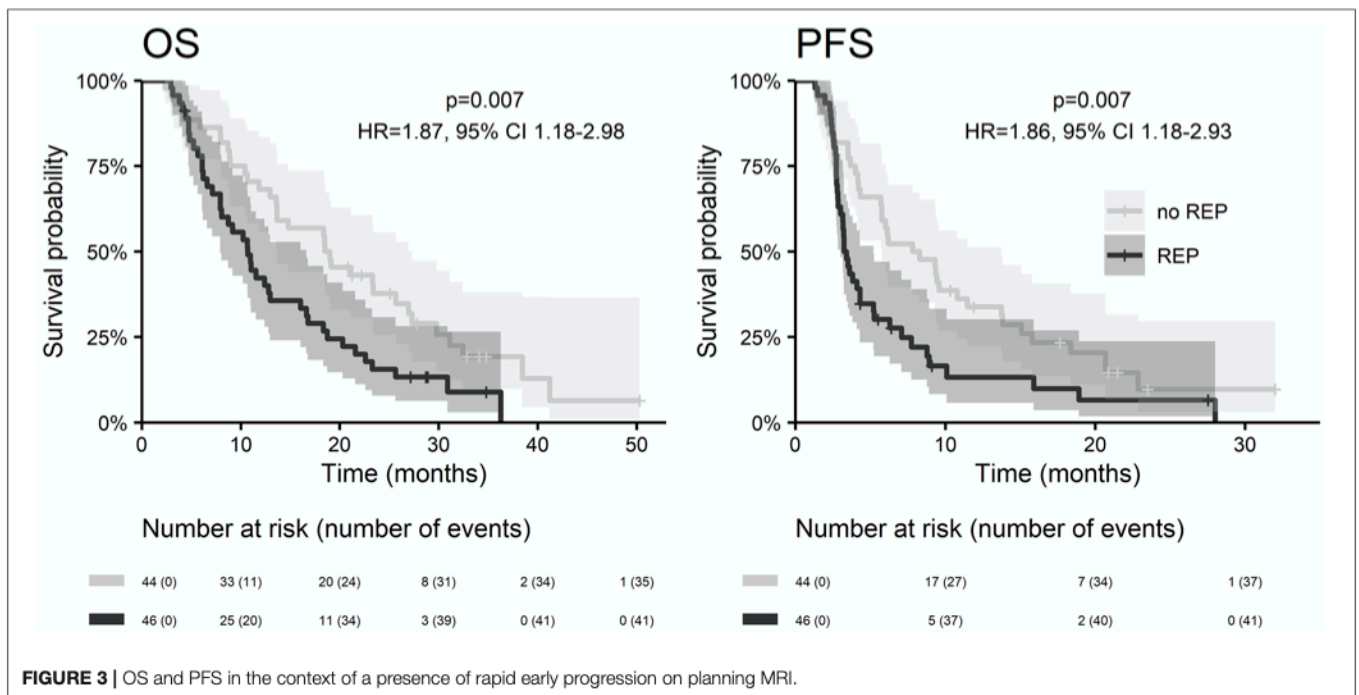


FIGURE 3 | OS and PFS in the context of a presence of rapid early progression on planning MRI.

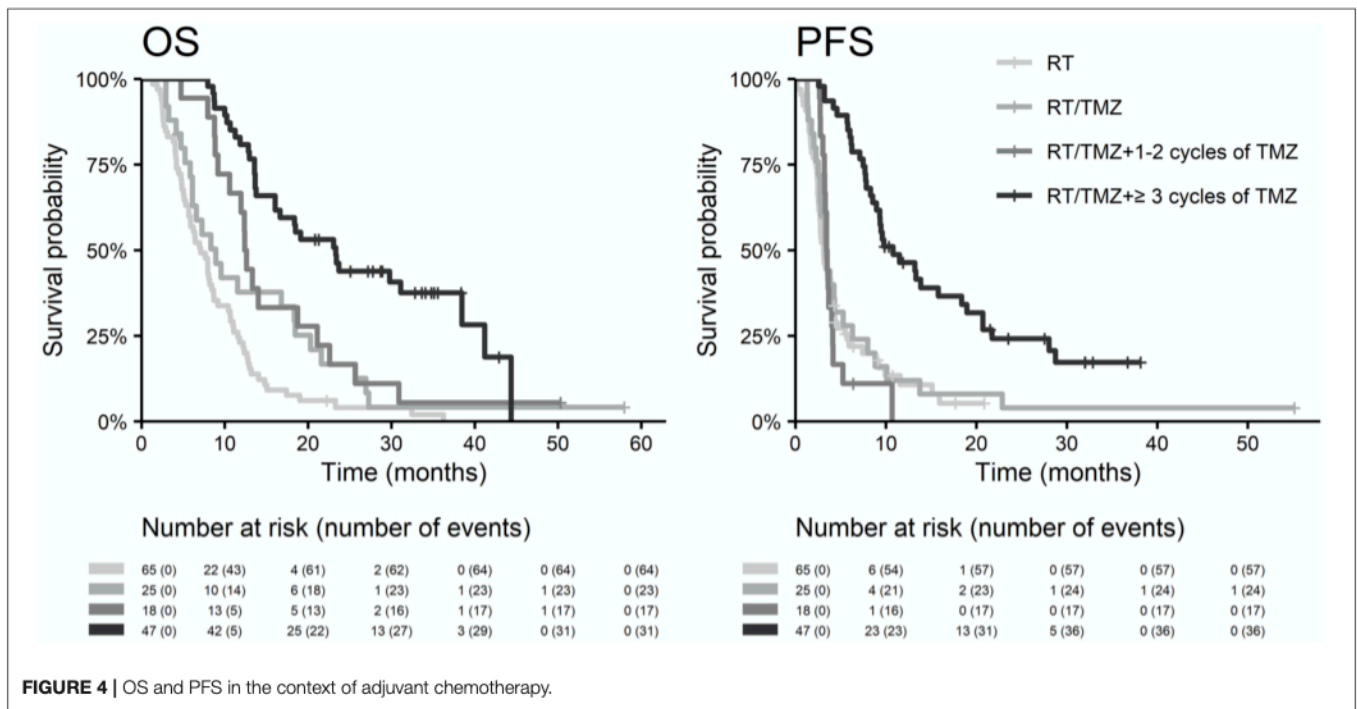


FIGURE 4 | OS and PFS in the context of adjuvant chemotherapy.

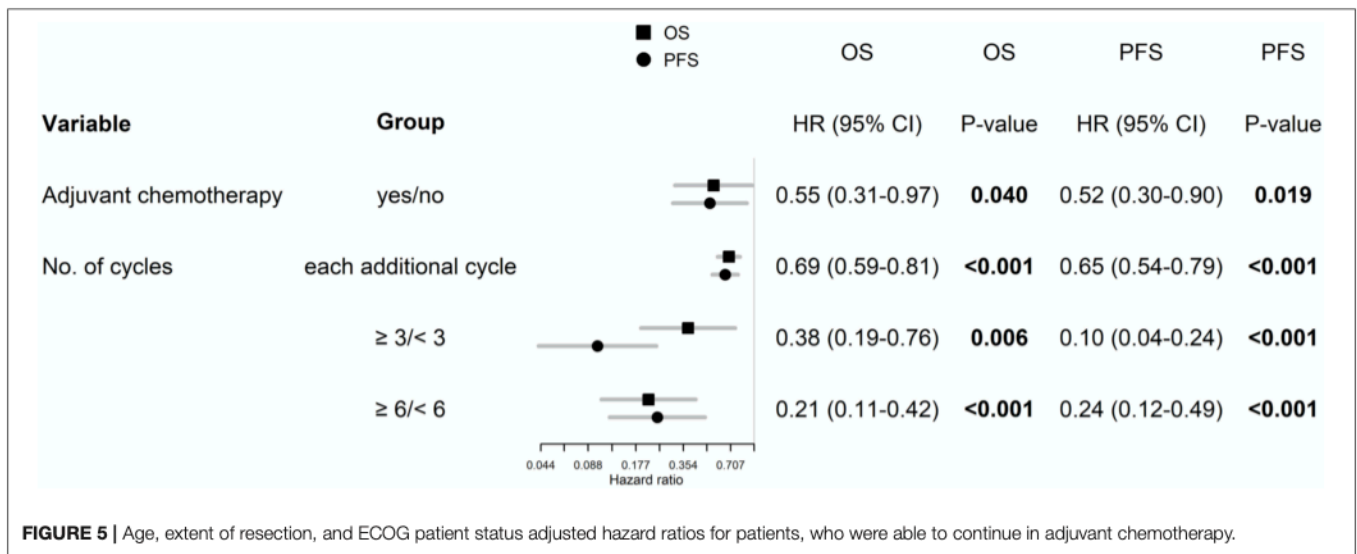


FIGURE 5 | Age, extent of resection, and ECOG patient status adjusted hazard ratios for patients, who were able to continue in adjuvant chemotherapy.

after surgery (28 vs. 27%), but lower 5-year survival (2 vs. 10%) compared to outcomes from original Stupp et al. trial (Table 4) (1, 11). In the recent evaluation of patients treated in 2014–2017, we confirmed the importance of known prognostic factors (except for MGMT methylation) and compared the overall survival to patients treated previously in 2003–2009 (86 patients). Similar to improvements observed in mentioned clinical trials, we also described increased survival in our cohort of patients treated outside of clinical trials (median overall survival increased from 13.8 to 16.0 months, the 2-year survival rate increased from 28 to 31% and 4-year survival increased from 2 to 10%), Table 4. This improvement in OS is also reflected by better

adjusted HR for treatment by Stupp regimen HR 0.45 (95% CI: 0.27–0.75, $p = 0.002$) in comparison to that reported in original Stupp paper [HR 0.63 (95% CI: 0.52–0.75) $p < 0.001$]. However, it should be acknowledged that the proportion of patients treated with adjuvant temozolomide increased significantly, mainly thanks to better toxicity management and improvements in general comprehensive cancer care. In the historical cohort, adjuvant chemotherapy after chemoradiotherapy was indicated in only 40% of patients, whereas in the current cohort it was already 72% (65/90), which is close to that in the Stupp and colleagues trial (78%) (1, 11). In addition, a subanalysis of 47 patients who underwent three or more cycles of adjuvant

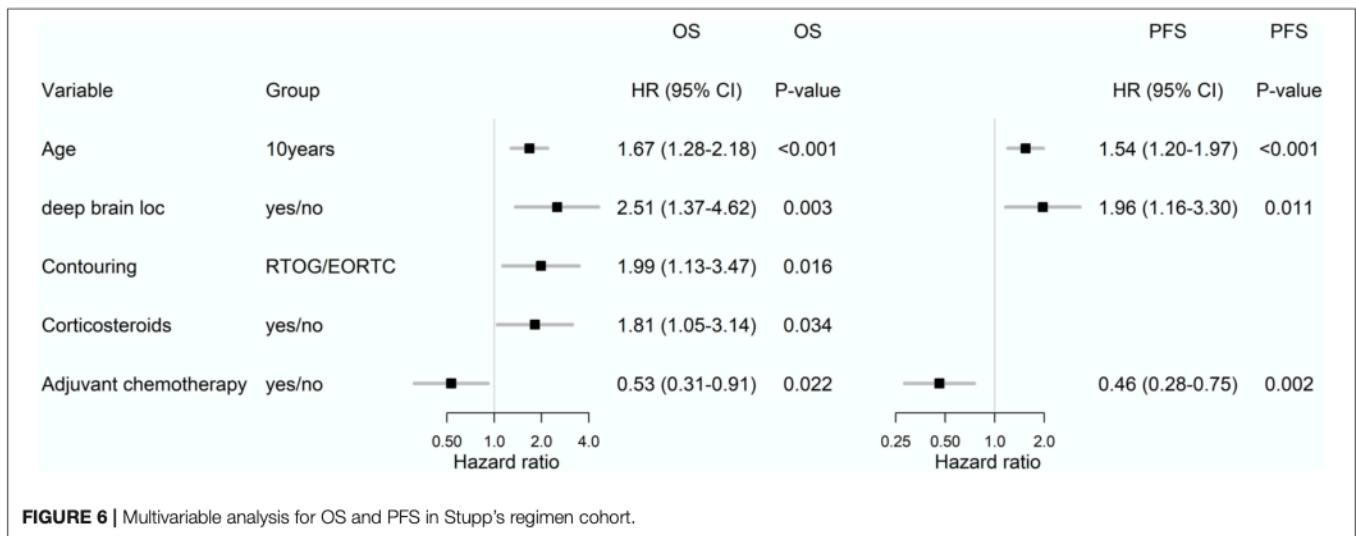


TABLE 4 | Survival outcomes (months) in comparison with previous cohorts.

	CHT/RT (MMCI 2014–2017) n = 90	CHT/RT (MMCI 2003–2009) n = 86	CHT/RT [Stupp trial (1)] n = 287
Median follow up (months)	34.8	NA	28
Overall survival			
Median	16.0	13.8	14.6
1-year	65%	58%	61%
2-year	31%	28%	27%
3-year	21%	7%	16%
4-year	10%	2%	12%
5-year	NA	2%	10%
Progression free survival			
Median	6.7	7.8	6.9
1-year	28%	32%	27%
2-year	14%	9%	11%

OS, overall survival; PFS, progression free survival; CHT/RT, chemoradiotherapy; MMCI, Masaryk Memorial Cancer Institute; NA, Not Available.

temozolomide revealed a significant increase in overall survival to 23.3 months and nearly 44% of patients achieved 2-year OS. From these results, it is evident that the continuation of adjuvant temozolomide is crucial. Premature discontinuation of chemotherapy should be avoided due to unclear findings at the first MRI follow-up after chemoradiotherapy (30–34). Attention should be paid to the differential diagnosis of pseudoprogression, with cooperation of radiation oncologist and neuroradiologist (35) as well as with employment of advanced imaging methods. Definitely, some patients (including those in original Stupp and colleagues trial) developed pseudoprogression, and wrong discontinuation of chemotherapy affected their survival. We can only assume the same bias rate in both historical and current cohort and thus relatively low influence on the overall survival analysis.

In the current analysis, we also addressed the issue of rapid early recurrence at planning MRI and its effect on overall survival (evaluated strictly only in patients who underwent postoperative MRI). The incidence of rapid early progression was 51% high, what incidence is in accordance with recent publications (36–39). We confirmed its significant negative prognostic effect on overall survival and progression-free survival (Figure 3). More aggressive treatment of this especially risky group of patients warrants further interest in future clinical trials.

The inherent limitation of this study is its retrospective nature. On the other hand, the methodology to obtain data describing the truly real clinical experience must be retrospective in nature. Thus, this represents both the strength as well as the limitation of this single institution study unbiased by inter-center variability. Definitely, there are enormous unmeasurable biases in the way the patients were treated during a decade (extent of surgery, demographic features, etc.) and these are likely different in the two time cohorts. Even more biases would be in the case we would aim to compare results with original trial by Stupp et al. (1) (patients enrollment between 2000 and 2002; patients enrolled in many institutions in many countries; ability to recover after brain surgery and ability to achieve better performance status also thanks to safer neurosurgery). For these reasons, we focused mainly on a comparison of our own two cohorts, and mentioned differences were acknowledged in statistical methodology. Some results (for example effect of RT target volumes contouring strategy on OS and observation of association between OS and number of temozolomide cycles in the adjuvant phase) warrant further detailed evaluation.

CONCLUSIONS

Age, performance status, extent of resection, the presence of rapid early recurrence before radiotherapy, MGMT gene promoter methylation, ability to finish concomitant chemoradiotherapy, and adjuvant chemotherapy significantly influence the prognosis of glioblastoma patients. According to an analysis of a recent group of patients treated outside of clinical trials with the Stupp's

regimen, we have shown a clear trend in extending overall survival over the last decade, despite the absence of a new treatment method. An important factor is the completion of the full Stupp's regimen. The most important is multidisciplinary cooperation and medical progress in both the area of diagnostics and individual treatment methods.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee, Masaryk Memorial Cancer Institute. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

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AUTHOR CONTRIBUTIONS

Conceptualization: TK, MS, RJ, JS, OS, and PS. Data curation: IS, PP, PF, VV, and OS. Formal analysis: RL, TK, AP, JS, and LH. Funding acquisition: RL, TK, and MH. Investigation: RL, TK, RB, PF, RJ, KM, MH, JS, and OS. Methodology: RL, TK, IS, RB, and JS. Project administration: LH. Supervision: PS. Validation: IS, AP, and PP. Writing—original draft: RL, TK, and IS. Writing—review and editing: TK, MS, OS, and PS. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Pre-Radiotherapy Progression after Surgery of Newly Diagnosed Glioblastoma: Corroboration of New Prognostic Variable

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Abstract: Background: The aim of this retrospective study is to assess the incidence, localization, and potential predictors of rapid early progression (REP) prior to initiation of radiotherapy in newly diagnosed glioblastoma patients and to compare survival outcomes in cohorts with or without REP in relation to the treatment. Methods: We assessed a consecutive cohort of 155 patients with histologically confirmed irradiated glioblastoma from 1/2014 to 12/2017. A total of 90 patients with preoperative, postoperative, and planning MRI were analyzed. Results: Median age 59 years, 59% men, and 39 patients (43%) underwent gross total tumor resection. The Stupp regimen was indicated to 64 patients (71%); 26 patients (29%) underwent radiotherapy alone. REP on planning MRI performed shortly prior to radiotherapy was found in 46 (51%) patients, most often within the

surgical cavity wall, and the main predictor for REP was non-radical surgery ($p < 0.001$). The presence of REP was confirmed as a strong negative prognostic factor; median overall survival (OS) in patients with REP was 10.7 vs. 18.7 months and 2-year survival was 15.6% vs. 37.7% (hazard ratio HR 0.53 for those without REP; $p = 0.007$). Interestingly, the REP occurrence effect on survival outcome was significantly different in younger patients (≤ 50 years) and older patients (> 50 years) for OS ($p = 0.047$) and non-significantly for PFS ($p = 0.341$). In younger patients, REP was a stronger negative prognostic factor, probably due to more aggressive behavior. Patients with REP who were indicated for the Stupp regimen had longer OS compared to radiotherapy alone (median OS 16.0 vs 7.5; HR = 0.5, $p = 0.022$; 2-year survival 22.3% vs. 5.6%). The interval between surgery and the initiation of radiotherapy were not prognostic in either the entire cohort or in patients with REP. Conclusion: Especially in the subgroup of patients without radical resection, one may recommend as early initiation of radiotherapy as possible. The phenomenon of REP should be recognized as an integral part of stratification factors in future prospective clinical trials enrolling patients before initiation of radiotherapy.

Keywords: glioblastoma; chemotherapy; radiotherapy; rapid early progression; overall survival

1. Introduction

The Stupp regimen is still the standard of care for patients with newly diagnosed glioblastoma, with only a few reports indicating possible improvement in the past decade [1,2]. Apart from the possible role of tumor treating fields [3], the update in treatment guidelines was mainly related to modifications of already known procedures (abbreviated chemoradiotherapy, or combination of temozolomide and lomustine) [4,5]. Prognostic and predictive biomarkers guide the indication for optimal treatment. Besides classical clinical prognosticators, biomarkers such as promoter methylation of the O6-methylguanine-DNA-methyltransferase (MGMT) gene or isocitrate dehydrogenase (IDH) 1 and 2 mutations moved into daily practice and became an integral part of diagnosis [6–9]. With the long-lasting lack of new effective therapeutics, further biomarkers for a suitable indication of the currently used modifications of temozolomide-based chemoradiotherapy are one way to improve the care of these patients.

The phenomenon of postoperative rapid early progression (REP) has only recently been explored with increasingly available magnetic resonance imaging (MRI) for both postsurgery and pre-radiotherapy (pre-RT) indication and is currently of high interest. REP diagnosis is based on a comparison of early postoperative MRI findings (up to 72 h postoperatively) and planning pre-RT MRI. Only a few studies retrospectively evaluated REP and indicated almost up to 50% risk of development of REP, regardless of the waiting time until the start of radiotherapy (RT) [10–12]. Clearly, these patients biased previous clinical trials, where no routine pre-RT MRI examination was performed. Currently, the treatment of these patients is not different from patients without REP, and if so, it is a purely individual approach.

The aim of this retrospective study is to evaluate the incidence and localization of REP in a consecutive cohort of patients treated, out of the frame of clinical trials (real-world evidence data). The aim is also to describe clinical factors associated with REP in glioblastoma and to describe the effect of REP and treatment on survival.

2. Materials and Methods

2.1. Patients and Treatment

A consecutive cohort of 155 histologically confirmed glioblastoma patients, who were indicated via a multidisciplinary neuro-oncology board to adjuvant or palliative radiotherapy between 01/2014 and 12/2017, were screened for eligibility to this retrospective study (Figure 1). All patients were

treated outside of clinical trials. Those with available early postoperative MRI (up to 72 h) evaluating the extent of surgery and those who had also performed pre-RT MRI were eligible for assessment of REP. The subgroup of patients who were indicated for the treatment according to the Stupp regimen was further analyzed in more detail. All patients signed standard informed consent to treatment and consent to processing their data for scientific purposes in a pseudonymized form. The study was approved by Institutional Review Board No. 2020/1206/MOU, JID: 315 453, approved on day month year. Institutional Review Board No. 2020/1206/MOU, JID: 315 453, approved on 18 June 2019.

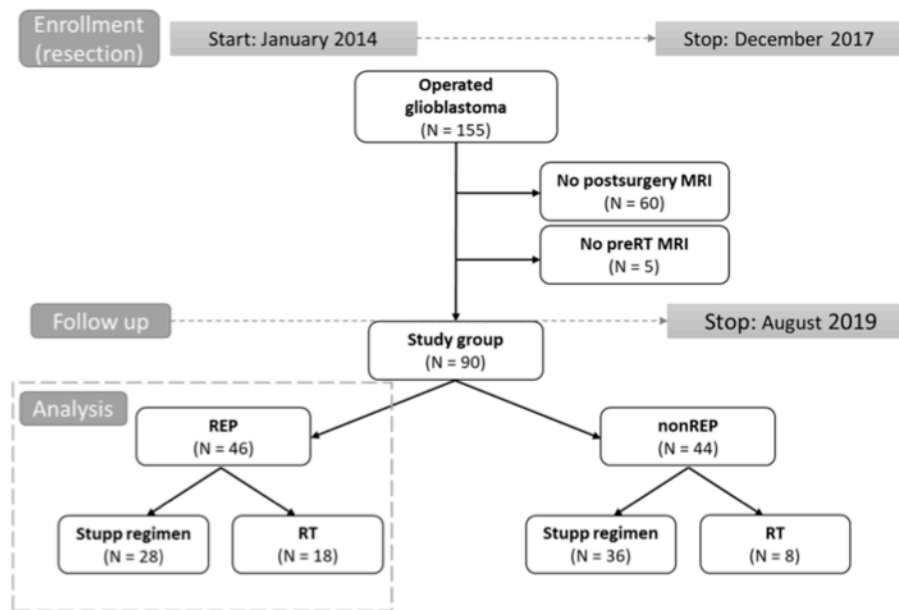


Figure 1. Design of study (flow chart). Grey dot line: time to study enrolment and follow up, respectively. Black line arrows: division into parts. Grey dot line box: cohort analyzed in more details.

Clinical and imaging data were retrieved from electronic medical records for further statistical analysis. Radiotherapy was performed in all patients. Planning pre-RT MRI (including postcontrast T1 weighted scan with submillimeter slices) was rigidly registered to planning CT scan for proper RT target and organs-at-risk definition. Individual prescription of RT dose and scheduling was guided mainly by the patient's performance status and by volume, size, shape, and location of the target volume. Both standard of care approaches in target volume definitions were employed in patients eligible for treatment by the Stupp regimen (60 Gy in 30 fractions), the Radiation Therapy Oncology Group (RTOG contouring approach) that defines two clinical target volumes accommodating hyperintensity at T2/FLAIR MRI in addition to T1 contrast-enhanced MRI [13], and the European Organization for Research and Treatment of Cancer (EORTC single-phase contouring approach) that defines one target utilizing mainly T1 post-contrast MRI [14]. In patients with REP, the single target EORTC approach was preferably performed. The RT plan was prepared employing the treatment planning system Eclipse™ (Varian medical systems, Palo Alto, CA, USA) and delivered on linear accelerators Varian Clinac iX or TrueBeam (Varian medical systems, Palo Alto, CA, USA). Abbreviated RT courses (for example, 15 × 2.7 or 10 × 3.4 Gy) were indicated according to the treating physician, reflecting the individual patient's performance status and disease.

Concurrent chemoradiotherapy and adjuvant chemotherapy were prescribed according to the original Stupp protocol [1]. Temozolomide (75 mg/m²) was administered on days 1 through 42 with concurrent RT (60 Gy). After 4 weeks, treatment was followed by the administration of temozolomide alone (150 to 200 mg/m²) on days 1–5 in six consecutive 4-week cycles or to progression. The prophylaxis against *Pneumocystis jirovecii* pneumonia was at the discretion of the treating physician. In patients with an abbreviated course of RT, concurrent chemotherapy was usually not indicated and was initiated

after the end of RT based on the patient's actual performance status. Treatment at progression was very individualized, with options for resurgery, reirradiation, temozolomide rechallenge, palliative chemotherapy (mostly lomustine), or symptomatic treatment.

2.2. Imaging Evaluation

All diagnostic MRIs were evaluated by two independent radiologists as part of the standard of care in our institution. In the case of discordance, patients were referred to the discussion on the neuro-oncology tumor board as well. Response to treatment was evaluated based on regular follow up MRI scanning. The first post (chemo) radiotherapy MRI was usually performed 4–6 weeks after the last RT session, followed by regular MRI every 3 months unless clinically indicated for earlier examination. No routine RANO criteria [15] were employed and MRI was visually evaluated by the servicing radiologist. Unclear findings (as was suspected pseudoprogression) were reviewed by a multidisciplinary neuro-oncology board, mostly with recommendations for earlier control exams with or without the change of treatment or with suggestions for advanced MRI methods [16].

The pre-RT MRI was retrospectively evaluated by an experienced radiation oncologist (TK) and doublechecked by a neuroradiologist (RB). Progression already presented on planning MRI was considered only in patients who had available early postsurgery (within 72 h) control MRI enabling a clear definition of eventual postsurgery residual disease. Criteria for REP were as follows: (1) increase in postsurgery residual disease (T1 weighted post contrast MRI) for $\geq 25\%$ in any dimension; (2) occurrence of a new enhancing lesion; (3) unambiguous progression of enhancing lesion (in multifocal glioblastomas where only some nodules were amenable to surgery). The localization of REP was categorized as follows: (1) progression of postsurgery residuum; (2) new enhancing satellite; (3) new enhancement in the wall of resection cavity; or (4) progression of tumor which was not operated on in patients with multicentric tumors.

2.3. Statistical Analysis

Patient and treatment characteristics were described using standard summary statistics, i.e., median and interquartile range (IQR) for continuous variables and frequency distributions for categorical variables. The comparison of these characteristics in patients with and without the occurrence of REP was performed using Fisher's exact test, a chi-squared test, or a Mann–Whitney test, as appropriate. Overall survival (OS) and progression-free survival (PFS) were considered as survival outcome. OS was defined as the time from the date of neurosurgery resection to the date of death from tumor cause. PFS was defined as the time from the date of initiation of RT until progression or death from tumor cause. Survival probabilities were calculated by the Kaplan–Meier method. Survival curves were compared using the log-rank test. The Cox proportional hazard model was used to perform the univariable and multivariable analysis. The proportional hazard assumption was verified based on scaled Schoenfeld residuals. Stepwise backward selection was performed to obtain characteristics independently associated with OS and PFS. Stratified models were used for the assessment of the effect of treatment or age in patients with and without the occurrence of REP. All statistical analyses were performed employing R version 4.0.0 [17], and the significance level of 0.05 was considered.

3. Results

3.1. Patients Characteristics

A total of 155 patients who were indicated for postoperative oncology treatment were screened for eligibility, and 90/155 (58%) met the inclusion criteria and had undergone both postsurgery as well as pre-RT MRI (Figure 1). The median age was 59 years, with 23% of patients being younger than 50 years. Gross total resection (GTR) was achieved in 39/90 (43%) patients, and 34/90 (38%) were in excellent overall performance status with Eastern Cooperative Oncology Group (ECOG) status 0. MGMT methylation was present in 26% and IDH mutation in 8% of patients (total of 53 evaluated

patients). The subgroup of 64 patients (64/90; 71%) was indicated for concurrent chemoradiotherapy and was further analyzed in detail. The other patients' diagnostic and treatment characteristics are summarized in Table 1; Table 2.

Table 1. Basic patients' characteristics of cohort with REP and non-REP.

Study Cohort (<i>n</i> = 90)	All	REP	Non-REP	<i>p</i> -Value
No. of patients	90 (100%)	46 (51%)	44 (49%)	
Age (years)				
median (IQR)	59.3 (51.1, 65.2)	60.0 (52.2, 67.8)	57.1 (50.6, 63.5)	<i>p</i> = 0.180
≤50	21 (23%)	10 (22%)	11 (25%)	<i>p</i> = 0.805
Men	53 (59%)	27 (59%)	26 (59%)	<i>p</i> > 0.999
Performance status (ECOG) and Karnofsky index				
ECOG 0 (KI 90–100%)	34 (38%)	17 (37%)	17 (39%)	<i>p</i> = 0.868
ECOG 1 (KI 70–80%)	49 (54%)	26 (57%)	23 (52%)	
ECOG 2 (KI 50–60%)	7 (8%)	3 (7%)	4 (9%)	
Tumor location				
deep brain location	21 (23%)	14 (30%)	7 (16%)	<i>p</i> = 0.136
Extent of resection				
GTR	39 (43%)	10 (22%)	29 (66%)	<i>p</i> < 0.001
STR	44 (49%)	31 (67%)	13 (30%)	
Partial resection or biopsy	7 (8%)	5 (11%)	2 (5%)	
Extent of resection				
GTR	39 (43%)	10 (22%)	29 (66%)	<i>p</i> < 0.001
Non-GTR	51 (57%)	36 (78%)	15 (34%)	
IDH status				
Mutated/evaluated	4/53 (8%)	1/24 (4%)	3/29 (10%)	
MGMT status				
Methylated/evaluated	14/53 (26%)	6/23 (26%)	8/30 (27%)	<i>p</i> > 0.999
Localization of REP				
Postsurgery residuum		31/46 (67%)		
New enhancing satellite		6/46 (13%)		
New enhancement in the wall of resection cavity		22/46 (48%)		
Not operated tumor in multicentric tumors		10/46 (22%)		

Abbreviations: REP—rapid early progression; ECOG—Eastern Cooperative Oncology Group; GTR—gross total resection; non-GTR—non gross total resection; STR—subtotal resection; MGMT—O6-methylguanine-DNA-methyltransferase; IDH—Isocitrate dehydrogenase; IQR—interquartile ratio.

Table 2. Patients' treatment.

Study Cohort (n = 90)	All (n = 90)	REP (n = 46)	Non-REP (n = 44)	p-Value
Time to RT initiation				
Median (weeks; IQR)	6.7 (5.9, 7.3)	6.6 (5.9, 7.1)	6.8 (5.8, 7.5)	p = 0.981
>6 weeks	56 (62%)	28 (61%)	28 (64%)	p = 0.830
Radiotherapy				
RT technique IMRT	89 (99%)	46 (100%)	43 (98%)	
RT technique other	1 (1%)	0 (0)	1 (2%)	
median dose (Gy; IQR)	60 (50, 60)	60 (43, 60)	60 (60, 60)	p = 0.024
pts. receiving ≥ 90% of prescribed dose	82 (91%)	43 (93%)	39 (89%)	p = 0.480
contouring approach EORTC	46 (51%)	30 (65%)	16 (36%)	p = 0.011
contouring approach RTOG	43 (48%)	16 (35%)	27 (62%)	
contouring unknown	1/90 (1%)	0/46 (0)	1/44 (2%)	
Chemoradiotherapy (Stupp regimen)				
No. of patients	64 (71%)	28 (61%)	36 (82%)	p = 0.037
median (days; IQR)	42 (30, 45)	41.5 (23, 43)	43 (39, 46)	p = 0.095
corticosteroids use	62 (69%)	35 (76%)	27 (61%)	p = 0.151
Adjuvant chemotherapy				
No. of patients	43 (48%)	16 (35%)	27 (61%)	p = 0.020
No. of cycles: median (IQR)	4.5 (2, 6)	3.5 (1, 6)	5 (3, 6)	p = 0.242
No. of cycles: ≥ 3	32/43 (74%)	8/16 (50%)	24/27 (89%)	p = 0.016
No. of cycles: ≥ 6	21/43 (49%)	7/16 (44%)	14/27 (52%)	p = 0.761
Treatment after progression				
No. of patients	42	22	20	p > 0.999
surgery	7 (17%)	4 (18%)	3 (15%)	
surgery + chemoradiotherapy	1 (2%)	0 (0)	1 (5%)	
surgery + chemotherapy	8 (19%)	2 (9%)	6 (30%)	
chemotherapy	18 (43%)	13 (59%)	5 (25%)	
reirradiation	6 (14%)	2 (9%)	4 (20%)	
reirradiation + chemotherapy	2 (5%)	1 (5%)	1 (5%)	

Abbreviations: IQR—interquartile ratio; IMRT—intensity modulated radiotherapy; EORTC—European Organization for Research and Treatment of Cancer; RTOG—Radiation Therapy Oncology Group; GBM—glioblastoma; CHT/RT—chemoradiotherapy; CHT—chemotherapy; RT—radiotherapy.

3.2. Rapid Early Progression

REP was presented in 46 out of 90 evaluated patients (51%). In the majority of patients, REP was presented as a progression of postsurgery residuum (31/46; 67%) or as a new enhancement in the wall of the resection cavity (22/46; 48%). Only 6/46 (13%) REP presented by a new enhancing lesion and 10/46 (22%) by the progression of the tumor, which was not operated on in patients with multicentric tumors. The occurrence of REP was significantly associated with the extent of resection (78% of patients with REP vs. 34% of patients without REP, after non-radical resection; $p < 0.001$). The other evaluated pre-RT diagnostic variables (age, sex, performance status, etc.) were not significantly associated with the development of REP (Table 1).

With a median follow up (measured from neurosurgery resection) of 34.1 months, the median OS was significantly longer in patients without REP (18.7 vs. 10.7 months; HR 0.53; $p = 0.007$) with corresponding 2-year survival 37.7% vs. 15.6%. A similar effect was observed for PFS (Figure 2). Interestingly, the REP occurrence effect on survival outcome is significantly different in younger patients (≤ 50 years) and older patients (> 50 years) for OS ($p = 0.047$) and non-significantly for PFS ($p = 0.341$). In younger patients (≤ 50 years), REP occurrence is a negative prognostic factor, probably in relation to more aggressive glioblastoma behavior at a younger age (Figure 3).

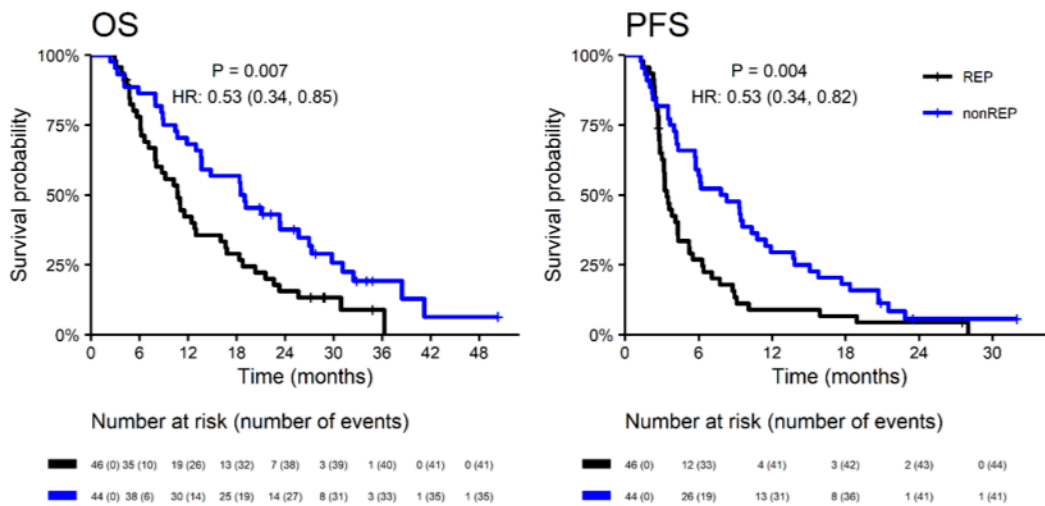


Figure 2. Overall survival and progression-free survival in patients with REP vs. non-REP. OS—overall survival; PFS—progression free survival; HR—hazard ration; REP—rapid early progression.

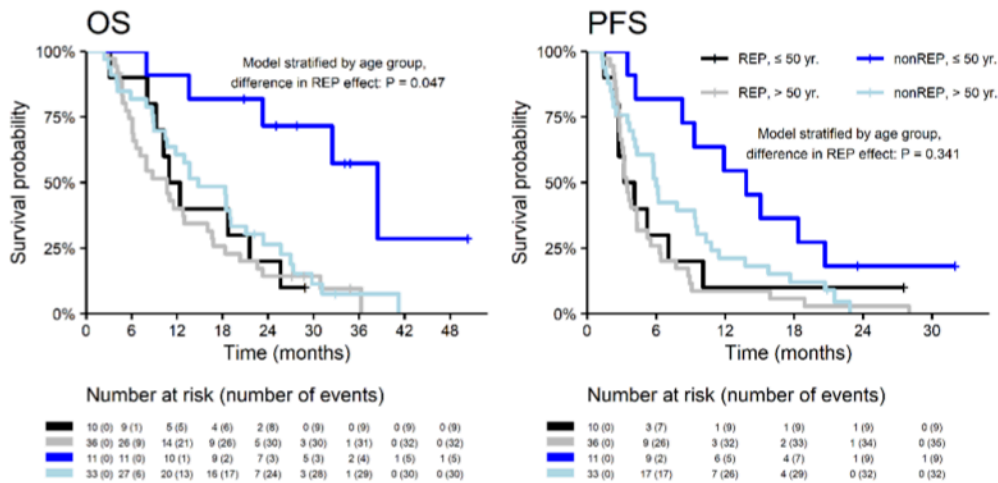


Figure 3. Overall survival and progression-free survival in patients with REP and non-REP in relation to age.

Indication to concurrent chemoradiotherapy (the Stupp regimen) was more common in the subgroup without REP (82% vs. 61%; $p = 0.037$), as was summarized in Table 2. OS and PFS were significantly better in patients indicated for the Stupp regimen in both subgroups with and without REP (Figure 4). The median OS of patients with REP who were indicated for the Stupp regimen was 16.0 (2-year OS 22.3%). The median OS of patients treated by RT alone was 7.5 months (2-year OS 5.6%) (Table 3). The model stratified by REP showed a 50% lower risk of death, and a 37% lower risk of progression in patients indicated concurrent chemoradiotherapy (OS: HR = 0.5, $p = 0.007$; PFS: HR = 0.63, $p = 0.060$).

The median time to initiation of radiotherapy was 6.7 weeks and was similar in both groups of patients (6.6 vs. 6.8 weeks in patients with and without REP, respectively). In the REP subgroup, both OS and PFS were similar in patients undergoing RT within six weeks after resection as in patients with a longer initiation time (Figure 5). Target definition for radiotherapy planning according to EORTC (the one same target for the whole course of RT) was more commonly employed in the subgroup of patients with REP (65%) vs. in patients without REP (36%; $p = 0.011$). Nevertheless, the OS of patients with REP did not differ with respect to contouring strategy (HR 0.9 for RTOG vs. EORTC; $p = 0.824$).

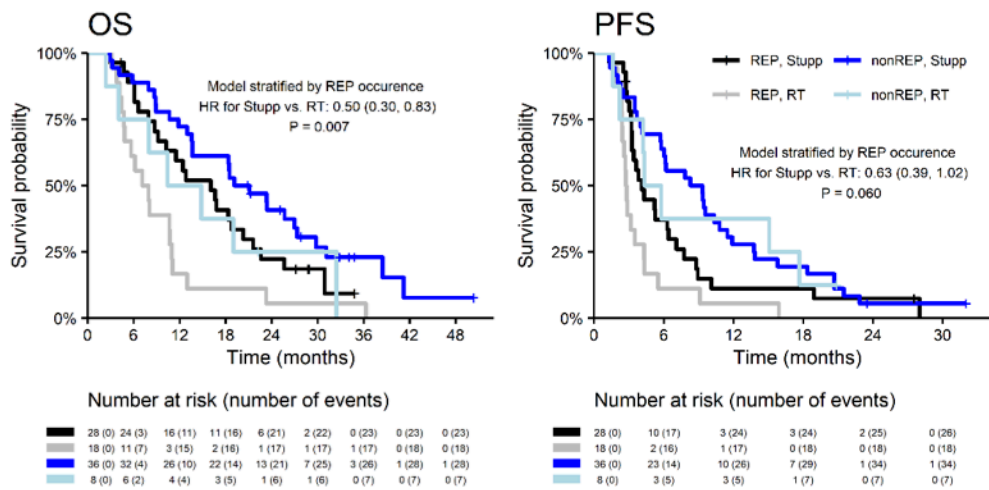


Figure 4. Survival outcomes of patients with REP and non-REP in relation to the treatment.

Table 3. Survival outcomes in patients with REP and non-REP in relation to the treatment.

	REP (n = 46)		Non-REP (n = 44)	
	Median follow up 31.9 (28.7, NA)		Median follow up 34.1 (32.9, NA)	
	Stupp regimen (n = 28)	RT (n = 18)	Stupp regimen (n = 36)	RT (n = 8)
Overall survival				
Median (months)	16.0 (10.2, 21.6)	7.5 (4.8, 11.0)	20.1 (13.6, 29.8)	12.6 (8.0, NA)
1-year	59.3 (43.4, 81.1)	16.7 (5.9, 46.8)	72.2 (59.0, 88.4)	50.0 (25.0, 100.0)
2-year	22.3 (11.0, 45.1)	5.6 (0.8, 37.3)	40.8 (27.3, 60.9)	25.0 (7.5, 83.0)
3-year	9.3 (1.9, 45.7)	5.6 (0.8, 37.3)	22.9 (11.9, 44.1)	0.0 (NA, NA)
Progression-free survival				
Median (months)	4.1 (3.2, 7.1)	2.8 (2.4, 4.3)	8.8 (5.8, 11.5)	5.0 (4.2, NA)
1-year	11.2 (3.8, 32.4)	5.6 (0.8, 37.3)	27.8 (16.4, 47.0)	37.5 (15.3, 91.7)
2-year	7.4 (2.0, 28.2)	0.0 (NA, NA)	5.6 (1.4, 21.4)	12.5 (2.0, 78.2)

Abbreviations: REP—rapid early progression; Stupp regimen—concomitant chemoradiotherapy and adjuvant chemotherapy with temozolomide; RT—radiotherapy; NA—Not Available.

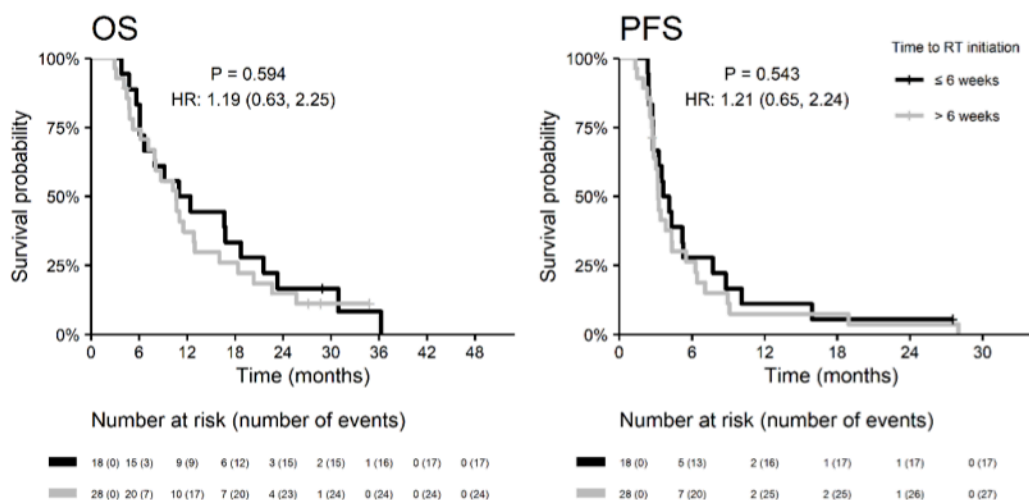


Figure 5. Survival outcomes of patients with REP in relation to the start of radiotherapy.

Based on univariable analysis of 46 patients with REP, the lower overall performance status (the median OS 16.8 vs. 11.0 vs. 5.8 months in patients with ECOG 0 vs. 1 vs. 2; $p = 0.011$), and indication to concurrent chemoradiotherapy (HR 0.50; $p = 0.022$ for OS) was positively associated with OS and PFS (Figure 6). REP presented as a progression of postsurgery residuum was a negative prognostic factor of OS with the borderline level of statistical significance (HR 1.9; $p = 0.068$). Deep brain tumor location was a significant negative prognostic factor for PFS (HR 2.4; $p = 0.014$), but not for OS (HR 1.0; $p = 0.948$). The other prognostic variables (age, sex, the extent of resection, MGMT status, the location of REP) were not significant in the univariable analysis (Figure 6). IDH mutation was not evaluated in a univariable analysis due to low numbers of positive patients. According to the multivariable analysis of patients with REP (Table 4), the extent of resection and Stupp regimen are independently associated with OS, and performance status and deep brain tumor location are independently associated with PFS.

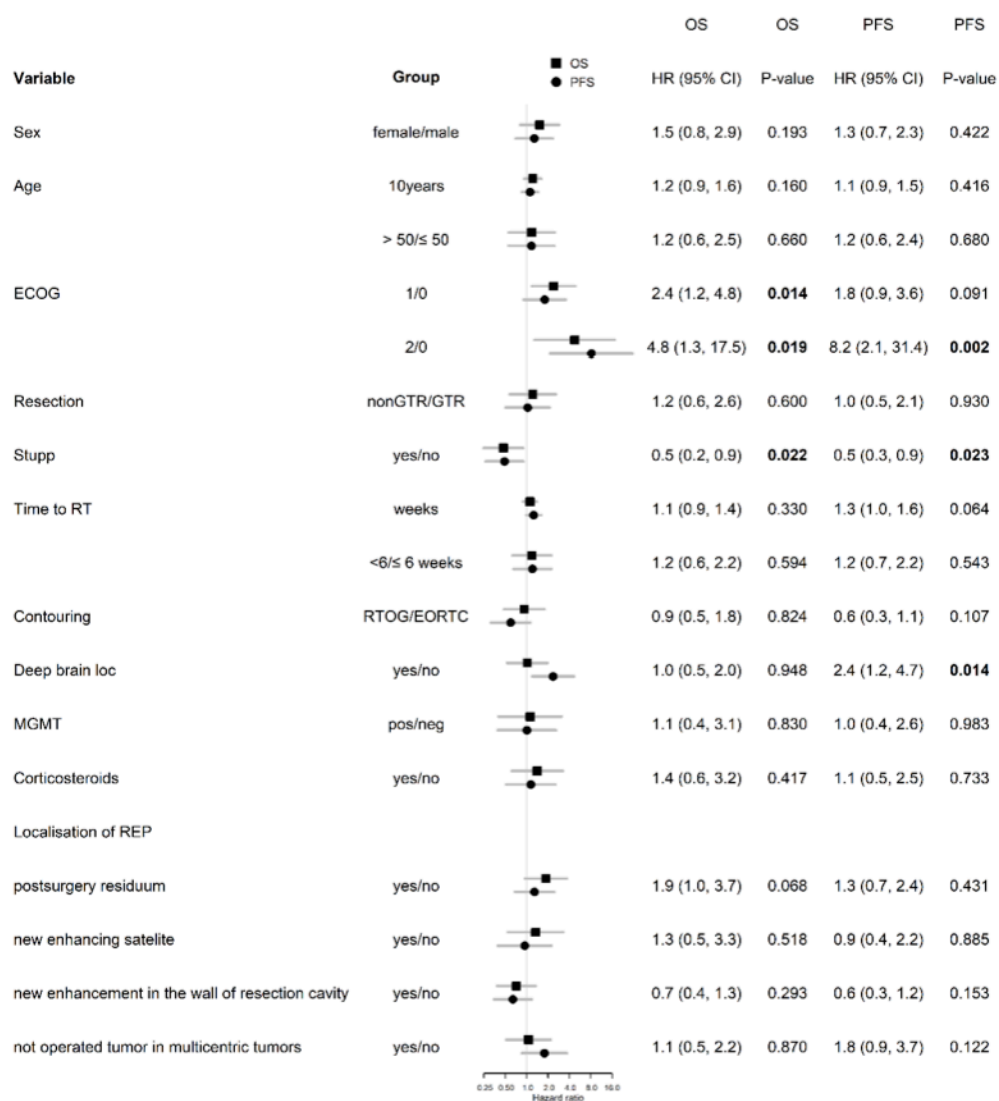


Figure 6. Univariable analysis in patients with REP. Number in bold are < 0.05.

Table 4. Multivariable analysis in patients with REP.

		OS		PFS	
		HR (95% CI)	<i>p</i> -Value	HR (95% CI)	<i>p</i> -Value
Performance status (ECOG)	1/0			2.3 (1.1,4.5)	0.033
	2/0			16.6 (3.9,70)	< 0.001
Extent of resection	non-GTR/GTR	2.2 (0.9,5.2)	0.088		
Stupp regimen	yes/no	0.3 (0.1,0.7)	0.003		
deep brain location	yes/no			3.1 (1.5,6.7)	0.003

Abbreviations: OS—overall survival; HR—hazard ratio; CI—confidence interval; PFS—progression free survival; ECOG—Eastern Cooperative Oncology Group; GTR—Gross total resection.

4. Discussion

A high proportion of glioblastoma patients indicated for adjuvant oncology treatment developed rapid early progression in this retrospective analysis of an unselected cohort of consecutive patients treated outside of clinical trials. About half of the patients (46/90; 51%) progressed between surgery and initiation of adjuvant RT, regardless of waiting time to RT initiation. High incidence of REP and reports of overall survival are in accordance with other retrospective published studies [10–12]. The only one clinical negative predictive factor for the development of REP in our cohort was non-radical surgery, confirming the overall prognostic value of surgical radicality in glioblastoma [1]. Further studies evaluating potential biomarkers of REP are highly warranted.

The question of optimal timing of RT initiation, the first logical argument for the risk of REP in a specific patient, is still unanswered. Published studies that evaluated this issue are inconclusive with different waiting times, ranging from 37 to 56 days after surgery [18–23]. Some reported no effect of waiting time on the OS. In the broad analysis of 2855 patients enrolled in 16 RTOG trials, Blumenthal et al. described even better outcomes in patients with the mild postponement of RT (4–6 weeks) comparing to early initiation of RT within 2 weeks after surgery [24]. One may assume the need for recovery from secondary edema and hypoxia to be a prerequisite for RT effect on radioresistant glioblastoma. However, considering glioblastoma aggressivity with doubling time reported about 24 days, it is recommended to avoid unnecessary delay in RT initiation [25–28].

Development of REP represents an important, and not yet described in detail, negative prognostic factor (median OS 10.7 vs. 18.7 months in our cohort). We confirmed other well-known prognostic factors, such as performance status and the ability to undergo the Stupp regimen. The question of eventual administration of chemotherapy for over 6 months remains to be answered. As expected, worse OS was in the subgroup of patients with REP who were treated by RT alone (OS 7.5 months).

The majority of patients with REP develop central progression within the initial lesion of the cavity. Modification of RT targeting and techniques including employment of planning PET may be another way how to improve the outcomes of this unfavorable group of patients [29,30]. Precise knowledge of tumor biology may also add to the guidance of optimal treatment (prediction for more invasive forms of glioblastoma and risk of distant satellites). MGMT promoter methylation is both a prognostic and predictive marker in an REP group of patients, as described by Palmer et al.: patients with both REP and MGMT methylation reached significantly longer survival compared to those with REP and MGMT intact (16.5 vs. 10.2 months, $p = 0.033$) [12]. In our cohort, we did not observe any role of MGMT ($p = 0.830$). However, only 23/46 (50%) patients with REP were examined, which could significantly affect our analysis. Palmer's study evaluated MGMT promoter methylation, however, there may be many different genetic mutations and molecular characteristics specific to a subset of patients that predispose to REP and poor treatment response. It can be assumed that other important molecular markers such as IDH and pTERT (Telomerase reverse transcriptase gene promoter) also influence the prognosis and rapid progression in patients with glioblastomas [31–33].

In our clinical practice, MGMT is more likely to be investigated in elderly patients and in patients unable to undergo intensive postoperative treatment. For all others, we indicate the Stupp regimen

regardless of MGMT methylation. It may be hypothesized that tumors with REP represent more aggressive disease, which may be associated with higher tumor mutation burden and neoantigens, relevant biomarkers for immunotherapy. On the other hand, unlike in other tumors, immunotherapy in glioblastoma did not prove clear effectivity so far, including immune checkpoint inhibitors or dendritic cell vaccines [34–36]. Analysis of REP patients may provide new insights into the biology of this aggressive tumor and potentially reveal new targets for cancer therapy.

An inherent limitation of our study is its retrospective nature, related also to limited possibility for molecular analyses. Ongoing work may provide more information, especially with the analysis of molecular biomarkers of REP. In future prospective studies, advanced MRI techniques such as MRI spectroscopy or diffusion-weighted MRI may play a role in the differential diagnosis of REP and postoperative changes, as does ischemia, for example.

5. Conclusions

The extent of surgery remains one of the most important prognostic factors in glioblastoma, affecting not only general OS but also the risk of REP development. Especially in the subgroup of patients without radical resection, one may recommend as early initiation of radiotherapy as possible. The phenomenon of REP should be recognized as an integral part of stratification factors in future prospective clinical trials enrolling patients before the initiation of RT.

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MiR-195, miR-196b, miR-181c, miR-21 expression levels and O-6-methylguanine-DNA methyltransferase methylation status are associated with clinical outcome in glioblastoma patients

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Glioblastoma multiforme (GBM) is the most frequently occurring primary malignant brain tumor; patients with GBM often have a very poor prognosis and differing responses to treatment. Therefore, it is very important to find new biomarkers that can predict clinical outcomes and help in treatment decisions. MicroRNAs are small, non-coding RNAs that function as post-transcriptional regulators of gene expression and play a key role in the pathogenesis of GBM. In a group of 38 patients with primary GBM, we analyzed the expression of eight microRNAs (miR-21, miR-128a, miR-181c, miR-195, miR-196a, miR-196b, miR-221, and miR-222). In addition, we examined the methylation status of O-6-methylguanine-DNA methyltransferase (MGMT) promoter by high-resolution melting analysis, as this has been shown to be a predictive marker in GBM. MGMT methylation status correlated with progression-free survival ($P = 0.0201$; log-rank test) as well as with overall survival ($P = 0.0054$; log-rank test). MiR-195 ($P = 0.0124$; log-rank test) and miR-196b ($P = 0.0492$; log-rank test) positively correlated with overall survival. Evaluation of miR-181c in combination with miR-21 predicted time to progression within 6 months of diagnosis with 92% sensitivity and 81% specificity ($P < 0.0001$). Our data confirmed that the methylation status of MGMT but also miR-21, miR-181c, miR-195, and miR-196b to be associated with survival of GBM patients. Above all, we suggest that the combination of miR-181c and miR-21 could be a very sensitive and specific test to identify patients at high risk of early progression after surgery. (Cancer Sci, doi: 10.1111/j.1349-7006.2011.02092.x, 2011)

Glioblastoma multiforme (GBM) is the most frequently occurring primary malignant brain tumor of astrocytic origin.⁽¹⁾ Despite the introduction of modern therapeutic approaches, this cancer remains generally associated with very poor prognosis.⁽²⁾ A significant benefit of overall survival (OS) has been achieved in patients treated with concomitant chemoradiotherapy with temozolomide (RT/TMZ), an alkylating agent. However, not all patients are sensitive to this therapy.^(3,4) Because of an extremely short median survival time of glioblastoma patients and diversity in therapy response, it is very important to identify new biomarkers that can be used in prognosis and prediction of therapeutic response and/or clinical outcome in GBM patients in order to rationalize treatment decisions.

MicroRNAs (miRNAs) are highly conserved, small, non-coding RNAs, 18–25 nucleotides in length, that function as post-transcriptional regulators of gene expression by silencing their mRNA targets. Bioinformatics tools estimate that miRNAs

regulate up to one-third of human genes including a significant number of oncogenes, tumor suppressor genes, and genes associated with the invasion, dissemination, and chemoresistance of tumors.⁽⁵⁾ Therefore, these molecules play significant roles in the pathogenesis of many cancers, including GBM.^(6,7) In the context of this tumor, recent published reports have proposed that some miRNAs that could be used to predict disease outcome and therapy response. As we previously described, miR-21, miR-128a, miR-181b, miR-181c, miR-221, and miR-222 were significantly altered in glioblastomas. Moreover, miR-181b and miR-181c were significantly downregulated in patients who responded to RT/TMZ compared to patients with progressive disease.⁽⁸⁾ In another study, miR-195, miR-455-3p, and miR-10a* were the most upregulated miRNAs in TMZ-resistant cell lines.⁽⁹⁾ MiR-196a and miR-196b expression levels are increased in glioblastomas relative to both anaplastic astrocytomas and normal brain tissues. Furthermore, patients with high miR-196 expression levels showed significantly poorer OS.⁽¹⁰⁾

Another frequently studied predictive marker in GBM is the O-6-methylguanine-DNA methyltransferase (MGMT).⁽¹¹⁾ This protein with DNA repair activity removes mutagenic O-6-alkylguanine induced by alkylating agents. Therefore, MGMT partially contributes to alkylating chemotherapy resistance, and epigenetic silencing of the *MGMT* gene by a promoter methylation has been shown to be an independent predictor of prognosis and response to RT/TMZ and adjuvant TMZ treatment of GBM patients.^(12–14)

The aims of this study were to quantify expression levels of eight miRNAs (miR-21, miR-128a, miR-181c, miR-195, miR-196a, miR-196b, miR-221, and miR-222) that have been previously described as associated with GBM pathogenesis in the clinical samples, and to determine the methylation status of MGMT gene promoter. The results were correlated to clinical data in order to obtain prognostic and predictive biomarkers for GBM patients treated with RT/TMZ and adjuvant TMZ.

Materials and Methods

Patients and treatment. The retrospective study included 38 patients with primary glioblastomas who were resected at the Department of Neurosurgery (University Hospital Brno, Brno, Czech Republic). After resection, patients underwent adjuvant

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therapy at the Masaryk Memorial Cancer Institute (Brno, Czech Republic) according to the standard Stupp protocol: radiotherapy (2 Gy per fraction for 6 weeks, total dose of 60 Gy) plus concomitant chemotherapy with RT/TMZ at 75 mg/m² daily, for 6 weeks. Nineteen patients received an adjuvant treatment with temozolomide (150–200 mg/m² for 5 days in 4-week cycles) (Table 1). Informed consent approved by the local Ethical Commission was obtained from each patient before the treatment. Clinical data were retrieved from the hospital's patient records.

Tissue sample preparation and nucleic acid extraction. The 38 tumor samples were obtained from surgically resected glioblastomas. As a control, six non-tumor samples of adult brain tissue were taken from areas surrounding arteriovenous malformations (AVM) and four commercially available RNAs from adult brain tissues (540005, total brain; 540117, frontal cortex; 540137, occipital cortex; 540135, striatum; Agilent-Stratagene, Santa Clara, CA, USA). Glioblastoma clinical samples were evaluated by two experienced neuropathologists. For subsequent analysis were used dissected formalin-fixed paraffin-embedded (FFPE) samples containing more than 90% tumor tissue. Small RNA-enriched total RNA was isolated using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Nucleic acid concentration and purity were controlled by UV spectrophotometry (A260:A280 > 2.0; A260:A230 > 1.8) using Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

Real-time quantification of miRNAs by stem-loop RT-PCR. Complementary DNA was synthesized from 10 ng small RNA-enriched total RNA using gene-specific primers and TaqMan MicroRNA Reverse Transcription kit according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Foster City, CA, USA). Real-Time PCR was carried out using the Applied Biosystems 7500 Real-Time PCR System in accordance with the TaqMan MicroRNA Assay protocol. The threshold cycle data were determined using the default threshold settings. All real-time PCR reactions were run in triplicate and average Ct and SD values were calculated.

Bisulfite conversion of DNA and high-resolution melting (HRM) analysis of MGMT promoter. Bisulfite conversion was carried out using the EpiTect Bisulfite Kit (Qiagen) with 1000 ng DNA per reaction. High-resolution melting was carried out on the LightCycler 480 (Roche, Mannheim, Germany) using a LightCycler 480 High Resolution Melting Master kit (Roche) with 30 ng bisulfite converted DNA on each reaction, 4 nM Mg²⁺, and previously described primers MGMT MS-HRM2.⁽¹⁵⁾ CpGenome Universal Methylated DNA and CpGenome Universal

Unmethylated DNA set (Millipore, Darmstadt, Germany) were used for dilution of standard samples (0, 10, 25, 50, 75, 90, and 100% methylated DNA). All HRM reactions were run in triplicate.

Statistical analysis. Expression data were normalized according to the expression of RNU6B (Assay no. 4373381; Applied Biosystems). Statistical analysis of differences between miRNA levels in glioblastomas and non-tumor adult brain tissues, and differences in therapy response and time to progression in relation to miRNA levels, were evaluated using the non-parametric Mann–Whitney *U*-test between two groups. Survival analyses were carried out by the Kaplan–Meier method and significance was calculated by the log–rank test. Sensitivity and specificity were evaluated and significance of the patient stratification according to a particular miRNA marker was assessed by Fischer's exact test. For all calculations we used MedCalc version 11.4.2.0 (MedCalc Software, Mariakerke, Belgium).

Results

Methylation status of MGMT promoter. Twenty-five percentage methylation status of MGMT promoter was used as cut-off for stratification of GBM patients into (un)methylated groups. The MGMT promoter was unmethylated in 26 cases (68%) and methylated in 12 cases (32%) (Table 2). In patients with methylated promoter of the *MGMT* gene, there was an observed benefit in OS (hazard ratio [HR] 0.4012; 95% confidence interval [CI] 0.2068–0.7783; *P* = 0.0054, log-rank test) as well as in progression-free survival (HR 0.4799; 95% CI 0.2516–0.9153; *P* = 0.0201, log-rank test) (Fig. 1). The MGMT methylation status was not associated with any of the miRNA expression levels analyzed in this study (Table 3).

Comparison of miRNA expression levels in GBM tissues and non-tumor brain tissue samples. Medians of relative expression levels of all examined miRNAs with their 25th and 75th percentile ranges, GBM samples and non-tumor brain tissue samples, as well as *P*-values indicating statistical significance of differences, are summarized in Table 3. Our data indicated significant overexpression of miR-21 and miR-196a/b in GBM samples compared to control non-malignant brain tissues, and downregulation of miR-181c, miR-221, miR-222, miR-195, and miR-128a. Of these, miR-128a showed the most significant change (*P* < 0.0001).

Correlation of miRNA expression levels with prognosis and prediction of response to RT/TMZ treatment in GBM patients. Higher levels of miR-195 (HR 0.4249; 95% CI 0.2167–0.8332; *P* = 0.0124, log-rank test) and miR-196b (HR 0.5470; 95% CI 0.2776–1.0776; *P* = 0.0492, log-rank test) expression have been significantly associated with longer OS of GBM patients. For these analyses, the 75th percentile range and median of relative expression levels have been used as cut-offs for miR-195 and miR-196b, respectively (Fig. 2). Significantly

Table 1. Characteristics of patients with glioblastoma multiforme who participated in this study (n = 38)

	Total n = 38	%
Age (years)		
≤50	14	37
>50	24	63
Median (range)	53 (28–67)	
Gender		
Male	19	50
Female	19	50
ECOG performance status		
0 and 1	36	95
2	2	5
Extent of resection		
Total	9	24
Subtotal	29	76

Table 2. Characteristics of patients with glioblastoma multiforme (n = 38), based on methylguanine-DNA methyltransferase methylation status

	Promoter status	
	Unmethylated	Methylated
No. of patients	26 (68%)	12 (32%)
Progression-free survival		
Median duration (months)	8.0 (3.0–24.0)	14.5 (3.0–27.0)
Rate at 6 months (%)	61.5	83.3
Overall survival		
Median duration (months)	13.0 (3.0–33.0)	22.5 (6.0–62.0)
Rate at 24 months (%)	11.5	58.3

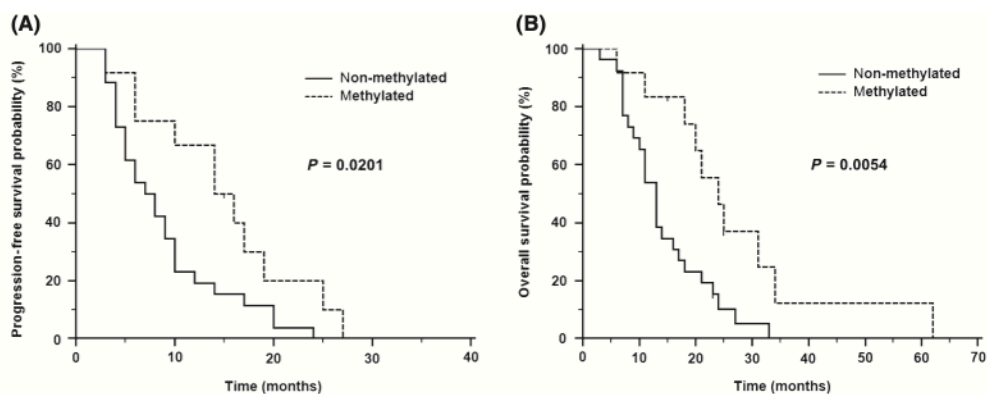


Fig. 1. Kaplan–Meier survival curves estimating progression-free survival (A) and overall survival (B) in patients with glioblastoma multiforme, according to methylguanine-DNA methyltransferase promoter methylation status.

Table 3. Comparison of normalized microRNA expression levels in glioblastoma multiforme and non-tumor brain tissues and their association with methylation status of methylguanine-DNA methyltransferase (MGMT) promoter

MicroRNA	Glioblastoma <i>n</i> = 38	Non-tumor brain tissue† <i>n</i> = 10	Fold change	<i>P</i> -value‡	MGMT association§ (<i>P</i>)
miR-21	78.5814¶ (33.1469–237.7333)	22.7989 (19.2862–30.8386)	3.45	0.02550	0.4142
miR-128a	0.01534 (0.005892–0.05036)	1.1798 (0.7614–7.7713)	0.01	<0.0001	0.8494
miR-181c	0.4805 (0.1345–0.9141)	2.2363 (0.6974–3.1595)	0.21	0.0005	0.4232
miR-195	5.4032 (0.8845–33.2029)	38.5964 (17.8927–46.8118)	0.14	0.0290	0.2928
miR-196a	0.14790 (0.06040–0.7335)	0.01511 (0.00022–0.04707)	9.79	0.0021	0.2573
miR-196b	0.09182 (0.01690–0.57340)	0.01978 (0.00022–0.11020)	4.64	0.0330	0.4795
miR-221	2.3737 (1.0943–6.7427)	23.2871 (8.2249–67.7547)	0.10	0.0001	0.9749
miR-222	7.0326 (3.2716–14.4534)	46.1839 (18.2944–99.2503)	0.15	0.0010	0.1092

†Non-tumor brain tissue from arteriovenous malformation surgeries (×6) and commercially available adult brain RNAs (×4). ‡Mann–Whitney *U*-test (bold indicates significance at *P* < 0.05). §Association with methylation status of MGMT promoter (Mann–Whitney *U*-test). ¶Median of expression level related to RNU6B with 25th and 75th percentiles.

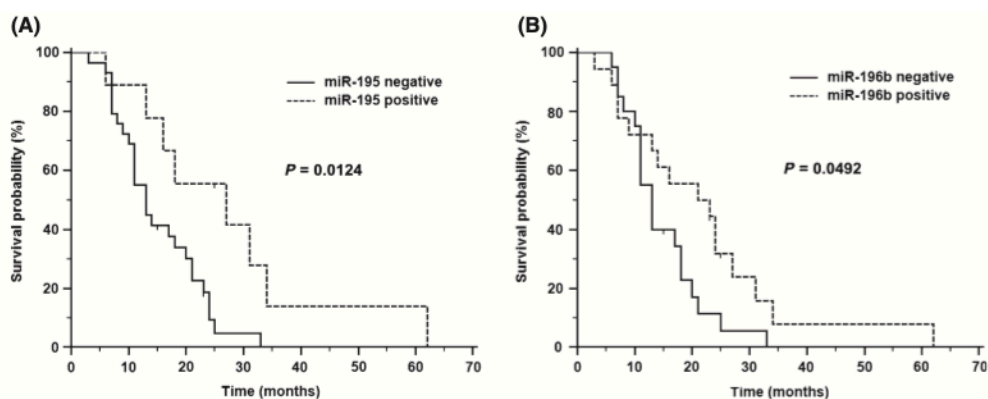


Fig. 2. Kaplan–Meier survival curves estimating overall survival in patients with glioblastoma multiforme, according to miR-195 (A) and miR-196b (B) expression levels.

higher miR-181c expression levels were observed in the group of patients with time to progression shorter than 6 months (TPP6) (*P* = 0.0010, Mann–Whitney *U*-test) (Fig. 3A). Identifi-

cally, miR-21 was significantly upregulated in this high risk group of patients (*P* = 0.0143, Mann–Whitney *U*-test) (Fig. 3B). Analysis of the miR-181c in combination with miR-21 (cut-offs

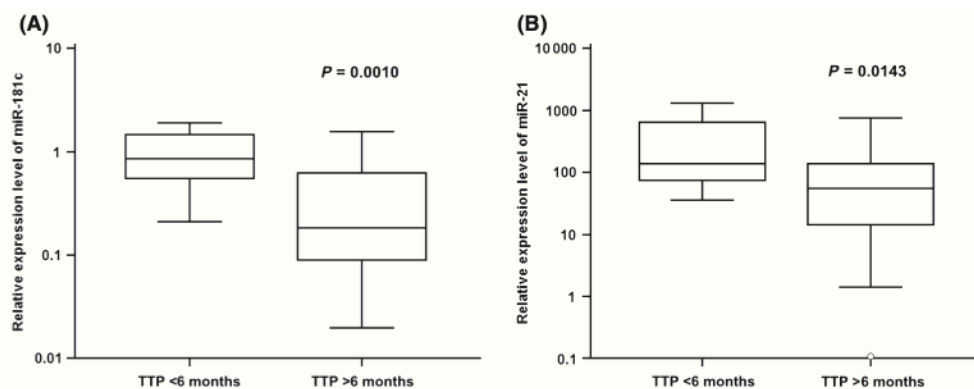


Fig. 3. Time to progression (TTP) up to 6 months in patients with glioblastoma multiforme, based on miR-181c (A) and miR-21 (B) expression levels. Central box represents values from the lower to upper quartiles (25th to 75th percentiles), and the middle line represents the median (Mann-Whitney *U*-test). Vertical line extends from minimum to maximum values.

were median and 25th percentile, respectively) predicted significantly TPP6 (sensitivity = 92%, specificity = 81%, $P < 0.0001$; see Table 4). However, neither prognostic nor predictive associations were found for miR-128a, miR-196a, miR-221, or miR-222.

Discussion

In this study, we have confirmed the impact of MGMT promoter methylation status on the favorable outcome after RT/TMZ therapy in GBM patients. Patients with MGMT promoter hypermethylation in >25% tumor DNA showed significantly longer OS as well as progression-free survival. These observations fully correlate with the results of many authors.^(12,16–18) The percentage of MGMT methylated samples has been smaller in our cohort (32%) than in other studies, where it ranged from 35% to 47%.^(8,12,17) However, we analyzed MGMT methylation status using the HRM method, which is a more suitable approach for methylation analysis in routine practice compared to methylation-specific PCR, used in most of the other published studies, which can easily generate PCR false-positive results.

There are several reports that discuss the use of miRNAs as potential predictive and prognostic factors in GBM.⁽⁶⁾ In this study, we analyzed the expression of eight miRNAs in primary GBM and we correlated the obtained data with clinical characteristics of GBM patients. In concordance with Guan *et al.*,⁽¹⁰⁾ we observed significantly lower levels of miR-196a,b in normal brain compared to glioblastoma tissue. This group also described a significant correlation between higher expression of miR-196a,b with poor survival in a group of GBM and anaplastic astrocytoma patients.⁽¹⁰⁾ Another study described miR-195 as one of the most upregulated miRNAs in TMZ-resistant GBM cell lines, and their knockdown led to reversal of TMZ resis-

tance and increased cytotoxicity of TMZ.⁽⁹⁾ Accordingly, we expected a negative correlation between miR-195 and miR-196a,b expression and OS. However, our analyses suggest that there is more likely an opposite association between miR-195 and miR-196b and OS in GBM patients, which is in agreement with several other reports in colorectal, hepatocellular, and adrenocortical cancers.^(19–22) The number of GBM patients in Guan's study was 39, which is comparable to the size of our group. Larger studies need to be carried out to establish the prognostic significance of miR-196b in glioblastoma. Interestingly, miR-196a showed no significance in OS in our study.

The miR-181 family has many predicted targets and some of these have been verified by *in vitro* functional analysis (HOXA11, TCL1, TGFBR1, and MAPK1).⁽⁸⁾ Therefore, it is a robust translational regulator and can mediate a number of genes in response to an acute cellular stress caused by a drug treatment or radiation. In this study, we have shown that higher levels of miR-181c is significantly associated with disease progression within 6 months. In fact, we confirmed results from our previous pilot study where we described positive correlation between lower miR-181c levels and the response to RT/TMZ in GBM.⁽⁸⁾ We did not observe an association between MGMT methylation status and miR-181c levels, nor with any other examined miRNAs. Our study also confirmed the upregulation of miR-21 in glioblastoma tissue, noted previously by others.^(8,23–26) MiR-21 is the most frequently explored miRNA in GBM, and it has been found to act as an oncogene. It is evident that this molecule influences multiple important components of oncogenic signaling pathways.⁽⁶⁾ Similar to miR-181c, we found that higher expression of miR-21 is associated with early progression. Moreover, the combination of miR-181c and miR-21 predicted progression within 6 months with 92% sensitivity and 81% specificity. This study also partially confirmed the downregulation of miR-221 and miR-222, described in our previous publication.⁽⁸⁾ In the current study, significant downregulation of miR-221, and a similar trend for miR-222, were observed. However, results from both of our studies conflict with another report showing miR-221 and miR-222 as overexpressed in primary GBM.⁽²³⁾ In our study, we combined tissue from AVM surgeries and commercially available RNAs from adult brain tissue as the control group. The miRNA expression changes tended to have identical trends in total RNA from AVM samples and commercially available adult brain RNAs, therefore, we integrated them together in further statistical analyses. In the study by Cifre *et al.*,⁽²³⁾ which reported opposite changes in miR-221/222 expression levels, peripheral tissue of glioblastomas were used as the control tissue. We suggest that this is not an ideal control tissue, and could partially explain the contradiction in results. Our data did

Table 4. Contingency table of glioblastoma patients stratified according to time to progression (TTP) and miR-21/miR-181c positivity

	No. of patients with TTP > 6 months	No. of patients with TTP < 6 months
miR-21 and miR-181c positivity	5	11
miR-21 and/or miR-181c negativity	21	1

not indicate the potential of miR-221/222 for survival prediction in GBM patients, as observed by Srinivasan *et al.*⁽²⁷⁾

In conclusion, we have verified two previous reports describing miR-128a deregulation in GBM compared to non-tumor adult brain tissue.^(8,23) Interestingly, this miRNA showed approximately 50-fold higher expression in non-tumor brain tissue than in GBM ($P < 0.0001$), suggesting its role in glioblastoma pathogenesis. Both miR-195 and miR-196b demonstrated prognostic significance, showing a positive correlation with OS. We suggest that the combination of miR-181c and miR-21 expression levels is a highly sensitive and specific indicator for identification of early progressing (high risk) patients, who would require specific attention, in order to improve their survival, through the use of more intensive therapy immediately after surgery. This fact is all the more important when bevacizumab is introduced for glioblastoma patients and evaluated in clinical trials combined with concomitant chemoradiotherapy.

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Disclosure Statement

The authors have no conflict of interest.

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PŘÍLOHA 7

Risk Score based on microRNA expression signature is independent prognostic classifier of glioblastoma patients

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Glioblastoma multiforme (GBM) is the most malignant primary brain tumor. The prognosis of GBM patients varies considerably and the histopathological examination is not sufficient for individual risk estimation. MicroRNAs (miRNAs) are small, non-coding RNAs that function as post-transcriptional regulators of gene expression and were repeatedly proved to play important roles in pathogenesis of GBM. In our study, we performed global miRNA expression profiling of 58 glioblastoma tissue samples obtained during surgical resections and 10 non-tumor brain tissues. The subsequent analysis revealed 28 significantly deregulated miRNAs in GBM tissue, which were able to precisely classify all examined samples. Correlation with clinical data led to identification of six-miRNA signature significantly associated with progression free survival [hazard ratio (HR) 1.98, 95% confidence interval (CI) 1.33–2.94, $P < 0.001$] and overall survival (HR 2.86, 95% CI 1.91–4.29, $P < 0.001$). O(6)-methylguanine-DNA methyltransferase methylation status was evaluated as reference method and Risk Score based on six-miRNA signature indicated significant superiority in prediction of clinical outcome in GBM patients. Multivariate Cox analysis indicated that the Risk Score based on six-miRNA signature is an independent prognostic classifier of GBM patients. We suggest that the Risk Score presents promising prognostic algorithm with potential for individualized treatment decisions in clinical management of GBM patients.

Introduction

Glioblastoma multiforme (GBM) is the most malignant primary brain tumor that arises by transformation of astrocytes. Because of its aggressive nature and common therapeutic resistance, GBM exhibits the worst prognosis among all gliomas. The median survival of patients is ~13 months from diagnosis; nevertheless, the survival ranges from 2.5

Abbreviations: FFPE, formalin-fixed paraffin-embedded; GBM, glioblastoma multiforme; MGMT, O(6)-methylguanine-DNA methyltransferase; miRNAs, microRNAs; OS, overall survival; PFS, progression-free survival; TCGA, The Cancer Genome Atlas.

to 70 months. Although the short- and long-term surviving patients with GBMs have histologically similar tumors, biological and molecular characteristics of these tumors vary significantly. This was evidenced by integrated genomic analyses of large set of GBMs, which identified clinically relevant molecular subtypes showing different treatment efficacy (1–3). Until recently, adjuvant chemotherapy with alkylating agents (temozolomide or carmustine) was the common GBM therapy following surgical resection and radiation with concomitant temozolomide (RT/TMZ). Currently, an angiogenesis inhibitor bevacizumab is evaluated in phase III clinical trials. Unfortunately, data suggest that bevacizumab in monotherapy improved progression free survival with preservation of quality of life and reduction of corticosteroids use, but did not improve overall survival (OS). Therefore, another agents, such as cilengitide, are used in combination with bevacizumab aiming to prolong OS (4,5). Thus, one of the important aims of GBM research is to find powerful prognostic biomarkers for GBM patients enabling sensitive prediction of clinical outcome and their suitability for implementation of new drugs.

One of the most modern and progressive approaches for molecular characterization of tumors today is based on microRNA (miRNA) expression profiling. miRNAs are highly conserved, small, non-coding RNAs, 18–25 nucleotides in length that function as post-transcriptional regulators of gene expression through silencing of their mRNA targets. Bioinformatic tools estimate that miRNAs could regulate up to 60% of human genes including a significant number of oncogenes, tumor suppressor genes and genes associated with the chemoresistance of tumors. Therefore, these molecules play significant roles in pathogenesis of many cancers, including GBM, and it is not surprising that their levels are frequently deregulated in tumor tissue (6,7). Moreover, some recent studies described miRNA signatures with ability to predict clinical outcome in GBM patients (6). From the analytical perspective, it is important to note that, due to their small size, miRNAs are subjected to significantly less degradation than mRNAs and, thus, also formalin-fixed paraffin-embedded (FFPE) tissues indicate sufficient quality for miRNA analyses (8).

The aim of this study was to define signature of miRNAs significantly deregulated in GBM tissues compared with non-tumor brain tissues, and to identify miRNA signature with ability to efficiently predict progression-free and survival OS of GBM patients treated with concomitant RT/TMZ. Potential of miRNA signature to predict clinical outcome of GBM patients was compared with O(6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation status as reference method and evaluated in multivariate model.

Material and methods

Patients

The retrospective one-center study included 58 patients with primary GBM who were surgically treated at the Department of Neurosurgery, University Hospital Brno. After resection of the tumor, patients underwent adjuvant therapy at the Masaryk Memorial Cancer Institute accordingly to the standard Stupp protocol; radiation (2 Gy per fraction for 6 weeks, total dose of 60 Gy) plus concomitant chemotherapy with temozolomide (75 mg/m² daily, for 6 weeks). After completion of the concomitant chemoradiotherapy, 31 patients received adjuvant temozolomide in monotherapy (150–200 mg/m² for 5 days in six cycles or until disease progression). Clinicopathologic characteristics of GBM patients are summarized in **Supplementary Table S1**, available at *Carcinogenesis* Online. Portions of the non-dominant anterior temporal cortex resected during surgery for intractable epilepsy of 10 patients were used as non-tumor control brain tissues. Control brain tissues have no signs of dysplastic changes. Informed consent approved by the local Ethical Commission was obtained from each patient before the treatment. Clinical data were retrieved from the hospital's patient records. The Cancer Genome Atlas (TCGA) dataset (485 GBM patients) was used for independent validation of the prognostic miRNA signature (2).

Tissue sample preparation and nucleic acid extraction

The GBM tissues samples and non-tumor brain tissue samples were surgically resected and immediately fixed in formalin and embedded in paraffin (FFPE). Histopathological diagnosis of GBM and evaluation of control brain tissues were performed independently by two experienced neuropathologists. Total RNA with enriched fraction of small RNAs was purified from FFPE samples by xylene deparaffinization and mirVana miRNA Isolation Kit (Ambion). miRNA extracted from FFPE samples are commonly used for high-throughput miRNA analyses (9). DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany). Nucleic acid concentrations and purities were controlled by UV spectrophotometry using Nanodrop ND-1000 (Thermo Scientific).

MicroRNA expression profiling

MicroRNA expression profiling was performed using TaqMan Low Density Array Human MicroRNA technology. In brief, 350 ng of total RNA was reverse transcribed into cDNA by the Taq-Man MicroRNA Reverse Transcription Kit and microRNA Megaplex RT set pool A and B version 3.0 (Applied Biosystems, Foster City, CA). The cDNA product was loaded into TaqMan Human MicroRNA A and B Cards Set version 3.0 (Applied Biosystems) enabling simultaneous quantification of 754 human miRNAs. TaqMan Low Density Array Assays and analysis were performed on the ABI 7900 HT Instrument (Applied Biosystems). All reactions were performed according to the standard manufacturers' protocols.

Bisulfite conversion of DNA and high-resolution melting analysis of MGMT promoter

Bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen, Hilden, MD) with utilization of 1000 ng DNA per reaction. High-resolution melting was performed on the LightCycler 480 (Roche, Germany) using LightCycler 480 High Resolution Melting Master kit (Roche) with utilization of 30 ng bisulfite converted DNA on each reaction, 4 nM Mg²⁺, and described previously primers MGMT MS-HRM2 (6,10). CpGenome Universal Methylated DNA and CpGenome Universal Unmethylated DNA set (Millipore, Germany) were used for dilution of standard samples (0%, 25%, 50%, 75% and 100% methylated DNA).

Cell cultures and growth conditions

The human GBM cell lines A172, T98G, U87MG and U251 were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg/ml penicillin, 100 µg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamin and 1 mM sodium pyruvate (all purchased from Invitrogen, Gibco) in 5% CO₂ at 37°C.

Transfection of GBM cells

Cells were transfected with 6 pmol of hsa-miR-31 mimic (assay ID MC12887, Life Technologies) or mirVana miRNA Mimic Negative Control (cat. no. 4464058, Life Technologies) oligonucleotides and equimolar concentration of Lipofectamine 2000 according to the manufacturer's recommendations (Life Technologies).

MTT assay

Cell viability of transfected cells was measured by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT, Sigma-Aldrich). GBM cells were seeded in 24-well plate at a density of 2 × 10⁴ (A172, T98G, U87MG) and 1 × 10⁴ (U251) cells per well 24 h prior to transfection with hsa-miR-31 mimic or miRNA mimic negative control oligonucleotides. Subsequently, 96 h after transfection, 60 µl of 5 mg/ml MTT solution in phosphate-buffered saline was added to each well, and plates were incubated for 1 h at 37°C. The precipitate was solubilized in 100% dimethyl sulfoxide (500 µl per well), and absorbance was measured on ELISA Multi-detection Microplate Reader (BIOTEK) at 570 nm wavelength. All experiments were run in tetraplicates.

Statistical analysis

Quantitative miRNA expression data were acquired by use of ABI 7900 HT SDS version 2.0.1 software (Applied Biosystems) (settings: automatic baseline, threshold 0.2) and, subsequently, normalized to the expression level of miR-1233 that showed the highest expression stability across all examined samples by use of GeneNorm and NormFinder algorithms. The relative miRNA expression levels were determined by 2^{-ΔCT} method, where ΔCTs were calculated as follows: ΔCT = CT_(miRNA of interest) - CT_(miR-1233). Normalized miRNA expression data were statistically evaluated in the environment of statistical language R (11) using the Bioconductor LIMMA package concerning miRNA profiling combined with hierarchical clustering (12).

To assess the miRNAs that were identified for survival prediction, a Risk Score formula for predicting survival was developed based on a linear combination of the miRNA expression level weighted by the regression coefficient

derived from the univariate Cox regression analysis (13,14). The Risk Score for each patient was calculated as follows: Risk Score = (-0.14745 * expression level of miR-224) + (0.09530 * expression level of miR-31) + (0.54293 * expression level of miR-454) + (0.21673 * expression level of miR-672) + (0.10605 * expression level of miR-885-5p) + (-0.05557 * expression level of miR-432*). Patients with high Risk Score are expected to have poor survival.

According to the Risk Score (cutoff value, 0.348), patients were stratified into a high-risk group and a low-risk group. The Risk Score threshold was set as a value, which significantly separates KaplanMeier survival curves. Cox proportional hazards regression analyses were performed to assess the independent contribution of the miRNA signature-based Risk Score and clinicopathologic variables to survival prediction (15). Our patient population was evaluated as large enough to allow multivariate survival analysis.

Viability was statistically analyzed using *t*-test and GraphPad Prism software. *P* ≤ 0.05 value was considered to be significant.

Results

MicroRNAs differentially expressed in glioblastoma and non-tumor brain tissues

We performed a genome-wide expression profiling of 754 human miRNAs in 58 GBM and 10 non-tumor brain FFPE tissue samples. LIMMA analysis revealed 108 significantly upregulated and 108 downregulated miRNAs in GBMs in comparison with non-tumor brain samples (*P* < 0.05) (see Supplementary Table S2, available at *Carcinogenesis* Online). Among them, 28 miRNAs showed *P* value < 10⁻⁹ and were able to discriminate GBMs and non-tumor brain samples with 100% sensitivity and 100% specificity (Figure 1). The most significantly upregulated miRNAs in GBM tissue were miR-21* and miR-155 (both *P* < 10⁻¹⁷; fold change = 8.44 and 4.59, respectively). On the other side, miR-220 and miR-1247 were the most significantly downregulated in tumor tissue (both *P* < 10⁻²¹; fold change = 9.15 and 8.35, respectively).

MGMT promoter methylation status

We have examined MGMT promoter methylation status in our cohort of GBM patients and have identified methylated promoter in 22 (38%) cases. Methylated promoter of MGMT was significantly associated with longer progression-free survival (PFS) (*P* = 0.0309, log-rank test; PFS medians of patients with methylated and non-methylated MGMT promoter were 9 and 6.75 months from diagnosis, respectively) (Figure 3A) and longer OS (*P* = 0.0202, log-rank test; OS medians of patients with methylated and non-methylated MGMT promoter were 18.5 and 12 months from diagnosis, respectively) (Figure 3B) in GBM patients. However, MGMT promoter methylation status has not reached statistical significance in multivariate Cox regression analysis.

Identification of miRNA prognostic signature

We used univariate Cox regression to analyze each miRNA as predictor of OS in 58 GBM patients and identified 15 miRNAs (*P* < 0.15), from which were subsequently, using bidirectional stepwise regression, selected six miRNAs (miR-31, miR-224, miR-432*, miR-454, miR-672 and miR-885-5p) (Supplementary Table S3, available at *Carcinogenesis* Online). This six-miRNAs signature was used to calculate the Risk Score for each patient (Figure 2). Higher Risk Score has been significantly associated with shorter PFS (*P* < 0.0001, log-rank test; median PFS for low-risk and high-risk patients were 9.4 and 4.4 months since diagnosis, respectively) (Figure 3C) and shorter OS (*P* < 0.0001, log-rank test; median OS of low-risk and high-risk patients were 16.2 and 7.5 months since diagnosis, respectively) (Figure 3D) in GBM patients.

Risk Score based on six-miRNAs signature is an independent prognostic factor

We performed univariate Cox regression analysis using clinical and molecular factors for whole set of 58 GBM patients and observed that the Risk Score based on six-miRNA signature and methylation status of MGMT gene promoter were significantly associated with OS and PFS. Moreover, PFS also correlated with adjuvant TMZ in monotherapy (Table I). Performance status and extent of resection were significantly associated with neither OS nor PFS. A multivariate Cox regression analyses showed that Risk Score based on six-miRNA

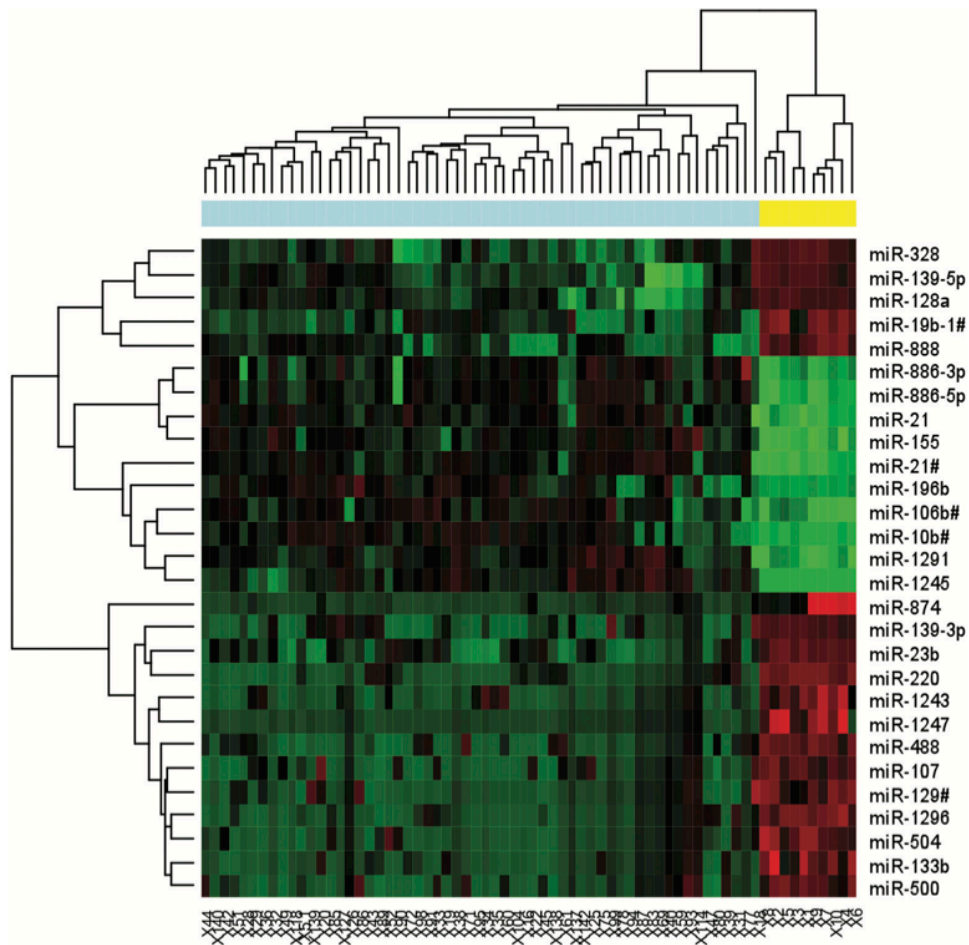


Fig. 1. Hierarchical clustergram discriminating GBM and non-tumor brain tissues according to differentially expressed miRNAs (blue color indicate GBM tissues, yellow color indicate non-tumor brain tissues, $P < 10^{-9}$).

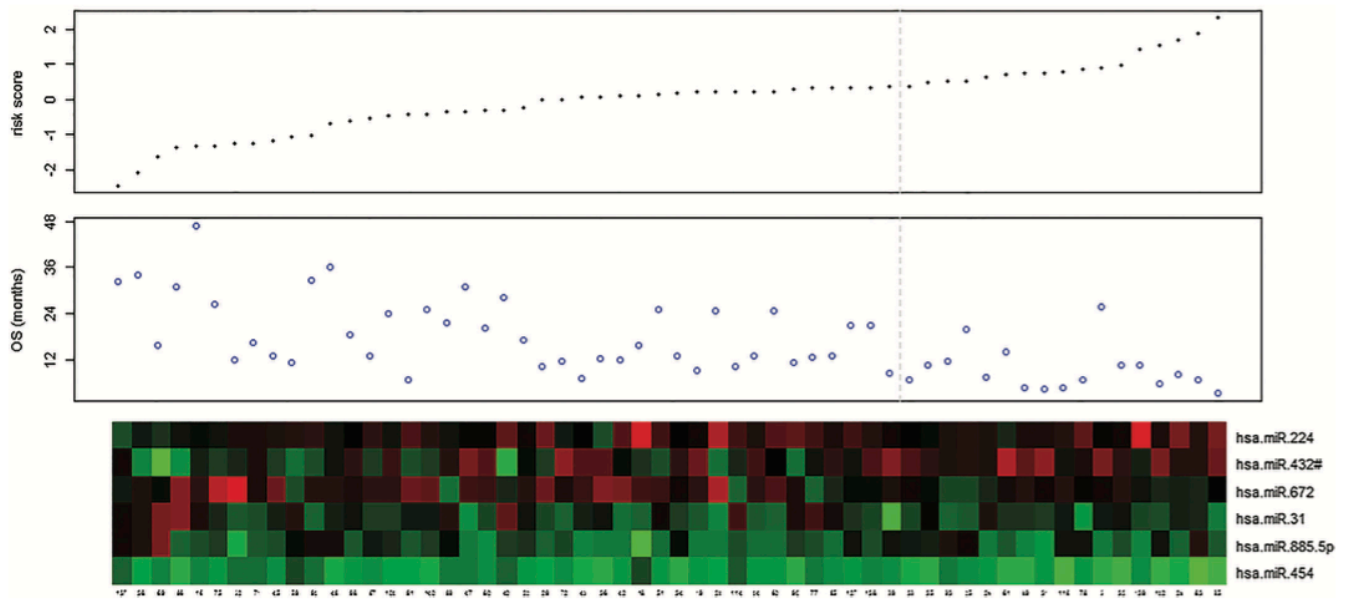


Fig. 2. Analysis of the 6-miRNA-based Risk Score for overall survival in GBM patients; top—Risk Score distribution, middle—overall survival of GBM patients, bottom—heat map of six-miRNA expression levels in GBM tissues, the black dotted lines on the top and middle represent the Risk Score cutoff value.

signature is independent prognostic factors in relation to both OS [hazard ratio (HR) 2.86; 95% confidence interval (CI) 1.914.29; $P < 0.001$] and PFS (HR 1.98; 95% CI 1.332.94; $P < 0.001$). As 2758

another independent factor associated with PFS was confirmed adjuvant TMZ in monotherapy (HR 0.56; 95% CI 0.320.97; $P = 0.039$) observed also in univariate Cox regression (Table I).

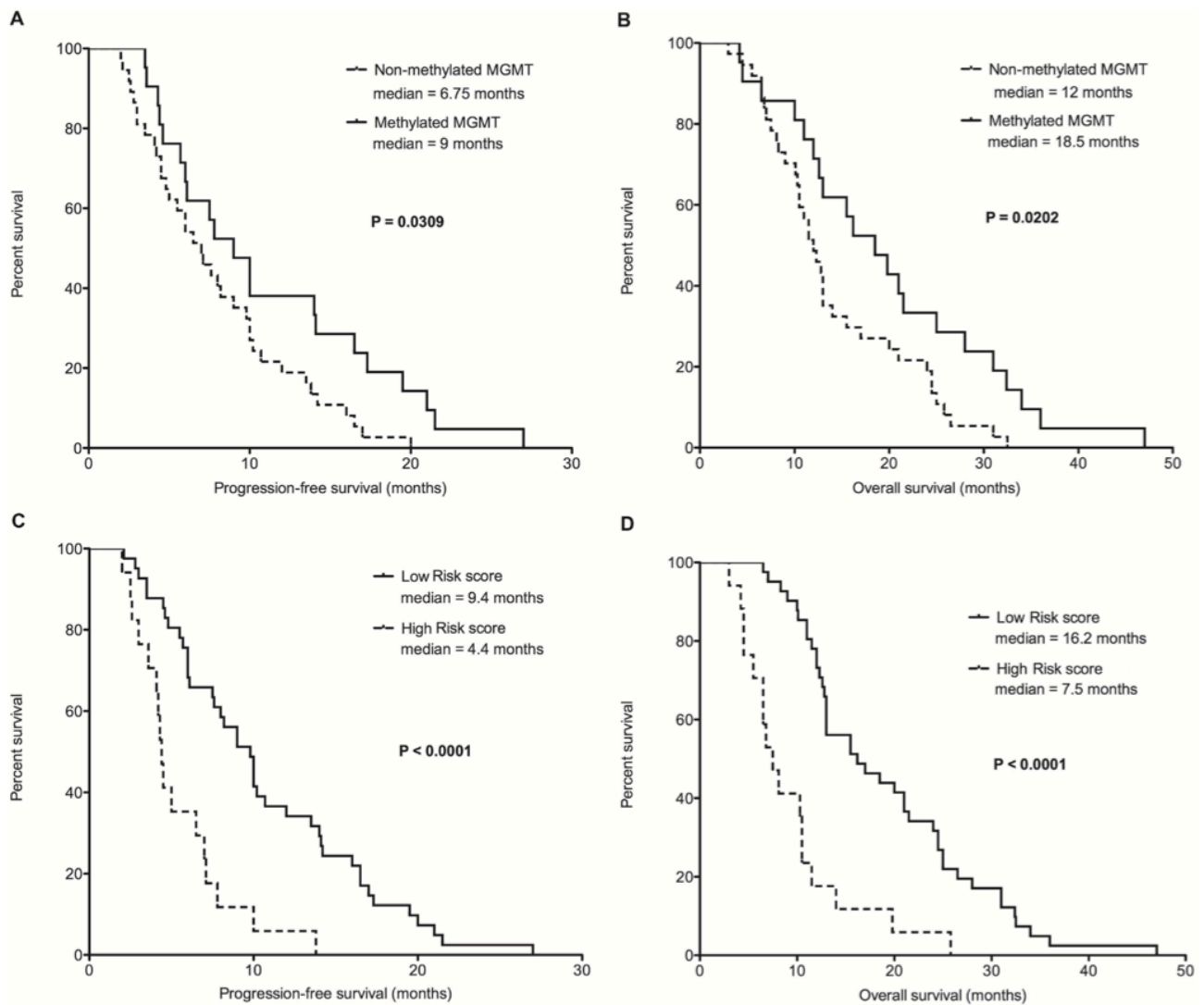


Fig. 3. Kaplan–Meier survival curves estimating PFS (A) and OS (B) in patients with GBM accordingly to MGMT methylation status; and PFS (C) and OS (D) in patients with GBM based on six-miRNA (miR-31, miR-224, miR-432*, miR-454, miR-672 and miR-885-5p) prognostic Risk Score.

Table I. Cox hazard regression analysis of common GBM prognostic factors and 6-miRNA-based Risk Score effects on survival of GBM patients

Factors	Univariate Cox regression			Multivariate Cox regression			Long-rank test <i>P</i>
	HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value	
Progression free survival							
Risk Score	2.00	1.37–2.91	<0.001	1.98	1.33–2.94	<0.001	<0.001
TMZ	0.48	0.28–0.82	0.007	0.56	0.32–0.97	0.039	
PS	1.69	0.70–4.04	0.242	2.17	0.89–5.30	0.090	
MGMT	0.54	0.23–0.96	0.035				
Overall survival							
Risk Score	2.72	1.84–4.02	<0.001	2.86	1.91–4.29	<0.001	<0.001
Extent of resection	1.00	0.49–2.04	0.991				
TMZ	0.64	0.37–1.08	0.096				
PS	1.79	0.76–4.22	0.186	2.31	0.96–5.57	0.061	
MGMT	0.51	0.29–0.91	0.022				

Bolded values are factors significant in multivariate cox regression analysis. CI, confidence interval; HR, hazard ratio; MGMT, methylation status of *O*⁶-methylguanine-DNA methyltransferase gene promoter; PS, performance status; TMZ, adjuvant temozolomide in monotherapy.

Validation of the six-miRNA prognostic signature by use of The Cancer Genome Atlas data

We used TCGA dataset of 485 GBM patients for whom OS information and miRNA expression profiles were available for validation of our

prognostic six-miRNAs signature. Unfortunately, only four miRNAs (miR-31, miR-224, miR-432* and miR-454-3p) from signature were represented in the TCGA dataset. First, we performed Z-score transformation on expression levels across the all GBM samples for each of

the aforementioned four miRNAs; then, the four-miRNAs signature was used for calculation of the individual Risk Score for each patient. By using the median value of the Risk Scores as the threshold, we divided GBM patients into high-risk and low-risk groups. Kaplan–Meier analysis confirmed that OS of the high-risk patients was significantly lower in comparison with low-risk patients ($P < 0.0115$, log-rank test) (Figure 4).

Ectopic expression of miR-31 decreases proliferation of GBM cells in vitro

We performed *in vitro* transient transfection of miR-31 mimic in A172, T98G, U87MG and U251 cell lines to investigate effect of miR-31 levels on GBM cells proliferation. MTT assay showed that miR-31 mimic transfection leads to the significant decrease of proliferation in all examined GBM cell lines when compared with control oligonucleotide ($P < 0.05$, *t*-test) (Figure 5).

Discussion

Comparison of miRNA expression profiles in GBMs and non-tumor brain tissues revealed a signature of 28 most significantly deregulated

miRNAs, which were able to precisely discriminate both of the investigated tissue categories accordingly to their origin. Nine miRNAs (miR-10b*, miR-21, 128a, miR-133b, miR-139-3p, miR-139-5p, miR-155, 196b and miR-328) were described previously to be deregulated in GBM (16–19). In this regard, the most frequently studied miRNA in cancer research and well-known oncogenic miRNA in GBM is miR-21. This miRNA was many times observed to be upregulated in GBM in comparison with non-tumor brain tissue and its expression level is positively correlated with increased grading of glioma tumors (16–19). These facts undoubtedly highlight miR-21 to be promising GBM biomarker. miR-21* (also called miR-21-3p) that shares the same stem-loop precursor as miR-21 was another significantly upregulated miRNAs in GBM. Although miR-21* is not as famous as its precursor counterpart, there are studies indicating that this molecule play a role in breast cancer, head and neck squamous cell carcinoma, and multiple myeloma (20–22). Moreover, recent data suggest that miR-21*, similarly to miR-21, positively regulate p-AKT level; and activation of AKT results in cell growth and survival of GBM cells (20,23,24). From one stem-loop precursor originate also miR-139-3p and miR-139-5p, which were both significantly down-regulated in GBM tissue in our study. These findings were several times confirmed previously in both GBM and some other cancers. In addition, miR-139-5p expression negatively correlates with survival of high-grade glioma patients. However, their functional participation on tumor cell biology remains unknown (25–27).

We have confirmed recently described reduction in expression levels of miR-128a, miR-133b and miR-328 in GBM tissue. From these, miR-128a has been the most frequently observed miRNA to be downregulated in GBM (6,17,18), where it is involved in negative regulation of mesenchymal signaling pathway and, thus, could be an useful biomarker of novel clinically relevant molecular taxonomy of GBM (1,28). In agreement with our results, several authors described downregulation of miR-328 in GBM and, moreover, there are studies showing negative correlation between miR-328 expression and malignant progression of gliomas and positive relationship with prognosis (17,29). This is probably due to the ability of miR-328 to participate on regulation of Wnt signaling pathway and/or ABCG2 expression (30,31). In concordance with our work, Silber et al. described down-regulation of miR-133b in GBM suggesting its tumor suppressive functioning (17). Decreased expression levels of miR-133b in other cancers and its tumor suppressive role mainly through targeting CXCR4 and EGFR signaling were recently published (32–34), which both were several times described also in relation to GBM molecular pathology.

Excepting miR-21, we observed higher expression levels of miR-10b*, miR-155 and miR-196b in GBM tissue in accordance with the earlier published works (6,16,17). Oncogenic functioning of miR-155 in GBM is well described. In addition to generally observed increased levels in tumor tissue, its expression inversely correlated with both OS and PFS in GBM patients (35–37). miR-155 regulates glioma cell proliferation, apoptosis, migration, invasiveness as well as chemoresistance to taxol through targeting MXI1 (antagonist of c-Myc), FOXO3 and/or GABA receptors (36–39). Much less is known about the two other upregulated miRNAs: miR-196b and miR-10*. High expression of miR-196b GBM tissue was recently observed, and upregulation of miR-196b confers a poor prognosis in GBM patients (40,41). miR-10* (miR-10b-3p), unlike its stem-loop precursor counterpart miR-10b-5p, is not well known and was not described in GBM yet and there are only a few references available in other cancers till now. This miRNA has been upregulated in saliva samples of esophageal carcinoma patients and in older melanoma patients (42,43). On the other hand, this miRNA has been downregulated in breast carcinoma and endometrial serous adenocarcinoma (44,45). If we consider miR-10b* to have similar functional properties as miR-10b, which functioning in cancer cell is well described, its role in GBM would be more probably oncogenic (46,47).

In the second part of our study, logistic regression revealed six-miRNAs signature (miR-31, miR-224, miR-432*, miR-454, miR-672 and miR-885-5p) that is associated with clinical outcome of GBM patients treated with chemoradiotherapy. Interestingly, all miRNAs, except miR-454, were significantly deregulated in tumor tissue

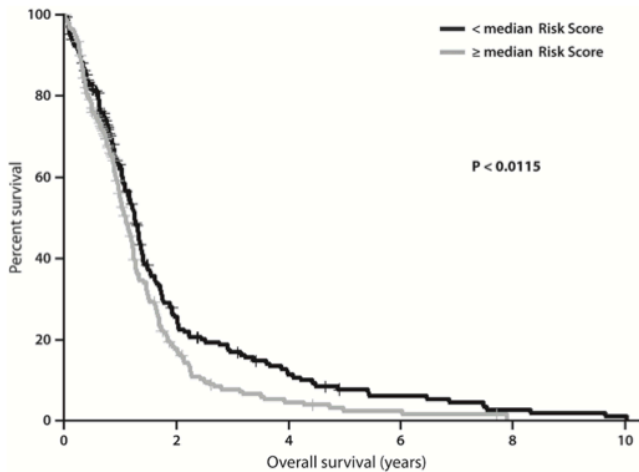


Fig. 4. KaplanMeier survival curves estimating OS in GBM patients from TCGA dataset accordingly to four-miRNA (miR-31, miR-224, miR-432* and miR-454-3p)-based Risk Score.

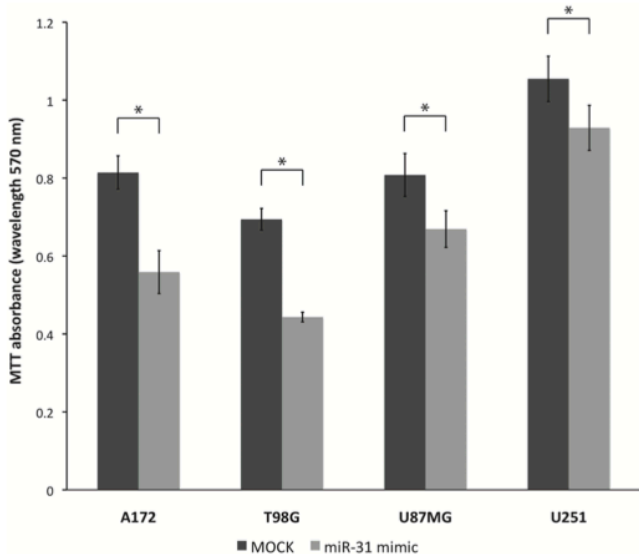


Fig. 5. MiR-31 replacement reduces proliferation of GBM cells A172, T98G, U87MG and U251. * indicates $P < 0.05$; 'dark' grey indicates mimic negative control (MOCK) transfected cells; 'pale' indicates miR-31 mimic transfected cells.

in our GBM patient cohort (Supplementary Table S2, available at *Carcinogenesis* Online). Three miRNAs (miR-31, miR-224 and miR-885-5p) were studied earlier in relation to gliomas (Supplementary Table S3, available at *Carcinogenesis* Online). In accordance with our findings, miR-224 was described to be significantly upregulated in glioma tissues and associated with survival of GBM patients (48). On the contrary, miR-31 and miR-885-5p showed lower expression levels in glioma cells, which also in agreement with our findings (47–50). Because of its significant downregulation in GBM tissues as well as its tumor suppressive character in relation to the GBM patient's survival, miR-31 was chosen for the following *in vitro* functional analyses. In accordance with the earlier observation, ectopic expression level of miR-31 led to the significant decrease of cell proliferation in all examined GBM cell lines indicating its tumor suppressive function in GBM. Moreover, in recent studies was observed that both miR-31 and miR-885-5p reduced migration and/or invasiveness of glioma cell lines suggesting their role in progression of gliomas and their prognosis (47–50). To our knowledge, miR-432*, miR-454 and miR-672 have not been observed till now as associated with any type of cancer.

Risk Score calculated on the basis of this six-miRNAs signature has been significantly associated with PFS and OS in KaplanMeier analyses. Our data showed that patients with higher Risk Score have statistically worse prognosis than patients with Risk Score under cut-off value. Our study confirmed well-described prognostic potential of methylation status of MGMT promoter in GBM patients, who underwent adjuvant concomitant RT/TMZ therapy. Ability of MGMT methylation status to predict clinical outcome of GBM patients was considerably lower in comparison with our six-miRNA-based Risk Score. Furthermore, univariate Cox regression analysis revealed that Risk Score and methylation status of MGMT gene promoter significantly correlate with OS and following multivariate Cox regression analysis confirmed Risk Score to be the independent prognostic factor. Similar results were reached also in relation to the PFS. Interestingly, common prognostic factors in GBM like performance status or extent of resection have not been associated with PFS and OS. This is probably due to the fact, that our cohort of GBM patients is highly homogenous and total/subtotal resection and performance status 1 or 2 comprised 86 and 90 percentages of cases, respectively.

Finally, we performed independent validation analysis of our results by use of TCGA dataset for OS prediction. Despite the fact that only four miRNAs (miR-31, miR-224, miR-432* and miR-454-3p) from six-miRNA prognostic signature were available in TCGA datasets for calculation of Risk Score, GBM patients having Risk Score below the median survived significantly longer time in KaplanMeier analysis. This confirmed our findings from explorative part of this study where higher Risk Score was associated with poor prognosis of GBM patients.

Overall, Risk Score based on six-miRNA signature showed significant superiority in prediction of clinical outcome of GBM patients when compared with MGMT methylation status as reference method. Multivariate Cox analysis indicated that the Risk Score based on six-miRNA signature is an independent prognostic classifier of GBM patients. Therefore, we suggest that the Risk Score presents promising prognostic algorithm with potential for individualized treatment decisions in clinical management of GBM patients.

Supplementary material

Supplementary Tables S1–S3 can be found at <http://carcin.oxfordjournals.org/>.

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PŘÍLOHA 8

Diagnostika, operační a systémová terapie metastáz solidních nádorů

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Sekundární mozkové nádory, mozkové metastázy, se vyskytují až u 30–40 % všech onkologických pacientů se solidními nádory, přičemž riziko jejich výskytu a incidence nadále narůstá. Cílem tohoto přehledového článku je poskytnout základní a zároveň aktuální informace o současných léčebných možnostech pacientů s mozkovými metastázami s výjimkou radioterapie, která je diskutována v samostatném, navazujícím, přehledovém článku. Snahou je, v souladu se zaměřením časopisu, poskytnout didakticky a prakticky podané informace o problematice sekundárních mozkových nádorů s důrazem na jejich využitelnost v denní klinické praxi. S rozšiřujícími se léčebnými možnostmi, včetně operačních výkonů a moderní cílené terapie a imunoterapie, lze do budoucna očekávat více personalizovaný přístup v léčbě tohoto onemocnění.

Klíčová slova: mozkové metastázy, neurochirurgie metastáz, cílená terapie, imunoterapie.

Diagnosis, surgery and systemic treatment of brain metastases

Secondary brain tumors, brain metastases, develop in up to 30–40% of all solid tumor cancer patients, with increasing incidence. The aim of this review is to provide both basic as well as current information on up-to-date treatment options for patients with brain metastases except of radiotherapy, which is discussed in separate, company review. The aim is, in accordance with the focus of the journal, to provide didactical and practical information on the issue of secondary brain tumors with an emphasis on their usefulness in daily clinical practice. With increasing therapeutic options, including surgery and advanced targeted therapy and immunotherapy, a more personalized approach to the treatment of this disease can be expected in the future.

Key words: brain metastases, neurosurgery, targeted therapy, immunotherapy.

Úvod do problematiky

Sekundární mozkové nádory (mozkové metastázy) vznikají metastazováním do mozku

z nejrůznějších primárních nádorů, kdy mezi nejčastější patří bronchogenní karcinom, nádory prsu, ledvin a maligní melanom (1). Jedná

se tedy o velice heterogenní skupinu pacientů, jejichž terapeutické možnosti jsou dány také typem a rozsahem extrakraniálního onemocnění.

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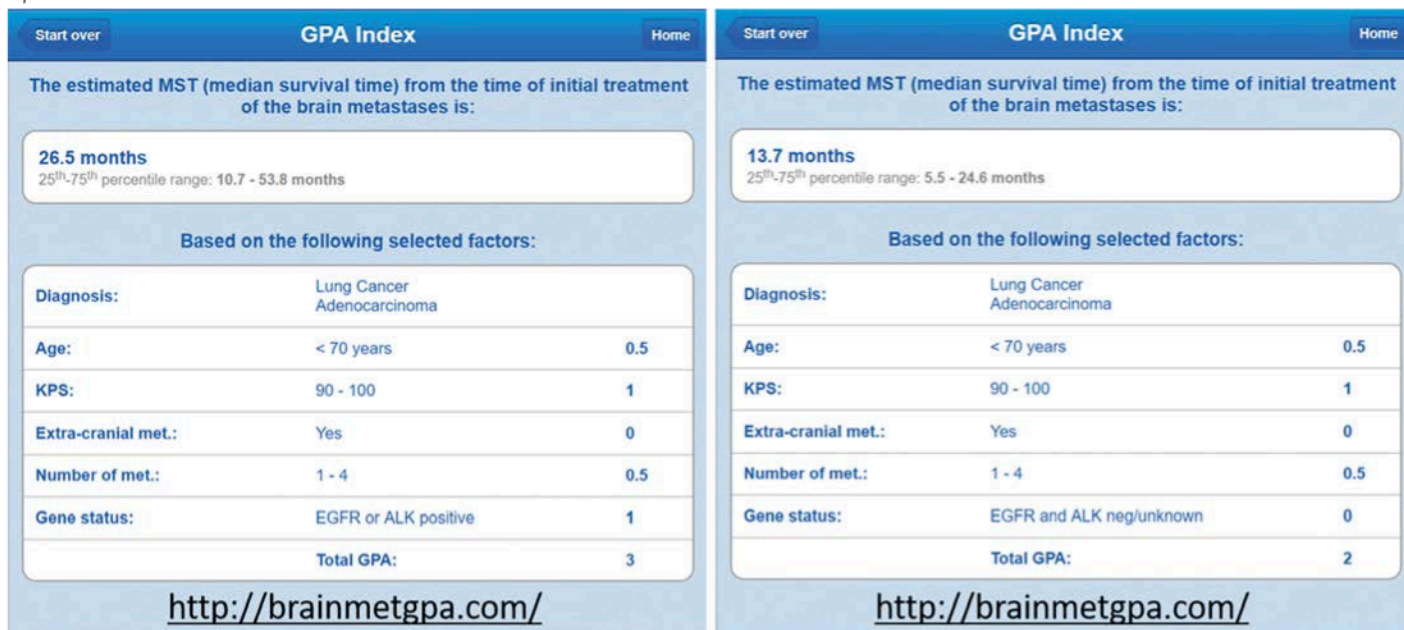
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Obr. 1. Náhled grafického výstupu z <http://brainmetgpa.com/> při modelovém odhadu přežití u pacienta s, respektive bez aktivačních mutací EGFR/ALK u plicního adenokarcinomu



Léčebný postup solitární mozkové metastázy tak bude odlišný u silně předléčeného pacienta s lokálně progredujícími bronchogenním nádorem a s mnohočetnými aktuálně progredujícími viscerálními metastázami a u jiné modelové pacientky s odléčeným nádorem prsu a nově diagnostikovanou solitární mozkovou metastázou jakožto jediným místem aktuální progresy onemocnění. Za základní studijní materiál lze považovat, kromě mnoha přehledových článků jako je například review od Archol a kol. publikované na začátku roku 2019 v Nature Reviews (2), oficiální doporučení evropské neuroonkologické asociace (EANO guidelines) z roku 2017 (3).

Cílem tohoto přehledového článku je poskytnout základní a zároveň aktuální informace o současných léčebných možnostech pacientů s mozkovými metastázami. Snahou je, v souladu se zaměřením časopisu Onkologie (společnost Solen), poskytnout didakticky a prakticky podané informace o problematice sekundárních mozkových nádorů s důrazem na jejich využitelnost v denní klinické praxi.

Mozkové metastázy jsou nejčastějším nádorovým onemocněním mozku s asi desetinásobnou incidencí v porovnání s primárními mozgovými nádory. Odhaduje se, že k rozvoji mozkových metastáz dochází až u 30 % pacientů se solidními nádory (4). Lze důvodně předpokládat, že incidence bude nadále vzrůstat díky větší dostupnosti magnetické rezonance a především díky rozšiřujícím se indikacím moderní systémové terapie (cílená léčba, imunoterapie),

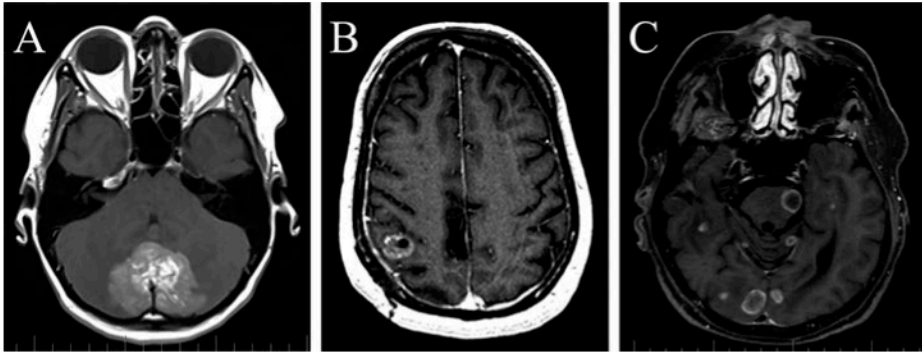
kteří však jen omezeně proniká do centrálního nervového systému (CNS), vedoucím k obecně delšímu celkovému přežívání diseminovaných pacientů, u kterých pak stoupá riziko další diseminace do této lokality.

Prognóza pacientů s mozgovými metastázami je i přes výše naznačené úspěchy moderní onkologické léčby nadále velice vážná s mediány celkového přežití udávanými v řádu měsíců. Existují ale velké rozdíly v přežívání jednotlivých pacientů, kdy lze u některých pacientů dosáhnout kombinovanou onkologickou léčbou přežití (a to s dobrou kvalitou života) až několika let (5). V souvislosti s reportováním klasických onkologických léčebných cílů (celkové přežití nebo přežití do progresu) je nutné poznamenat, že se v případě léčby mozkových metastáz jedná vždy o léčbu s paliativním záměrem (s tzv. krátkodobým nebo tzv. dlouhodobým paliativním záměrem), kdy není hlavním cílem prodloužení celkového přežití, ale udržení, nebo dokonce zlepšení, kvality života a neurokognitivních funkcí (6). Teprve v posledních letech jsou k dispozici výsledky randomizovaných studií, které jsou přímo designované k posouzení vlivu jednotlivých léčebných intervencí na kvalitu života jakožto primárního cíle těchto studií (7, 8).

Vzhledem k popisovaným rozdílům v přežívání pacientů je odhad prognózy u konkrétního pacienta s nově diagnostikovanou metastázou extrémně důležitý při rozvaze o nevhodnějším diagnosticko-terapeutickém postupu. V této souvislosti je nutno, především pro neurochi-

urgie a radioterapeutu, zdůraznit vhodnost konzultace ošetřujícího klinického onkologa, který zná pacienta dlouhodobě, včetně detailní historie tolerance předchozí onkologické léčby. Navíc je důležité znát, jaké jsou další možnosti systémové onkologické léčby. V denní rutinní praxi jsou také neocenitelnou pomůckou nejrůznější prognostické skórovací systémy umožňující u daného pacienta validní odhad celkového přežití (9). Nejznámějším, přes dvacet let užívaným, systémem je RPA (Recursive Partitioning Analysis – statistická metoda multivariační analýzy), pomocí kterého lze zhodnocením celkového výkonnostního stavu (Karnofsky index), věku, kontroly primárního onemocnění a přítomnosti extrakraniálních metastáz rozdělit pacienty do tří skupin s predikcí mediánu přežití 7,1 měsíce, 4,2 měsíce a 2,3 měsíce (10). V současné době existuje bezpočet dalších skórovacích systémů, zohledňujících mimo jiné i molekulárně biologické charakteristiky daných nádorů (např. aktivační mutace EGFR u nemalobuněčných plicních nádorů, mutace BRAF V600 u melanomu). Pro denní ambulantní praxi lze doporučit online dostupné systémy. Příkladem je DS-GPA (disease specific graded prognostic assessment) dostupný online na <http://brainmetgpa.com/> (11). Tak například odhad celkového přežití u pacienta (věk < 70 let, Karnofsky 90, přítomny extrakraniální metastázy a jedna mozková metastáza) s plicním adenokarcinomem a aktivačními EGFR a ALK mutacemi je 26,5 měsíců, ale bez těchto mutací pouze 13,7 měsíců (náhled grafického

Obr. 2. Příklady MR nálezu mozkových metastáz. A: objemná metastáza v mozečku, B: drobná metastáza v centrální krajině vpravo, C: mnohočetné metastázy supra- a infratentoriálně



výstupu z <http://brainmetgpa.com/> na obrázku 1). Další rozvoj individuálních prognostických indexů je nutný u specifických skupin pacientů, jako jsou např. pacienti po metastazektomii, kdy je popisována výrazná genetická diverzifikace při srovnání molekulárního profilu původního primárního tumoru a metastatického onemocnění (12). Na základě této skutečnosti je tedy poměrně obtížné přesněji predikovat přežití. I přes zmiňované individualizované prognostické indexy (pro pacienty po metastazektomii, pro pacienty s primárním nádorem plic, melanomem apod.) je nutné zdůraznit obecné prognostické faktory platné pro všechny pacienty. Především se jedná o celkový stav pacienta (jakožto samostatné kritérium determinující kohortu pacientů s nejhorší prognózou v již zmiňovaném RPA skórovacím systému), nebo rozsah nově diagnostikovaného, případně již léčeného extrakraniálního onemocnění. V případě kontrolovaného extrakraniálního onemocnění se dá předpokládat také větší pravděpodobnost kontroly intrakraniálního onemocnění, kdy mohou mít tito pacienti benefit z agresivnější léčby mozkových metastáz, včetně více sofistických metod radioterapie s efektem příznivějšího profilu nežádoucích účinků (alterace neurokognitivních funkcí). Tato problematika je blíže diskutována v navazujícím článku o radioterapii mozkových metastáz, jež bude následovat.

Klinické příznaky a diagnostika mozkových metastáz

Velká variabilita v klinických příznacích je dána mimo jiné lokalizací metastázy (např. centrální krajina, mozeček, temporální lalok dominantní hemisféry) a přítomností perilezionálního otoku, který je pro metastázy typický, nebo např. zakrvácením, které se častěji vyskytuje u některých histologických typů. Velikost metastázy

nekoreluje přímo s velikostí otoku, který může být velice rozsáhlý i u malých solitárních lézí a naopak nemusí být přítomen u velkých metastáz (obrázek 2A). Mezi typické příznaky patří ložisková iritační (epilepsie) nebo zániková (paréza, hypestezie) neurologická symptomatologie a/nebo intrakraniální hypertenze projevující se bolestmi hlavy, závratěmi, nevolností a zvracením, případně dvojitým viděním (13). Na druhou stranu, mozkové metastázy mohou být dlouho asymptomatické a pacienti jsou diagnostikováni již s rozsáhlým postižením CNS. Dalšími symptomy mozkových metastáz jsou náhlé neurokognitivní a psychické změny u pacienta. Na možnost mozkových metastáz je nutno pomyslet vždy při objevení se uvedených symptomů u onkologických pacientů, především u výše jmenovaných primárních nádorů.

Mozkové metastázy jsou nejčastěji diagnostikovány pomocí zobrazovacích metod (CT, MR) indikovaných pro typické neurologické symptomy, případně v průběhu stagingu nádorového onemocnění u asymptomatických pacientů. Staging CNS je doporučován u pacientů s nově diagnostikovaným malobuněčným plicním karcinomem. U nemalobuněčných plicních karcinomů je provádění vstupního stagingu v oblasti CNS diskutabilní, lze ho doporučit u lokálně velmi pokročilých nálezů, případně u výskytu extrakraniálních metastáz.

V rámci akutní diagnostiky je často indikováno CT vyšetření, nicméně za zlatý standard nutno považovat vyšetření magnetickou rezonancí, kdy je preferovanou sekvencí T1 vážené zobrazení s aplikací kontrastní látky. Nejčastěji je pozorována kortikosubkortikálně lokalizovaná kulovitá sytící se léze s ostrou hranicí oproti okolní tkáni a s perifokálním edémem. Nález na MR je často určující v rozvaze o nevhodnějším terapeutickém postupu (obrázek 2). MR zob-

razování je také důležité v rámci sledování po léčbě. Nejznámější RECIST (Response Evaluation Criteria in Solid Tumors) kritéria, používaná především při hodnocení léčebné odpovědi extrakraniálních lézí, mají určité limity v hodnocení mozkových metastáz (především neberou v potaz neurologické symptomy nebo užívání kortikoidů). RANO (Response Assessment in Neuro-Oncology) pracovní skupina je multidisciplinární mezinárodní uskupení pracující na definování a zavedení systémů hodnocení léčebné odpovědi v oblasti CNS. Bylo publikováno několik doporučení stran mozkových a leptomeningeálních metastáz (14, 15), kdy je zohledněn také celkový stav pacienta, nebo užívání kortikoidů, včetně stavu extrakraniálního onemocnění (RANO-BM, tabulka 1). Zvláštní pozornost je potřeba věnovat hodnocení prvních MR snímků po cílené stereotaktické vysokodávkované radioterapii a radiochirurgii, kdy je často pozorován fenomén pseudoprogrese (16). V rutinní praxi je tedy při hodnocení MR nálezu důležitá spolupráce radiologa a ošetřujícího klinického onkologa, respektive radioterapeuta.

Chirurgická léčba mozkových metastáz

Neurochirurgický výkon je nejčastěji indikován u pacientů s chirurgicky dostupnou solitární metastázou, kteří jsou v dobrém celkovém stavu a s absencí extrakraniálního onemocnění, resp. v případech dobře kontrolovaných extrakraniálních metastáz, případně u pacientů s perspektivou další systémové terapie. Především u pacientů s objemnou metastázou a velkým perifokálním edémem je intervence spojena s velkým klinickým zlepšením v důsledku dekomprese. Zvláště u takovýchto pacientů nelze od alternativní léčebné metody – radioterapie – očekávat větší klinický benefit. Operační výkon v takovém případě také umožní snížení nebo vysazení kortikoterapie. Při rozhodování o indikaci chirurgického řešení je vhodné vzít v potaz také možnosti daného onkologického pracoviště (např. dostupnost stereotaktické radioterapie), jelikož chirurgické řešení solitární metastázy následované WBRT je doporučováno jako preferovaná léčba před pouhým WBRT (17). Nezanedbatelným výstupem neurochirurgického výkonu je získání tkáně pro histologické vyšetření, což je důležité nejen u pacientů s neznámým primem onemocnění, ale i u všech

ostatních pacientů vzhledem k možnosti molekulární subtypizace onemocnění mozku. Do budoucna lze očekávat čím dál větší uplatnění cílené léčby mozkových metastáz, kdy aktualizace molekulárního profilu může mít prediktivní význam s identifikací nových terapeutických cílových molekul. Cílem operačního řešení je ale především zachování a/nebo zlepšení kvality života pacienta. Možnosti operačního řešení je tak nutno zvažovat s ohledem na elokventnost (funkční důležitost) dané lokality, možnosti jiné terapie a především s ohledem na celkový stav a prognózu pacienta. Operace metastázy v elokventní oblasti mozku tak v absolutní většině není indikována k operačnímu řešení (metastázy v elokventních oblastech mozku, obrázek 2B).

Naopak často z odstranění nádoru klinicky výrazně profitují pacienti s metastázou v mozečku, zejména pokud je současně přítomen obstrukční hydrocefalus, ne zřídka postupem je právě selektivní resekce cerebelární metastázy s pokračováním léčbou pomocí radioterapie. Na většině neurochirurgických pracovišť je hlavním morfologickým kritériem k indikaci operační/neoperační léčby kromě lokalizace také velikost metastázy (> 3 cm) a dále jejich celkový počet. Zcela výjimečně neurochirurg provede resekci 2 metastáz najednou, tento postup preferuje, pokud je možno použít stejný přístup nebo neměnit polohu pacienta při jednodobé operaci, například při resekci metastázy v zadní jámě lební a okcipitálně. Urgentní načasování operace, kromě resekce v zadní jámě lební, vyžaduje i deteriorace neurologického stavu při prokrvácení metastázy. Vhodná je i individuální domluva neurochirurga s radioterapeutem (zejména v případě plánování SRS), v případě velké cystické složky metastázy, kdy neurochirurg re-

sekcí její stěny a zmenšením celkového objemu nádoru (zejména pokud solidní část zasahuje do elokventních oblastí) připraví podmínky pro stereotaktické vysokodávkované ozáření.

Pooperační léčba je blíže diskutována v samostatném přehledovém článku navazujícím na tuto práci.

Systémová léčba mozkových metastáz

Systémová terapie mozkových metastáz je obecně velice limitovaná, jednak předléčeností většiny pacientů, jednak přítomností hematoencefalické bariéry, která brání efektivnímu průniku většiny léčiv s protinádorovým účinkem do mozku. Proto je téměř vždy nejprve zvažována možnost operace nebo radioterapie (SRS, SRT, WBRT). O tom, zda se rozhodneme indikovat také chemoterapii, cílenou léčbu nebo moderní imunoterapii rozhoduje vždy několik faktorů. K základním patří celkový stav pacienta a jeho spolupráce, rozsah mozkového postižení, možnosti chirurgické či radioterapeutické léčby, kontrola extrakraniálních metastáz a samozřejmě i pravděpodobnost ovlivnění dalšího průběhu celého onemocnění. Základním předpokladem intrakraniální účinnosti systémové léčby je vedle prostupnosti léku přes hematoencefalickou bariéru také její vysoká orgánově specifická efektivita. V případě chemoterapie lze zvážit indikaci cytostatik u chemosenzitivních malignit, jako jsou germinální nádory, malobuněčný plicní karcinom, případně karcinom prsu (např. cisplatina, karboplatina, etoposid, cyklofosfamid, ifosfamid, vinkristin, 5-fluorouracil, kapecitabin). Naopak u pacientů s primárně chemorezistentními tumory, mezi něž patří maligní melanom nebo hepatocelulární karcinom, je indikace paliativní

chemoterapie sporná, protože celkové přežití zásadně neovlivní. V případě moderních léků z oblasti cílené léčby a imunoterapie s checkpoint inhibitory je situace složitější. Dříve proběhlé velké randomizované klinické studie buďto pacienti s mozkovými metastázami nezařazovaly nebo metastázy musely být ošetřeny chirurgicky či radiochirurgicky a nesměly být aktivní (symptomatické). Doporučení byla proto dříve nejasná. S postupem času se ale ukázalo, že moderní cílená léčba a imunoterapie může být u pacientů s mozkovým postižením stejně efektivní jako u extrakraniálních metastáz, a to i bez předchozí operace nebo radioterapie. Nejdříve se objevovaly pozitivní výsledky z retrospektivních analýz malých souborů. Nyní již máme u některých diagnóz výsledky i z prospektivních studií.

V případě pacientů s nemalobuněčným plicním karcinomem lze jmenovat například EGFR tyrozinkinázový inhibitor 3. generace osimertinib nebo ALK inhibitor alectinib (18). U pacientek s HER2 pozitivním nádorem prsu (19) (kde je relativně větší riziko rozvoje mozkových metastáz) nutno zmínit studii LANDSCAPE, jednoramennou prospektivní studii hodnotící efekt tyrozinkinázového inhibitoru lapatinibu podávaného v kombinaci s kapecitabinem u pacientek s HER2 pozitivními karcinomy prsu a mozkovými metastázami (20). Objektivní odpověď na léčbu byla pozorována u 66 % pacientek (20). U maligního melanomu s mozkovými metastázami máme dnes k dispozici výsledky jak studie s cílenou léčbou při mutaci onkogenu BRAF, tak s imunoterapií (21).

Lepší výsledky lze očekávat u pacientů s asymptomatickým mozkovým postižením. Dle studie 2. fáze COMBI-MB s dabrafenibem a trametinibem dosahuje četnost intrakraniálních

Tab. 1. Přehled RANO-BM kritérií hodnocení mozkových metastáz (14)

	Kompletní odpověď	Částečná odpověď	Stabilizace nemoci	Progrese
Cílové léze	Žádné	≥ 30% zmenšení součtu nejdelších rozměrů vzhledem k výchozímu stavu	< 30% zmenšení vzhledem k výchozímu stavu, ale < 20% zvětšení součtu nejdelších rozměrů vzhledem k nádoru	≥ 20% zvětšení součtu nejdelších rozměrů vzhledem k nádoru ¹
Necílové léze	Žádné	Stabilní nebo zlepšené	Stabilní nebo zlepšené	Jednoznačně zhoršení ¹
Nová(é) léze ²	Žádné	Žádné	Žádné	Přítomna(y) ¹
Kortikosteroidy	Žádné	Stabilní nebo snížení	Stabilní nebo snížení	Nerelevantní ³
Klinický stav	Stabilní nebo zlepšení	Stabilní nebo zlepšení	Stabilní nebo zlepšení	Zhoršení ¹
Potřebné splnění požadavků	Vše	Vše	Vše	Jakýkoliv ³

U pacientů s více jak jednou měřitelnou lézí je celkem až 5 metastáz (5 největších) označeno za cílové léze.
 1) Progrese nastává, je-li splněno toto kritérium. 2) Nová léze je ta, která se neobjevuje na předchozích snímcích a je viditelná minimálně ve dvou projekcích. Pokud je nová léze nejednoznačná, například vzhledem k malé velikosti, může se zvážit pokračování léčby a to, zdali nová léze představuje skutečnou progresi, se objasní během sledování. Pokud opakovaný sken s jistotou potvrdí novou lézi, měla by být konstatována progrese k datu původního vyšetření, které lézi odhalilo. Při imunoterapii nové léze samy o sobě nedefinují progresi. 3) Navýšení dávky kortikosteroidů samo o sobě není bráno při hodnocení progrese v potaz bez přetrvávajícího zhoršení klinického stavu.

odpovědí 58 % (4 % kompletních remisí) (22). Dostatečně efektivní se jeví i imunoterapie, jejíž nespornou výhodou je větší šance na dlouhodobou léčebnou odpověď. Dle publikovaných výsledků studií 2. fáze (ABC trial a CheckMate 204) se jeví jako nejúčinnější kombinace ipilimumabu a nivolumabu (četnost odpovědí 46–56 %, četnost intrakraniálních kompletních remisí 17–26 %) (23, 24).

Do budoucna lze předpokládat rozšiřování indikací k podávání moderních přípravků systémové terapie, které spolu s dalším rozvojem neurochirurgických a radioterapeutických postupů diskutovaných v tomto přehledovém článku, povede k další optimalizaci léčby pacientů s mozkovými metastázami. Určitou limitací mohou být pravidla úhrady moderní léčby.

Podpůrná a symptomatická léčba

Nedílnou součástí je také podpůrná a symptomatická terapie. Mezi systémovou léčbu se řadí také kortikoterapie, která je indikována prakticky u všech pacientů, minimálně v době diagnózy mozkových metastáz (2, 25). I když jsou kortikoidy v neuroonkologii používány již několik desítek let, nejsou k dispozici jednoznačná data a doporučení stran správného dávkování. U jednotlivých pacientů je potřeba brát v potaz také četné nežádoucí účinky, především při dlouhodobém užívání (hyperglykemie, proximální myopatie, gastritida, perforace střeva, infekce, orální kandidóza, osteopenie, psychické změny, trombembolické příhody, cushingoidní

habitus a další). V průběhu kortikoterapie se tak standardně indikuje také preventivní podávání inhibitoru protonové pumpy (např. omeprazolu 20 mg 1x denně nalačno). Dávka kortikoidů (nejčastěji používaného dexametazonu s nejvýhodnějším poměrem mezi glukokortikoidním a mineralokortikoidním účinkem) je tak vždy individuální a závisí na typu, velikosti a lokalizaci metastáz a na rozsahu okolního vazogenního edému. Snahou je vždy najít nejnižší účinnou dávku steroidů, která ještě dostačuje ke kontrole symptomů. V některých případech, například v průběhu vysokodávkové cílené radioterapie, se doporučuje přechodně dávku kortikoidů navýšit. Není jednota v dělení celkové dávky s podáváním několikrát v průběhu dne. Obecně se doporučuje, vzhledem k přirozeným diurnálním rytmům, podávání kortikoidů v ranních hodinách. Poločas rozpadu 36–54 hodin opravňuje podávání léku pouze jednou denně, byť je v praxi celková dávka často dělena.

Dalším lékem užívaným v terapii edému je osmotické diuretikum manitol. Jeho antiedematozní efekt v mozku je kombinovaný, za prvé zlepšuje mozkový perfuzní tlak v důsledku přechodného zvýšení srdečního výdeje, za druhé způsobuje reflexní mozkovou vazokonstrikci a za třetí působí bezprostředním plasma-expanzivním efektem, kdy osmotický gradient mezi plasmou a mozkovou buňkou vede k přesunu vody do systémového oběhu, a tím dojde ke snížení otoku mozku. U symptomatického pacienta (rozsáhlý edém s vyjádřeným syndromem nitrolební hypertenze, případně

s hemiparézou a horšícím se stavem vědomí) je možností například následující postup (dle aktuálních doporučených postupů Masarykova onkologického ústavu v Brně; www.mou.cz): intravenózní dexametazon 16–24 mg/den (resp. 8 mg 2–3x denně), případně lze na začátku aplikovat bolus (např. 40 mg i.v.). K tomu lze indikovat brzké podání manitolu rychle podanou infuzí (1,0–2,0 g/kg t.h.m. / den, tj. např. u 70 kg člověka 70 až 140 g/den). V praxi se nejčastěji jedná o podání 200 ml 15 % Manitolu rychlou i.v. infuzí první 3 dny á 8 hod, čtvrtý den á 12 hod, pátý den 1x a poté vysadit. Při takové terapii je nutno sledovat bilanci tekutin, iontoqram (Na+, K+), TK, glykémii u diabetiků.

Další detailnější rozbor podpůrné a symptomatické terapie je již nad rámec tohoto textu. Odkázat lze alespoň na aktuální doporučení stran antiepileptické profylaxe a kortikoterapie z března 2019, simultánně publikované v hlavních časopisech onkologické (Journal of Clinical Oncology) a neuroonkologické (Neuro-Oncology) obce (26, 27).

Tato práce byla částečně podpořena

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» PŘEHLEDOVÉ ČLÁNKY

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PŘÍLOHA 9

MicroRNAs and glioblastoma: roles in core signalling pathways and potential clinical implications

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Abstract

MicroRNAs (miRNAs) are endogenously expressed small non-coding RNAs that act as post-transcriptional regulators of gene expression. Dysregulation of these molecules has been indicated in the development of many cancers. Altered expression levels of several miRNAs were identified also in glioblastoma. It was repeatedly found that miRNAs are involved in important signalling pathways, which play roles in crucial cellular processes, such as proliferation, apoptosis, cell cycle regulation, invasion, angiogenesis and stem cell behaviour. Therefore, miRNAs represent promising therapeutic targets in glioblastoma. In this review, we summarize the current knowledge about miRNAs significance in glioblastoma, with special focus on their involvement in core signalling pathways, their roles in drug resistance and potential clinical implications.

Keywords: glioblastoma • microRNAs • prognosis • prediction • therapeutic targets

Introduction

Glioblastoma is the most frequently occurring primary malignant brain tumour, with an incidence of 355 new cases per 100,000 Caucasians per year. Despite introduction of modern therapeutic approaches, this cancer remains associated with very poor prognosis characterized by median overall survival less than 1 year [1,2]. For glioblastoma, rapid diffuse and infiltrative growth with high level of cellular heterogeneity associated with therapeutic

resistance is typical. There are also multiple genetic alterations characteristic for glioblastoma. Primary glioblastoma arising de novo is often characterized by EGFR amplification and PTEN mutations, whereas TP53 mutations are typical for secondary glioblastoma developing from lower-grade astrocytomas. Nevertheless, this distinction is not absolute and both glioblastoma types may harbour other genetic and chromosomal changes, for example, the loss of heterozygosity (LOH) 10q is the most frequent aberration in both primary and secondary glioblastomas [3,4].

miRNAs are small non-coding RNAs, 22-nt in length that guide post-transcriptional gene silencing of their mRNA targets. miRNAs are encoded by genes that are presumably transcribed into long primary miRNAs (pri-miRNAs) by RNA polymerases II/III [5]. Afterwards, RNase III enzyme converts pri-miRNAs into pre-miRNAs

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hairpin transcripts. At last, pre-miRNAs are processed into mature miRNAs that are incorporated into a ribonucleoprotein complex RISC. Within RISC, miRNAs act as specific determinants, whereas protein components achieve target mRNA silencing [6]. Deregulation of miRNAs can affect carcinogenesis if their mRNA targets are encoded by oncogenes or tumour suppressor genes; overexpression, silencing or switching off specific miRNAs have been described in carcinogenesis of glioblastoma. Up-regulation of mature miRNA may occur as a consequence of transcriptional activation or amplification of the miRNA encoding gene, whereas silencing or reduced expression may result from deletion of a particular chromosomal region, epigenetic silencing, or defects in their biogenesis [7]. In this review, we summarize recent work on miRNAs, with emphasis on their alterations and roles in glioblastoma pathogenesis and their potential used as disease biomarkers or novel therapeutic targets.

miRNAs signatures of glioblastoma tissue and cell lines

Global analysis of miRNA expression profiles of both glioblastoma tissues and glioblastoma cell lines allowed to identify a group of miRNAs with significantly altered expression in this tumour. When primary glioblastoma tissue was compared to non-malignant brain tissue, expression levels of nine miRNAs were significantly increased, whereas levels of four miRNAs were decreased (Table 1). The most significant results indicated strongly overexpressed miR-221, and down-regulated, miR-128a, miR-181a, miR-181b and miR-181c from a set of brain-enriched miRNAs [8]. The same authors performed miRNA expression analysis of 10 glioblastoma cell lines using identical methodic approach and data analysis. Interestingly, miRNAs underexpressed in glioblastoma cell lines generally confirmed primary tumour data, whereas only miR-21 and miR-221 that were overexpressed in tumours were deregulated also in the cell lines [8].

In another study, Slaby *et al.* evaluated expression profiles of eight miRNAs in glioblastoma tissues compared to non-malignant brain tissues from areas surrounding arteriovenous malformation (AVM). In glioblastoma tissue, only miR-21 and miR-125b were overexpressed, whereas six miRNAs were down-regulated (Table 1) [9]. In contrast to previous study, approximately four-fold lower levels of miR-221/222 were observed in glioblastomas in comparison to the adult 'normal-like' brain tissue. Authors discussed that it is likely that the brain tissue, although excised from the margin of resection material, contained traces of micro-capillaries from around the AVM. It is generally known that very high levels of miR-221/222 are found in endothelial cells. This could be responsible for the apparently low levels of miR-221/222 in glioblastomas despite their absolute levels being comparable to previously published reports [9].

Taken together, only miR-21 and miRNA-181 family were significantly and consistently altered in all three studies (Table 1).

Involvement of miRNAs in core glioblastoma signalling pathways

Glioblastoma development has been linked to progressive acquisition of mutations in genes with a crucial role in cell growth, proliferation and programmed cell death. As shown in many different studies, miRNAs might perfectly fit and integrate model of glioblastoma pathogenesis by controlling its core signalling pathways [3].

EGFR and PI3K/AKT signalling pathways

The epidermal growth factor receptor (EGFR) signalling network contributes to promotion and progression of a broad spectrum of solid tumours; it is a promising and, at least for some tumours, a validated target for anticancer therapy. Stimulation of the EGFR and, subsequently, KRAS signalling, lead to activation of numerous signal transduction molecules initiating a cascade of downstream effectors that mediate tumour growth, survival, angiogenesis and metastasis [10].

Several recent reports have also identified up-regulation of miR-21 in glioblastoma tissue [8,9]. Consequently, mechanistic studies identified mRNA targets of miR-21 among important components of the EGFR signalling pathway. Glioblastoma cell lines U251 (mutant PTEN) and LN229 (wild-type PTEN) showed a decreased expression of EGFR, activated AKT, Cyclin D and Bcl-2 after treatment by miR-21-specific antisense oligonucleotide [11]. Although miR-21 is known to regulate PTEN and down-regulation of miR-21 led to increased PTEN expression, the glioblastoma suppressor effect of antisense-miR-21 is most likely independent of PTEN status because U251 has mutated PTEN [11,12]. Oncogenic phenotype indicates also miR-26a, which is highly up-regulated in glioblastoma tissue [8]. Phenomenon of PTEN down-regulation followed by AKT activation was described after transfection of glioblastoma cells with the primary transcript of miR-26a-2. Similarly, the miR-26a mimics decreased PTEN protein levels and increased AKT phosphorylation [13,14]. Modulation of AKT signalling cascade using miRNAs in glioblastoma cell lines was described also in Nan *et al.* In this study, transfection of miR-451 mimicked reduced expression levels of Akt1, Cyclin D1, MMP-2, MMP-9 and Bcl-2. By contrast, miR-451 down-regulation led to increase in p27 levels. According to phenotypic experiments, miR-451 inhibited invasive ability, induced cell cycle arrest in the G0/G1 phase, delayed the progression of cell cycle, inhibited cell proliferation and induced apoptosis in glioblastoma cells *in vitro*. To conclude, it seems that miR-451 affects glioblastoma cells *via* regulation of the PI3K/AKT signalling pathway [15].

Another miRNA involved in the EGFR signalling pathway is miR-7. Kefas *et al.* published that miR-7 directly inhibited EGFR expression *via* its 3'-UTR and independently suppressed the AKT pathway *via* targeting upstream regulators, such as IRS-1 and IRS-2. Moreover, transfection with miR-7 oligonucleotides decreased viability and invasiveness of primary glioblastoma cell

Table 1 miRNAs significantly de-regulated in human glioblastoma tissues and glioblastoma cell lines

	Ciafre <i>et al.</i> (2005)			Slaby <i>et al.</i> (2010)		
	miRNA	C/P ratio	L/B ratio (P)	miRNA	Fold change (P)	
Up-regulated	miR-9-2	1.88–10.16				
	miR-10b	1.97–13.6				
	miR-21	1.81–9.3	1.61 (0.008)	miR-21	8.35 (<0.001)	
	miR-23a		6.22 (<0.001)			
	miR-23-b		3.28 (0.043)			
	miR-24-1		1.83 (<0.001)			
	miR-24-2		1.88 (<0.001)			
	miR-25	1.99–3.6				
	miR-123	1.9–2.45				
	miR-125b-1	2.19–2.73		miR-125b	1.45 (0.502)	
	miR-125b-2	1.95–2.88				
	miR-130a	2.11–5.3				
	miR-191		1.92 (0.008)			
	miR-220		1.68 (0.020)			
	miR-221	1.84–4.8	5.34 (<0.001)			
	miR-222-prec		2.43 (0.039)			
	Down-regulated	miR-125b-1		0.31 (0.043)		
		miR-125b-2		0.39 (0.047)		
		miR-128a	0.34–0.56		miR-128a	0.03 (<0.001)
miR-128b			0.53 (0.008)			
miR-181a		0.082–0.56	0.35 (<0.001)	miR-181a	0.4 (0.073)	
miR-181b		0.098–0.56	0.34 (0.005)	miR-181b	0.28 (0.036)	
miR-181c		0.096–0.56	0.49 (0.040)	miR-181c	0.29 (0.043)	
miR-197			0.33 (0.040)			
				miR-221	0.25	
				miR-222	0.22	

C/P ratio represents the range of ratio between tumour samples values (C: centre of the tumour) and the control samples values (P: peripheral brain area from the same patient). L/B ratio represents the ratio between averaged cell line samples values (L) and the control sample values (B); P value is presented (t-test [8], Mann-Whitney U-test [9]).

lines [16]. Webster *et al.* confirmed that miR-7 down-regulates EGFR mRNA and protein expression in glioblastoma cell lines via two of the three predicted sites, and induces cell cycle arrest and apoptosis. Furthermore, these authors also described Raf1,

another member of the EGFR signalling pathway, as a direct target of miR-7 in cancer cells [17].

Godlewski *et al.* published that miR-128 expression significantly reduced glioma cell proliferation *in vitro* and correspondingly

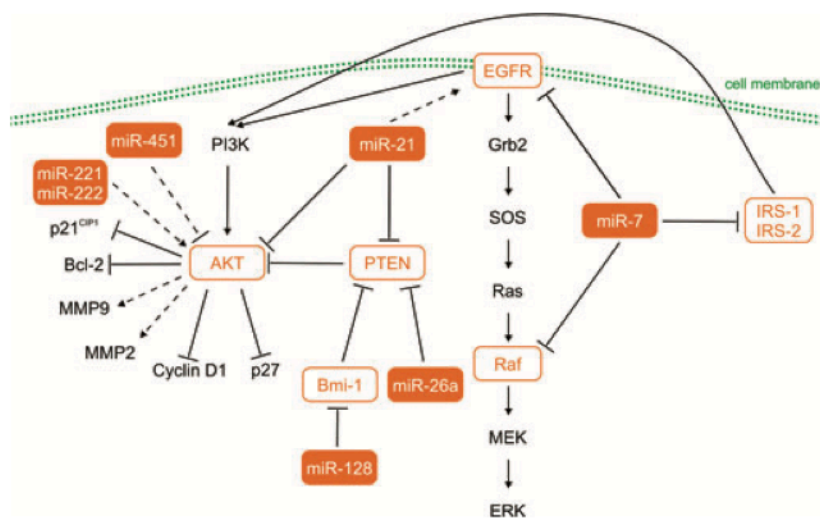


Fig. 1 MiRNAs involved in EGFR and PI3K/AKT signalling pathways. EGFR: epidermal growth factor receptor; AKT: serine/threonine protein kinase Akt; PTEN: phosphatase and tensin homologue; Bmi-1: polycomb ring finger oncogene; Raf: raf kinase, effector of Ras; IRS1/2: insulin receptor substrate 1/2; PI3K: Phosphatidylinositol 3 kinase; MMP9/2: matrix metalloproteinase 9/2; p27: cyclin-dependent kinase inhibitor 1B (p27, Kip1); p21: cyclin-dependent kinase inhibitor 1A (p21, Cip1); Bcl-2: B-cell CLL/lymphoma 2; Grb2: growth factor receptor-bound protein 2; SOS: son of sevenless homologue 1; MEK: mitogen-activated protein kinase kinase 1; ERK: extracellular signal-regulated kinase. Dashed lines indicated indirect regulation, solid lines indicate direct regulation.

glioma xenograft growth *in vivo*. This effect was explained by direct regulation of the Bmi-1 mRNA 3'-UTR, through a single miR-128 binding site. Bmi-1 expression was significantly up-regulated and miR-128 was down-regulated compared to normal brain. In addition, miR-128 expression leads to a decrease in H3K27 methylation and modulation of cellular pathways, especially p21^{CIP1} and Akt, involved in cell cycle arrest and survival [18]. Some of the investigations supported by revelation that Bmi-1 transcriptionally down-regulates expression of the tumour suppressor PTEN in tumour cells through direct association with the PTEN locus [19].

Finally, miR-221 and miR-222 were revealed using bioinformatics analysis as potential regulators of many target genes involved in AKT signalling pathway. Up-regulation of miR-221/222 resulted in remarkable increase of p-Akt and significant changes in expression of Akt-related genes in glioma cells. Consequently, miR-221/222 overexpression increased glioma cell proliferation and invasion *in vitro* and induced glioma growth in a subcutaneous mouse model. These results suggest that miR-221/222 enhance glioma malignant phenotype *via* activation of the AKT signalling pathway mediated by regulation of common gene expression (Fig. 1) [20].

p53, TGF- β and apoptotic signalling pathways

Papagiannakopoulos *et al.* reported that p53, TGF- β and mitochondrial apoptotic networks are de-repressed in response to miR-21 knockdown. They published a panel of genes involved in particular pathways and simultaneously modulated by miR-21 treatment. From this panel, p63, JMY, TP53BP2, HNRPK, TOPORS, IGFB3, APAF1, PPIF, TGFBR2/3, DAXX, HNRNPK were predicted to be direct targets of miR-21 that can stabilize p53 protein levels by interfering with MDM2 and/or act as p53 transcriptional cofactors [21]. Inhibition of miR-21 increased also endogenous levels of PDCD4 in human glioma cell lines and

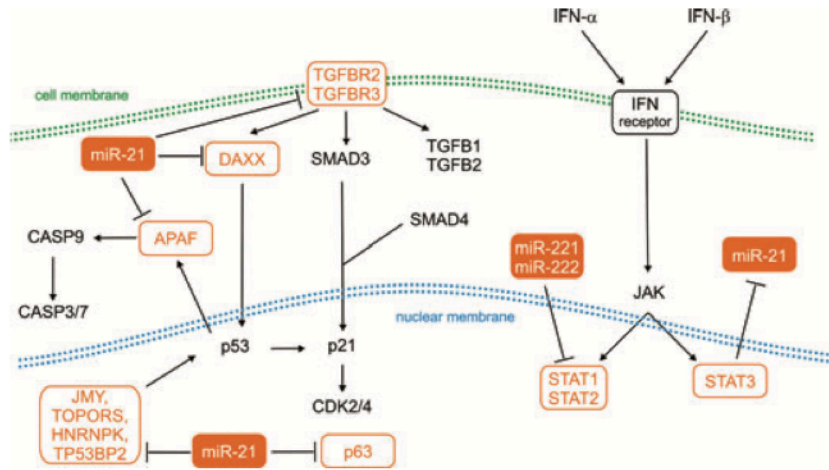
activated caspases 9 and 3, which may be mediated by modulating multiple potential target genes, such as TIMP3 [22,23]. Protein PDCD4 inhibits translation by its interaction with the factor that initiates translation of eIF4A and eIF4G. PDCD4 also inhibits proliferation *via* activation of p21^{CIP1} [6]. In addition, specific inhibition of miR-21 led to elevated levels of RECK and TIMP3 and therefore reduced MMP activities *in vitro* and in model of gliomas in nude mice. Consequently, down-regulation of miR-21 decreased migratory and invasive abilities in glioma cells (Fig. 2) [24].

IFN- α /IFN- β signalling pathways

Interferons (IFNs) are cytokines released by lymphocytes that have antiviral, antiproliferative and immunomodulatory effects. They are connected with the JAK-STAT (Janus kinase-Signal Transducer and Activator of Transcription) signalling cascade and allow communication between cells to trigger protective defences of the immune system leading to eradication of affected cells [25].

Insight into transcriptional regulation of miRNAs through both intracellular and extracellular mechanisms is one of the fundamental ideas leading to understanding oncogenesis. Ohno *et al.* investigated the possibility that IFN- β may induce or down-regulate cellular miRNAs in human gliomas. They analysed the effect of IFN- β treatment on miR-21 expression in glioma cells and intracranial glioma xenografts. Systematic delivery of IFN- β markedly reduced the level of miR-21 in all glioma cells. The pri-miR-21 transcript levels decreased 6 hrs after the addition of IFN- β and began to recover after 48 hrs. These results indicate that decrease in the levels of miR-21 is the result of transcriptional suppression. In contrast, the addition of the STAT3-specific inhibitor increased the level of miR-21 and inhibited IFN- β -mediated suppression of miR-21, suggesting that miR-21 expression is negatively regulated by STAT3 [5].

Fig. 2 MiRNAs involved in TGF- β and IFN- α /IFN- β signalling pathways. TGFBR2/3: transforming growth factor β receptor 2/3; TGFB1/2: transforming growth factor β 1/2; DAXX: death-domain associated protein; SMAD3/4: SMAD family member 3/4; APAF: apoptotic peptidase activating factor; CASP3/7/9: caspase 3/7/9; p53: tumour protein p53; p21: cyclin-dependent kinase inhibitor 1A (p21, Cip1); p63: tumour protein p63; CDK2/4: cyclin-dependent kinase 2/4; JMY: junction mediating and regulatory protein, p53 cofactor; TOPORS: topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase; HNRNPK: heterogeneous nuclear ribonucleoprotein K; TP53BP2: tumour protein p53 binding protein, 2; IFN: interferon; STAT1/2/3: signal transducer and activator of transcription 1/2/3; JAK: Janus kinase. Dashed lines indicated indirect regulation, solid lines indicate direct regulation. EGFR: epidermal growth factor receptor.



Another study revealed miR-221 and miR-222 as possible regulators of IFN pathways. Using the KEGG pathway databases and BioCarta, Zhang *et al.* found that the IFN- α signalling pathway was the most significant pathway modulated by genes with the most different expression after knockdown of miR-221 and miR-222. The authors showed that STAT1 and STAT2 expression and phosphorylation were up-regulated in U251 cells with silenced miR-221/222. Tyrosine phosphorylation of STAT1 and STAT2 was present in the nucleus after repression of the same miRNAs. These data illustrate a mechanism of STAT1/2 up-regulation under the transcriptional control of IFN- α signalling after knockdown of miR-221/222 cluster in U251 glioma cells (Fig. 2) [26].

Notch signalling pathway

Notch signalling is critical in stem cell maintenance and cell survival, as well as in cell fate decisions such as neuronal *versus* glial fate in the developing nervous system. Therefore, it is not surprising that this pathway plays a key role in brain tumours, including glioblastoma [27].

miR-326 is associated with Notch signalling pathway in glioblastomas. This miRNA was first identified among a set of miRNAs expressed in neurons and further noted on a list of miRNAs elevated in zebrafish embryos treated with a Notch inhibitor [28,29]. To find potential miRNA mediators of Notch effect in glioma, Kefas *et al.* performed miRNA microarray analysis of glioma tumour stem cells transfected with Notch-1 siRNA. In these Notch-1 knockdown cells, miR-326 was one of the miRNAs significantly increased when compared to control transfected cells. Therefore, it was indicated that miR-326 is suppressed by Notch activity. However, pre-miR-326 transfection caused substantial decrease in both Notch-1 and Notch-2 protein as was shown by immunoblotting. This paper showed

that another Notch pathway components are inhibited by miR-326. It was observed that miR-326 induces apoptosis and decreases glioma cells proliferation, viability and invasiveness of glioblastoma stem cell-like lines. Furthermore, miR-326 transfection also reduced glioma cell tumourigenicity *in vivo* [27]. Considering that the expression of miR-326 down-regulated the Hedgehog stem cell pathway in medulloblastoma cells, authors tested the effects of this miRNA on Hedgehog activity in a glioma line using the Gli-1 promoter reporter plasmid. Unfortunately, expected effects were not observed in this case; this may reflect distinct roles for miR-326 in these pathways in different cancers [30].

Computational target gene prediction identified pyruvate kinase type M2 (PKM2) as another target of miRNA-326. PKM2 has recently been shown to play a key role in cancer cell metabolism. It is crucial for aerobic glycolysis and provides a growth advantage for tumour cells [31]. To investigate whether PKM2 might be a functionally important target of miR-326, Kefas *et al.* used RNA interference to knockdown PKM2 expression in glioma cells. Transfection of established glioma and glioma stem cells with PKM2 siRNA reduced their growth, cellular invasion, metabolic activity, ATP and glutathione levels and activated cAMP-activated protein kinase. Levels of PKM2 negatively correlated with levels of miR-326, suggesting regulatory relationship of PKM2 and miR-326 [32]. Among others, all of these results showed that efficient delivery of miR-326 has therapeutic potential against both glioma stem-like cells and established glioma lines.

Li *et al.* studied the role of miR-34a in human brain tumours with a special focus on glioblastomas. They found that miR-34a inhibits Notch-1 and Notch-2 protein expression and 3'-UTR reporter activities as well as CDK6 and c-Met protein expression in glioma cells. They observed for the first time that average pre-miR-34a expression is down-regulated in human glioblastoma tissues

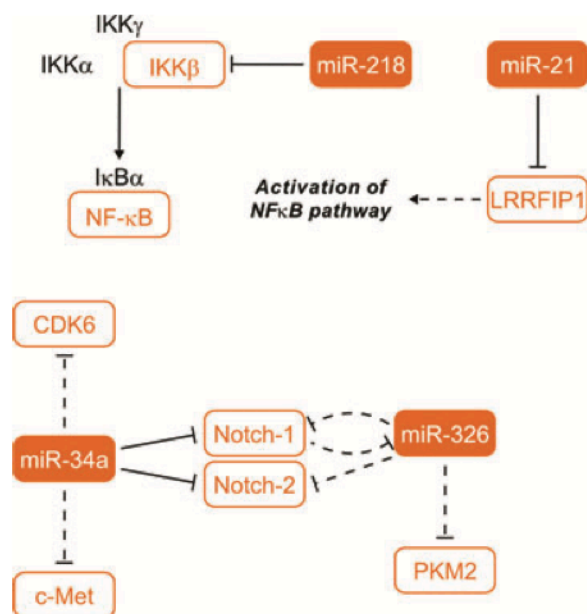


Fig. 3 MiRNAs involved in notch and NF- κ B signalling pathways. IKK- $\alpha/\beta/\gamma$: inhibitor of κ B kinase $\alpha/\beta/\gamma$; NF- κ B: nuclear factor of κ B; I κ B: inhibitor I κ B; LRRFIP1: leucine rich repeat (in FLII) interacting protein 1; CDK6: cyclin-dependent kinase 6; c-MET: met proto-oncogene (hepatocyte growth factor receptor); PKM2: pyruvate kinase, muscle. Dashed lines indicated indirect regulation, solid lines indicate direct regulation. EGFR: epidermal growth factor receptor.

when compared to normal human brain [33]. Other studies showed that miR-34a expression was higher in wild-type p53 glioblastoma tissues compared to mutant p53 glioblastoma; miR-34a acts as a tumour suppressor in p53-mutant glioma cells U251, partially through regulating SIRT1 [34]. Transfection of miR-34a into tested glioblastoma cell lines strongly inhibited cell proliferation, cell cycle, cell survival, cell invasion and *in vivo* glioblastoma xenograft growth; however, the treatment did not affect human astrocyte cell survival and cell cycle. Forced c-Met and Notch-1/2 expression partially rescued the effects of miR-34a on the cell cycle and cell death in gliomas, respectively [35] (summarized in Fig. 3).

NF- κ B signalling pathway

Nuclear factor-kappa B (NF- κ B) is the transcription factor with pleiotropic activity owing to its central roles in various biological processes. Aberrant activation of NF- κ B signalling pathway has been proved to be important for invasiveness and metastatic capacity of tumours through up-regulation of matrix metalloproteinases (MMPs) and transcription factors regulating E-cadherin, such as Snail, Twist or Slug. A critical component in NF- κ B regulation is the I κ B kinase (IKK- β) complex [36,37].

Song *et al.* identified miR-218 expression in glioma cells lines and in human primary glioma tissues was substantially down-reg-

ulated, when compared to miR-218 expression in normal human astrocytes and normal brain tissues. Forced up-regulation of miR-218 dramatically reduced the migratory speed and invasive ability of analysed cells. Ectopic expression of miR-218 down-regulated matrix MMP-9 and reduced NF- κ B transactivity at transcriptional level, whereas inhibition of miR-218 enhanced the expression of MMP-9 and transcriptional activity of NF- κ B. Authors demonstrated that miR-218 could inactivate NF- κ B/MMP-9 signalling by directly targeting the 3'-UTR of the IKK- β [37].

miR-21 was revealed as another post-transcriptional regulator involved in NF- κ B signalling pathway in glioblastoma. Combining target prediction by bioinformatics with expression profiling, Li *et al.* identified LRRFIP1 gene, which was remarkably up-regulated in miR-21-knockdown cells, as a candidate target gene of miR-21. Further, through sequence analysis, they found that LRRFIP1 mRNA carried a putative miR-21 binding site. Further analyses confirmed LRRFIP1 as a direct target of miR-21. Moreover, their data suggest that miR-21 likely contributes to vespid resistance through depression of LRRFIP1 expression, leading to the reduction of cytotoxicity of chemotherapeutic drugs through activation of the NF- κ B pathway [38] (summarized in Fig. 3).

Single nucleotide polymorphisms and miRNAs: risk factors for glioblastoma

In gliomas, only one polymorphism found in mature miRNA sequence, specifically a polymorphism of miR-196a (rs11614913), has been studied so far. Published data suggest that the CC genotype of miR-196a (rs11614913) polymorphism is associated with decreased risk of glioma in the Chinese population (OR = 0.74, 95% CI: 0.56–0.98). Significant association was observed also between these genotypes and risk of particular glioma subgroups: patients over 18 years (OR = 0.73, 95% CI: 0.55–0.98), male glioma patients (OR = 0.69, 95% CI: 0.48–0.99) and patients with high-grade glioma-glioblastoma (OR = 0.58, 95% CI: 0.37–0.91). In contrast to other tumours, such as lung cancer and breast cancer [39,40], data in glioblastoma showed opposite association between miR-196a genotype and cancer risk. This may be related to the diversity on the tissues origin and characteristic molecular alterations in different cancers [41].

miRNAs in glioblastoma prognosis and prediction of therapeutic response

Clinical significance of miRNA expression profiles in glioblastoma has not been explored very much. Nevertheless, 16 candidate

miRNAs were published to associate with malignant behaviour of gliomas (miR-196a, miR-15b, miR-105, miR-367, miR-184, miR-196b, miR-363, miR-504, miR-302b, miR-128b, miR-601, miR-21, miR-517c, miR-302d, miR-383, miR-135b). Among them, miR-196a and miR-196b indicated the highest level of significance ($P = 0.0038$ and 0.0371 , respectively). Both miRNAs showed increased expression levels in glioblastomas relative to anaplastic astrocytomas and normal brain tissues. Higher level of miR-196 transcript significantly correlated with poorer survival as demonstrated by the Kaplan–Meier method ($P = 0.0073$); moreover, multivariate analysis showed that these expression levels were independent predictors of overall survival in glioblastoma patients ($P = 0.021$; HR, 2.81) [42]. Malzkorn *et al.* investigated the miRNA expression profiles in four patients with primary WHO grade II gliomas that spontaneously progressed to WHO grade IV secondary glioblastomas. They identified 12 miRNAs (miR-9, miR-15a, miR-16, miR-17, miR-19a, miR-20a, miR-21, miR-25, miR-28, miR-130b, miR-140 and miR-210) showing increased expression and two miRNAs (miR-184 and miR-328) showing reduced expression upon tumour progression. Validation experiments on an independent series of primary low-grade and secondary high-grade astrocytomas confirmed miR-17 and miR-184 as interesting candidates contributing to glioma progression [43].

Treatment of malignant gliomas remains one of the greatest challenges facing oncologists today through a frequent resistance to both chemo- and radiotherapeutics and short survival [44]. Important question for management of glioblastoma patients is the possibility of predicting therapeutic outcome. The miRNA expression profiles of glioblastoma tissues have shown association of miR-181b and miR-181c with response to concomitant chemoradiotherapy with temozolomide (RT/RMZ). miR-181b and miR-181c were significantly down-regulated in glioblastoma tissue of patients who responded to RT/TMZ ($P = 0.016$ and 0.047 , respectively) in comparison to patients with progressive disease [9].

miRNAs involved in drug resistance of glioblastoma

Temozolomide is an oral alkylating agent, which is frequently used for the treatment of glioblastoma. To explore the mechanism of resistance to TMZ, Shi *et al.* found that overexpression of miR-21 in glioblastoma cells could significantly reduce TMZ-induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity [45]. The miR-21 inhibitor could also enhance the chemosensitivity of human glioblastoma cells to paclitaxel *via* inhibition of STAT3 expression and phosphorylation. Moreover, the same treatment by miR-21 antisense oligonucleotides led to enhanced cytotoxicities of vepesid [12,21]. The results of glioblastoma *in vitro* experiments showed also other miRNAs involved in the TMZ resistance. miR-195, miR-455–3p and miR-10a* were the three most up-regulated miRNAs in the drug resistant glioblastoma cell line (U251R) [46].

Multidrug resistance protein ABCG2 (ATP-binding cassette sub-family G member 2) is the target gene for miR-328. Li *et al.* observed that miR-328 is underexpressed in many cancers including glioblastoma and contributes to tumour chemoresistance through ABCG2, which is highly expressed in glioblastoma cells [47]. Another research group reported the possible impact on the therapeutic effect by transfection of miR-451 in combination with imatinib mesylate treatment. Up-regulation of miR-451 led to differentiation of glioblastoma stem cells [48].

miRNAs as potential therapeutic targets

The association of miRNA deregulation with pathogenesis and progression of malignant disease illustrates great potential of utilizing miRNAs as targets for therapeutic intervention. The basic strategy of current miRNA-based treatment studies is either to antagonize the expression of target miRNAs with antisense technology or to restore or strengthen the function of given miRNAs to inhibit the expression of certain protein-coding gene. There is a number of experimentally, *in vitro* or/and *in vivo*, proved miRNAs presenting potential therapeutic targets in glioblastoma, which were mentioned in the context of altered signalling pathways (*e.g.* miR-21, miR-451, miR-7, miR-128, miR-221/222).

Considering angiogenesis, which is critical in most solid tumours, including glioblastoma, miR-296 has been demonstrated to be up-regulated in glioblastoma-associated endothelial cells [49]. This miRNA promotes angiogenesis by down-regulating HGS (hepatocyte growth factor-regulated tyrosine kinase substrate), an inhibitor of pro-angiogenic receptors VEGFR2 and PDGFR β (vascular endothelial growth factor and platelet-derived growth factor receptor β , respectively). This study indicated the potential of anti-angiogenic therapy of glioblastoma by delivery of a miR-296 inhibitor [49].

Although siRNAs allow specific knockdown of individual gene targets, miRNAs result in a broad reduction of gene expression being affected. The ability of individual miRNAs to target multiple genes/pathways could be a major advantage, especially given studies indicating the therapeutic necessity of simultaneously targeting multiple pathways in glioblastoma [50]. Unfortunately, there are several major challenges to overcome before the application of miRNA-based treatment. First, the multitargeting nature of miRNAs gives the risk of unintended off-target effects that need to be carefully evaluated. Secondly, the expression of target gene may be controlled by several different miRNAs, which may compromise the effect of miRNA-based treatment. Finally, there is still lack of miRNA delivery system with enough specificity and efficacy.

Conclusions

The discovery of miRNAs has substantially changed the view on gene expression regulation, and new findings over the past few

years have catapulted miRNAs into the centre of cancer molecular biology. It is now evident that dysregulation of miRNAs is an important step in the development of many cancers, including glioblastoma. Several studies based on expression profiling have proven there are significant changes of miRNA expression levels in glioblastomas in comparison to adult brain tissue; these expression levels identified groups of miRNAs with potential of prognostic stratification and prediction of responses to chemoradiotherapy in glioblastoma patients. To improve our knowledge of role of miRNAs in glioblastoma core signalling pathways, functional effects of particular miRNAs were successfully studied. The results of these studies suggest a great potential and relevance of miRNAs as a novel class of therapeutic targets and possibly powerful intervention tools in glioblastoma.

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Conflict of interest

The authors declare no conflict of interest.

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PŘÍLOHA 10

Review

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Cerebrospinal fluid microRNAs as diagnostic biomarkers in brain tumors

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Abstract: Cerebrospinal fluid (CSF) is a body fluid that has many important functions and is in direct contact with the extracellular environment of the central nervous system (CNS). CSF serves as both the communication channel allowing the distribution of various substances among the CNS cells and the storage facility for the waste products these cells release. For these reasons, CSF is a potential source of diagnostic biomarkers of many CNS diseases, including brain tumors. Recent studies have revealed that CSF also contains circulating microRNAs (miRNAs), short non-coding RNAs that have been described as biomarkers in many cancers. However, CSF miRNAs are difficult to detect, which is why researchers face major challenges, including technological difficulties in its detection and its lack of standardization. Therefore, this review aims (i) to highlight the potential of CSF miRNAs as diagnostic, prognostic and predictive biomarkers in brain tumors, and (ii) to summarize technological approaches for detection of CSF miRNAs.

Keywords: biomarker; brain cancer; cerebrospinal fluid (CSF); diagnosis; microRNA; prognosis.

Introduction

With an incidence rate of around 22 cases per 100,000 people, primary brain tumors account for less than 2% of all malignancies. However, the malignant forms account for about 33% of primary brain tumors, with an estimated 5- and 10-year relative survival rates of 34.4% and 28.8%, respectively. A prognosis and the patient's estimated survival depend on the tumor type. The most favorable prognosis is associated with pilocytic astrocytoma (with a 5-year survival rate of 94.2%), followed by malignant meningioma (65.2%) and lymphoma (29.2%). Patients with glioblastoma (GBM) have only a 5.1% 5-year survival rate [1]. Occurring in 20%–40% of adult cancer patients, metastases form another large group of brain tumors. Although the survival rate of brain metastasis patients is generally low, the prognosis varies among individual cases [2].

Diagnosis plays a crucial role in making the prognosis and choosing the best therapy for the diagnosed brain tumor. Despite significant recent advances in the diagnosis of brain tumors, such as various modifications of imaging methods followed by histopathological examinations, the diagnosis is still limited by a tumor's size and localization as well as the heterogeneity of its tissue [3]. Hence, we need to develop new powerful diagnostic approaches that, together with the existing methods, would increase the accuracy of brain tumor diagnosis and thereby the survival of patients.

Promising diagnostic markers in many solid cancers seem to be circulating biomolecules that are altered in body fluids such as blood, urine or saliva. Nevertheless, the localization of brain tumors and the presence of the blood-brain barrier are deemed responsible for preventing the release of tumor-specific molecules into the above-mentioned body fluids [4]. Cerebrospinal fluid (CSF), which bathes the central nervous system (CNS) and is in direct contact with brain neoplasms, seems to be a suitable source of diagnostic biomarkers [5, 6].

MicroRNAs (miRNA) are short non-coding RNAs that generally bind to the 3' untranslated regions of messenger RNAs (mRNAs) and repress protein translation [6, 7].

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miRNAs may regulate over 50% of all human genes, and each miRNA can control hundreds of mRNA targets. The deregulated expression of miRNAs is closely associated with the pathology of many cancers, including brain tumors [8]. Moreover, circulating miRNAs (c-miRNAs) have been found in several types of human body fluids, CSF being one of them. These miRNAs might be both secreted by the cells in extracellular vesicles and be released by necrotic cells as naked oligonucleotides.

Many authors have also suggested that c-miRNAs participate in intercellular communication and may influence molecular cell processes, such as growth, invasion and drug resistance in recipient target cells [4, 9]. An increasing number of studies on the altered levels of specific miRNAs in CSF of brain tumor patients have clearly shown that CSF miRNAs are promising diagnostic biomarkers. These results, however, are far from conclusive, mainly because of their technological variations, but also due to other serious limitations which we discuss below.

In this review, we summarize the diagnostic relevance of miRNAs in CSF for brain tumor patients and the technological approaches for their detection.

Methodical approaches used for miRNA detection in CSF

Detection of miRNA in CSF is a complex process that requires the following consecutive steps: CSF management and storage; isolation and expression analysis of miRNA; and final data normalization, analysis and interpretation. Unfortunately, the whole process can be affected by many factors and has not yet been standardized (Figure 1).

CSF management and storage

In the first and critical step of CSF management, a sample of patients needs to be collected. This should be performed by experienced physicians using the standard lumbar puncture, external ventricular drain or cisternal aspiration at the time of craniotomy [10]. To avoid bias of the subsequent analyses due to the preanalytical factors, a standardized protocol for collection of CSF samples should be established. Such a protocol has already been proposed by Teunissen et al. in 2009 and 2011 [11, 12]. These authors highlighted three crucial points for the protocol to work. First, as the volume of CSF can influence the concentration of biomarkers, the volume of CSF withdrawal

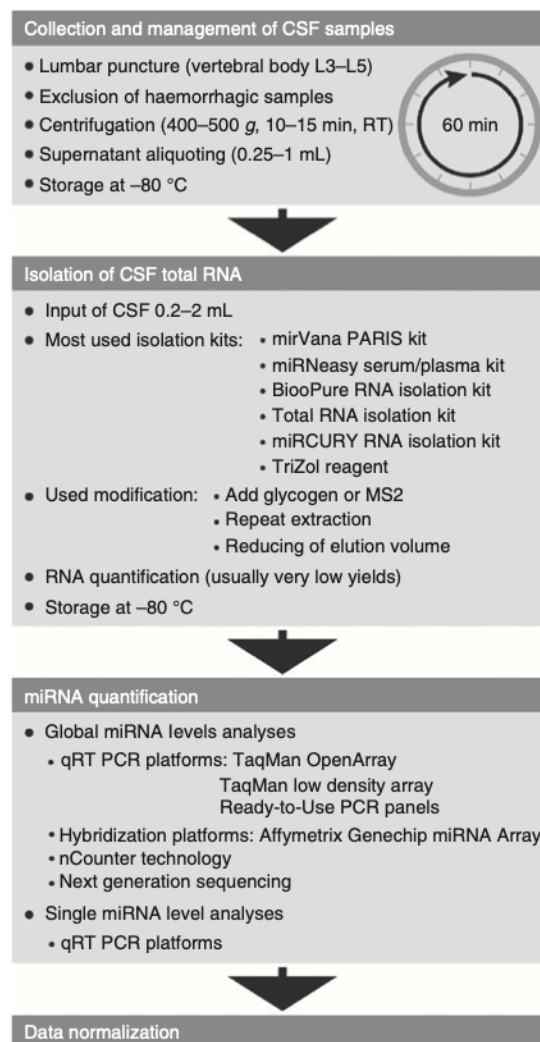


Figure 1: Summary of the most common techniques used for detection of CSF microRNAs.

should be at least 12 mL. An inadequate total volume of the sampled CSF can lead to complications during the lumbar puncture, like brain herniation; the adequate CSF volume should then be assessed individually for each patient and the corresponding diagnosis [2]. Second, a lumbar puncture should be performed between the L3 and L5 vertebrae. Third, CSF samples containing blood-derived cells or even skin cells must be excluded. In an independent study, Müller et al. [13] confirmed the importance of this point by showing that even after centrifugation of samples before analysis, the number of blood-derived cells influenced the expression of miRNAs. In this context, Kirschner et al. [14] detected 136 hemolysis-susceptible miRNAs (e.g. miR-21, miR-106a, miR-92a, miR-17, miR-16 and miR-451). Similarly, Bache et al. [15] found that levels of miR-16-5p and miR-451a as well as the average levels for all miRNAs in each sample strongly correlated with the net

absorbance of oxyhemoglobin and hemoglobin in CSF of subarachnoid hemorrhage (SAH) patients. Therefore, the collected CSF should be tested for the presence of hemoglobin or cells. The time from the CSF withdrawal to laboratory processing and storage of samples is also important and should thus be documented.

Next, the conditions of centrifugation for removing the debris, which should be done up to 60 min after the lumbar puncture, should be standardized at 400 g for 10 min at room temperature. However, in most of the existing studies, CSF samples are centrifuged at 500 g. More extensive and longer centrifugation is used to separate CSF extracellular vesicles and extract RNA from them. Akers et al. [10] used a different approach: they filtered the collected samples using a 0.8 μm pore size membrane filter. Finally, although miRNAs seem to be stable over a wide range of storage temperatures and resistant to repeated freeze-thaw [16], the samples should be protected against any biochemical changes and supernatants should be aliquoted and stored at -80°C as soon as possible.

miRNA isolation

As the concentration of miRNAs in CSF is low, their extraction is challenging. A few studies have compared the efficiency of currently available commercial kits for the purification of RNA from CSF. This process still lacks standardization, and thus it is not easy to choose the most efficient method.

Burgos et al. tested four commercially available RNA isolation kits for CSF samples: *mirVana* miRNA isolation kit, *mirVana* PARIS kit (both Thermo Fisher Scientific), BioPure RNA isolation reagent (Bio Scientific), and miRNeasy mini kit (Qiagen). They followed the manufacturer-provided protocol with one modification, for the phenol/chloroform phase separation. The authors performed two consecutive RNA extraction steps from an organic phase to maximize RNA recovery and, in both these extractions, measured the total and miRNA concentrations. The tested kits gave different yields, with the highest obtained by the *mirVana* PARIS kit (± 9 and 25 ng/mL of total RNA from first extraction; ± 6 and 20 ng/mL of total RNA from second extraction) and the BioPure RNA isolation reagent (± 10 and 10 ng/mL; ± 1 and 40 ng/mL). In addition, the *mirVana* PARIS kit returned the greatest amount of spike-in cel-miR-39, measured by qPCR [17]. qPCR is commonly used for indirect quantification when RNA is in so small a concentration that RNA yield is immeasurable [18]. Thus, as the *mirVana* PARIS kit gave the greatest concentration of small RNAs and had

a user-friendly protocol, it comes as no surprise that the authors selected this kit as the most suitable one for small RNA sequencing. They also found that in some cases the combination of the two extractions almost doubled the recovery of the total RNA yield. Finally, they also estimated correlations of miRNA yields isolated using the *mirVana* PARIS kit with the 0.5- to 1.25-mL starting CSF volumes. The next-generation sequencing (NGS) analysis confirmed high positive correlations (Spearman's correlation over 0.95) between the starting volume of CSF and the levels of miRNAs with the coverage higher than five reads. The same experiment, but with RNAs extracted by the BioPure RNA isolation kit, showed similar associations as the experiment with the *mirVana* PARIS kit did. Thus, 0.5 mL of CSF should be a sufficient starting extraction volume to achieve reproducible results of miRNA analyses [17].

To summarize our discussion of the methods of extraction of small RNA from CSF, several modifications of the manufacturer-provided protocols have been suggested. In McAlexander et al.'s [19] study, which used the miRCURY: Biofluids kit (Exiqon), the increasing input volumes of CSF from *Macaca nemestrina* showed a quantitative recovery of four endogenous miRNAs. But as kits vary in the input of biofluid, it seems reasonable to choose the optimal input volume for a particular combination of biofluid and method [19]. Some studies have also supplemented the RNA extraction kit with glycogen or exogenous RNA (east tRNA or MS2 phage RNA) as the carrier. McAlexander et al. used pure glycogen, which – as an inert carrier – does not affect the downstream assays. Unlike pure glycogen, RNA carriers might cause non-specific hybridization or amplification in the quantification assays. The authors also studied how glycogen affects RNA yield in blood plasma samples. The results were inconsistent because adding glycogen increased RNA yields only in the case of some extraction kits [19].

Bache et al. [15] successfully used the MS2 carrier during the RNA isolation process handled by the miRCURY RNA isolation kit – Biofluids (Exiqon). The RNA obtained was then analyzed using high-throughput real-time PCR. However, for RNA isolation in the explorative phase of the project, the authors used the total RNA purification kit supplied by the Norgen Biotek Corp. [15]. In our experiments, the urine microRNA purification kit, also supplied by the Norgen Biotek Corp., was the most sufficient method for isolating miRNA from CSF, but only after reducing the elution volume from 50 μL to 20 μL and extending elution time to 20 min before centrifugation. However, these preliminary results need further studies and thus have not yet been published.

Finally, low concentrations of total and small RNA greatly decrease the accuracy of the standard methods used for the quantification and quality control of RNA, such as the Nanodrop and Qubit technologies. Hence, alternative methods might work better, e.g. the automated capillary electrophoresis, i.e. Bioanalyzer (Agilent). One can even use a special chip for a small RNA analysis, able to detect RNA at as low concentration as 50 pg/ μ L. Unfortunately, the price-achievement ratio of this chip is far from outstanding. This problem of inaccurate measurement of RNA quality and quantity might be alleviated by using the optimum volumes for the procedure chosen [4, 20].

miRNA expression analysis

Final quantification of miRNAs in the extracted RNA samples is very important. Simply put, imprecise miRNA levels detected in the experiment can greatly affect the diagnostic power of these molecules. Although the techniques of CSF miRNA quantification do not differ from the commonly used approaches, the low RNA concentrations of the samples examined should always be taken into account.

The miRNA expression analyses are divided into two major groups: single miRNA analyses and global (or whole-genome) miRNA profiling. Currently, levels of individual miRNAs in CSF are measured exclusively using modified real-time PCR technologies. Among them, the most common approach is based on the specific reverse transcription with the stem-loop primers followed by the real-time PCR using the TaqMan detection system (Thermo Fisher Scientific) [16, 21, 22]. Baraniskin et al. [16] confirmed the reproducibility of this method. It is not the only suitable approach, however; another one is based on PCR with the universal reverse transcription followed by SYBR Green quantitative PCR with miRNA specific forward and reverse locked nucleic acid (LNA)-modified primers (Exiqon) [15]. Another study used digital PCR handled according to the TaqMan chemistry protocol for miRNA levels analyses in CSF. The results, consistent with the simultaneously performed NGS analysis, proved that the digital PCR experiments worked correctly [23]. This PCR technology seems suitable only for low-concentrated RNA samples and samples with undefined normalizer molecules, such as CSF.

For the global miRNA profiling, four high-throughput platforms are currently available, namely, hybridization arrays, technologies based on real-time PCR, nCounter platform and NGS. These platforms differ in the number of miRNAs they can detect, sensitivity, specificity, dynamic

range, quantity of input material and total cost. Thus, all the circumstances of the profiling need to be considered before selecting the adequate technology.

Several studies have compared the high-throughput technologies for the CSF miRNA analysis. Sørensen et al. performed small RNAseq of RNA samples from CSF and detected 246 miRNAs, of which 71 were found in over 90% of the patients examined. Simultaneously, the authors used the Exiqon qPCR arrays, which enabled them to analyze 372 miRNAs in one experimental run, in which they detected 210 miRNAs. Nevertheless, only 21 molecules occurred in over 90% of the investigated samples. Both qPCR and small RNAseq detected all but one differentially expressed miRNAs, but in general the qPCR-based technology showed a lower detection rate [24].

Bache et al. analyzed the global miRNA expression in CSF using the TaqMan Low Density Array (TLDA; Thermo Fisher Scientific) with 754 miRNAs on two independent cards. They detected 168 miRNAs in over 60% of healthy patients (128 miRNAs in all healthy patients) and 216 in over 60% of SAH patients (SAH; 155 miRNAs in all SAH patients). These 155 miRNAs were then validated using the Exiqon technology, with which only 74 miRNAs were detected in over 60% of the healthy patients (19 miRNAs in all healthy patients) and 100 in over 60% of the SAH patients (60 miRNAs in all SAH patients) [15]. These different numbers of the detected miRNAs by the two technologies may result from the preamplification step that precedes the TLDA analysis.

Table 1 summarizes available studies that analyze CSF miRNAs using high-throughput approaches.

Data normalization

The last issue to address about miRNA profiling of CSF concerns how to normalize raw data. This is a challenging but important problem to solve, especially in the context of circulating miRNAs. Various technical and biological factors affect miRNA levels in body fluids. Examples of the technical factors are variability induced during collection and storage of the clinical specimens, the approach to miRNA extraction and the methods of final miRNA quantification. Examples of the biological factors are the amount of miRNAs in tissues, the intensity of their secretion into body fluids, the form of circulating miRNAs affecting their ability to cross various barriers and the stability of miRNA. It is because of these various factors that miRNA raw data need to be normalized. This normalization has been broadly recognized as one of the key factors in efficient miRNA analysis in CSF [35].

Table 1: A summary of the studies analyzing global microRNA expression profiles in CSF samples.

Diagnosis	High-throughput technology	Number of detectable miRNAs ^b	Number of detected miRNAs	Normalization method	CSF RNA extraction kit	CSF management	References
AD Controls	TOA ^a	1178	441 Cq ≤ 34	2 ^{-ΔCq} miR-21, -24, -328, -99b, -1274B, let-7c	mirVana PARIS kit (Thermo Fisher Scientific)	LP 4 mL of CSF 1600 g, 4 °C, 15 min ^d	[25]
SZ, NOS SCAD	TLDA-A ^a	NS	248 (an average 86 per sample); Ct < 30	NS	7.5 mL of CSF by Asuragen, Inc	15–25 mL of CSF	[18]
AD Controls	TLDA-A+B ^a	754	144 ± 66 per sample Mean Ct = 32.4 ± 0.5	Mean of all detected miRNAs in one plate	2 mL of CSF mirVana PARIS kit	LP L3/L4 or L4/L5 centrifuged	[26]
STBI Controls	TLDA-A+B ^a	792	An average 75 per sample	NS	200 μL of CSF miRNeasy serum/plasma kit (Qiagen)	NS	[27]
RRMS ND	TLDA-A+B ^a	754	62 in more than 75% samples Ct < 37	Mean restricted normalization method	300 μL of CSF mirVana PARIS kit	400 g, 19 °C, 15 min ^d	[28]
SAH Healthy donors	TLDA-A+B ^a	754	168 in >60% of healthy patients 216 in >60% of SAH	Values were normalized to the sample mean	200 μL of CSF Total RNA isolation kit (Norgen Biotech)	External ventricular drain 500 g, 10 min ^d	[15]
NS	Exiqon	151	74 in >60% of healthy patients 100 in >60% of SAH	NS	200 μL of CSF miRCURY RNA isolation kit Biofluids (Exiqon) + MS2	LP	[29] ^{Exo}
RRMS CIS, IND NIND	TLDA-A+B	746	132 in one from three groups; Ct < 35	2 ^{-ΔCt} RNU44 and RNU6B	mirNeasy serum/plasma kit	2000 g, 10 min ^d LP	[30]
NS	TLDA-A+B ^a	754	88	2 ^{-ΔCq} cel-miR-39, -54, -238	500 μL of CSF miRCURY RNA isolation kit	440 g, RT, 10 min ^d	
NS	NGS-TruSeq	Unlim	281 in all samples CPM ≥ 5	NS	0.5–1.5 mL of CSF mirVana PARIS kit & BioPure RNA isolation kit (Bio Scientific)	LP centrifuged NS	[17]
AD, PD Controls	NGS-TruSeq	Unlim	428 Average > 5 reads	NS	1 mL of CSF mirVana PARIS kit	NS	[31]
IS Controls	NGS-TruSeq	unlim	246 71 in more than 90% of samples	Trimmed mean of M-values	100 μL of CSF TriZol reagent (Thermo Fisher Scientific)	LP > 5 mL of CSF 2000 g, 4 °C, 15 min ^d	[24]
	Exiqon	372	210 21 in more than 90% of samples	2 ^{-ΔCq} Mean of Cq miR-15a-5p, -21-5p, -23a-3p, -23b-3p, -99a-5p, -125b-5p, -145-5p, -204-5p, -320a	200 μL of CSF miRCURY RNA isolation kit Biofluids		

Table 1 (continued)

Diagnosis	High-throughput technology	Number of detectable miRNAs ^b	Number of detected miRNAs	Normalization method	CSF RNA extraction kit	CSF management	References
Healthy donors	NGS–TruSeq	Unlim	397 in exosomes 337 in supernatant (at least in one sample)	CPM	miRNeasy mini kit	LP 7 mL of CSF 500 g, 10 min ^d	[23] ^{Exo}
UPTBI	Affymetrix	5818 ^c	NS	NS	TriZol reagent	LP 6–8 mL of CSF 3000 g, 10 min ^d	[32]
Controls	Affymetrix	NS	NS	NS	TriZol reagent	LP	[33]
<2 years						CSF was stored at –80 °C, thawed on ice and centrifuged 2000 g, 15 min	
>70 years patients							
<2 years patients	NGS–TruSeq	Unlim	Small RNAs mapping to 4004 unique locations	NS			
GBM	TOA ^a	754	NS	NS	miRCURY RNA isolation kit	Ventricular/lumbar drain or cisternal aspiration 1100 g, 30 min ^d	[34] ^{Exo}

Diagnosis: AD, Alzheimer's disease; SZ, schizophrenia; SCAD, schizoaffective disorder; NOS, psychosis not otherwise specified; STBI, severe traumatic brain injury; RRMS, relapsing remitting multiple sclerosis; ND, other neurological diseases; SAH, subarachnoid hemorrhage; NS, non-specified; CIS, clinically isolated syndrome; NIND, noninflammatory neurologic disease; IND, inflammatory neurologic disease; PD, Parkinson's disease; IS, ischemic stroke; UPTBI, unconscious patients after traumatic brain injury; BBT, benign brain tumor; MBT, malignant brain tumor; NBT, non brain tumor; GBM, glioblastoma. High-throughput technology: TOA, TaqMan OpenArray; TLDA–A+B, TaqMan low density array – cards A and B; Exiqon, Exiqon LNA technology; NGS–TruSeq, Next Generation Sequencing (TruSeq small RNA library preparation kit); Affymetrix, Affymetrix Genechip miRNA 3.0 arrays; ^aPre-amplification step. Number of detectable miRNAs: ^baccording to the authors; ^cpremature and mature microRNAs. CSF management: LP, lumbar puncture; ^dcentrifugation of CSF prior to the freezing. Reference: Exo, global miRNA expression profiling in CSF exosomes.

Several methods of CSF miRNA normalization have already been proposed. The first one is normalization by spike-in miRNA, which is added to the samples before the RNA extraction and quantitative analysis. This exogenous miRNA is normally absent in the organism studied (i.e. *Caenorhabditis elegans* [*C. elegans*] or plant miRNA), so its level reflects the technological variability only. The miRNA spike-in normalization has worked well in several studies comparing and optimizing the methods of CSF miRNAs detection. Burgos et al. [17] used quantitative analysis of spiked-in *C. elegans* miRNA cel-miR-238 to compare yields of small RNAs obtained in two consecutive extractions from CSF. Analyzing the effect of the starting CSF volume on the quantitative recovery of RNA yield, McAlexander et al. [19] spiked cel-miR-39 into the lysis/denaturant buffer before adding it to CSF. According to the authors, synthetic RNA should not be added directly to CSF because endogenous RNases would degrade it immediately. For normalization of sample-to-sample variation, Bergman et al. [30] used exogenous miRNA spike-in from *Arabidopsis thaliana* ath-miR-159a and *C. elegans* cel-miR-54, cel-miR-39 and cel-miR-238; the authors added synthetic miRNAs to each denatured CSF sample.

This approach, however, does not reflect the biological factors and possible variations in the CSF management prior to the RNA extraction. To take account of them, data can be normalized using the most stable endogenous miRNAs or another short RNA that is not associated with the pathogenic condition. Recent works have identified several such endogenous controls in CSF, for instance, Baraniskin et al. (who used miR-24) [16], Teplyuk et al. (miR-24 and miR-125) [22], Denk et al. (let-7c, miR-21, miR-24, miR-99b, miR-328 and miR-1274B) [25], Sørensen et al. (miR-15a-5p, miR-21-5p, miR-23a-3p, miR-23b-3p, miR-99a-5p, miR-125b-5p, miR-145-5p, miR-204-5p and miR-320a) [24], and Gui et al. (RNU44 and RNU6B) [29]. Nevertheless, it is not easy to choose the best normalizer, and no RNA molecule shows stable levels in all pathological conditions and CSF components, a conclusion drawn by Akers et al. [36]. In their study, miR-125 and miR-24 were inappropriate references for the quantitative miRNA analyses of extracellular vesicles in CSF.

A third approach, possible in high-throughput analyses, is to normalize levels of individual miRNAs according to the selected parameters based on comprehensive characterization of the sample. In other words, small RNA-seq raw data are generally expressed in reads per million (RPM), i.e. specifically mapped reads to individual miRNA divided by the total number of aligned reads and multiplied by one million. This approach was successfully used by Yagi et al. [23]. To estimate the relative

miRNA levels from small RNA-seq data, the trimmed mean of M-values normalization method (TMM) can also be used. It estimates scale factors between the analyzed samples; these scale factors can then be used in the statistical methods for differential expression analysis [37]. This approach was used by Sørensen et al. [24] for the CSF miRNA analysis.

The simplest approach to normalizing raw data obtained by hybridization high-throughput platforms, like Affymetrix, is to re-scale each chip in an experiment by its total intensity. Many computational approaches can be used for such normalization, such as using selected genes instead of the entire gene set or scaling the individual intensities so that the median or mean value of intensities are identical within a single array or across all arrays.

Many normalization strategies are available and new ones will be developed in the future because choosing the most suitable approach to normalize the miRNA data for the method in use is crucial to draw unbiased conclusions.

CSF miRNAs as biomarkers in brain tumors

During the last decade, several studies have shown that deregulated levels of CSF miRNAs might be associated with malignant tumors of the CNS and might thus represent a novel group of possible diagnostic biomarkers. Many researchers have also focused on the use of CSF miRNAs as prognostic and predictive biomarkers of therapy response in patients with the CNS malignancies.

Diagnostic markers

In 2011, Baraniskin et al. [16] analyzed levels of six candidate miRNAs (miR-15b, miR-19b, miR-21, miR-92a, miR-106b and miR-204) in CSF samples obtained from 23 patients with primary CNS B-cell lymphoma (PCNSL) and 30 control patients with various neurologic disorders. These miRNAs were selected based on the two following criteria: (i) they are expressed by lymphoma cells at moderate or high levels and (ii) they show low or undetectable concentrations in CSF derived from the control patients. Only miR-19b, miR-21 and miR-92a showed significantly higher levels in the CSF samples from the patients with PCNSL than those from the control patients. Moreover, 22 of 23 PCNSL patients (95.7%) were correctly identified according to the levels of the above three miRNAs [16]. Several months later, the same authors published another study, in which miR-15b

and miR-21 were higher in CSF samples from patients with glioma than in those from the control patients. On the other hand, CSF samples from patients with glioma had higher levels of miR-15b and lower levels of miR-21 than the CSF sample from the PCNSL patients [21].

Subsequently, Teplyuk et al. reported that miR-10b was detected in most of CSF of GBM patients (89%) and patients with leptomeningeal and brain metastases of both lung and breast carcinomas (81%). This miRNA was, however, not detected in patients with various non-neoplastic neurological diseases. Furthermore, miR-21 level was higher in most of the CSF samples taken from GBM and metastatic patients than in the control CSFs. MiR-10b was expressed in most extracranial tissues and in peripheral blood serum. This miRNA was absent in both brain tissue and CSF of patients without pathologically diagnosed malignancy. This observation might suggest that, in physiological conditions, miR-10b and other miRNAs cannot pass the blood-brain barrier and, thus, CSF miRNAs might reflect a specific brain miRNA signature. Metastatic cells might bring their own miRNA signature to CSF, as miR-10b is abundant in lung and breast tissues and is present in the CSF of breast and lung cancer patients with the CNS metastases. To confirm this presumption, the authors evaluated the levels of miR-200a, miR-200b, miR-200c and miR-141 in the CSF samples from GBM and metastatic brain cancer patients and from healthy donors. Comparing these two patient groups is relevant because the miR-200 family is highly expressed in a primary carcinoma but not in normal brain or GBM and the other primary brain tumors. The levels of the miRNAs examined were higher in most of the CSF samples from the patients with leptomeningeal and brain metastases, but not from the GBMs or healthy donors. These results show that the miR-200 family could be a promising biomarker able to classify brain metastases and primary brain cancers [22].

Using NanoString nCounter assay, Drusco et al. looked for possible diagnostic biomarkers of all CNS tumors across CSF miRNAs. They divided CSF samples from 34 neoplastic and 14 healthy patients into seven groups (GBM, medulloblastoma, lung metastasis, breast metastasis, primary CNS lymphoma, benign and normal) and performed data analysis as well as comparisons among all groups. Results were further validated using qRT-PCR and hybridization *in situ*. The CSF levels of miR-125b, miR-223, miR-451, miR-711 and miR-935 exhibited different patterns between the examined groups, which suggests that these miRNAs might be efficient diagnostic biomarkers of the CNS malignancies [38].

Grotzer et al. analyzed CSF miRNAs in medulloblastoma and control patients. They detected 1254 miRNAs

in the tumor CSF samples, and 86 of these miRNAs were differentially expressed between the groups. The authors also used culture medium *in vitro* to analyze miRNAs excreted by the medulloblastoma cells. Fifty-seven detected miRNAs were specific to the metastasis-related cell lines which represent the most aggressive medulloblastoma subtypes. Interestingly, the three miRNAs associated with metastases (miR-125a, miR-125b and miR-1290) which were over-represented in the culture medium of cancer metastasis-related cell lines were also detected in the CSF samples from the medulloblastoma patients [39].

Finally, Akers et al. analyzed miR-21 levels in extracellular vesicles (EV) extracted from CSF samples from 13 GBM patients and 14 non-tumor control patients. miR-21 ranged from 0.14 to 1.04 copies per EV in the GBM samples and from $5.26 \cdot 10^{-4}$ to $1.48 \cdot 10^{-1}$ copies per EV in the control patients. The cut-off (<0.25) copy per EV classified the GBM patients with 87% sensitivity and 93% specificity. The authors also measured the levels of miR-21 in EV depleted CSF of the GBM patients, but miR-21 was undetectable [36]. Yagi et al. [23] confirmed, by NGS, that miRNA in CSF of the healthy donors is more abundant in the exosomal fraction than in the supernatant.

Table 2 summarizes studies comparing CSF microRNA levels among brain tumors.

Prognostic and predictive markers

Teplyuk et al. were the first to study whether levels of CSF miRNAs reflects the activity of the GBM and metastatic brain cancers. Members of neither the miR-10b nor miR-200 families were detected in the CSF samples from cancer patients in remission. Similarly, miR-21 was detected at significantly lower levels in cancer patients in remission than in patients with progressing GBM or metastatic brain cancer. To examine whether levels of specific CSF miRNAs are associated with disease status and activity as well as tumor response to the therapy, miRNAs were quantified in the CSF of GBM and lung cancer patients under erlotinib treatment. During the treatment, the CSF levels of members of both the miR-10b and miR-200 families increased in patients with relapsing non-small cell lung carcinoma. However, the miRNA levels decreased after increasing the treatment dosage. The miR-21 levels in the CSF samples from the GBM patients with pseudo-progression were similar to the levels in the CSF samples from the non-neoplastic patients, whereas the miR-10b levels were higher in the first set of patients than in the second one. On the other hand, disease progression was accompanied by a notable increase in the miRNA CSF

Table 2: CSF microRNAs with significantly different levels between brain tumors and non-tumor brain tissue.

Compared brain tumors	microRNA levels in CSF ^a	References
Glioblastoma vs. non-tumor	↑miR-451, ↑miR-223, ↑miR-125b, ↑miR-10b, ↑miR-21	[22, 34, 38]
Glioblastoma vs. lymphoma	↑miR-711	[38]
Glioblastoma vs. medulloblastoma	↑miR-223, ↑miR-711, ↓miR-125b	[38]
Glioblastoma vs. brain metastasis	↑miR-223, ↓miR-200a, ↓miR-200b, ↓miR-200c, ↓miR-141	[22, 38]
Medulloblastoma vs. non-tumor	↑miR-451, ↑miR-223, ↑miR-125b	[38]
Medulloblastoma vs. lymphoma	↑miR-711, ↑miR-125b	[38]
Medulloblastoma vs. metastasis	↑miR-125b	[38]
Metastasis vs. non-tumor	↑miR-451, ↑miR-223, ↑miR-125b, ↑miR-10b, ↑miR-21, ↑miR-200a, ↑miR-200b, ↑miR-200c, ↑miR-141	[22, 38]
Metastasis vs. lymphoma	↑miR-711, ↑miR-223	[38]
Lymphoma vs. non-tumor	↑miR-21, ↑miR-19b, ↑miR-92a	[16]
Glioma (WHO II–IV) vs. non-tumor	↑miR-21, ↑miR-15b	[21]
Glioma (WHO II–IV) vs. PCNSL	↓miR-21, ↑miR-15b	[21]
Glioma (WHO II–IV) vs. brain metastasis	↓miR-21, ↑miR-15b	[21]

^aCSF miRNA level is related to the first diagnosis between the two compared brain tumors. ↑ significantly higher CSF miRNA level, ↓ significantly lower CSF miRNA level.

levels. In summary, Teplyuk et al.'s. [22] study indicates that CSF miRNAs may serve as biomarkers of response to the therapy and brain cancer progression.

Shi et al. examined CSF samples from recurrent glioma patients to detect miRNAs associated with this cancer, aiming to test whether these miRNAs can serve as prognostic biomarkers. For this purpose, they compared the miR-21 levels in CSF, blood serum and exosome. While blood serum-derived exosomal miR-21 levels of the glioma patients did not differ from the non-tumor control group, CSF miR-21 levels of exosomal origin were higher in the glioma patients. The CSF exosomal miR-21 levels reflected the presence of both tumor recurrence with anatomical site preference and spinal/ventricle metastasis. Therefore, the authors suggested that exosomal miR-21 might be a promising diagnostic and prognostic biomarker of glioma [40]. Finally, Tumilson et al. [41] observed that the increased levels of miR-21 in CSF were associated with a poor prognosis. Moreover, they suggested that CSF miR-21 has potential as a predictive biomarker of temozolomid resistance.

Conclusions

More often than not, prognoses for patients with brain malignancies are unfavorable. Not only does the prognosis depend on a tumor, it can also vary among patients with the same tumor type. Like in other cancers, early and accurate diagnosis is crucial for effective treatment but is seldom easy, mainly due to the tumor's localization or heterogeneity, common in gliomas. For these reasons, there

is a clinical need for new biomarkers of brain tumors that would allow for precise diagnosis, prognosis and prediction of a response to therapy. These biomarkers should be stable, and their analysis should use common methods (like qRT-PCR) that provide simple, reproducible and fast analysis. From this perspective, brain tumors associated with miRNAs released and detected in CSF seem promising molecules, so it is not surprising that more and more research on this topic is being published. Despite the promising published data indicating CSF miRNAs as specific and sensitive brain tumor biomarkers, technical issues of the analysis are hurdles still to overcome. This is exactly why further research should focus on these technical issues, before even thinking of clinical implementation of CSF miRNAs.

Given that the CSF miRNAs are so promising as diagnostic, prognostic and predictive biomarkers of therapy response in brain tumor patients, we should continue our efforts to solve the technical hurdles and better understand the molecular mechanisms leading to the cellular secretion of miRNAs into body fluids.

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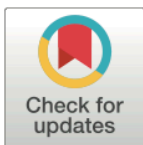
MicroRNA isolation and quantification in cerebrospinal fluid: A comparative methodical study

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Abstract

Associated with the pathogenesis of many cancers, including brain tumors, microRNAs (miRNAs) present promising diagnostic biomarkers. These molecules have been also studied in cerebrospinal fluid (CSF), showing great potential as a diagnostic tool in patients with brain tumors. Even though there are some biological and technological factors that could affect the results and their biological and clinical interpretation, miRNA analysis in CSF is not fully standardized. This study aims to compare several RNA extraction and miRNA quantification approaches, including high-throughput technologies and individual miRNA detection methods, thereby contributing to the optimization and standardization of quantification of extracellular miRNAs in CSF. Such knowledge is essential for the potential use of miRNAs as diagnostic biomarkers in brain tumors.

Introduction

Primary brain tumors and brain metastases annually affect close to 40 patients per 100,000 persons worldwide, a still growing incidence rate [1, 2]. Prognosis and therapy depend on the brain tumor type, so an early and accurate diagnosis can significantly affect the quality of life and the survival of patients. Unfortunately, a diagnosis of brain cancer is often limited by the localization and heterogeneity of the tumor tissue. Like in the other cancers, when tissue diagnosis is impossible because of the tissue's localization or a lack of precision, liquid biopsies are promising diagnostic approaches. Specifically in brain cancers, frequent discussions have focused on the diagnostic utilization of cerebrospinal fluid (CSF). Bathing the central nervous system, CSF is in direct contact with all brain components, including neoplasms, and thus it is a source of many potential biomarkers [3].

Perspective CSF biomarkers seem to be circulating microRNAs (miRNAs). MiRNAs constitute a class of single-stranded non-coding RNAs, about 18–25 nucleotides in length, which post-transcriptionally regulate gene expression, thus being key players in the regulation of all

cellular processes. Usually tissue-specific, miRNAs are involved in the pathogenesis of many diseases, including brain tumors [4]. Circulating miRNAs have been detected in almost all human body fluids, such as blood plasma and serum, urine, saliva, tears, and cerebrospinal fluid [5]. Moreover, levels of selected circulating miRNAs have been repeatedly described to be associated with specific tumors, grades, stages, prognosis, and therapy response in cancer patients. Interestingly, miRNAs are highly stable and resist extreme conditions, such as ribonuclease activity, repeated freezing and thawing, boiling, low and high pH, and long-term storage at room temperature [5]. Recent studies have shown that deregulated levels of CSF miRNAs are associated with malignant tumors of CNS [6–9]. Qu *et al.* have also showed that miR-21 level in CSF enabled to identify glioma patients with higher sensitivity and specificity in comparison with the plasma/serum miR-21 level [10]. Taken together, analysis of circulating miRNAs in CSF seems to be a promising tool leading to the refinement of current brain tumor diagnostics [11, 12]. Unfortunately, such analysis in human body fluids can be affected by many biological and technological factors, thus posing quite a challenge. Here we compare several approaches and protocols for RNA extraction from CSF and for conducting high-throughput and individual miRNA analyses in CSF.

Material and methods

Clinical samples

In this study, we included CSF samples collected from 10 glioblastoma (GBM) patients and 10 non-tumor donors (patients with hydrocephalus). From each person, a sample of 3–5 ml of CSF was collected through lumbar puncture (between the L3 and L5 vertebrae). The samples were taken in 2016 at the Department of Neurosurgery, The University Hospital Brno (Brno, the Czech Republic). CSF samples containing blood cells were excluded. All the patients signed informed consents for the use of CSF and clinical data for research purposes. The study was approved by the local Ethics Committee at The University Hospital Brno.

CSF handling and sample preparation

CSF samples were centrifuged at 500g for 10 min at 4°C (Eppendorf 5810 R, Germany), and the supernatant were aliquoted to 1 ml tubes and stored at -80°C till further analyzed. For the first step of this study, that is, to select the most efficient method for RNA extraction from CSF, two GBM and two control independent CSF pools were prepared, each pool made from five different CSF samples. To prevent repeated thawing, the pools were aliquoted into tubes according to the volumes required for particular RNA isolations.

RNA isolations

We compared four different commercially available RNA purification kits: Urine microRNA purification kit (Norgen Biotek, Canada), miRNeasy Serum/Plasma kit (Qiagen, Germany), miRVANA miRNA Isolation Kit (Ambion, Austin, TX, USA), and Trizol reagent (Thermo Fisher Scientific, USA). The manufacturers' protocols were followed except for the following modifications: In the case of Norgen, both 1 ml and 0.5 ml of CSF were used as an input volume for RNA extraction, and in the case of Qiagen and Ambion, 50 ng of glycogen (a co-precipitant and carrier) per isolation was added or omitted. At the elution step, samples were incubated on the column for 20 min at RT (except for the miRVANA kit, in which the elution buffer is heated to 96°C); the volume of elution buffer was in all cases modified to 30 µl. After adding of lysis buffer, 3.5 µl of miRNeasy Serum/Plasma Spike-In Control (1.6×10^8 copies/µl, *C. elegans* miR-39 miRNA mimic, Qiagen, Germany) were added to each sample and mixed

thoroughly. The spike-in controls were diluted according to the manufacturers' protocols. Fig 1A and 1B show the workflow of CSF RNA extraction optimization.

miRNA expression analysis

Spike-in detection. The RNA samples supplemented with miRNeasy Serum/Plasma Spike-In Control were transcribed using miScript II RT Kit (Qiagen, Germany), according to the manufacturer's protocol. Real-time PCR was performed by miScript SYBR Green PCR Kit and Cel_miR-39_1 miScript Primer Assay (both Qiagen, Germany), using LightCycler 480 Instrument II (Roche, Switzerland), according to Qiagen's protocol.

RT-qPCR and digital PCR. Reverse transcription was performed by TaqMan Reverse Transcription Kit (Applied Biosystems, USA) with stem-loop miRNA specific primers (hsa-miR-10a-5p; ID 000387 and hsa-miR-196a-5p; ID 241070 and miR-16; ID 00391). Real-Time PCR was performed using TaqMan Universal PCR Master Mix, NoUmpErase UNG (Applied Biosystems) and LightCycler 480 Instrument II (Roche, Switzerland). All steps were performed according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the Fit point method of the LightCycler quantification software.

Digital PCR (dPCR) was performed using QuantStudio 3D Digital PCR 20K Chip Kit + Master mix v1 on a QuantStudio 3D Digital PCR Instrument (all ThermoFisher Scientific, USA), according to the manufacturer's protocol.

High-throughput profiling methods. MiRCURY Exiqon Human PCR Panel I (miRCURY LNA, Exiqon, Denmark) was applied after Exiqon's standardized protocol, using Exi-LENT SYBR Green master mix, miRCURY LNA, microRNA Ready-to-Use PCR, Human Panel I, V4-R, Universal cDNA Synthesis Kit II, 8–64 rxns, miRCURY LNA, UniSp6 RNA spike-in control primer set v2 (all Exiqon, Denmark), and LightCycler 480 Instrument II (Roche, Switzerland).

TaqMan Low Density Arrays (Applied Biosystems, USA) were performed with the preamplification step, according to Applied Biosystems's protocol, using TaqMan MicroRNA Reverse Transcription Kit, Megaplex RT Primers, Megaplex PreAmp Primers, Human Pool Set v3.0, TaqMan PreAmp Master Mix, TaqMan Array Human MicroRNA A+B Cards Set v3.0 and TaqMan Universal PCR Master Mix, No AmpErase UNG, 2x (all Applied Biosystems, USA)

Next generation sequencing was performed using the NextSeq 500 (Illumina, USA) technology, with CleanTag Small RNA Library Preparation Kit (TriLink, Biotechnologies, L-3206) applied for library preparation and NextSeq 500/550 High Output Kit v2, 75 cycles (Illumina, USA) applied for sequencing run, according to the manufacturer's protocol.

Data normalization. All real-time PCR reactions were run in triplicate, and for each sample the average threshold cycle and standard deviations (SDs) were calculated. Ct values were transformed using the $2^{-\Delta Ct}$ method ($\Delta Ct = Ct(\text{miRNA}) - 40$). The Wilcoxon pair test and the t-test were used to compare the efficiencies of the extraction methods. Spearman correlation was used to analyze relationships between the miRNA quantification approaches. All the analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA, USA), with the significance level of 0.05.

Results

Comparison of RNA extraction kits for miRNA isolation from cerebrospinal fluid

Using the four commercially available RNA extraction kits, we obtained very low RNA concentrations/yields from CSF—too low to use them for their comparison. Therefore, to evaluate the

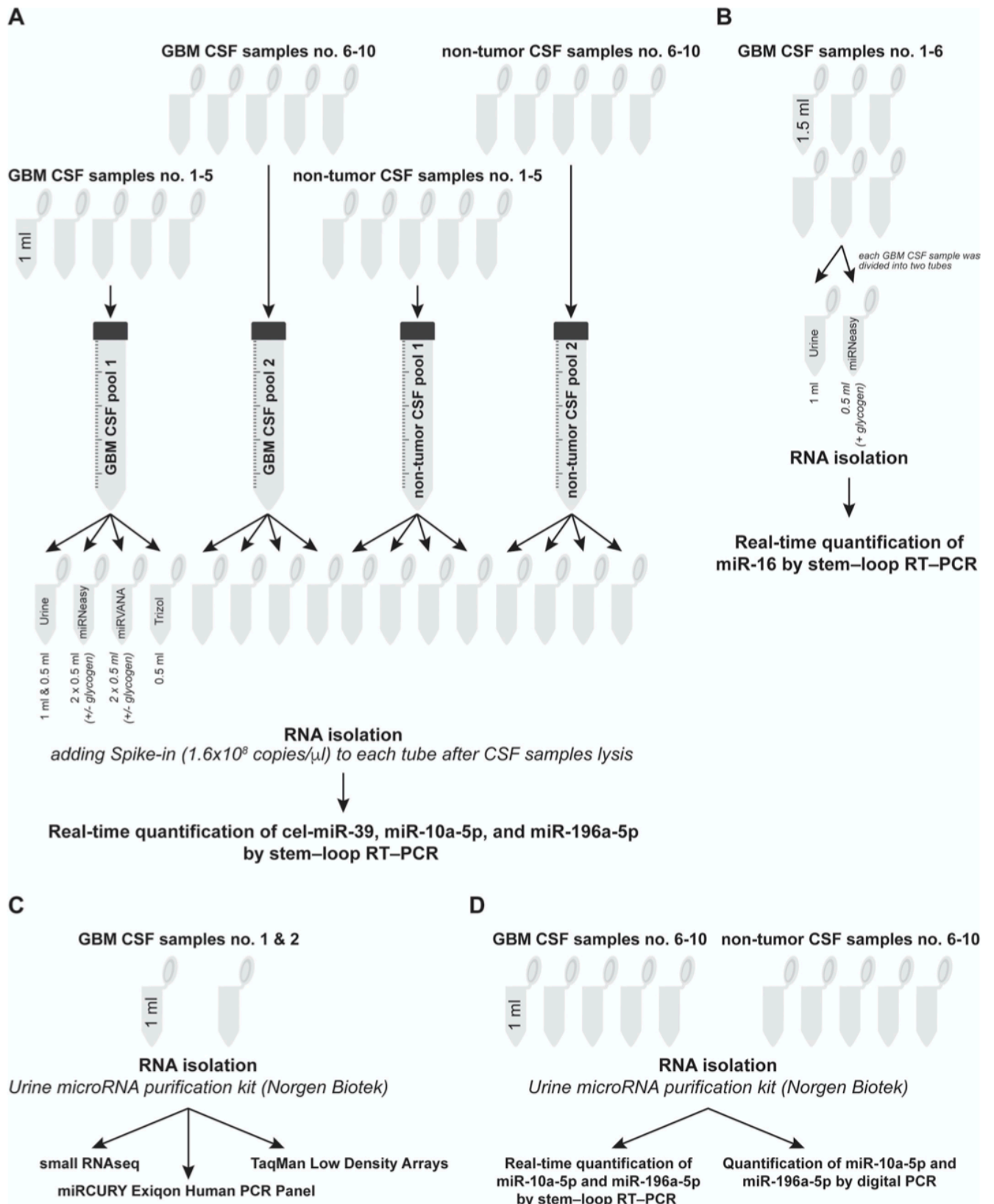


Fig 1. An illustrated workflow of RNA extraction optimization (A, B), high-throughput miRNA analysis (C), and the selection of miRNA analysis (D) methods. <https://doi.org/10.1371/journal.pone.0208580.g001>

efficiency of miRNA extraction, we used real-time PCR quantification of cel-miR-39 spike-in control and two endogenous miRNAs (miR-10a-5p and miR-196a-5p). We chose these two particular miRNAs after a preliminary experiment, in which they showed significantly higher levels in GBM CSF samples compared to non-tumor CSF samples (data not shown). The highest levels of the miRNAs analyzed were detected in the RNA samples extracted using the Norgen kit ($p < 0.001$). The examined miRNA levels did not significantly differ between RNA samples extracted from both 0.5 and 1 ml of CSF (Fig 2A, 2B and 2C). Moreover, miR-16-5p (which was selected based on a previous study, in which it had detectable levels in both glioma [Ct means 26.28] and control CSF samples [Ct means 28.69] [7]) was quantified in six RNA samples extracted from independent GBM CSFs the using the Norgen and Qiagen kits supplemented with glycogen. CSF RNA samples extracted using the former kit showed significantly higher levels of miR-16 than those extracted using the latter kit ($p = 0.0313$ in the Wilcoxon pair test, Fig 1D).

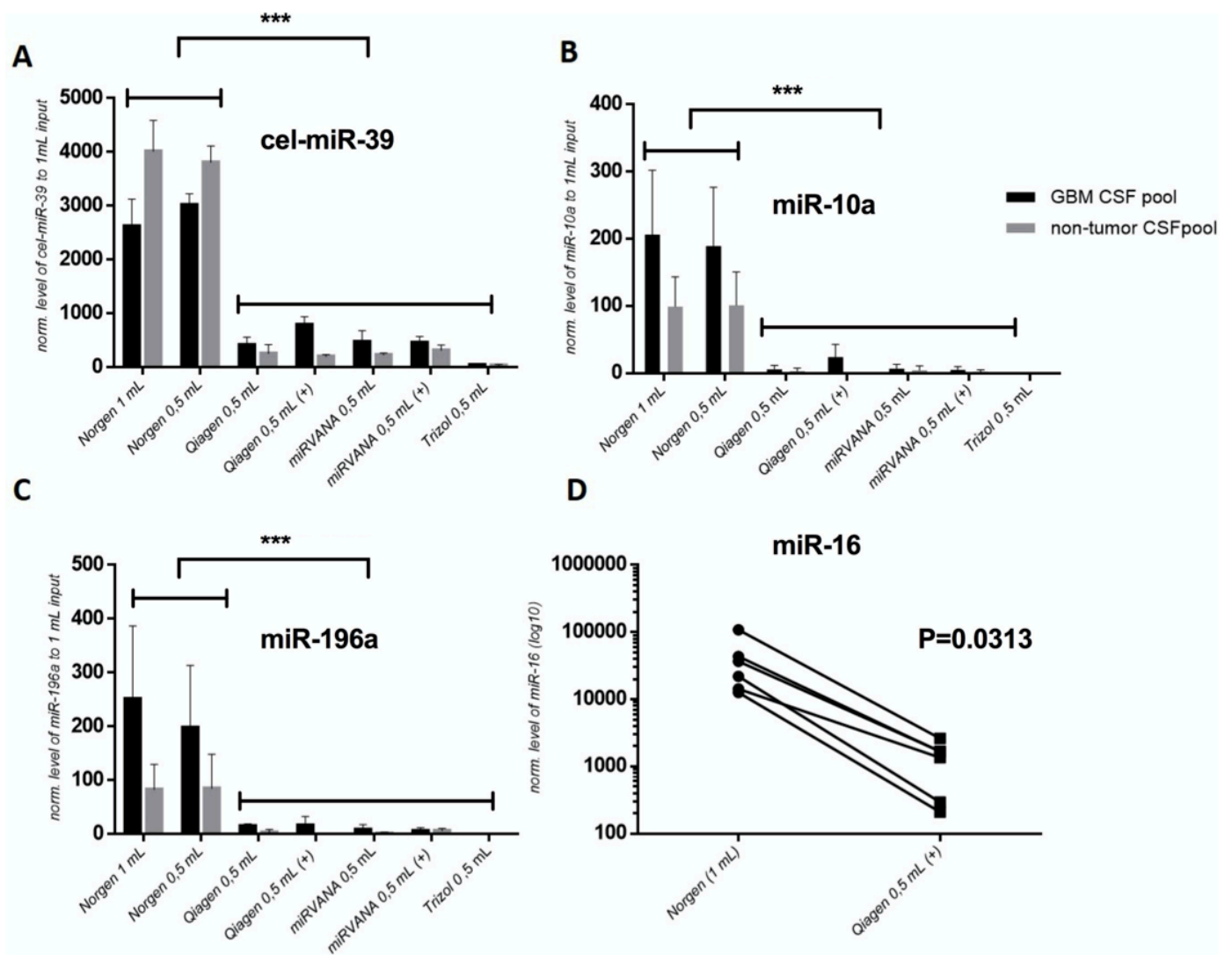


Fig 2. A comparison of selected CSF miRNA levels in RNA samples extracted by four RNA isolation kits with various protocol modifications, including different volumes of CSF input (1 ml and 0.5 ml) and adding (+) or omitting of glycogen during extraction. Levels of cel-miR-39 (A), miR-10a (B), and miR-196a (C) were analyzed using Real-Time PCR in RNA samples extracted from two GBM and two non-tumor CSF pools. Levels of miR-16 (D) were analyzed in paired RNA samples extracted from six independent CSF samples.

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Table 1. A comparison of the selected high-throughput technologies for miRNA profiling in cerebrospinal fluid and the number and quantity of miRNAs detected in the study.

Method	NGS		TLDA (A+B Card) with pre-amplification		miRCURY LNA (Panel I) without pre-amplification	
Sample	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B
The number of possibly detected miRNAs	unlimited		754		372	
The number of detected miRNAs	369 [§]	272 [§]	283 [#]	241 [#]	16 [#]	47 [#]
Median of reads or Ct values of detected miRNAs*	31 (12/137)	18 (6/93)	29.7 (26.9/32.4)	30.8 (27.6/32.8)	33.7 (32.7/34.4)	33.3 (31.5/34.4)

*Ct < 35

[†] 25/75% percentiles of the number of detected miRNAs

[§] number of raw reads ≥ 1

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Comparison of high-throughput technologies for miRNA profiling in CSF

The NGS-based technology detected the most miRNAs in both analyzed samples: 369 (median of 31) and 272 (median of 18) miRNAs with at least one raw read per sample (Table 1, Fig 1C). Between the two examined real-time-PCR-based methods, TaqMan Low Density Arrays (TLDA; ThermoFisher Scientific) with a preamplification step was more effective, with 283 (median Ct value of the detected miRNAs of 29.7) and 241 (median of 30.8) detected miRNAs of the 754 pre-designed miRNAs. The Exiqon technology without a preamplification step detected only 16 (median Ct value of the detected miRNAs of 33.7) and 47 (median of 33.3) of the 372 pre-designed miRNAs. S1 Table lists miRNAs detected with at least two of the above technologies. Venn diagrams (Fig 3A, 3B, 3C and 3D) show the numbers of miRNAs detected by the high-throughput technologies compared. Small RNAseq analysis detected most individual miRNAs (Fig 3A and 3B), a number dramatically reduced when comparing only miRNAs pre-designed in TLDA (Fig 3C and 3D).

The results of the PCR-based technologies and those of the NGS platform were only weakly correlated in both samples examined (Fig 4). Specifically, correlation coefficients between NGS platform and Exiqon technology reached 0.35 and 0.43 in samples A and B, and those between the NGS platform and the TLDA method reached 0.26 and 0.13, respectively.

Comparison of real-time PCR and digital PCR technologies for quantification of individual miRNAs in CSF

Based on our previous experiences with these miRNAs, we selected miR-10a-5p and miR-196a-5p for quantification using the real-time PCR and digital PCR technologies. These analyses were performed in CSF samples collected from five patients with primary GBM and five healthy donors (Fig 1D). According to Spearman correlation, the results of the PCR-based technologies and the NGS platform were highly correlated. Specifically, the correlation between digital PCR and NGS reached 0.85 in miR-10a-5p and 0.92 in miR-196a-5p (Fig 5A and 5B). Similar correlation was observed between real-time PCR and NGS (r = 0.88 in miR-10a-5p and 0.86 in miR-196a-5p; Fig 5C and 5D).

Discussion

To find RNA extraction method providing the highest miRNA levels from CSF samples, we compared four commercially available RNA isolation kits, following the recommended protocol and with its small modifications related to glycogen supplementation, CSF input volumes,

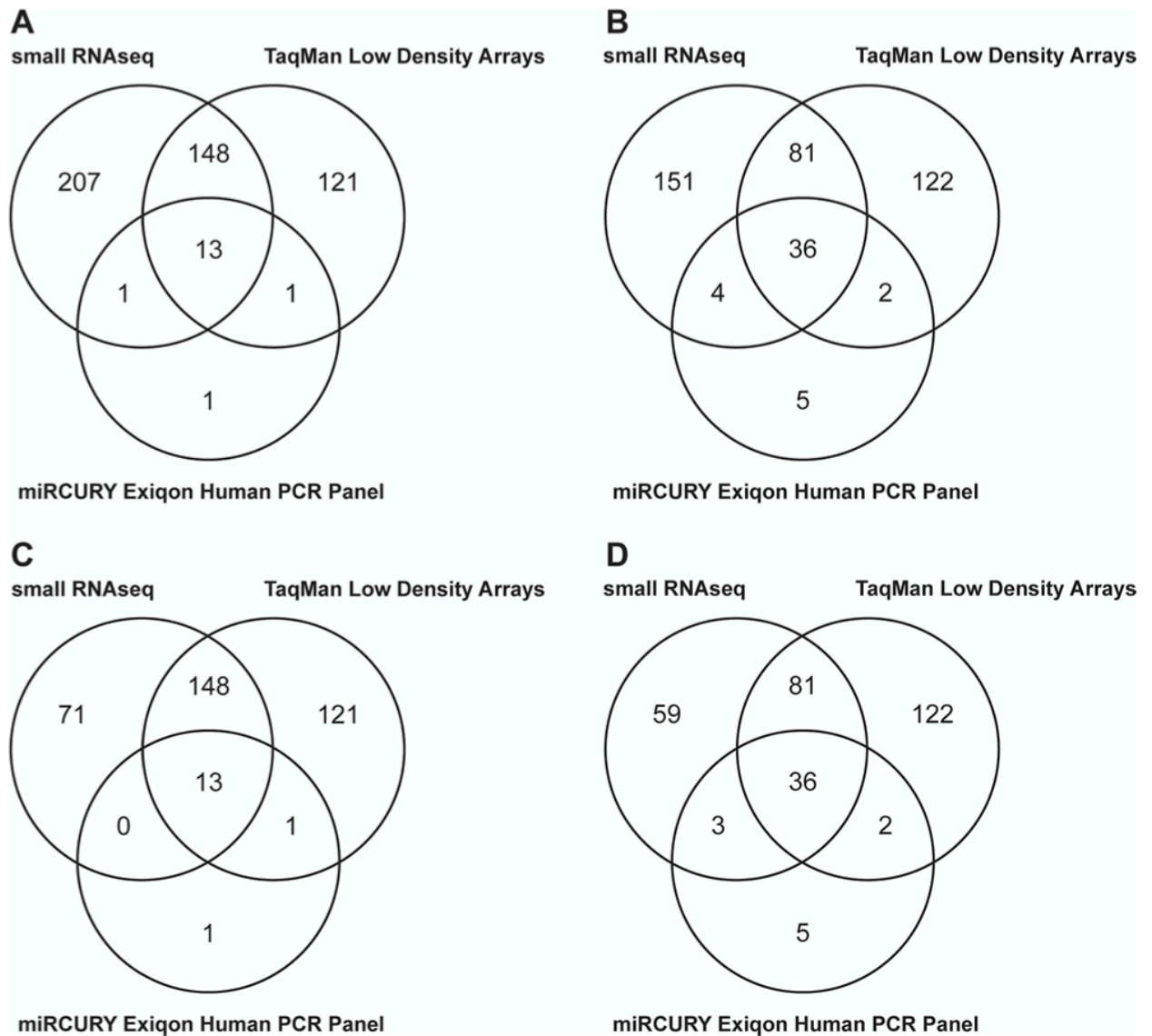


Fig 3. Venn diagrams showing an overlapping of detected miRNAs between different high throughput technologies, applying all the detected miRNAs in CSF sample A (A) and CSF sample B (B), and applying only a set of TLDA pre-designed miRNAs in CSF sample A (C) and CSF sample B (D).

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and time of RNA elution [13, 14]. Unfortunately, the RNA yields obtained were undetectable by common fluorospectrophotometer-based methods, such as the Nanodrop and Qubit technologies (both ThermoFisher).

Therefore, using the real-time PCR technology to evaluate extracted miRNA levels, we quantified three endogenous miRNAs (miR-16, miR-10a-5p, and miR-196a-5p) and exogenous cel-miR-39, which were added during RNA extraction process. To avoid cel-miR-39 degradation by endogenous RNases, we added it into the sample after the lysis step [15]. In our hands, Urine microRNA Purification kit from Norgen, which eventually eluted into 30 µl of elution buffer after 20 minutes of incubation on the column, showed the highest cel-miR-39 recovery rate. The same extraction protocol led to the highest levels of all the endogenous miRNAs analyzed. Based on these results, we suggest Urine microRNA Purification kit from

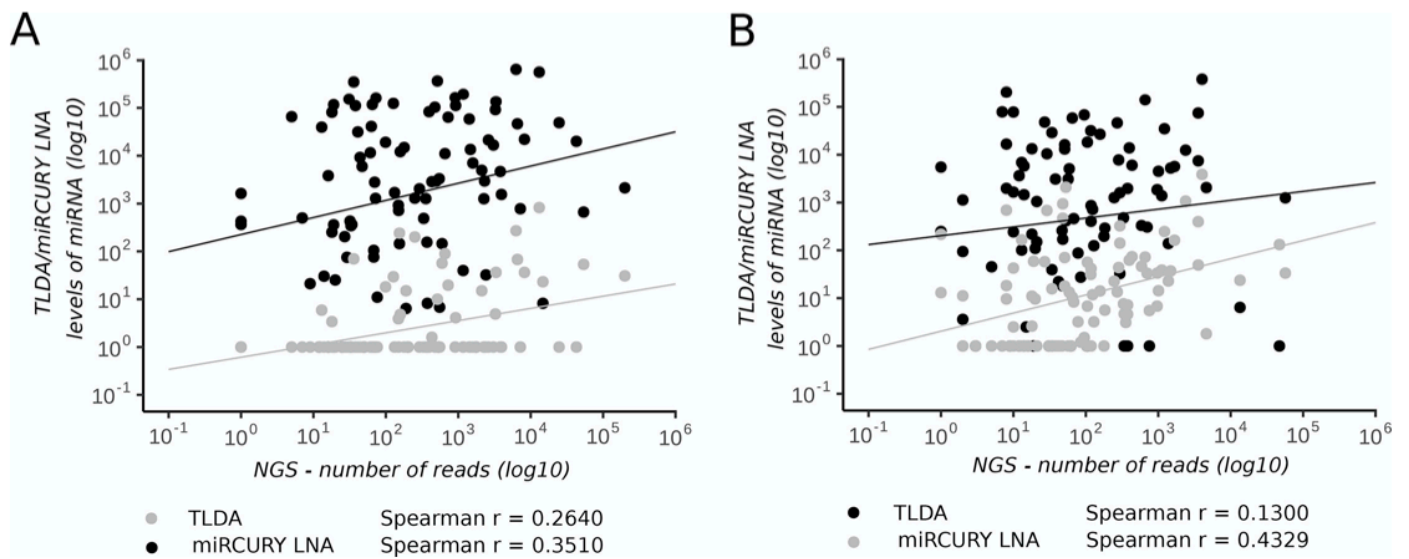


Fig 4. Correlation analyses of miRNA levels detected using the Exiqon and TLDA approaches and the NGS platform in (A) CSF sample A and (B) CSF sample B.

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Norgen to be the most appropriate for miRNA extraction from CSF samples. Thus, we used this kit for RNA extraction in the following analyses. However, levels of spike-in cel-miR-39 varied between the pools. This may be due at least in part to the fact that we made a new dilutions of spike-in from a highly concentrated stock several times during the study. When the most efficient RNA isolation approach (Norgen) was used, the differences between pools ranged from 25–30%, which when expressed in Ct values means dCt less than 0.4 between pools. It should be recommended to prepare and use only the one dilution of spike-in cel-miR-39 for the whole experiment to eliminate technological variability in its quantification.

The potential of CSF miRNAs to serve as the accurate brain tumor biomarkers depends on methodological approaches used for their quantification. Unfortunately, methods commonly used for high-throughput miRNA profiling require a higher RNA input than RNA yields recovered from CSF samples. Moreover, these methods are optimized for RNA specimens extracted from cells and tissues. A better option is the manufacturing protocol supports RNA extracted from blood plasma/serum samples. However, there is no commercially available method for CSF miRNA quantification. In this regard, miRNA profiles in cell/tissue, blood plasma/serum, and CSF samples show significantly different patterns. Specifically, Iwuchukwu *et al.* analyzed 782 known miRNAs (Exiqon) in plasma and CSF samples and identified significantly more miRNAs in CSF than plasma [16]. Sorensen *et al.* [17] reported similar results. Akers *et al.* [18] found more specific miRNAs in glioblastoma tissue than in CSF [18].

Different distributions and proportions of miRNAs in total RNA yields can affect the accuracy of miRNA analysis. For high-throughput miRNA analysis in CSF samples, we compared two real-time-PCR-based technologies and an NGS platform in two independent CSF samples collected from GBM patients. NGS detected the most miRNAs. The PCR methods were more limited by the low RNA input because the number and proportion of detected miRNAs increased rapidly when a preamplification step was included. On the other hand, correlation analysis of miRNA levels detected using all three high-throughput approaches showed lower correlation between NGS and PCR with a preamplification step than that without it. Thus, it seems that a preamplification step preceding the final real-time PCR analysis biased the results. Since NGS is able to detect not only miRNAs but also other small RNA classes (including

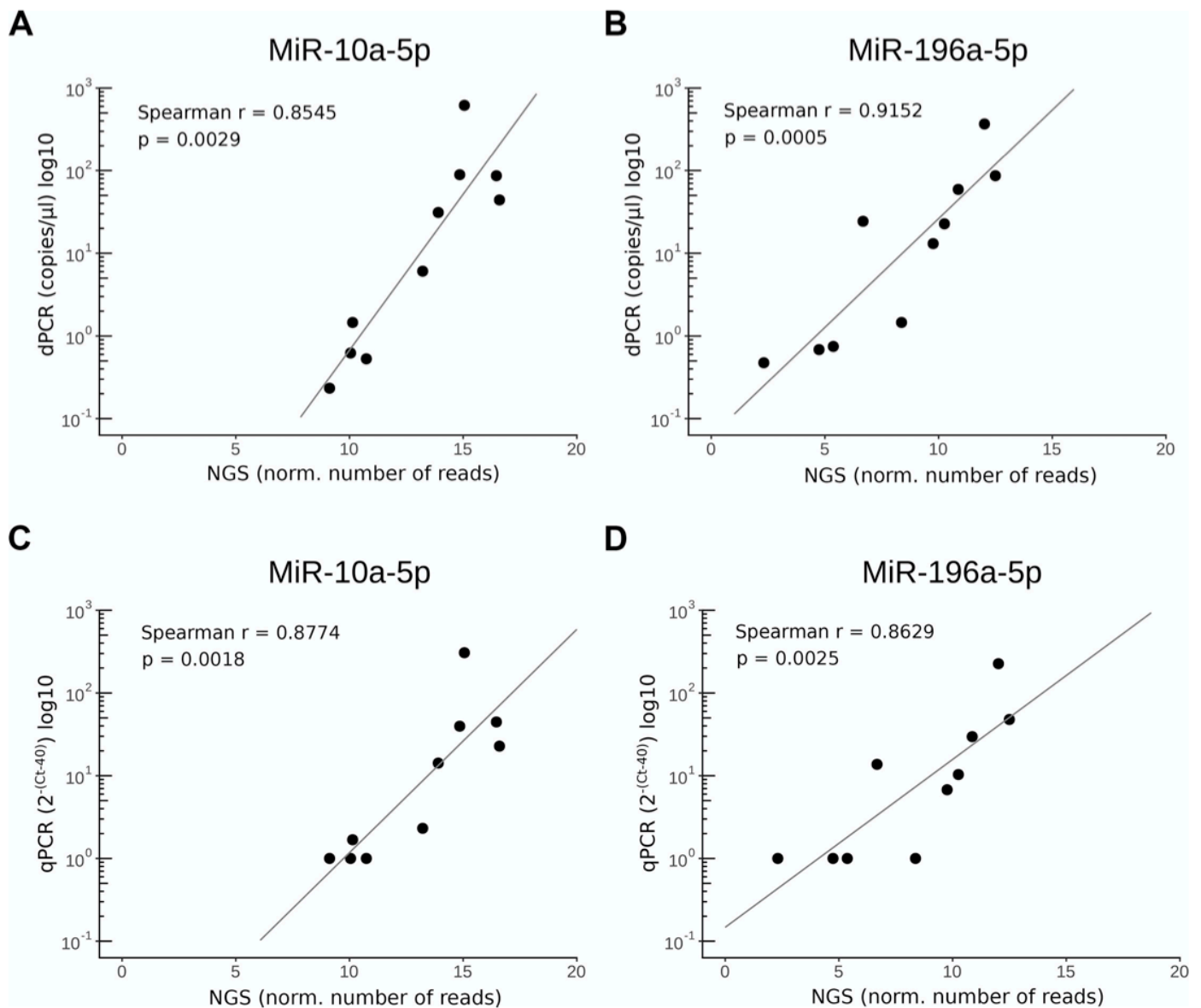


Fig 5. Correlation analyses of miR-10a-5p and miR-196a-5p levels detected using (A,B) digital PCR and (C,D) real-time PCR technologies and NGS platform in CSF samples.

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PIWI-interacting RNAs [19]) and to determine their isoforms [20], we suggest that a NGS platform is the most suitable for the analysis and quantification of miRNAs in CSF samples. The feasibility of this method for miRNA analysis in CSF samples was previously examined and confirmed by Burgos *et al.* [13].

We compared real-time PCR with digital PCR. Although real-time PCR is nowadays the most established method of miRNA expression analysis, it has some limitations. Its main weaknesses are low sensitivity and accuracy in low-copy template detection [21] and complicated raw data normalization (especially in body fluids), all of which can bias final results. On the other hand, Conte *et al.* showed dPCR to be accurate, reproducible, and reliable—and thus more appropriate for the identification and quantification of miRNAs in body fluids [22]. In

this study, we have compared miR-10a-5p and miR-196a-5p levels detected by real-time PCR and dPCR with NGS data in ten independent CSF samples. Although the results of the methods were highly correlated, our data suggest that real-time PCR is not able to precisely distinguish samples with lower than ten miRNA copies. Based on our results and those published in other studies, we suggest that dPCR is a more suitable method for the quantification of individual miRNAs in CSF samples.

There is no consensus on the best normalization approach, a challenging issue since analysis of miRNA levels in body fluids is affected by many technical and biological factors. In CSF samples, several methods have already been suggested, including exogenous spike-in miRNAs, reference endogenous small RNAs, and global mean normalization approaches used in high-throughput analyses. However, all these methods have limitations. Spike-in miRNAs do not reflect biological factors. Endogenous small RNAs that are stably expressed in cells—such as RNU 44, RNU 48, and RNU 6B—show varying levels in individual CSF samples. Moreover, they do not fully reflect the biogenesis of circulating miRNAs [18]. Despite this, recent studies have suggested some promising reference circulating miRNAs (miR-24, miR-125, let-7c, miR-21, miR-24, miR-99b, miR-328 and miR-1274B, miR-15a-5p, miR-21-5p, miR-23a-3p, miR-23b-3p, miR-99a-5p, miR-125b-5p, miR-145-5p, miR-204-5p, and miR-320a) [7, 8, 23, 24]. Even though another study has already disproved some of these miRNAs as useful reference molecules for the normalization of CSF miRNA levels [25], using them remains a promising approach to objectivize results of CSF miRNA analysis.

Conclusion

Circulating CSF miRNAs seem to be promising biomarkers that could help to refine current brain tumor diagnostics. However, analysis of these small non-coding RNAs in CSF is still not fully standardized, and many factors can bias the results. Thus, optimization and standardization of individual steps in this analytical process could bring CSF miRNAs closer to clinical use. After comparing several RNA extraction methods, we suggest that the Urine microRNA purification kit provided by Norgen Biotek company is the most appropriate kit for miRNAs extraction from CSF samples. Further, our data show small RNaseq and digital PCR to be suitable methods for CSF miRNA quantifications.

Supporting information

S1 Table. MicroRNAs d with at least two of the tested high-throughput technologies. (XLSX)

Author Contributions

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Formal analysis: Tana Machackova, Marek Vecera, Petra Vychytilova-Faltejskova, Martin Smrcka, Ondrej Slaby.

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Writing – original draft: Alena Kopkova, Jiri Sana, Ondrej Slaby.

Writing – review & editing: Martin Smrcka, Ondrej Slaby.

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Article

Cerebrospinal Fluid MicroRNA Signatures as Diagnostic Biomarkers in Brain Tumors

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Abstract: Central nervous system (CNS) malignancies include primary tumors that originate within the CNS as well as secondary tumors that develop as a result of metastatic spread. Circulating microRNAs (miRNAs) were found in almost all human body fluids including cerebrospinal fluid (CSF), and they seem to be highly stable and resistant to even extreme conditions. The overall aim of our study was to identify specific CSF miRNA patterns that could differentiate among brain tumors. These new biomarkers could potentially aid borderline or uncertain imaging results onto diagnosis of CNS malignancies, avoiding most invasive procedures such as stereotactic biopsy or biopsy. In total, 175 brain tumor patients (glioblastomas, low-grade gliomas, meningiomas and brain metastases), and 40 non-tumor patients with hydrocephalus as controls were included in this prospective monocentric study. Firstly, we performed high-throughput miRNA profiling (Illumina small RNA sequencing) on a discovery cohort of 70 patients and 19 controls and identified specific miRNA signatures of all brain tumor types tested. Secondly, validation of 9 candidate miRNAs was carried out on an independent cohort of 105 brain tumor patients and 21 controls using qRT-PCR. Based on the successful results of validation and various combination patterns of only 5 miRNA levels (miR-30e, miR-140, let-7b, miR-10a and miR-21-3p) we proposed CSF-diagnostic scores for each tumor type which enabled to distinguish them from healthy donors and other tumor types tested. In addition to this primary diagnostic tool, we described the prognostic potential of the combination of miR-10b and miR-196b levels in CSF of glioblastoma patients. In conclusion, we performed the largest study so far focused on CSF miRNA profiling in patients with brain tumors, and we believe that this new class of biomarkers have a strong potential as a diagnostic and prognostic tool in these patients.

Keywords: glioblastoma; meningioma; brain metastases; microRNA; cerebrospinal fluid

1. Introduction

Malignancies of the central nervous system (CNS) consist of primary tumors and secondary tumors that originate in different parts of a body and occur in CNS as brain metastasis. These two groups of CNS tumors count almost 40 patients per 100,000 persons worldwide and the incidence

rate is still growing. The main types of primary brain tumors include gliomas, ependymomas and meningiomas [1,2]. Gliomas originate from glial cells and are classified by histopathological and molecular features into four classes, more generally into low grade gliomas (LGG, WHO I and II), and high-grade gliomas (HGG, WHO III and IV), when the most common glioma is glioblastoma multiforme (GBM) [3]. GBM, with the incidence rate of 4.7–5.7 cases per every 100,000, is also one of the most aggressive brain tumors, and even after therapy, the median survival time is only around 14.6 months [4]. On the other hand, meningiomas are in most cases slowly growing tumors and represent most common adult primary brain tumors, characterized by almost two times more frequent occurrence in females than in males. According to the World Health Organization (WHO) classification they are divided into three grades (grade I, grade II, also referred as atypical meningioma and grade III). The majority of grade I is benign and counts almost 80% of all meningiomas. Compared to a good prognosis of grade I, atypical meningiomas grow and progress more rapidly and represent about 15%. Meningiomas grade III are rarer and they occur around 2% and show the most aggressive behaviour [5–7]. Brain metastases are also one of the most frequently occurring brain malignancies with poor overall survival [8].

Following the fact that prognosis and therapy depends on detecting the brain tumor type early and accurate diagnosis is crucial, this could significantly affect life quality as well as survival of the patients. Current diagnosis approaches are based on imaging methods such as computed tomography (CT) and magnetic resonance (MRI) with subsequent histological examination of biopsy. Nevertheless, these approaches are limited by brain tumor localization and heterogeneity. Therefore, it is still necessary to look for diagnostic approaches and biomarkers that are at the same time robust, sensitive and specific, and whose collection is not very invasive. The use of biomarkers found in body fluids (liquid biomarkers) appears to be a suitable approach for detecting a variety of pathological conditions including cancer. Cerebrospinal fluid (CSF), which bathes all the CNS and is in direct contact with any possible pathological components, is considered as the ideal source of these biomarkers for detecting brain tumors [9,10].

MicroRNAs (miRNAs) are single stranded, non-coding RNA which are 18–25 nucleotides in length, and post transcriptionally regulate gene expression. These molecules are usually tissue specific and involved in the pathogenesis of many diseases [11]. Circulating miRNAs were found in almost all human body fluids including CSF and they seem to be highly stable and resist extreme conditions [12]. Moreover, several studies have shown that deregulated levels of CSF miRNAs are associated with malignant tumors of CNS [13–15]. Taken together, analysis of miRNAs in CSF of brain tumor patients might help to develop a new diagnostic platform enabling more precise diagnostic approaches.

2. Material and Methods

2.1. Collection of Clinical Samples and CSF Processing and Storage

CSF samples were collected from the Department of Neurosurgery, University Hospital Brno, Czech Republic. Informed consent approved by the local Ethical Committee of University Hospital Brno (ethic code: 14-08-27-01) on 27 August, 2014, was obtained from each patient before the lumbar puncture. In the discovery phase, 89 CSF samples taken from 32 glioblastoma, 14 low-grade glioma, 11 meningioma, 13 brain metastasis patients, and 19 non-tumor patients were used for small RNAseq analysis. Subsequently, 126 CSF samples were used for the validation phase (41 glioblastoma, 8 low-grade glioma, 44 meningioma, 12 metastasis patients and 21 non-tumor patients) (summarized in Table 1). Briefly, 4–6 mL of CSF samples were obtained during the lumbar puncture between the L3 and L5 vertebrae before surgical intervention in brain tumor patients or during standard therapy management of patients with normal-pressure hydrocephalus (non-tumor patients). CSF samples containing blood-derived cells were excluded. Subsequently, CSF samples were centrifuged at 500× g for 10 min at 4 °C (Eppendorf 5810 R, Hamburg, Germany), and the supernatant were aliquoted to

1 mL tubes and stored at $-80\text{ }^{\circ}\text{C}$. The sample processing took no more than one hour. In glioblastoma patients we also collected follow-up clinical data and information on overall survival (OS).

Table 1. Groups of patients included in this study.

Group	Discovery Cohort	Validation Cohort
	N = 89	N = 126
controls (hydrocephalus)	19	21
glioblastoma	32	41
low-grade glioma	14	8
meningiomas	11	44
brain metastasis	13	12

2.2. RNA Isolation

Urine microRNA Purification Kit (Norgen Biotek, Thorold, ON, Canada) was used for isolations of all CSF samples according to manufacturer's protocol with few modifications: (i) at the elution step, samples were incubated for 20 mins on the column, (ii) we decreased the volume of elution solution to 20 μL , (iii) elution step was repeated twice with the same sample.

2.3. Small RNA Sequencing

Library preparation was performed by CleanTag Library preparation kit (Trilink Biotechnologies, L-3206, San Diego, CA, USA) according to manufacturer's protocol. The maximum volume of RNA sample was always added to reaction. Libraries were purified by Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA). The sequencing analysis was performed by Next 500/550 High Output v2 Kit with 75 cycles using the NextSeq 500 instrument (both Illumina, San Diego, CA, USA). For miRNA mapping and analysis, an online tool Chimira (Enright Lab at EMBL-EBI, Cambridge, UK) was used. Obtained data were subsequently statistically evaluated in the environment of statistical language R using the Bioconductor edgeR and DESeq2 package.

2.4. cDNA Synthesis and qRT-PCR

In the validation phase of the study, cDNA synthesis was performed by TaqManTM Advanced miRNA cDNA Synthesis kit followed by qRT-PCR using TaqManTM Fast Advanced Master Mix with individual TaqMan Advanced miRNA assays (all ThermoFisher Scientific, Waltham, MA, USA) on the QuantStudioTM 3D Digital PCR Instrument (ThermoFisher Scientific, Waltham, MA, USA). All reactions were held according to manufacturer's protocol.

2.5. Data Analysis

All real-time PCR reactions were run in triplicates and average threshold cycle and SD values were calculated. $2^{-\Delta\text{Ct}}$ method ($\Delta\text{Ct} = \text{Ct}(\text{miRNA}) - \text{Ct}(\text{average}(\text{let-7i-5p}, \text{miR-151a-3p}, \text{miR-423-3p}))$) was used for Ct values normalization. Reference miRNAs let-7i-5p, miR-151a-3p, and miR-423-3p were chosen based on the analysis of small RNAseq data using algorithms geNorm and NormFinder. LogFC was calculated as logarithm of ratio between specific miRNA average expressions of two statistically compared groups. All analyses (Mann-Whitney non-parametric tests, ROC analyses, Kaplan-Meier and long-rank test) were performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA, USA). *p*-values of <0.05 were considered statistically significant.

For discovery of diagnostic signatures (DS), and prognostic miRNA combination, logistic regression was performed. Successfully validated miRNAs were introduced into a bidirectional stepwise logistic regression model and the final model was taken as that which maximizes the Akaike information criterion. Formulas for calculation of Diagnostic Scores (DS):

$$\text{Brain tumors DS} = -1.742 + (\text{miR-30e} \times 1.139) + (\text{miR-140} \times -2.320);$$

$$\begin{aligned} \text{Glioblastoma DS} &= -2.876 + (\text{let-7b} \times -1.823) + (\text{miR-21-3p} \times 4.380) + (\text{miR-10a} \times 2.244); \\ \text{Meningioma DS} &= 2.472 + (\text{let-7b} \times -0.064) + (\text{miR-21-3p} \times -10.826) + (\text{miR-10a} \times -1.278); \\ \text{Brain metastasis DS} &= -2.571 + (\text{let-7b} \times 1.746) + (\text{miR-21-3p} \times 11.672) + (\text{miR-10a} \times -1.114). \end{aligned}$$

3. Results

In the discovery phase of the study, we successfully performed small RNA sequencing of 89 CSF samples collected from patients with brain tumors and hydrocephalus (non-tumor controls). When CSF miRNA profiles from glioblastoma patients were compared to CSF from controls, we identified 25 miRNAs to be significantly deregulated ($p < 0.001$, Table 2, Figure 1A). Low-grade glioma miRNA profiles significantly differed from controls in levels of 14 miRNA ($p < 0.1$, Table 5, Figure 1B). In CSF of meningioma and brain metastasis patients 12 miRNAs ($p < 0.01$) and 14 miRNAs ($p < 0.001$) identified to differentially expressed, respectively (Tables 3 and 4, Figure 1C–D). Based on the fold-change, significance specificity, and uniqueness for various tumor types, we selected 9 miRNAs (let-7a, let-7b, miR-10a, miR-10b, miR-21-3p, miR-30e, miR-140, miR-196a and miR-196b) to be validated in CSF specimens of independent groups of patients (41 GBMs, 8 low-grade gliomas, 44 meningiomas, 12 brain metastases and 21 non-tumor patients). Results of the validation phase are shown in Figure 2. We also proposed the Diagnostic Scores (DS) for each tumor type and the schema for stratification of brain tumor and non-tumor patients (Figure 3A), and glioblastoma, meningioma and brain metastasis patients (Figure 3B), based on a detection of miR-30e and miR-140, and let-7b, miR-21-3p and miR-10a in CSF respectively. Through ROC analysis we identified the DS thresholds enabling to stratify patients with the highest sensitivity and specificity. Specifically, DS threshold -1.883 was calculated based on CSF levels of miR-30e, and miR-140 enabled stratification of brain tumor patients and non-cancer donors with the sensitivity 76% and specificity 75% (Figure 3A). DS thresholds -0.525 , 0.033 and -2.164 were calculated based on CSF levels of let-7b, miR-21-3p and miR-10a enable stratify GBM (sensitivity 73% and specificity 75%), meningioma (sensitivity 73% and specificity 72%) and brain metastasis (sensitivity 75% and specificity 71%) from other brain tumor types (Figure 3B). In addition to this primary diagnostic approach, we described prognostic potential of the combination of miR-10b and miR-196b levels in CSF of glioblastoma patients (Figure 4). Whereas the median overall survival (OS) in patients with miR-10b/miR-196b high levels was 9 months, in patients with low levels the median OS was 16.5 months.

Table 2. MicroRNAs with the most significantly different levels in cerebrospinal fluid of glioblastoma patients in comparison to controls ($p < 0.001$) supplemented with additional miRNAs tested in the validation phase of the study listed at the bottom of the table (in italics). All miRNAs selected for the validation phase are in bold; and logFC = binary logarithm of Fold Change.

Genes	logFC	Average Expression	<i>p</i> -Value	Adjusted <i>p</i> -Value
miR-196a-5p	4.22	9.76	<0.00001	<0.00001
miR-4306	3.99	1.75	<0.00001	<0.00001
miR-10a-5p	2.64	14.78	<0.00001	<0.00001
miR-4791	-4.01	3.37	<0.00001	<0.00001
miR-30c-5p	-2.20	7.87	<0.00001	<0.00001
miR-1255b-5p	3.18	1.41	<0.00001	<0.00001
miR-30e-5p	-1.21	10.67	<0.00001	<0.00001
miR-549a	4.06	3.71	<0.00001	<0.00001
miR-10b-5p	2.21	16.15	<0.00001	<0.00001
miR-196b-5p	3.76	5.32	<0.00001	<0.00001
miR-199b-3p	1.53	14.09	<0.00001	<0.00001
miR-127-3p	-1.79	7.98	<0.00001	0.00027
let-7b-5p	1.13	17.84	<0.00001	0.00011
miR-574-5p	1.45	10.21	<0.00001	0.00027
miR-152-3p	1.39	10.42	<0.00001	0.00027
miR-1247-3p	2.43	0.43	<0.00001	0.00033

Table 2. Cont.

Genes	logFC	Average Expression	p-Value	Adjusted p-Value
miR-944	3.55	3.33	<0.00001	0.00033
let-7c-5p	1.22	15.75	<0.00001	0.00022
miR-224-5p	3.05	2.21	<0.00001	0.00039
miR-4454	−3.39	5.10	<0.00001	0.00039
miR-335-5p	3.38	4.92	<0.00001	0.00039
miR-17-3p	2.84	2.29	<0.00001	0.00039
miR-365b-5p	2.96	1.62	<0.00001	0.00059
miR-10b-3p	3.10	2.45	<0.00001	0.00072
miR-10a-3p	3.71	4.25	<0.00001	0.00078
<i>miR-140-5p</i>	1.89	4.77	0.01080	0.05594
<i>miR-21-3p</i>	1.14	1.97	0.11732	0.27104

Table 3. MicroRNAs with the most significantly different levels in cerebrospinal fluid of meningioma patients in comparison to controls ($p < 0.001$) supplemented with additional miRNAs tested in the validation phase of the study listed at the bottom of the table (in italics). All miRNAs selected for the validation phase are in bold; and logFC = binary logarithm of Fold Change.

Genes	logFC	Average Expression	p-Value	Adjusted p-Value
miR-196a-5p	4.01	9.76	<0.00001	0.00001
miR-10a-5p	2.82	14.78	<0.00001	0.00003
miR-549a	4.69	3.71	<0.00001	0.00059
miR-196b-5p	4.06	5.32	<0.00001	0.00103
miR-199b-3p	1.65	14.09	0.00001	0.00109
miR-101-3p	1.56	11.93	0.00001	0.00118
miR-152-3p	1.62	10.42	0.00001	0.00137
miR-10a-3p	4.37	4.25	0.00003	0.00315
miR-148a-3p	1.70	11.92	0.00005	0.00489
miR-140-5p	3.39	4.77	0.00008	0.00695
miR-1247-5p	3.26	1.55	0.00011	0.00841
miR-205-5p	4.45	4.29	0.00014	0.00953
<i>miR-10b-5p</i>	1.77	16.15	0.00039	0.01893
<i>let-7b-5p</i>	0.69	17.84	0.01600	0.13214
<i>let-7c-5p</i>	0.83	15.57	0.00830	0.09333
<i>miR-30e-5p</i>	−0.05	10.67	0.84957	0.95825
<i>miR-21-3p</i>	0.75	1.97	0.44158	0.76889

Table 4. MicroRNAs with the most significantly different levels in cerebrospinal fluid of brain metastases patients in comparison to controls ($p < 0.001$) supplemented with additional miRNAs tested in the validation phase of the study listed at the bottom of the table (in italics). All miRNAs selected for the validation phase are in bold; and logFC = binary logarithm of Fold Change.

Genes	logFC	Average Expression	p-Value	Adjusted p-Value
miR-5100	−5.28	2.33	<0.00001	0.00011
miR-92a-3p	2.50	13.27	<0.00001	0.00011
miR-143-3p	1.86	14.69	<0.00001	0.00023
miR-196a-5p	3.34	9.76	<0.00001	0.00033
miR-196b-5p	4.08	5.32	<0.00001	0.00033
miR-490-3p	−4.82	1.37	<0.00001	0.00036
miR-1247-5p	3.70	1.55	<0.00001	0.00036
miR-199b-3p	1.67	14.09	<0.00001	0.00036
miR-21-3p	4.22	1.97	0.00001	0.00062
miR-3607-3p	−4.21	0.79	0.00001	0.00067
miR-205-5p	4.87	4.29	0.00001	0.00067
miR-532-5p	2.77	8.05	0.00001	0.00067
miR-381-3p	3.24	5.97	0.00001	0.00067

Table 4. Cont.

Genes	logFC	Average Expression	p-Value	Adjusted p-Value
miR-10a-5p	2.28	14.78	0.00001	0.00067
miR-10b-5p	1.72	16.15	0.00041	0.00919
let-7b-5p	0.43	17.84	0.11602	0.38548
let-7c-5p	0.39	15.75	0.21442	0.51985
miR-140-5p	2.60	4.77	0.00247	0.03024
miR-30e-5p	-0.47	10.67	0.08626	0.32384

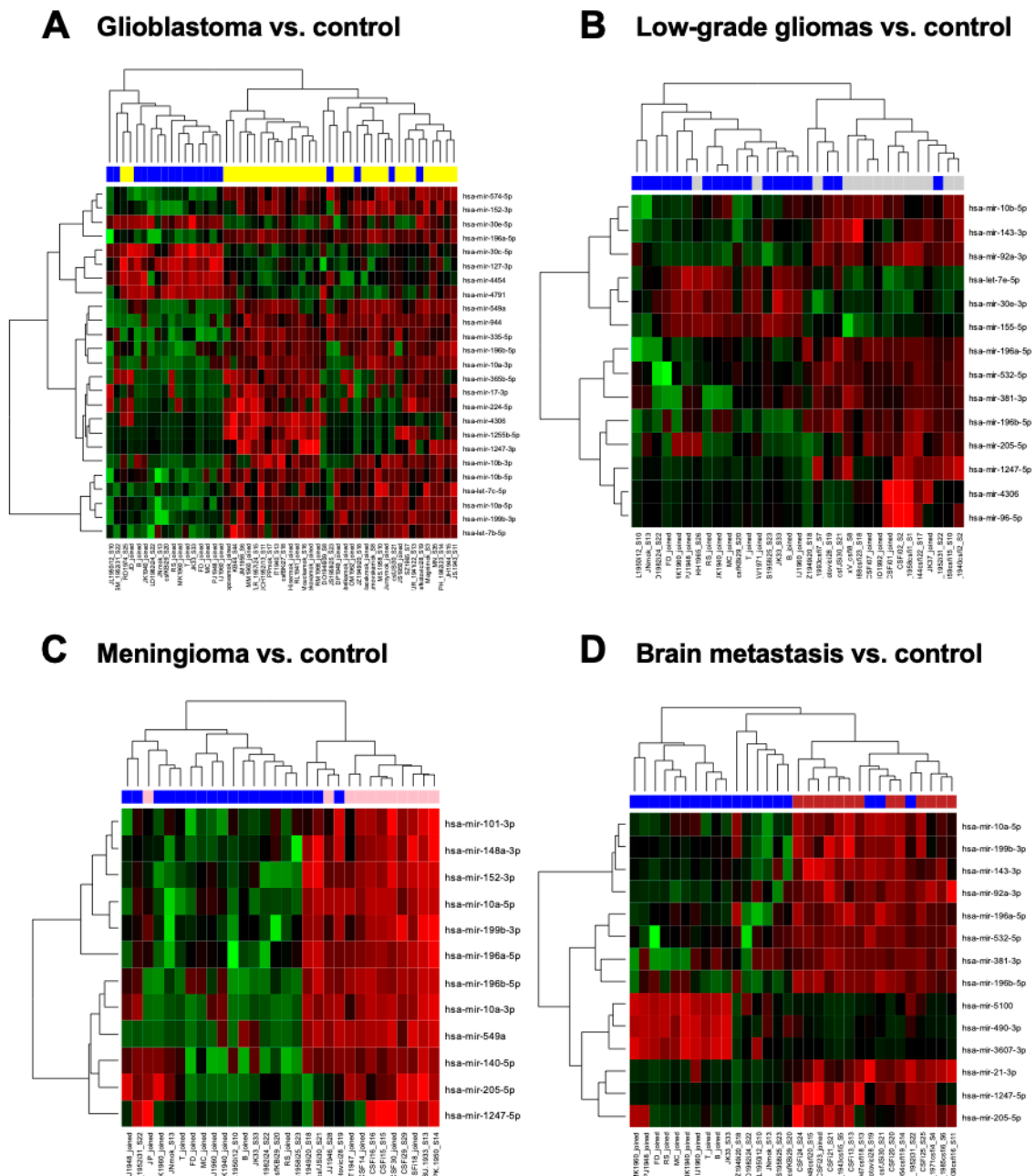


Figure 1. Hierarchical clustering based on cerebrospinal fluid (CSF) miRNA expression profiles of glioblastomas and controls (A); low-grade gliomas and controls; (B) meningiomas and controls (C); and brain metastases and controls (D). Blue color always indicates CSF specimen collected from control individual. A gradient of green and red colors is used in the heatmap (green color indicates lower expression whereas red color indicates higher expression of individual miRNAs in analyzed samples).

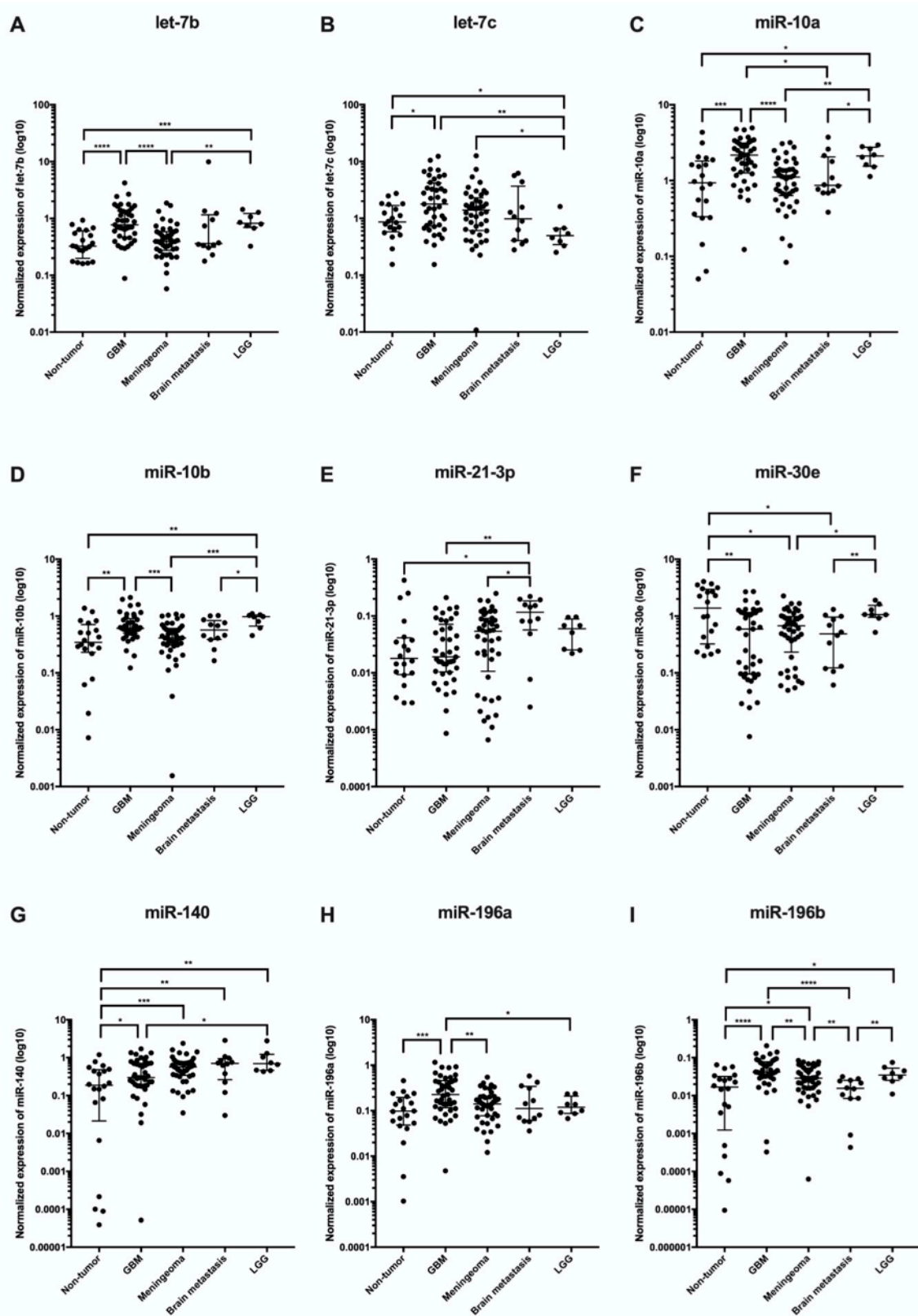


Figure 2. Validation of candidate cerebrospinal fluid miRNA biomarkers (A let-7b, B let-7c, C miR-10a, D miR-10b, E miR-21-3p, F miR-30e, G miR-140, H miR-196a, I miR-196b). In controls, patients with glioblastoma (GBM), meningioma, brain metastasis, and low-grade glioma (LGG).

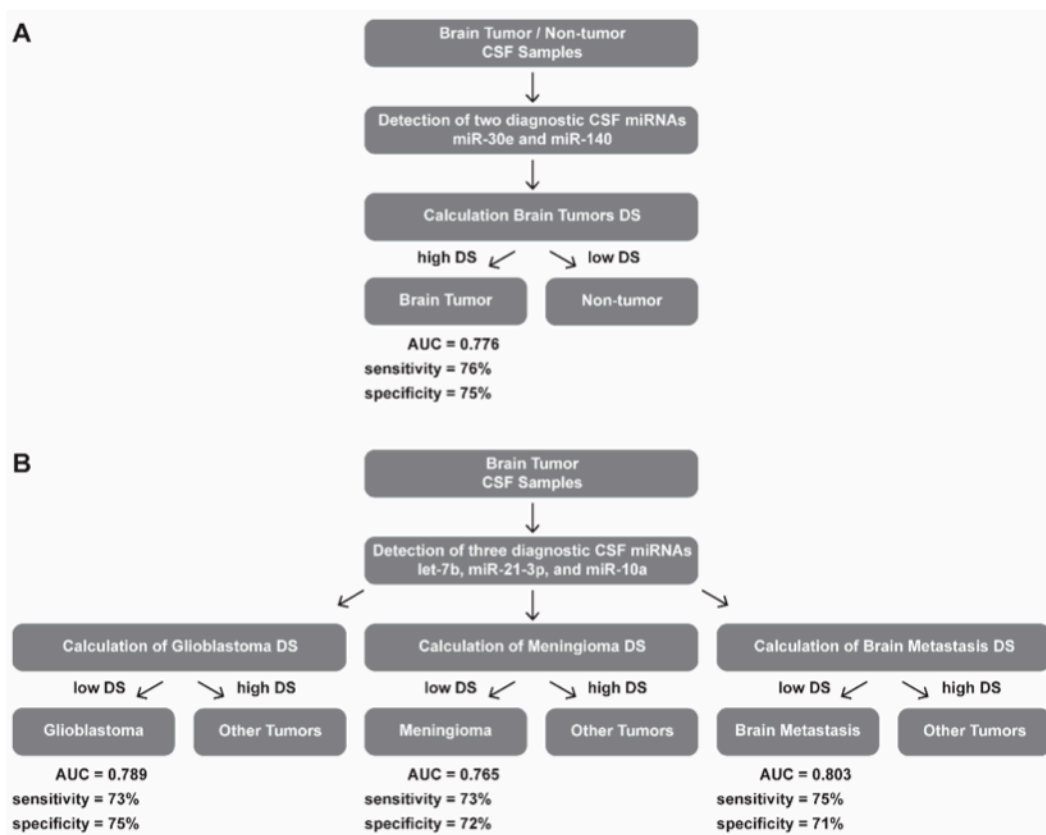


Figure 3. Diagnostic schemas for brain tumor patients stratification of (A) brain tumor and non-tumor patients; and (B) glioblastoma, meningioma and brain metastasis patients based on detection of selected miRNAs in CSF. DS = Diagnostic Score; AUC = Area Under Curve; and CSF = Cerebrospinal fluid.

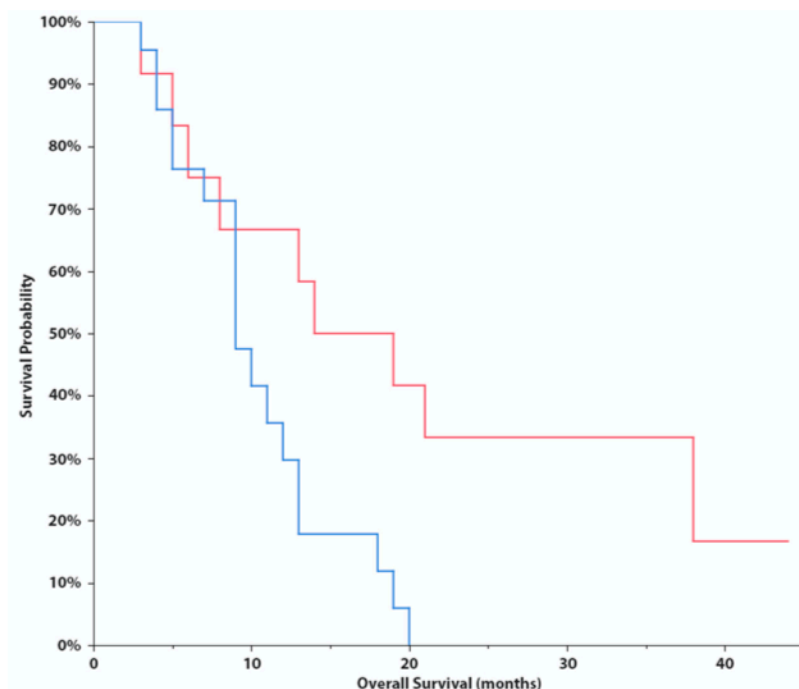


Figure 4. Kaplan-Meier survival curves estimating overall survival in patients with glioblastoma according to combined cerebrospinal fluid levels of miR-10b and miR-196b (low levels in red - median OS = 16.5 months; and high levels in blue - median OS = 9 months; $p = 0.0170$, Log-Rank test).

Table 5. MicroRNAs with the most significantly different levels in cerebrospinal fluid of low grade glioma patients in comparison to controls ($p < 0.01$) supplemented with additional miRNAs tested in the validation phase of the study listed at the bottom of the table (in italics). All miRNAs selected for the validation phase are in bold; and logFC = binary logarithm of Fold Change.

Genes	logFC	Average Expression	<i>p</i> -Value	Adjusted <i>p</i> -Value
miR-381-3p	3.37	5.97	0.00000	0.00334
miR-205-5p	4.31	4.29	0.00011	0.03741
miR-92a-3p	1.88	13.27	0.00013	0.03741
miR-532-5p	2.28	8.05	0.00031	0.05360
miR-1247-5p	2.72	1.55	0.00032	0.05360
miR-155-5p	−2.15	8.63	0.00049	0.06435
miR-196a-5p	2.45	9.76	0.00054	0.06435
miR-196b-5p	2.94	5.32	0.00087	0.07357
miR-96-5p	1.79	−0.24	0.00148	0.09360
miR-4306	2.55	1.75	0.00157	0.09360
miR-30e-3p	−1.36	10.03	0.00139	0.09360
miR-143-3p	1.27	14.69	0.00088	0.07357
hsa-let-7e-5p	−0.87	11.75	0.00143	0.09360
miR-10b-5p	1.58	16.15	0.00088	0.07357
<i>let-7b-5p</i>	<i>0.25</i>	<i>17.84</i>	<i>0.36461</i>	<i>0.77936</i>
<i>let-7c-5p</i>	<i>−0.06</i>	<i>15.75</i>	<i>0.86022</i>	<i>0.95823</i>
<i>miR-10a-5p</i>	<i>1.46</i>	<i>14.78</i>	<i>0.00520</i>	<i>0.14588</i>
<i>miR-140-5p</i>	<i>2.24</i>	<i>4.77</i>	<i>0.00914</i>	<i>0.20606</i>
<i>miR-21-3p</i>	<i>1.00</i>	<i>1.97</i>	<i>0.27474</i>	<i>0.73439</i>

4. Discussion

The overall aim of our study was to identify specific CSF miRNA patterns that could differentiate among brain tumors in the largest cohort of patients published so far. From a translational perspective, our aim was to identify new biomarkers that can aid borderline or uncertain imaging results onto the diagnosis of CNS malignancies, avoiding most invasive procedures such as stereotactic biopsy or biopsy. Therapeutic strategies could be planned in advance improving patients' quality of life. Moreover, the identification of such biomarkers could help in finding alternative therapeutic targets. Based on the knowledge that CSF is the CNS biological fluid, that it flows only in the CNS, and it is easily collectable by a spinal tap at the lumbar cisternae level, we also hypothesized that CSF would be the ideal biological fluid to find CNS biomarkers [15]. On the other hand, miRNAs have demonstrated their great ability to classify human cancers [16,17] and to be very stable RNAs in CSF [9]. CSF also has the advantage to contain fewer miRNAs than blood plasma or serum, which are, instead, flowing throughout the body and, thus, less tissue specific and more vulnerable to contaminations from blood cellular components.

Our results indicate a very good potential of CSF miRNAs in primary diagnostics of brain tumors and their potential supportive value in the diagnostic process in cases with borderline or uncertain imaging results. We identified CSF miRNA signatures for all studied cancer types. Some of the miRNAs identified in our study were already described by others, for instance miR-10b increased in CSF of GBM patients [14] or miR-21 in CSF of patients with brain metastasis [14,15]. Until now, there was no study published which focused on miRNA levels in CSF of meningioma patients.

In the validation phase of our study, we confirmed very good reproducibility and robustness of CSF miRNAs as biomarkers. We successfully validated all miRNAs identified by small RNA sequencing to have significantly (adj. $p < 0.05$) different levels in CSF of glioblastoma cases also by use of qRT-PCR method. In cases of meningioma we confirmed 2 out of 5 miRNAs, and in brain metastasis 2 out of 6 miRNAs were independently validated. We suppose that lower validation success in meningiomas and brain metastasis is caused by smaller cohorts in both explorative and validation phases in comparison with GBM. Last but not least, we successfully validated 4 out of 6 miRNAs in

low-grade gliomas with different levels ($p < 0.05$) in the exploratory phase. We used these miRNAs to establish a diagnostic schema for brain tumor patient stratification based on the detection of only five miRNAs in CSF. Moreover, we were able to show that 2 miRNAs measured in pre-operatively collected CSF indicated prognostic functioning in patients with glioblastoma (Figure 4). This could potentially present clinically very important information since in cases with borderline resectable tumors, prognostic information could also be considered as a factor for the decision making process regarding surgical intervention. We also believe that another potential clinical application of our observations is an improvement of the low-grade glioma diagnosis since occurrence of glioblastoma CSF miRNA profiles in these cases could be considered an indicator of the presence of high-grade focuses which could be “overlooked” within the standard diagnostic process. Specifically, our results indicate that let-7c, miR-140 and miR-196a show significantly different levels in glioblastoma and low-grade glioma patients’ CSF. Although a lot of studies have described possible functions of miRNAs at the cellular and molecular levels, there are only a few studies focused on the cell-free miRNAs to be brain tumor biomarkers, and none from them describe our successfully validated miRNAs as potentially diagnostic biomarkers. Only Regazzo et al., detected let-7c levels in pre-surgery blood serum obtained from GBM, WHO II-III glioma and meningioma patients and healthy donors. They did not observe any differences among examined groups, so this corresponds with previous conclusions that CSF seems to be a more sensitive diagnostic biofluid in comparison with blood plasma and serum in brain tumors [18,19]. Many more studies have been published in relation to tumor brain biomarkers and tissue miRNAs. Among all, miR-196a and miR-196b showed increased expression levels in GBMs relative to both anaplastic astrocytomas and normal brain tissues, which is consistent with our results since both miRNAs had significantly higher levels in CSFs from GBM patients than from non-tumor donors. Moreover, miR-196a seems to be associated with glioma progression and the prognostic role of miR-196b was suggested in GBM patients [20]. Another two studies described increasing tissue expression levels of miR-196a upon progression of low-grade gliomas to the GBM [21,22]. In accordance with our results, miR-10b was upregulated in GBM tissue compared to brain tissue of non-neoplastic controls. However, we did not observe different CSF levels of miR-10b between GBM and WHO I-III gliomas like Visani et al. [23]. Whereas our study shows significantly higher levels of let-7b in CSF from GBM patients in comparison to non-tumor donors, in GBM tissues the levels were described to have lower expression of let-7b [24] indicating active release of this tumor suppressive miRNA by glioblastoma cells. Similarly, miR-140 showed increased expression upon progression of WHO grade II to glioblastomas [25] whereas our results indicate higher CSF levels of miR-140 in low-grade glioma patients. However, analysis of these small non-coding RNAs in CSF is still not fully standardized and there are many factors that could bias the results. Thus, optimization and standardization of individual steps of the whole analytical process could bring CSF miRNAs closer to the clinical utilization [26].

5. Conclusions

In conclusion, we performed the largest study so far focused on CSF miRNA profiling in patients with brain tumors. We described significant differences in CSF miRNA levels in patients with all tested tumor types by the use of small RNA sequencing which is the most comprehensive method of miRNA profiling. The majority of the miRNA candidates we have also successfully validated in independent cohorts of patients by standard qRT-PCR method. Based on our results, we believe that CSF miRNAs have a strong potential as the diagnostic and prognostic biomarkers in patients with brain tumors.

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MicroRNAs involved in chemo- and radioresistance of high-grade gliomas

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Abstract High-grade gliomas (HGGs) are malignant primary brain tumors of glial cell origin. Despite optimal course of treatment, including maximal surgical resection followed by adjuvant chemo- and/or radiotherapy, the prognosis still remains poor. The main reason is the commonly occurring chemo- and radioresistance of these tumors. In recent years, several signaling pathways, especially PI3K/AKT and ATM/CHK2/p53, have been linked to the resistance of gliomas. Moreover, additional studies have shown that these pathways are significantly regulated by microRNAs (miRNAs), short endogenous RNA molecules that modulate gene expression and control many biological processes including apoptosis, proliferation, cell cycle, invasivity, and angiogenesis. MiRNAs are not only highly deregulated in gliomas, their expression signatures have also been shown to predict prognosis and therapy response. Therefore, they present promising biomarkers and therapeutic targets that might overcome the resistance to treatment and improve prognosis of glioma patients. In this review, we summarize the current knowledge of the functional role of miRNAs in gliomas resistance to chemo- and radiotherapy.

Keywords microRNA · Glioma · Chemoresistance · Radioresistance

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Introduction

Gliomas account for approximately 80 % of all primary malignant brain tumors; despite improvements in clinical care over the last 20 years, they still remain associated with high mortality. Prognosis of patients with glioblastoma multiforme (GBM), the most common histological subtype of high-grade gliomas (HGGs), is very poor with median survival of 12–15 months from diagnosis (CBTRUS 2012) [1, 2]. Current management of HGGs is based on a maximal surgical resection and adjuvant chemoradiotherapy, which leads to inhibition of the cell cycle and increased apoptosis of tumor cells [3, 4]. However, HGGs are well known for early recurrence, which is caused by chemo-radioresistance of some of the tumor cells. Thus, several mechanisms of glioma resistance have been described; the most important of which is the deregulation of signaling pathways such as PI3K/AKT [5, 6] and ATM/CHK2/p53 [7]. Another significant component of therapeutic resistance of gliomas is the presence of cancer stem cells (CSCs) and signaling pathways related to the maintenance of the stem cell-like phenotype (OCT4, SOX2, Notch, and Nanog). This assumption is supported by recent findings that CSCs generally show a higher level of resistance in comparison to non-CSCs [8, 9]. Therefore, another means to overcome the HGGs' resistance is to regulate pathways associated with cancer stem cells.

MicroRNAs (miRNAs) are emerging as an important class of endogenously produced, short (approximately 22 nt) noncoding RNAs. They have the ability to negatively regulate gene expression at the posttranscriptional level by their interaction with 3'-untranslated region of target mRNA [10]. The binding of a miRNA and its target site does not require a perfect base complementarity, and thus, one miRNA can regulate multiple targets. Alternatively, a single gene can be regulated by several miRNAs. The discovery that within miRNA targets is a significant number of

oncogenes and tumor suppressors that are deregulated in many cancers, has highlighted miRNAs as important regulators in cancer cell biology and as promising biomarkers or therapeutic targets. Recently, a number of studies have focused on a relationship between miRNAs and signaling pathways involved in GBM and HGGs pathogenesis [11]. In this review, we summarize present knowledge about miRNAs with emphasis on their role in glioma chemoradioresistance.

MiRNAs involved in radioresistance

The therapy of glioma is typically initiated by surgical resection, followed by radiotherapy to eliminate the rest of the tumor cells. Radiotherapy is focused on one important property of cancer cells—an immense proliferation. Ionizing radiation (IR) causes water ionization within the cells and so gives rise to the production of reactive radicals, which subsequently interact with DNA and disrupt the phosphate DNA backbone. DNA strand breaks caused by this interaction can be either repaired or can lead to cell cycle arrest. Depending on the response to the therapy, we can observe a long-term effect of IR which is manifested as senescence of the tumor cells, or a short term effect which is cell death via apoptosis [12]. Relapse of the tumor after radiotherapy is common, and then, tumor often progresses into more aggressive forms associated with poor prognosis and resistance to further treatments [13]. It was previously described in several studies that IR triggers DNA repair mechanisms and activates several signaling pathways such as PI3K/AKT [14] or ATM/Chk2/p53 [7] that subsequently leads to higher proliferation, invasivity, and survival of glioma cells (Fig. 1). In the past several years, there has been an increasing number of studies published describing miRNAs as important regulators of different pathways involved in development of gliomas as well as their role in the resistance to the treatment.

PI3K/AKT/mTOR pathway

Deregulation of PI3K/AKT/mTOR pathway occurs frequently in GBM [15]. Physiologically, the PI3K signaling pathway is critical for normal brain development; however, it has also been found to be hyperactivated in brain tumors [16, 17]. Epidermal growth factor receptor (EGFR) play an important role as an activator of this pathway since mutations in EGFR lead to tumor cell proliferation, increased survival, angiogenesis, and metastasis. Activation of EGFR allows for phosphorylation of PIP2 via PI3K to PIP3. Subsequently, PIP3 activates AKT, an important regulator of various processes in the cell. Downstream signaling components of AKT belong to regulators of the cell cycle i.e.,

glycogen synthase kinase 3, proliferation—nuclear factor kappa-B (NFκB), or mammalian target of rapamycin (mTOR) and apoptosis (BAD, MDM2, Caspase9) [17]. As indicated, activation of AKT can result in cell growth and cell survival which are closely linked to treatment resistance. These capabilities make AKT an attractive therapeutic target.

One of the ways of targeting the PI3K/AKT pathway and its downstream components for intervention is offered by miRNAs. Study of miRNA expression profiles after IR exposure in the U87MG glioblastoma cell line showed downregulation of miR-181a. Based on the computational analysis and predictive tools, BCL-2, a downstream protein of AKT signaling, was chosen as a direct target for further validation. Transient overexpression of miR-181a sensitized the U87MG cell line to the IR and led to downregulation of mRNA and protein level of BCL-2 which is not only associated with radioresistance but also plays a protective role against apoptotic cell death and is frequently overexpressed in human tumor cells [18, 19]. Another miRNA involved in the AKT signaling is miR-21, which is generally classified as an oncomiR. MiR-21 was one of the first identified miRNAs to play an important role in glioma pathogenesis with an antiapoptotic effect on HGGs. Computational analysis revealed phosphatase and tensin homolog (*PTEN*), a direct negative regulator of AKT, as a target gene of miR-21. However, after downregulation of miR-21, the expression of EGFR and BCL2 was decreased, AKT and cyclin D were activated, but the status of PTEN appeared to be independent of miR-21 [20]. In another study, PTEN was found to be regulated directly by miR-26a [21]. MiR-26a alone is able to transform cells by promoting GBM cells growth in vitro and in vivo. Cell growth is enhanced either by decreased PTEN, RB1, or MAP3K2/MEKK2 protein expression, which subsequently leads to increased AKT activation and promotes proliferation, or by decrease of c-JUN N-terminal kinase-dependent apoptosis [22].

Additional miRNAs linked to AKT regulation are miR-7 and miR-451 [23, 24]. The involvement of miR-7 in this pathway was evaluated on U251 and U87 glioma cell lines. Ectopic overexpression of miR-7 attenuated EGFR and AKT expression and radiosensitized both glioma cell lines. Furthermore, it prolonged radiation-induced γH2AX foci formation and led to downregulation of DNA-dependent protein kinases (DNA-PKs). This indicates a relationship between delayed DNA damage repair and increased radiation-induced cell killing by overexpression of miR-7, which leads to downregulation of the EGFR-AKT signaling network [14]. AKT also plays an important role in DNA repair after IR through controlled DNA-PK expression. After irradiation, recognition of DNA double-strand breaks during nonhomologous end joining is carried out

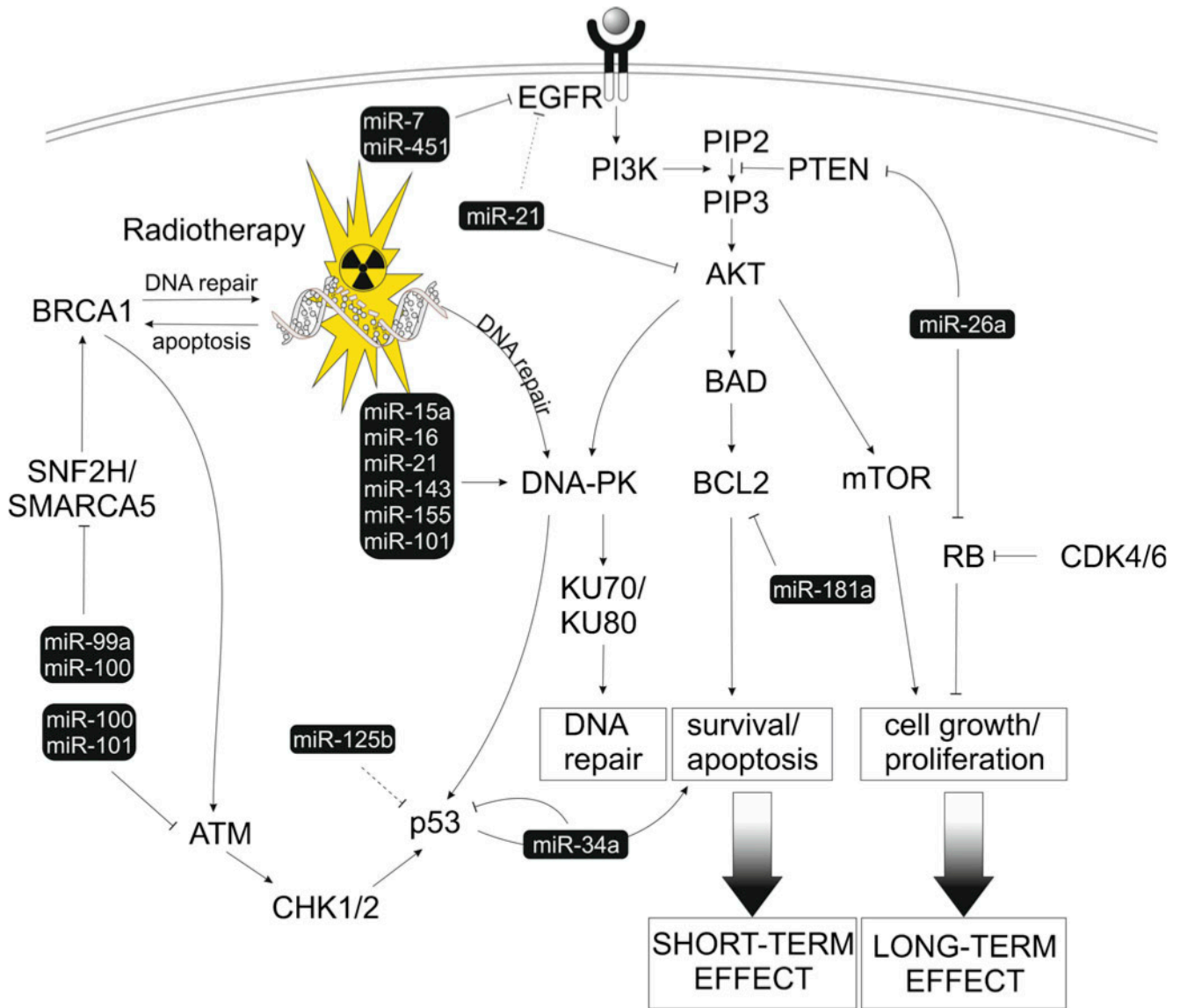


Fig. 1 MiRNAs and pathways involved in radioresistance of gliomas. After IR which causes DNA damage, several pathways are activated to protect tumor cells from apoptosis and give them the ability of uncontrolled growth and proliferation. Among these pathways belong the PI3K/AKT and ATM/Chk2/p53 pathways which are tightly connected to and regulated by several miRNAs. The main player of the first pathway is AKT, which is, inter alia, responsible for the

activation of DNA-PK and also for DNA repair, then it activates antiapoptotic factor BCL2 or mTOR, which ensures cell growth and proliferation. ATM/CHK2/p53 pathway, which is activated by BRCA1 after DNA damage is important for cells as well. The survival and cell apoptosis is dependent on its downstream proteins activation, such as CHK1/2 and p53

by the heterodimers Ku70/Ku80, which are activated by the DNA-PKs [25, 26]. It seems that miR-7 could be a useful therapeutic target for overcoming the radioresistance of human cancers with activated EGFR-PI3K-AKT signaling [14].

ATM/Chk2/p53 pathway

Loss of ATM/Chk2/p53 pathway components accelerates glioma development and contributes to radiation resistance. In response, IR cells activate the sensor kinases ATM, ATR,

and DNA-PKs that phosphorylate multiple downstream mediators, including the checkpoint kinases Chk1 and Chk2, which lead to checkpoint initiation in cell cycle and/or to apoptosis [7]. A lower level of ATM was observed in the M059J radiosensitive cell line when compared to the M059K radioresistant cell line due to deficiency in DNA-PK. This effect might be caused by overexpression of miR-100, which was predicted to be a direct regulator of ATM [27]. After IR of both M059K and M059J cell lines, several miRNAs were upregulated: miR-17-3p, miR-17-5p, miR-19a, miR-19b, miR-142-3p, and miR-142-5p. Moreover,

miR-15a, miR-16, miR-21, miR-143, and miR-155 were found to be upregulated only in the M059K cell line with normal DNA-PK activity [28]. These findings are supported by a study focused on lymphoblastoid cells, where low-dosage IR also caused upregulation of miR-15a, miR-16, and miR-21 in cells with a normal status of p53 [29]. Furthermore, miR-143 was found to directly target fragile histidine triad (FHIT), which is often downregulated in epithelial tumors. Cells with homozygous deletion of FHIT show higher resistance to multi-DNA damage inducers, including IR. Overexpression of miR-143 protects cells from DNA damage-induced killing by downregulation of FHIT expression leads to significant G2-phase arrest [30]. Increased level of miR-155 was also found in lung cancer cells. This miRNA protected the cells against IR and inhibition of this miRNA led to sensitization of cells to radiation [31]. Overall, these observations point to the involvement of miRNAs in the different responses of GBM cells to treatment by IR, which are often seen in brain tumors, especially in HGGs [29].

MiR-101 is another miRNA associated with the protein levels of ATM and DNA-PK in the U87MGD cell line. Upregulation of miR-101 by lentiviral transduction sensitized tumor cells to radiation *in vitro* and *in vivo* [32]. These observations are supported by a study on lung cancer cell lines, which demonstrated that ectopic expression of miR-101 could sensitize human tumor cells to radiation by targeting ATM and DNA-PK [33].

For repair of double-strand breaks nascent after irradiation, chromatin remodeling protein complexes are required. MiR-99 expression was described to correlate with sensitivity to IR as it targets the SWI/SNF chromatin remodeling factor SNF2H/SMARCA5, a component of the ACF1 complex, which plays an important role in double-strand break repair [34]. Moreover, it has been elucidated that reduction of BRCA1 level at the DNA damage site was the result of downregulation of SNF2H, which was caused by induction of miR-99a and miR-100. These observations were further supported by an experiment where ectopic expression of the miR-99 family in cells reduced the rate of overall efficiency of repair by both homologous recombination and nonhomologous end joining [34, 35].

ATM encodes a protein kinase that acts as a tumor suppressor. This kinase is activated by IR damage of DNA, it stimulates DNA repair, and blocks cell cycle progression. One of the mechanisms of ATM function is by ATM dependent phosphorylation of p53, which either arrests the cell cycle at a restriction point to allow for the DNA damage repair or leads to the apoptosis of damaged cells. The p53 is indeed a central regulator of cell response to stress and it has to be tightly regulated [36]. Bioinformatics suggested that miR-125b is a negative regulator of p53-induced apoptosis during cell stress [37]. Likewise, miR-

34a acts as a tumor suppressor in p53-mutant glioma cells U251. Overexpression of miR-34a, which is transcriptionally activated through p53, led to cell growth inhibition, cell cycle arrest in G0-G1, induction of apoptosis, and significantly reduced migration and invasion capabilities. Such events could also be due to regulation of SIRT1, which is predicted to be a direct target of miR-34a [38]. This is also supported by a study, where high dosage of IR lead to induction of miR-34 and reduced the p53 expression level [39]. Effects of miRNA modulation on radioresistance are summarized in Table 1.

MiRNAs involved in chemoresistance

Temozolomide (TMZ) is a commonly used alkylating agent in the treatment of gliomas; however, after prolonged exposure a development of resistance is frequently observed. Unfortunately, there is little known about the signaling pathways affected by TMZ [40]. Cytotoxicity of TMZ is based on methylation of guanines in DNA at the O⁶ position and also on the base-pair mismatch with thymine. Glioma cells frequently have activated repair mechanisms to overcome this substitution through DNA repair enzyme O⁶-guanine-DNA methyltransferase (MGMT) and become resistant to TMZ exposure [41]. In nonresistant glioma cells, TMZ induces cell death partly by the fact that the MGMT promoter is hypermethylated [42]. Interestingly, miR-181 family members have a capacity to regulate MGMT and so, increase the chemosensitivity to TMZ. Silencing of the miR-181 family led to increased levels of MGMT, suggesting that the miR-181 family could be involved in posttranscriptional regulation of MGMT and could also be used as a predictive factor of the response to TMZ [43, 44]. An experiment with the chemoresistant glioma cell line U118 indicated a functional relationship between TMZ and survival pathways PI3K/AKT and ERK1/2 MAPK. These pathways are activated during treatment with TMZ; however, after inhibition of these pathways, the chemoresistance was partially eradicated [40] (Fig. 2).

As mentioned above, many studies described the overexpression of miR-21 in gliomas [45-50]. Downregulation of miR-21 in GBM cells led to repression of cell growth, increased cellular apoptosis and cell cycle arrest, which might enhance the chemotherapeutic effect in cancer therapy. Overexpression of miR-21 in glioma cell line U87 protected the cells against TMZ-induced apoptosis via decreasing the BAX/BCL-2 ratio and Caspase-3 activity, thereby contributing to chemoresistance [47, 48]. An opposite effect was observed for miR-221/222 downregulation in the same cell line, where it caused an increase of sensitivity to TMZ and apoptosis, independently of p53 status. Simultaneously, an increase in expression of apoptotic factors BAX, cytochrome c, and cleaved-

Table 1 Effect of miRNA modulation on radioresistance

miRNA	Up-/downregulation	Effect on radioresistance	Direct and indirect targets	References
miR-181a	↓	+	BCL-2	[18, 19]
miR-7	↓	+	EGFR, AKT, DNA-PKs	[14]
miR-143	↑	+	FHIT	[28, 30]
miR155	↑	+	GABRA1	[31, 70]
miR-101	↓	+	ATM, DNA-PK	[32]
miR-99	↑	-	SNF2H/SMARCA5	[34]
miR-100	↑	-	SNF2H/SMARCA5	[35]
mir-34a	↑	+	p53, SIRT	[38, 39]
miR-17-3p	induction after IR		GalNT7, vimentin, MDM2	[28, 71, 72]
miR-17-5p	induction after IR		E2F1, PTEN	[28, 71, 72]
miR-19a	induction after IR		PTEN, TNF- α	[28, 73-75]
miR-19b	induction after IR		CUL5	[28, 73]
miR-142-3p	induction after IR			[28]
miR-142-5p	induction after IR			[28]

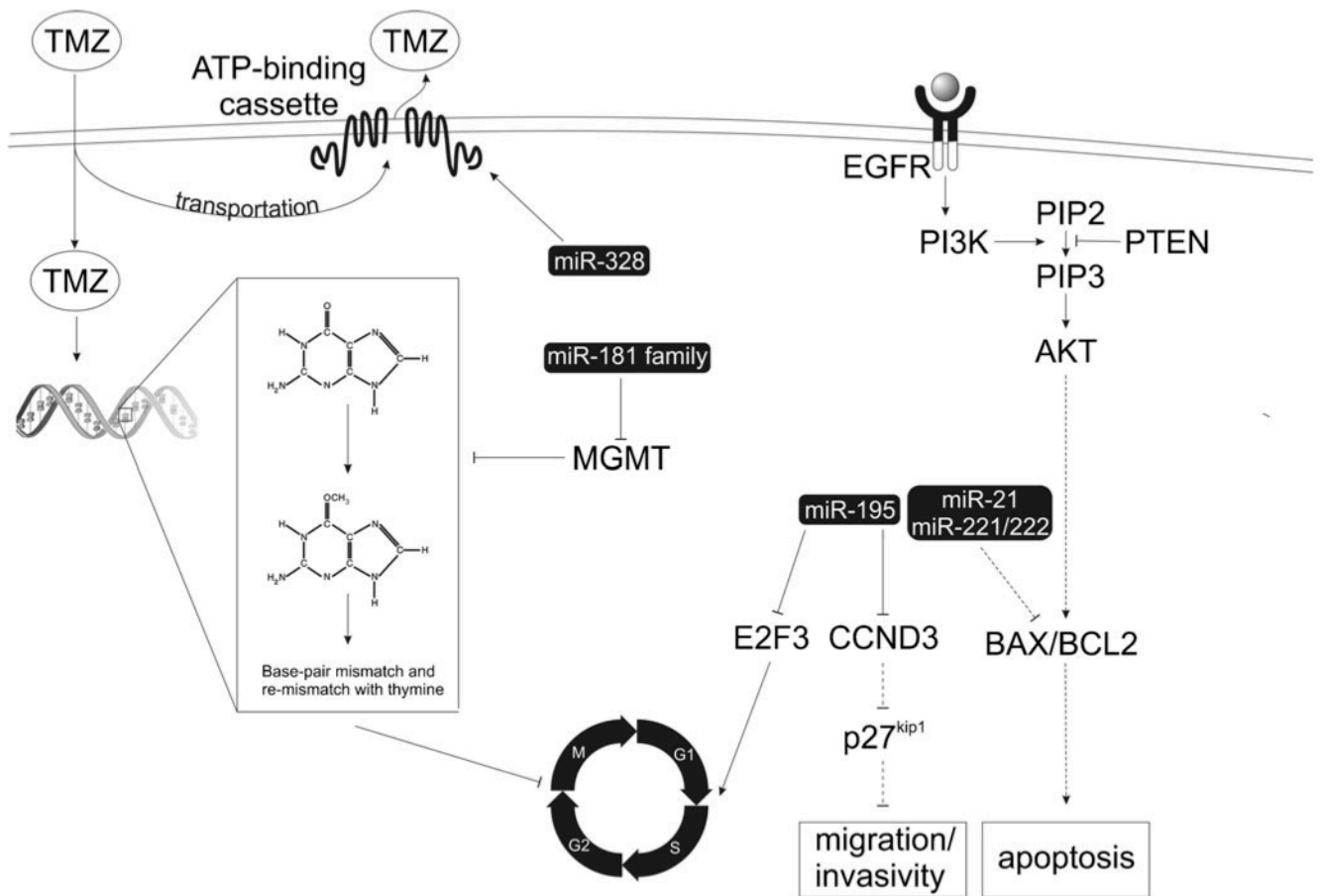


Fig. 2 MiRNAs involved in chemoresistance of gliomas. Glioma cells have several possibilities on how to overcome the treatment of alkylating agents such as TMZ. They evolved special mechanisms, i.e., O⁶-methylguanine methyltransferase (MGMT) enzyme, which is able to excise the nucleotides in DNA base-pair mismatch, and so, neutralize the effect of TMZ. This is directly controlled by miR-181 family. Another approach is by ATP-binding cassette transporters,

which contribute equally to chemotherapy resistance by transport and efflux of anticancer drugs. The inhibition of their activity is mediated by miR-328. Moreover, proteins which are activated as a secondary consequence of TMZ treatment can be regulated by miRNAs, and so, inhibit the survival of the cells (miR-21, miR-221, and miR-222) or arrest cell cycle by inhibition of transcription factors (miR-195)

caspace-3 was observed [51]. In the TMZ resistant GBM cell line U251R, the expression levels of miR-195, miR-455-3p, and miR-10a(*) were increased in comparison to the U251MG TMZ sensitive cell line. Inhibition of miR-455-3p or miR-10a(*) showed a modest cell killing effect in the presence of TMZ compared to the suppression of miR-195, which in combination with TMZ, strongly enhanced cell death [52]. An inverse correlation was found between E2F transcription factor 3 (E2F3) and cyclin D3 (CCND3) protein levels and expression of miR-195 in glioma cells, suggesting that these proteins might be direct targets of miR-195. Downregulation of E2F3 can suppress the transcription of cell cycle related genes and cause accumulation of cells in G1 phase and inhibition of cell cycle progression. As for CCND3, its suppression mediated by miR-195 can upregulate the expression of cytoplasmic p27^{Kip1}, which negatively regulates G1 phase and so affects the expression of other proteins involved in cell migration, resulting in repression of GBM cells invasion [53].

Besides TMZ, there are other therapeutic agents used in the treatment of gliomas, such as VM-26 (Teniposide). MiR-21 was found to mediate chemoresistance to VM-26 in U373MG cells, where suppression of miR-21 led to enhancement of cytotoxicity of VM-26. *LRRFIP1* was confirmed and validated as a direct target of miR-21, whose product is an inhibitor of NFκB factor, a downstream effector of PI3K/AKT signaling [50]. In another study, an anti-sense-miR-21 oligonucleotide significantly improved the effect of 5-FU on U251 glioma cells and increased apoptosis with a simultaneous decrease of migration ability [46].

There are other likely mechanisms which are involved in the chemoresistance of glioma cells. One of these mechanisms is based on the activity of proteins from ATP-binding cassettes family, which are able to transport various molecules across cellular membranes, i.e., they are able to export antitumor drugs or their metabolites from cells [9]. ATP-binding cassette, a subfamily of G member 2 protein family (ABCG2), is highly expressed in GBM and is associated with multidrug resistance. MiR-328 contributes to the chemosensitivity by decrease of ABCG2 expression. Thus, inhibition of ABCG2 by miRNA can lead to better treatment outcomes for patients [54]. Effects of miRNA modulation on radioresistance are summarized in Table 2.

Glioma initiating cells/stem cells and their role in resistance

Over the past several years, there has been an intense discussion about the effect of glioma stem cells (GSCs) on the chemo-/radioresistance of gliomas and their early relapse [55]. GBM is a very heterogeneous tumor that contains cells in different states of differentiation. On the top of the cell hierarchy are probably GSCs, which have the ability of self

renewal, differentiation into progeny cancer cells, and unlimited proliferation, as well as initiation of neurospheres' growth in vitro and generation of highly malignant tumors in NOD/SCID mice. Similarly to normal stem cells, GSCs express markers such as CD133, nestin, OCT4, SOX2, and Nanog [56]. Inhibition of Sonic hedgehog (SHH) and Notch pathways enhanced sensitivity of CD133 positive (CD133+) glioma cells to the TMZ therapy [57]. SHH has been found to play a role in GSCs, especially in maintaining their undifferentiated stem cell state. Notch signaling is active in GSCs and participates in GBM pathogenesis [9]. The resistance of GSCs to TMZ is thought to be associated with higher expression of MGMT, which is able to repair the TMZ-induced DNA damage. Furthermore, Notch and SHH were shown to be upregulated in GSCs and their inhibition led to increased susceptibility of these cells to TMZ treatment (Fig. 3).

Similar results were obtained for ABCG2, which, as mentioned above, is implicated in the TMZ resistance. The activation of other ATP-binding cassettes ABCC3 and ABCC6 with inhibitor of differentiation 4 (ID4), led to the chemoresistance of cells, whose resistance was mediated by SOX2 induction. It was found that ID4 enhances the expression of SOX2 through miR-9* suppression, which represses SOX2 by targeting its 3'-untranslated region [58]. Similar to miR-9*, miR-145 seems to be the key factor of reduction of CSCs-like properties and chemoresistance, through the direct downstream inactivation of SOX2 and OCT4. MiR-145 has the capacity to suppress the self renewal and initiating properties of GSCs in vitro. The capability of miR-145 to suppress the tumorigenesis, stemness, and survival rate synergistically with radio- and chemotherapy was also proved in vivo by the use of polyurethane-short branch polyethylenimine in orthotopic GBM-CD133⁺-transplanted immunocompromised mice [59].

Furthermore, miR-34a, transcriptionally activated through p53, suppresses proliferation and induces apoptosis in GBM cells by decreasing the expression of direct target gene *Notch-1* [60, 61]. This tumor suppressor acts also as a direct regulator of c-Met, Notch-1, and Notch-2. MiR-34a enhanced expression induced GSCs differentiation and inhibited glioma xenograph growth in vivo. These findings suggest that miR-34a is a regulator of multiple oncogenic pathways in brain tumors and in differentiation of CSCs [62, 63].

It was further shown that *Notch-1* knockdown led to miR-326 upregulation. Moreover, miR-326 alone suppressed expression of Notch-1 and reduced its protein level and activity, which indicates a feedback loop. Transfection of miR-326 into GSCs lines was cytotoxic and caused reduction of tumorigenicity in vivo [64].

One of the first targets in CSCs was EGFR pathway, which is associated with CSCs via induction of self renewal

Table 2 Effect of miRNA modulation on chemoresistance

miRNA	Up-/downregulation	Effect on chemoresistance	Direct and indirect targets	References
miR-181	↓	–	MGMT	[43, 44]
miR-21	↑	–	BAX, BCL-2	[47, 48]
miR-221/222	↓	–	BAX, cyt c, Caspase3	[50]
miR-195	↑	+	E2F3, CCND3	[51]
miR-455-3p	↑	+		[53]
miR-10a(*)	↑	+	EPHX1, BRD7	[52]
miR-328	↑	–	ABCG2	[54]

and resistance to chemo- or radiotherapy [9]. MiR-146a constitutes an endogenous feedback system to counteract the oncogenic potential of dysregulated signaling pathways, such as activation of EGFR and inactivation of PTEN in gliomas. Downregulation of miR-146a caused an enhancement of GSCs formation and supported a tumor growth. Thus, miR-146a seems to be involved in regulation of GSCs stem-like properties [65].

As described above in glioma tumors, EGFR and its downstream AKT signaling play an important role in the resistance to the therapy and is frequently deregulated also in GSCs. Higher expression level of BMI-1, a downstream protein of AKT signaling, was detected in tumor-derived neurospheres, suggesting that BMI-1 contributes to GSCs proliferation [34]. BMI-1 is classified as an oncogene and stem cell renewal factor. Regulation of BMI-1 is mediated by miR-128, the overexpression of which significantly

reduced glioma cell proliferation in vitro and also glioma xenograft growth in vivo. MiR-128 caused significant downregulation of BMI-1 expression which led to the decrease of histone methylation (H3K27me(3)), AKT phosphorylation, and upregulation of p21(CIP1) levels [55].

Furthermore, it was shown that transfection of miR-124 or miR-137 into CD133+ human GBM-derived stem cells (SF6969) induced morphological changes and marker expressions consistent with neuronal differentiation. It is very well known that cells' self renewal and differentiation are closely related to the cell cycle. Therefore, it is interesting that ectopic expression of miR-124 and miR-137 also induced G1 cell cycle arrest in U251 and SF6969 GBM cells, which was associated with decreased expression of cyclin-dependent kinase 6 (CDK6) and phosphorylated RB (pSer 807/811) proteins [66]. Another study found miR-125b to be implemented in the cell

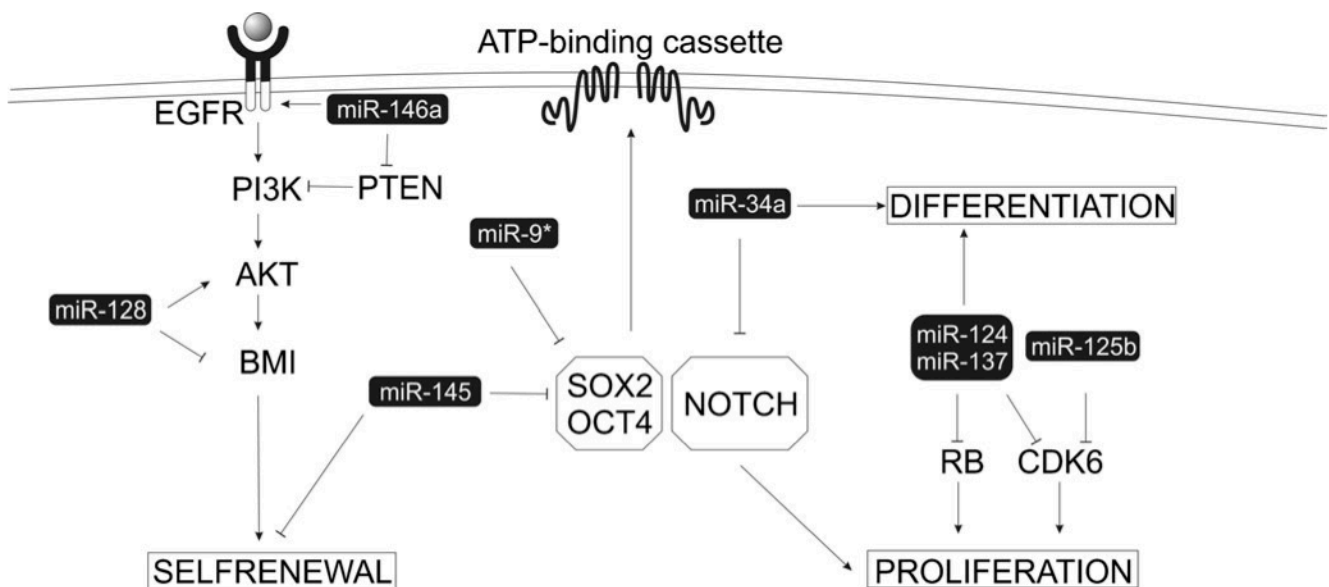


Fig. 3 MiRNAs and glioma stem cells. In GSCs (glioma stem cells) ATP-binding cassette family plays a crucial role in multidrug resistance. Their members, such as ABCC3 and ABCC6, can be activated by SOX2 where miR-9* regulates its expression. The simplified principle of the GSC defense from the effects of IR is their low proliferation activity. This is caused by cell cycle inhibition through miR-124 and

miR-137 which downregulates RB and CDK6 proteins important for the G1/S transition. The IR affects only cells which rapidly proliferate. On the other hand, GSCs have the capacity for self renewal via activation of EGFR-signaling pathway and its downstream proteins where miR-128, miR-145, and miR-146a play important roles as their regulators

cycle as it is necessary for stem cell fission to bypass the normal G1/S checkpoint and make stem cells insensitive to chemotherapy signals, which normally arrest the cell cycle at the G1/S transition. Moreover, miR-125b was associated with inhibition of CDK6 and CDC25A expression, which are necessary for Cyclin/CDK complexes activation, and thus cause cell cycle arrest [67].

MiRNAs as predictive/prognostic biomarkers in glioma translational research

One of the main goals of the HGGs research is to discover new powerful predictive and/or prognostic biomarkers, enabling a higher level of therapy individualization. MiRNAs seem to be suitable candidates for clinical biomarkers as was shown by several recent studies that focus on a link between miRNA and overall survival and time to progression in HGGs, mainly in GBM patients. Srinivasan and co-workers analyzed ten selected miRNAs in 222 tumor samples of GBM patients treated with concomitant chemoradiotherapy and an adjuvant TMZ. Their findings showed three protective miRNAs (miR-20a, miR-106a, and miR-17-5p), which were upregulated in long-term surviving patients, and seven risk miRNAs (miR-31, miR-221, miR-222, miR-148a, miR-146b, miR-200b, and miR-193a), which were conversely upregulated in short-time surviving patients [68]. In the same year, another retrospective study was published focusing on expression profiles of 35 GBM patients, who underwent an adjuvant chemoradiotherapy. The aim of this study was to discover miRNAs associated with different prognostic groups of GBM patients. Expression levels of miR-3163 (fold change (FC)=2.0, $p=0.05$), miR-539 (FC=0.5, $p=0.001$), miR-1305 (FC=0.5, $p=0.05$), miR-1260 (FC=0.5, $p=0.03$), and let-7a (FC=0.3, $p=0.02$) were significantly deregulated between patients with short- and long-time survival [69]. In our recent study, miR-195 ($p=0.0124$) and miR-196b ($p=0.0492$) were positively correlated with the overall survival of GBM patients treated with concomitant chemoradiotherapy and adjuvant TMZ. Moreover, evaluation of both miR-181c and miR-21 enabled identification of a group of patients, which relapsed in 6 months after surgery and concomitant chemoradiotherapy with TMZ (sensitivity 92 %, specificity 81 %, $p<0.0001$) [69]. The response to therapy in HGGs patients treated according to NCCN guidelines is usually evaluated in the context of survival, and it is not possible to exactly divide prognostic and predictive components of evaluated biomarkers. However, several identified predictive/prognostic miRNAs were previously functionally associated with chemo- and radioresistance of glioma cells in vitro (miR-181c, miR-21, miR-221/222, miR-195, miR-146a) indicating predictive properties of these miRNAs.

Conclusion

High-grade gliomas are the most common primary malignant brain tumors characterized by unfavorable prognosis, which is generally linked to their early relapse, occurring even after radical surgery and intensive oncology treatment. This is primarily caused by resistance of these tumors to the adjuvant chemoradiotherapy. Therefore, overcoming the resistance is nowadays one of the main challenges in HGGs research. In the past few years, several signaling pathways have been associated with decreased sensitivity of glioma cells to radiation and chemotherapy. Many genes involved in these pathways were shown to be regulated by miRNAs, which play a role in glioma pathogenesis. MiRNAs with regard to their wide regulatory capability are promising therapeutic targets which may enable the overcoming of glioma resistance to adjuvant chemo- and/or radiotherapy. Their potential use could lead to a more effective treatment, improved prognosis, and prolonged survival of HGGs patients.

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Conflicts of interest None.

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PŘÍLOHA 14

MiR-338-5p sensitizes glioblastoma cells to radiation through regulation of genes involved in DNA damage response

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Abstract Glioblastoma multiforme (GBM) is the most aggressive form of brain tumor. Despite radical surgery and radiotherapy supported by chemotherapy, the disease still remains incurable with an extremely low median survival rate of 12–15 months from the time of initial diagnosis. The main cause of treatment failure is considered to be the presence of cells that are resistant to the treatment. MicroRNAs (miRNAs) as regulators of gene expression are involved in the tumor pathogenesis, including GBM. MiR-338 is a brain-specific miRNA which has been described to target pathways involved in proliferation and differentiation. In our study, miR-338-3p and miR-338-5p were differentially expressed in GBM tissue in comparison to non-tumor brain tissue. Overexpression of miR-338-3p with miRNA mimic did not show any changes in proliferation rates in GBM cell lines (A172, T98G, U87MG). On the other hand, pre-miR-338-5p notably decreased

proliferation and caused cell cycle arrest. Since radiation is currently the main treatment modality in GBM, we combined overexpression of pre-miR-338-5p with radiation, which led to significantly decreased cell proliferation, increased cell cycle arrest, and apoptosis in comparison to irradiation-only cells. To better elucidate the mechanism of action, we performed gene expression profiling analysis that revealed targets of miR-338-5p being Ndfip1, Rheb, and ppp2R5a. These genes have been described to be involved in DNA damage response, proliferation, and cell cycle regulation. To our knowledge, this is the first study to describe the role of miR-338-5p in GBM and its potential to improve the sensitivity of GBM to radiation.

Keywords Glioblastoma multiforme · GBM · Radiation resistance · miRNA · miRNA338-5p

Andrej Besse and Jiri Sana contributed equally to this work.

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Introduction

Glioblastoma multiforme (GBM) is one of the most common and most malignant brain tumors of astroglial origin. GBM accounts for 15–20 % of all primary intracranial tumors with incidence of 3–4 new cases per 100,000 individuals. Although the incidence of GBM is rather low, the mortality is high as the tumors are extremely aggressive, giving the patients without any therapy dismal prognosis of only 3–6 months. During the past years, only modest improvements were done in the treatment of GBM. As for now, current standard therapy, including maximal surgical resection followed by concomitant chemoradiotherapy with temozolomide (TMZ) and adjuvant TMZ, can extend the median of overall survival only approximately to 12–15 months from the time of diagnosis [1, 2]. Despite aggressive treatment regimens centered on radiotherapy, tumor recurrence is nearly universal; therefore, modulation of radiation resistance is of significant interest [3]. Regrettably, the treatment has only supportive character able to extend the time of tumor recurrence followed by death [4]. Short interval of GBM recurrence suggests the presence of cells with the ability to survive the treatment without major damage. The resistant cell population is characterized by altered signaling pathways responsible for DNA repair and stability, such as ATM or PI3K-PTEN-Akt-mTOR, that are important for tumorigenesis [5–10]. Furthermore, the resistant cells have active signaling pathways responsible for DNA repair such as NHEJ and homologous recombination [7, 11].

With the discovery of RNA interference and microRNAs (miRNAs), increased evidence suggested the contribution of miRNAs in molecular biology of tumors; furthermore, there is a solid body of evidence about miRNAs' involvement in chemo- and/or radioresistance [12, 13]. MiRNAs are small non-coding RNAs with a length of approximately 22 nucleotides, which are able to post-transcriptionally regulate gene expression. They were shown to regulate multiple cellular processes, either physiological or pathological, such as apoptosis, proliferation, angiogenesis, differentiation, etc. [14]. In addition to these well-studied mechanisms of tumor pathology, recent studies have linked the acquisition of cancer resistance and increased DNA repair ability with altered expression of miRNAs [12, 13]. MiR-338 was found to be a brain-specific miRNA [15]. Additionally, it has been shown in vitro to be able to control proliferation and metastasis in neuroblastoma and oral squamous cell carcinoma [16, 17]; however, its role in GBM is still unclear. Therefore, to determine whether miR-338 has a role in GBM, we analyzed its expression in GBM samples and performed in vitro functional studies on three cell lines (A172, T98G, and U87MG) with a transiently upregulated level of both mature strands 3p/5p. The effect of miR-338-3p/5p was evaluated at the level of cell proliferation, apoptosis, and cell cycle distribution and mainly sensitivity of GBM cells to radiation.

Material and methods

Patients' characteristics

Expression of miR-338-3p and miR-338-5p strands was evaluated in a set of 40 formalin-fixed paraffin-embedded (FFPE) samples of patients with primary GBM, who underwent tumor surgical resection at the Department of Neurosurgery, University Hospital Brno; St. Anne's University Hospital Brno; and University Hospital Ostrava, Czech Republic (Table 1). As a non-tumor control, brain tissue of non-dominant anterior temporal cortexes resected during surgery for intractable epilepsy of 10 patients was used. Control brain tissues did not show any signs of dysplastic changes. An informed consent form approved by the local ethical committees was obtained from each patient before the treatment.

Cell culture

GBM primary cell lines A172, T98G, and U87MG were obtained from ATCC and cultivated in culture medium DMEM (Sigma-Aldrich, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % non-essential amino acids, 2 mM L-glutamine, 4.5 g/l D-glucose, 110 mg/l sodium pyruvate, 100 µg/ml streptomycin, and 100 U/ml penicillin (all Gibco, CA, USA). Cells were cultivated at 37 °C in humidified atmosphere containing 5 % CO₂.

Transient transfection

GBM cell lines were seeded 24 h before transfection on 24-well plates in a concentration of 2.5×10^4 cells/500 µl in DMEM supplemented with 10 % FBS without antibiotics. Transfection was performed using 10 nM of miR-338-3p and miR-338-5p mimics (pre-miR-338-3p ID: MC10716,

Table 1 Baseline characteristics of patients involved in the study

	Total (n = 40)	Percentage
Age		
≤50	18	45
>50	22	55
Gender		
Male	24	60
Female	16	40
Karnofsky performance status		
0 and 1	36	90
2	4	10
Extent of resection		
Total	7	17.5
Subtotal	29	72.5
Partial	4	10

pre-miR-338-5p ID: MC12825; Applied Biosystems, USA) or negative control (pre-miR-negative control; Applied Biosystems, USA) and an equimolar concentration of Lipofectamine® RNAiMAX (Invitrogen, USA). Effectivity of transfection was measured after 24 h using quantitative real-time PCR (qRT-PCR). For transfection of cells seeded on 60-mm dishes, the amount of reagents and cells was multiplied by 10.

RNA extraction

Total RNA enriched for a small RNA fraction was isolated from FFPE patients' samples by xylene deparaffinization, followed by purification by MirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's protocol. Nucleic acid concentrations and purities were evaluated by UV spectrophotometry using Nanodrop ND-1000 (Thermo Fisher Scientific, USA). RNA with sufficient purity evaluated by ratios $260/280 > 1.8$ and $260/230 > 1.8$ was used for further study.

Quantitative real-time PCR

Evaluation of specific miRNA expression was performed using TaqMan miRNA individual assays: hsa-miR-338-3p, hsa-miR-338-5p, RNU44, and hsa-miR-1233 for normalization (ID: 002252, ID: 002658, ID: 001094, ID: 002768; Applied Biosystems, USA); TaqMan MicroRNA Reverse Transcription kit; and 2× TaqMan Universal Master Mix II, no UNG, according to the manufacturer's protocol. For patients' FFPE sample normalization, miR-1233 was chosen as it was the most stable across all the samples [18], while for in vitro analyses, RNU44 was chosen as it was the most stable across all the experiments. Evaluation of gene expression from total RNA was performed by TaqMan High Capacity cDNA Reverse Transcription kit, 2× TaqMan Gene Expression Master Mix, and individual gene expression assays for NDFIP1, RHEB, PPP2R5A, and GAPDH for normalization (ID: Hs00228968_m1, ID: Hs00950800_m1, ID: Hs00196542_m1, cat. n.: 4326317E; Applied Biosystems, USA). All reactions were performed according to the standard manufacturers' protocols. Analysis was performed on 7500 Real-Time PCR System using fixed threshold settings (Applied Biosystems, USA).

Radiation treatment

For irradiation of cells, OGL-1 VF γ -radiant (Černá Hora, Czech Republic) with the source of radiation Cs-137 γ -radiation (2 Gy min^{-1}) was used. For in vitro experiments, an optimal dosage of radiation of 5 Gy was chosen.

MTT proliferation assay

The proliferation of GBM cell lines (A172, T98G, and U87MG) was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) proliferation assay. Cells were seeded on 24-well plates and incubated overnight, then transfected with miRNA mimics (10 nM) and negative control miRNA (10 nM), respectively. After 24 h of incubation, the medium was changed with $500 \mu\text{l}$ of fresh medium supplemented with 10 % FBS and antibiotics. For the irradiated cells, radiation was applied 24 h post-transfection. Proliferation of cells was evaluated 24, 48, 72, and 96 h post-treatment. Plates were read on Synergy HT Multi-Mode Microplate reader at a wavelength of 570 nm.

Cell cycle distribution and apoptosis measurement

Cells were cultivated on 60-mm dishes. The distribution of cells in specific cell cycle stages was evaluated by assessment of DNA content by flow cytometry 48 h post-transfection/radiation. Cells were stained with propidium iodide (PI) and analyzed on the flow cytometer BD FACS Canto II (BD Biosciences, USA) based on DNA content. Results were analyzed using FACS Diva software. To evaluate the number of cells undergoing apoptosis, cells were seeded on 60-mm plates and, 48 h post-transfection/radiation, stained by Annexin V: PE Apoptosis Detection Kit I (BD Biosciences, USA) according to the manufacturer's protocol. Measurement was performed on the flow cytometer BD FACS Canto II (BD Biosciences, USA). Results were analyzed using FACS Diva software.

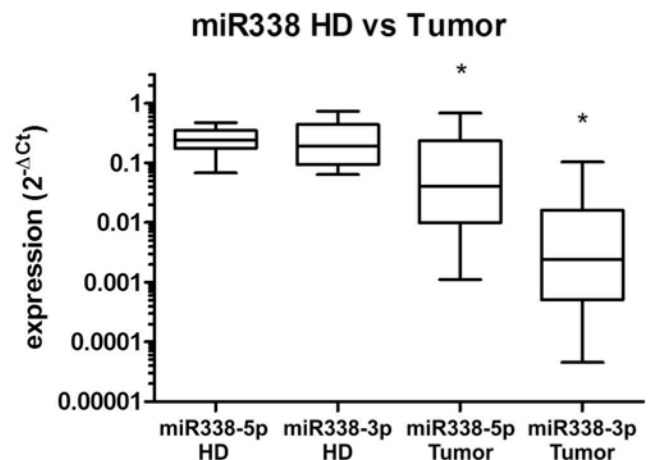


Fig. 1 Expression of miR-338-3p and miR-338-5p in GBM patients' samples compared to that of healthy tissue (HT); significance $p < 0.05$ is represented by *

Gene expression profiling

Total RNA with purity ratio $260/280 > 1.7$ and integrity (RIN) > 7.5 measured by Agilent 2100 Bioanalyzer, (Agilent, USA) was transcribed into cDNA and amplified using WT Expression Kit (Affymetrix, USA); then, it was labeled and hybridized on the Affymetrix GeneChip Human Gene ST 1.0 array and processed through complete Affymetrix workflow (Affymetrix,

USA). The whole-genome expression data, Affymetrix raw data (.cel files), were normalized using the robust multichip average (RMA) algorithm from ‘oligo’ Bioconductor package in R version 3.0.1. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus [19] and are accessible through the GEO Series accession number GSE 75789 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75789>).

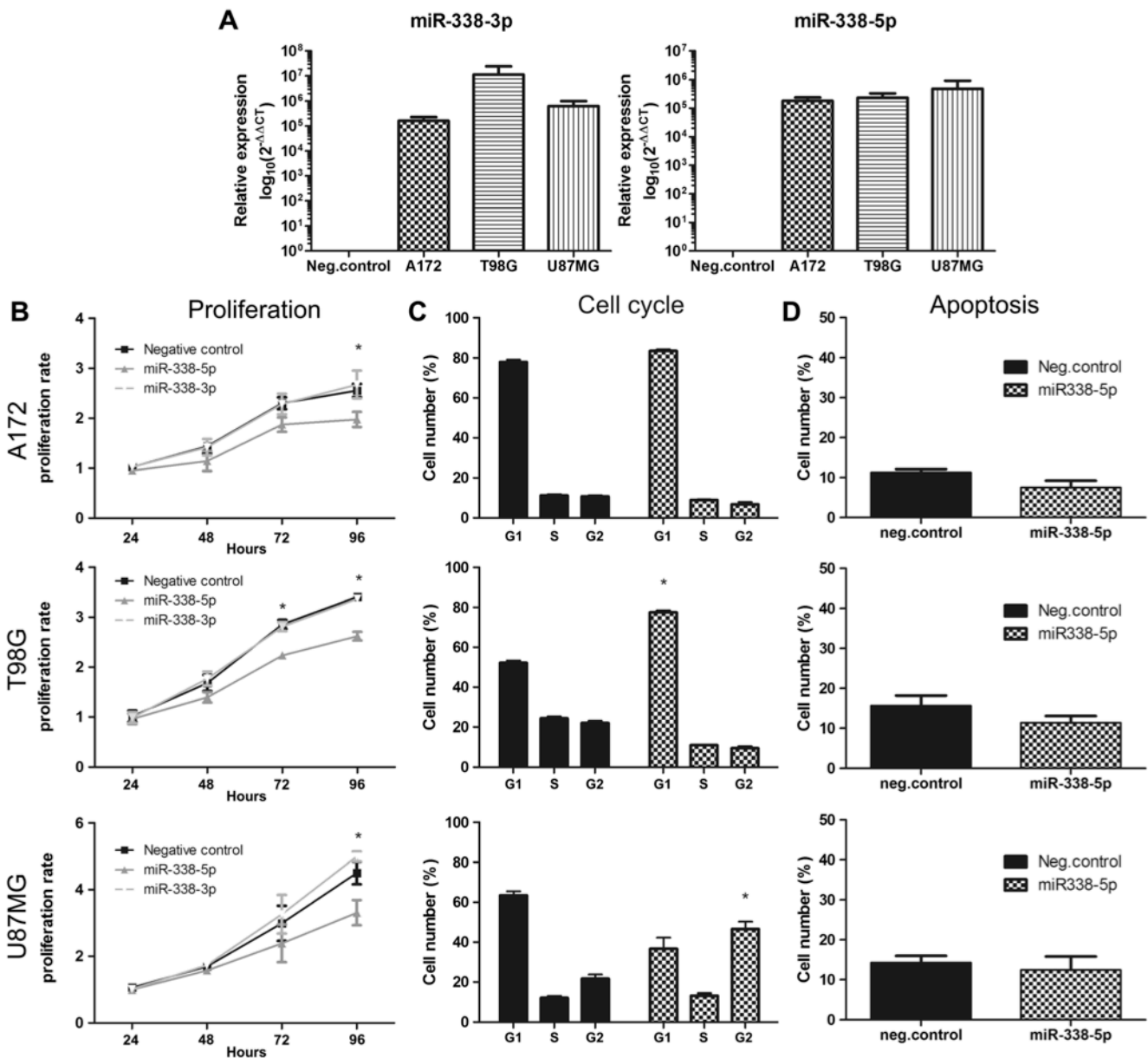


Fig. 2 **a** Expression levels of miR-338-5p 24 h after transfection. Normalization was performed using $2^{-\Delta\Delta CT}$ equation with RNU44 as an endogenous control. Negative control is expressed as a baseline, and expression of miRNAs in all cell lines is depicted as a fold change related to the negative control ($p < 0.05$). **b** Viability of A172, T98G, and U87MG cell lines 24 to 96 h post-transfection with pre-miRNA-338-3p and pre-miRNA-338-5p oligonucleotides, expressed as a fold change of

absorbance normalized to the negative control at the 24-h time point. **c** Quantity of apoptotic cells (%) in A172, T98G, and U87MG cell lines 48 h post-transfection with pre-miR-338-5p. **d** Cell cycle distribution expressed as a percentage of cells in the G1, S, and G2 phases of A172, T98G, and U87MG cell lines transfected with pre-miR-338-5p 48 h post-transfection. All data are presented as mean value \pm SD of three independent experiments, where significance $p < 0.05$ is represented by *

Statistical evaluation

For patients’ samples, relative miRNA expression levels were determined by $2^{-\Delta\Delta CT}$ method, where ΔCTs were calculated as follows: $\Delta CT = CT(\text{miRNA of interest}) - CT(\text{miR-1233})$. For normalization of in vitro analyses, equation $2^{-\Delta\Delta CT}$ was used, in which ΔCTs were calculated as follows: $\Delta CT_{(\text{target gene})} = CT_{(\text{target gene})} - CT_{(\text{endogenous control})}$, where for miRNA normalization RNU44 and for messenger RNA (mRNA) normalization GAPDH expression was used. Data were statistically analyzed with GraphPad Prism v.5 software. Statistical significance in in vitro experiments was evaluated by Mann–Whitney *U* test for miRNA experiments and by two-tailed paired *t* test for target genes. *p* values below 0.05 were considered as statistically significant. Normalized data from gene expression profiling were clustered using hierarchical clustering based on Euclidian distance. Differential expression analysis was performed using LIMMA R-package. Genes were declared differentially expressed with a fold-change cutoff greater than 2 and *p* value below 0.05 [20].

Results

Expression of miR-338-3p and miR-338-5p in patients’ samples

Expression of miR-338-5p and miR-338-3p was evaluated in 40 primary GBM patients’ samples and 10 control brain samples. The level of miR-338-5p as well as miR-338-3p was significantly downregulated ($p < 0.05$) in tumor samples as compared with control samples (Fig. 1), which suggested involvement of this miRNA in the GBM pathogenesis. To further elucidate the role of these miRNAs, we performed in vitro functional analyses on GBM cell lines.

In vitro functional assay of miR-338-5p and miR-338-3p

MiR-338-5p and miR-338-3p levels were upregulated by transient transfection of GBM cell lines A172, T98G, and U87MG using miRNA mimics and negative control. Twenty-four hours post-transfection, levels of miRNAs were

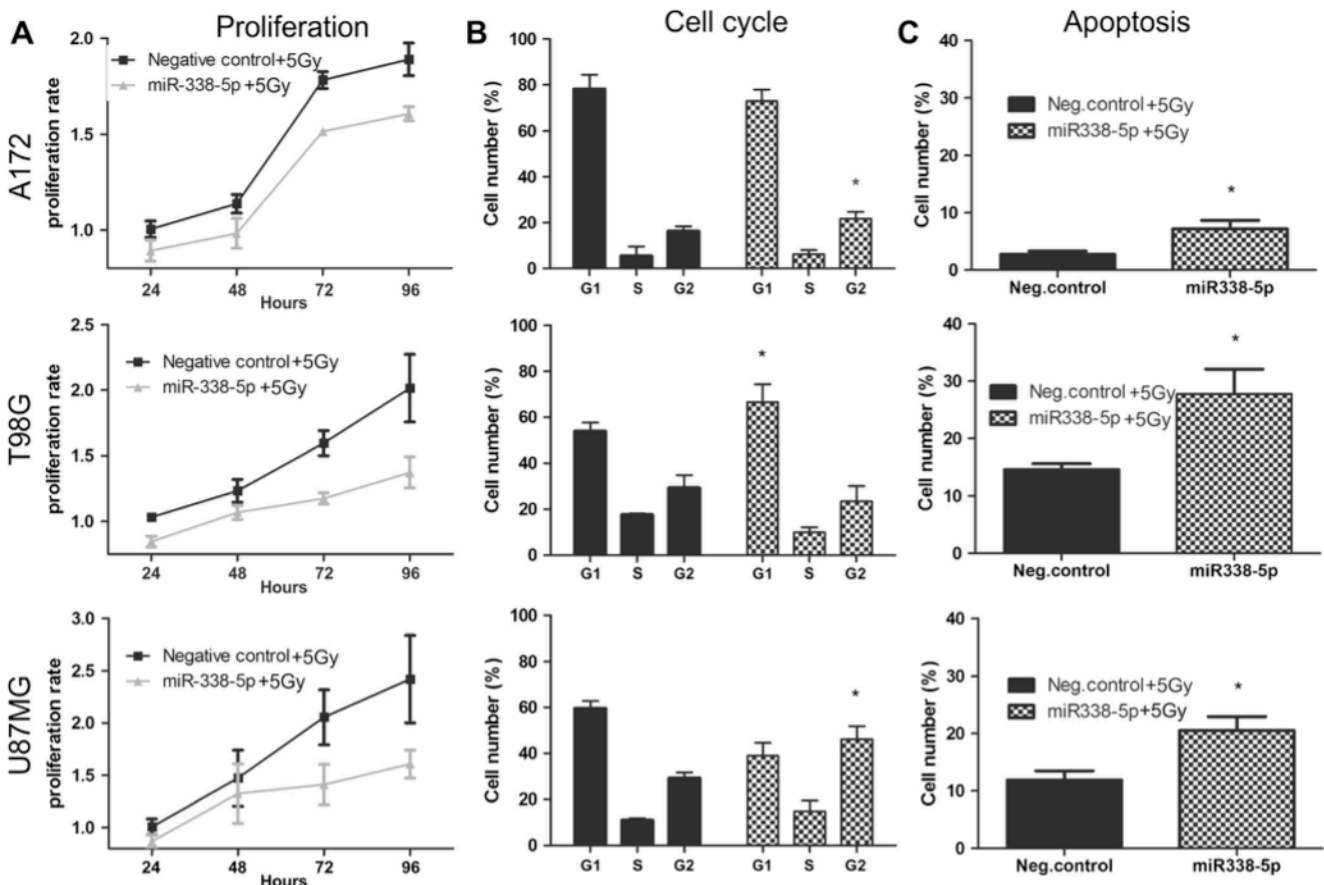


Fig. 3 Combination of miR-338-5p overexpression with radiation. **a** Viability of A172, T98G, and U87MG cell lines 48 to 120 h post-transfection with pre-miRNA-338-3p and 24 to 96 h after irradiation, expressed as a fold change of absorbance normalized to the negative control at the 24-h time point post-radiation. **b** Cell cycle distribution of A172, T98G, and U87MG cell lines transfected with pre-miR-338-5p

48 h post-radiation, expressed as a percentage of cells in the G1, S, and G2 phases. **c** Quantity of apoptotic cells (%) in A172, T98G, and U87MG cell lines 48 h after transfection with pre-miR-338-5p and irradiation. All data are presented as mean value \pm SD of three independent experiments, where significance $p < 0.05$ is represented by *

evaluated using qRT-PCR specific primers and probes and compared to negative control. Overexpression of miR-338-5p and miR-338-3p was confirmed across all three cell lines ($p < 0.05$) (Fig. 2a).

Subsequently, analysis of the proliferation rate was performed using MTT colorimetric assay. Overexpression of miR-338-3p caused no viability changes compared to negative control, while overexpression of miR-338-5p inhibited cell viability in A172 after 96 h (22.7 ± 7.6 %), T98G after 72 h (22.7 ± 7.6 %) and 96 h (23.1 ± 3.3 %), and U87MG after 96 h (24.9 ± 7.5 %) (all $p < 0.05$) (Fig. 2b). To assess the cause of viability dropdown, apoptosis of cells as well as cell cycle distribution was evaluated 72 h post-transfection. Measurement of apoptosis by Annexin V: PE staining did not show any significant changes in apoptosis between miR-338-5p-transfected cells and negative control in all three cell lines (Fig. 2d). However, analysis of cell cycle distribution in miR-338-5p-transfected cells revealed significant arrests in the G1 phase for T98G (25.37 ± 1.77 %) and the G2 phase for U87MG (24.87 ± 2.89 %) cell lines (all $p < 0.05$) (Fig. 2c, Supplementary Figure S1).

In vitro combination of miR-338-5p overexpression and radiation in GBM cell lines

A172, T98G, and U87MG GBM cell lines were irradiated with 5 Gy 24 h after miR-338-5p overexpression, and functional analyses evaluating cell viability, apoptosis, and cell cycle distribution were performed. All three cell lines showed a significant decrease in proliferation after irradiation ($p < 0.05$). More specifically, the decrease in proliferation in A172 72 h post-radiation was 14.9 ± 3.2 % and 96 h post-radiation 14.7 ± 5.7 %. For the T98G cell line, the decrease in proliferation 72 h post-radiation was 26.2 ± 4.7 % and 96 h post-radiation 31.5 ± 5.1 %, and for U87MG, the decrease in proliferation 72 h post-radiation was 31.4 ± 1.4 % and 96 h post-radiation 32.9 ± 5.7 (Fig. 3a).

Further analyses of cell cycle distribution and apoptosis 72 h after irradiation (96 h post-transfection) showed that the observed dropdown in cell proliferation was caused partly by cell cycle arrest. More specifically, there was an increase of cells in the G2 phase for A172 (5.41 ± 1.14 %, $p < 0.05$), in the G1 phase for T98G (12.69 ± 4.92 %, $p < 0.05$), and also in the G2 phase for U87MG (16.91 ± 7.41 %, $p < 0.05$) in miR-338-5p-transfected cells (Fig. 3b, Supplementary Figure S2). However, opposite to the results observed without irradiation, there was a significant increase in the apoptosis rate in all three cell lines transfected with miR-338-5p: 4.33 ± 1.52 % in A172, 13.10 ± 4.27 % in T98G, and 8.57 ± 2.46 % in U87MG as compared with cells transfected with the negative control (all $p < 0.05$) (Fig. 3c). A low level of apoptotic cells in the A172 cell line can be explained by different sensitivity to the radiation [21]. In order to elucidate the mechanism of observed changes in viability and cell cycle distribution, global gene expression profile (GEP) analysis was performed.

Gene expression profiling of GBM cell lines after miR-338-5p overexpression

A172, T98G, and U87MG GBM cell lines were transfected with the miR-338-5p mimic and negative control. Twenty-four hours post-transfection, total RNA was isolated and gene expression profiling was performed. Ten differentially expressed genes were found across all three cell lines—NDFIP1, C5orf34, ATAD1, DCP2, BLZF1, DPY19L1, FAM168B, PPP2R5a, RHEB, and GNAQ (with $FC \geq 2$, $p < 0.001$)—between miR-338-5p-overexpressed cells and cells transfected with the negative control. From this gene set, suitable candidates for further validation were chosen based on miRanda, miRDB, miRWalk, and TargetScan prediction databases of 3'UTR of miRNA and RNA interaction. According to the match of miRNA–mRNA interaction in all four databases, NDFIP1, RHEB, and PPP2R5a genes were chosen (Fig. 4).

Furthermore, the regulatory effects of miR-338-5p on NDFIP1, RHEB, and PPP2R5a expression levels were validated by qRT-PCR. Twenty-four hours post-transfection with miR-338-5p mimics, the expression of all three studied genes was significantly downregulated in comparison with the negative control. Specifically, the decrease was for (i) NDFIP1— 49.5 ± 4.8 % in A172, 58.8 ± 10.4 % in T98G, and 50.9 ± 2.1 % in U87MG; (ii) RHEB— 54.3 ± 9.9 % in A172, 42.6 ± 4.5 % in T98G, and 54.5 ± 7.8 % in U87MG; and (iii) PPP2R5a— 11.1 ± 10.9 % for A172, 35.2 ± 17.0 % for T98G, and 22.2 ± 6.3 % for U87MG

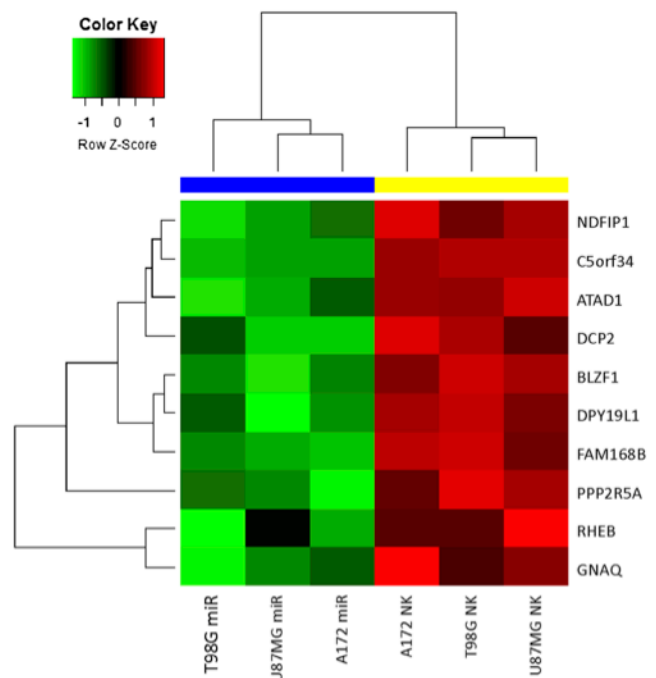


Fig. 4 Hierarchical clustering presented by a heatmap of differentially expressed genes in A172, T98G, and U87MG cell lines 24 h after miR-338-5p overexpression at the significance level $p < 0.001$ with $FC > 2$ normalized using the RMA algorithm. Red color represents upregulated expression, while green color stands for downregulated genes

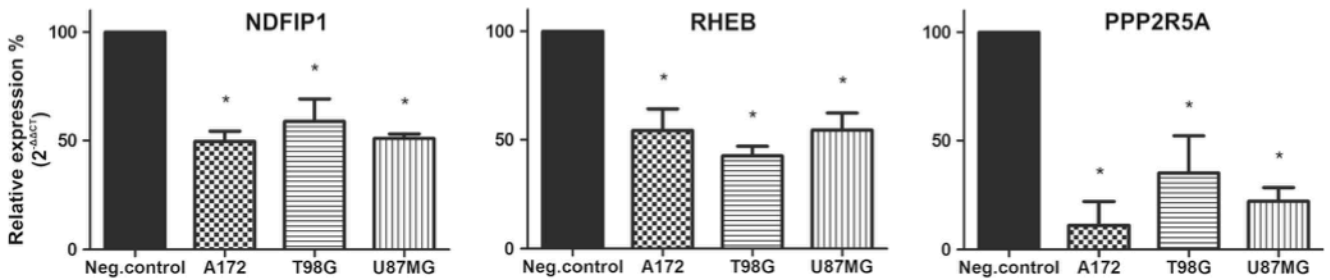


Fig. 5 Validation of the downregulation of selected genes NDFIP1, RHEB, and PPP2R5a 24 h after pre-miR-338-5p transfection. Normalization was performed using $2^{-\Delta\Delta CT}$ equation to GAPDH as an

endogenous control. Negative control is expressed as baseline (100 %), and expression of genes in all cell lines is depicted by percentage of decrease ($p < 0.05$)

(Fig. 5). As these observations revealed two genes (NDFIP1 and PPP2R5a), which are involved in DNA stability and repair, we decided to demonstrate the impact of the combination of miR-338-5p overexpression and irradiation in GBM cell lines.

Discussion

MiR-338-5p is one of the isoforms of miR-338, which is a brain-specific miRNA [15]. In the current study, we found miR-338-5p and miR-338-3p to be significantly downregulated in GBM patients compared to the control group. Ectopic overexpression

of miR-338-5p in GBM cell lines A172, T98G, and U87MG caused a significant decrease of cell viability and proliferation rate, while miR-338-3p did not show any effect. The observed different impact might be explained by the fact that miR-338-3p is involved mainly in the differentiation processes [22], what is not known for miR-338-5p. A decrease of cell proliferation in all three cell lines was presumably caused by the significant cell cycle arrest, as there was no sign of increased apoptosis. We assume that the observed dropdown in cell proliferation after miR-338-5p increase was presumably due to targeting and thus decreasing Rheb mRNA, which is a well-known player in the mTOR signaling pathway promoting cell growth [23].

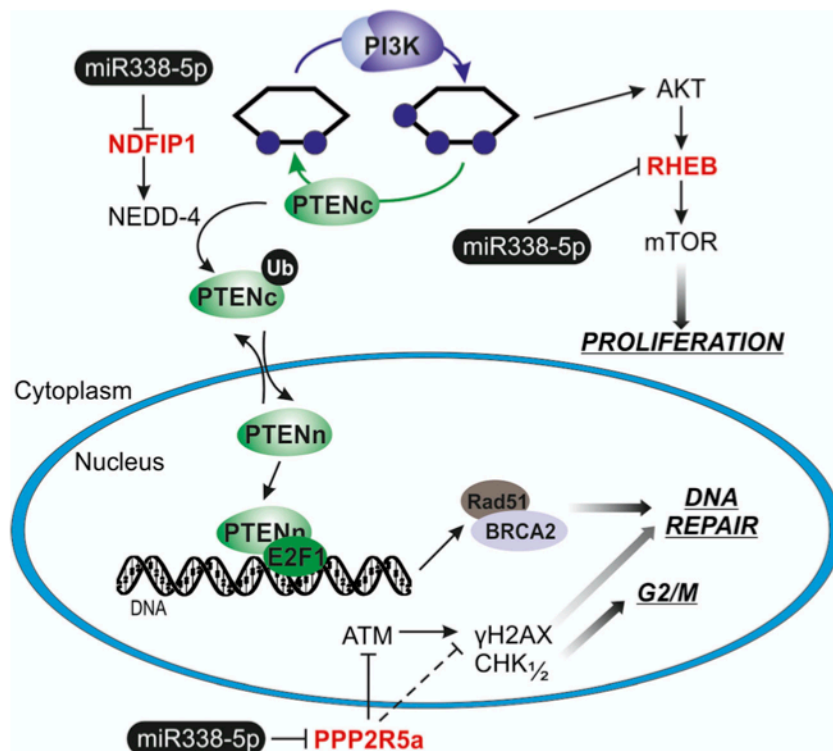


Fig. 6 Proposed model of miR-338-5p involvement in cell proliferation. MiR-338-5p inhibits RHEB GTPase, an important activator of mTOR serine/threonine kinase that is involved in the activation of protein synthesis and growth. NDFIP1 as well as PPP2R5a is involved in the genomic integrity and cell cycle progression. NDFIP1 acts as an E3 ligase promoting PTEN monoubiquitination, which is necessary for its

translocation to the nucleus. There, monoubiquitinated PTEN helps to coordinate E2F1 transcription factor and transcriptional regulation of Rad51 and BRCA2. This results in the control of DSB repair. PPP2R5a, a serine/threonine phosphatase, has been directly implicated in the negative regulation of γ -H2AX and ATM, and DNA damage response signals

As one of the main therapeutic modalities in GBM is radiotherapy, we decided to combine miR-338-5p overexpression with irradiation. Compared to the experiments without irradiation, we observed significant cell cycle arrest in miR-338-5p-transfected cells. However, contrary to previous experiments, we observed an increase of apoptosis in miRNA-treated cells in parallel to the negative control. Further, two of the three predicted/validated target genes of miR-338-5p are NDFIP1 and PPP2R5a, which are closely connected to genomic instability and promotion of DNA damage response [24, 25]. Therefore, we hypothesize that the increase of apoptosis after miR-338-5p overexpression and irradiation can be explained by decreased genomic instability caused by NDFIP1 and PPP2R5a downregulation. NDFIP1 is an E3 ligase which, together with RAB5, is responsible for PTEN ubiquitination and nuclear trafficking [26]. Besides the involvement of PTEN in the regulation of the PI3K/Akt signaling pathway in the cytoplasm, it is also important in the nucleus to coordinate E2F1 and RAD51 transcription. Nuclear import of PTEN is reached by its monoubiquitination by NDFIP1. Stabilization of PTEN in the nucleus results in the control and activation of DNA repair response to double-strand breaks (DSB) [26, 27]. As the irradiated cells are not able to activate DNA damage response, they are predetermined to apoptosis. Furthermore, not only inhibition of NDFIP1 promotes genome instability but also decrease of the serine/threonine phosphatase family PPP2R2A, PPP2R2D, PPP2R5A, and PPP2R3C, which are critical effectors of the homologous recombination repair through modulation of ATM phosphorylation [28]. Induction of DSB initiates phosphorylation of the histone H2A family H2AX to generate γ -H2AX, which is important for the maintaining of genome stability [29, 30]. Downregulation of the phosphatase family leads to delay of γ -H2AX formation and subsequent recruitment of DNA repair proteins, which results in the sensitivity to DNA damage inducers [23]. The proposed mechanism of action of miR-338-5p in the regulation of cell functions such as viability, cell cycle progression, and apoptosis of GBM cells is depicted in Fig. 6.

So far, this is the first report focused on miR-338-5p regulatory function in GBM and its role in radiation. We confirmed different expression of miR-338-3p and miR-338-5p in GBM in comparison to non-tumor brain tissue. We showed its impact on viability and cell cycle distribution and potential to sensitize GBM cell lines to radiation. Furthermore, we propose, based on the global gene expression profiling, the mechanism of function of miR-338-5p via the targets NDFIP1, RHEB, and PPP2R5a. However, we are aware that further studies are needed to elucidate fully the mechanism behind its functioning in GBM cells.

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Compliance with ethical standards Informed consent was obtained from each patient before the treatment.

Conflicts of interest None

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PŘÍLOHA 15

Global MicroRNA Expression Profiling Identifies Unique MicroRNA Pattern of Radioresistant Glioblastoma Cells

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Abstract. Glioblastoma multiforme (GBM) is the most aggressive intracranial tumor characterized with infaust prognosis. Despite advances in neurosurgical and radiotherapeutic techniques and chemotherapy, the median overall survival ranges between 12–15 months from diagnosis. The main cause of treatment failure is considered the presence of tumor cells resistant to conventional therapy, mainly radiotherapy. MicroRNAs (miRNAs) are small, non-coding RNAs that function as post-transcriptional regulators of gene expression and have been repeatedly proven to play important roles in pathogenesis and biological features of many cancers, including GBM and its radioresistant phenotype. In our study, we established radioresistant cells from the commonly used human GBM cell lines T98G, U87MG and U251. Consequently, we performed global miRNA expression profiling in both radioresistant and parental cell lines and identified 113 miRNAs with significantly different expression ($p < 0.05$) between these two groups (73 miRNAs were up-regulated, 40 miRNAs were down-regulated). Some of these miRNAs have been previously described in relation to ionizing radiation, and others were herein identified for the first time. We believe that after deeper functional investigation of identified miRNAs in relation to radioresistance, these miRNAs present potential predictive biomarkers or therapeutic targets in GBM.

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Key Words: Glioblastoma, radioresistance, microRNA.

Glioblastoma multiforme (GBM) is a highly aggressive primary brain tumor of astrocytic origin accounting for nearly 50% of all gliomas with incidence of 3–4 new cases per 100,000 individuals (1, 2). Although metastases are unusual, GBM is characterized by infiltrative growth and early recurrences. Current standard therapeutic protocol consists of maximal surgical resection followed by concomitant chemoradiotherapy with temozolomide and adjuvant temozolomide in monotherapy. Unfortunately, GBM is often chemo-radioresistant and thus, adjuvant therapy fails. The prognosis of GBM patients is very poor with median overall survival ranging between 12 and 15 months from diagnosis (1, 2). A lot of effort has been spent in researching of both suitable biomarkers that could predict response to the therapy as well as drugs and therapeutic targets that could overcome GBM resistance. Over the past decade, involvement and functional roles of microRNAs (miRNAs) in GBM radioresistance were described (3, 4). MiRNAs are short, approximately 18–25 nucleotides long, non-coding single-stranded RNAs with an ability to post-transcriptionally regulate gene expression. Due to this feature, miRNAs are able to regulate multiple cellular processes, either physiological or pathological, such as apoptosis, proliferation, cell cycle, angiogenesis, differentiation. A number of miRNAs have been also identified to be deregulated in various cancers including GBM and some of them were associated with clinical outcomes and radioresistance (5–7). However, involvement of miRNAs in the molecular processes of cell response to the ionizing radiation in glioma is still poor understood. Therefore, we established radioresistant GBM cell lines in which we have subsequently performed global miRNA expression profiling in order to identify miRNAs deregulated in these cells and associated with resistance to the ionizing radiation in GBM. We believe that after deeper investigation

of functional roles of these miRNAs in radioresistance *in vitro*, they present potential predictive biomarkers or therapeutic targets in GBM.

Materials and Methods

GBM cell lines and establishment of radioresistant cells. GBM cell lines U251, T98G, and U87MG as well as their radioresistant counterparts were cultivated in DMEM medium enhanced by 10% heat-inactivated fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin, 4.5 g/l D-glucose, 2 mM L-glutamine, 1% nonessential amino acids, and 10 mg/l sodium pyruvate. Cells were cultivated on 100-mm culture dishes at 37°C in humidified atmosphere containing 5% CO₂.

All three GBM cell lines were firstly grown to approximately 60% confluence and irradiated with 2 Gy (Cs-137 γ-radiation, 2 Gy/min). When cells reached about 90% confluence, they were subcultured into new dishes. The cells were treated again with 2 Gy when they reached 60% confluence. This procedure was repeated until total dose was 32 Gy. After that, cells were irradiated by the same process but dose 4 Gy was used instead of 2 Gy. This procedure was repeated until total dose of ionizing radiation was 60 Gy. Parental cells were cultured under the same conditions without ionizing radiation treatment. Multiply irradiated cells were named with suffix -R (Radioresistant), giving U251-R, T98G-R, U87MG-R. Parental cells were named with suffix -C (Control). For all assays, there was at least a four-week interval between the last fractionated irradiation and the experiment.

Colony formation assay. Diluted cell suspension was seeded into 6-well plates, incubated overnight, irradiated by dose 4 Gy, and cultured until cells form colonies. Subsequently, cells were fixed in 4% paraformaldehyde (30 min/RT) and visualized by 0.5% crystal violet (20 min/RT). The colonies were counted using GelCount (Oxford, USA). Calculation of surviving fractions (SF) of cells after irradiation was performed using the equation $SF = \text{colonies counted} / (\text{cells seeded} * PE)$. Plating efficiency (PE) is the ratio of the number of colonies to the number of cells seeded, which were not treated by radiation (8). Experiments were repeated three times; each experiment was performed in triplicates. Statistical evaluations of data obtained from colony formation assay were performed on GraphPad Prism 5 software by a one-tailed non-parametric *t*-test.

MiRNA microarray analysis. Small RNA enriched total RNA was isolated using the Direct-zol RNA MiniPrep Kit (Zymo Research, USA). Nucleic acid concentrations and purities were controlled by UV spectrophotometry using Nanodrop ND-1000 (Thermo Scientific). To assess miRNA expression in radioresistant and control GBM cell lines, the samples were analyzed with Affymetrix GeneChip miRNA 3.0 arrays (Affymetrix, Santa Clara, CA, USA) containing 1733 probe sets for human mature miRNAs. Experiments were performed according to the Affymetrix standardized protocol for miRNA 3.0 arrays. Intensity values for each probe cell were calculated using Affymetrix GeneChip Command Console (AGCC). Quality control of the microarray was performed with the Affymetrix miRNA QC Tool, version 1.1.1.0.

Microarray expression data analysis. All data were pre-processed and further analyzed by the software packages included in the R/Bioconductor (9). Pre-processing was performed by the RMA method with default parameters as implemented in the Bioconductor

package oligo (10). All data were log₂-transformed. To identify differentially expressed miRNAs, the LIMMA approach (11) for paired samples was applied with additional Benjamini-Hochberg correction of *p*-values.

Results

Radioresistance analysis. Prior to miRNA expression analyses in the examined GBM cell lines, their resistance to ionizing radiation was evaluated. The colony formation assay has confirmed that all three GBM cell lines, which have received a total dose of 60 Gy, form significantly higher number of colonies after 4 Gy irradiation in comparison to the parental control GBM cell lines (Figure 1). In concrete, cell lines U251-R, T98G-R, and U87MG-R form on average 3-times ($p=0.0395$; Figure 1C), 3.5-times ($p=0.0363$; Figure 1F), and 1.6-times ($p=0.0277$; Figure 1I) more colonies than their parental cell lines U251-C, T98G-C, and U87MG-C, respectively.

Global miRNA expression analysis of radioresistant and parental GBM cell lines. Global miRNA expression analyses followed by statistical comparison of three pairwise radioresistant and parental GBM cell lines identified 113 significantly differently expressed miRNAs between these two groups ($p<0.05$). From these, 73 miRNAs were up-regulated and 40 miRNAs down-regulated in radioresistant cells; 21 miRNAs have shown the *p*-value less than 0.01. The most up-regulated miRNA in radioresistant GBM cells was identified as miR-145 while the most down-regulated was miR-1271 (Table I).

Discussion

Radiotherapy plays an important role in oncological treatment of GBM patients. Nevertheless, this brain tumor is well known for its frequent resistance to ionizing radiation and, thus, adjuvant therapy often fails and GBM develop early recurrences. This results in a bad prognosis associated with short survival of GBM patients. In the presented study, we compared miRNA expression profiles in radioresistant and parental stable GBM cell lines U251, T98G, and U87MG. Our aim was to use global miRNA expression profiling and identify miRNAs that might be closely associated with radioresistance of GBM cells and, thus, could be promising predictive markers, as well as potential therapeutic targets in GBM patients.

Firstly, we established three GBM cell lines treated with total dose of 60 Gy. Following evaluation of their resistance to the ionizing radiation confirmed significantly higher potential of these cell lines to form colonies after 4-Gy experimental treatment compared to the parental control cell cultures. Similar results were published by the Yang *et al.* who induced radioresistance in U251 GBM cell line by the fractionated ionizing radiation exposure in total dose 62 Gy (12).

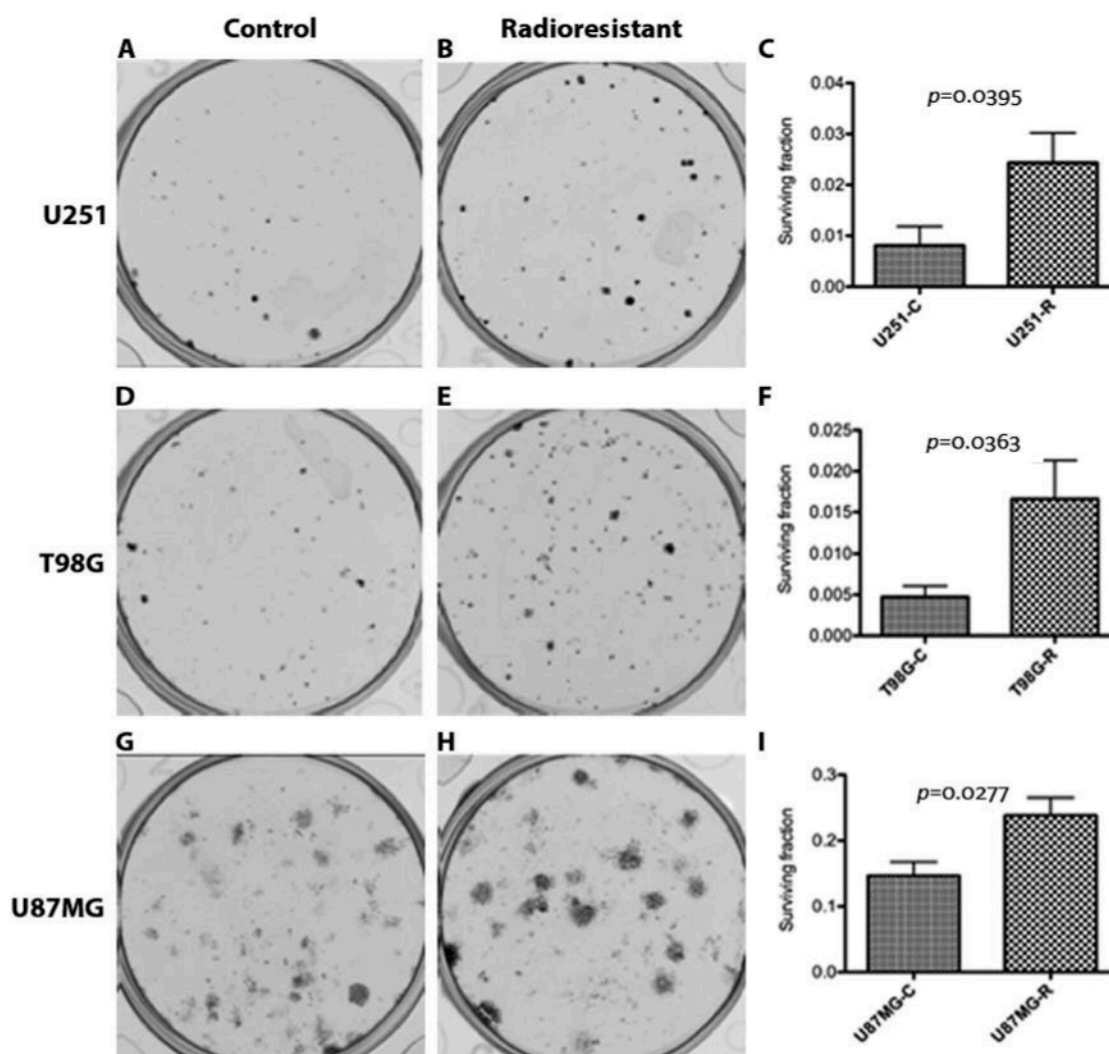


Figure 1. Colony formation ability of 4 Gy radiation treated U251-C/R (A, B), T98G-C/R (D, E) and U87MG-C/R (G, H) GBM cell lines. Surviving fraction of (C) U251-C/R, (F) T98G-C/R and (I) U87MG-C/R GBM cell lines after 4 Gy radiation treatment (one-tailed non-parametric t-test).

The main aim of our work was to compare miRNA expression profiles in pairwise radioresistant and parental control GBM cell lines and identify miRNAs with significantly different expression levels between these groups. We identified 113 miRNAs with significantly different expression in radioresistant GBM cells ($p < 0.05$). Many of these miRNAs have been previously observed to be linked with radiation treatment, including miR-29, miR-218, miR-145, miR-204, miR-126, miR-146b-5p, miR-31, miR-302a and miR-452 (13-22). Focusing on these miRNAs, several previous studies have been focused on the discovery of molecular mechanisms responsible for the tumor radioresistance caused by these miRNAs, mainly in oropharyngeal area. Lynam-Lennon *et al.* observed, similarly

like in our study, that miR-31 is significantly down-regulated in isogenic model of radioresistance in oesophageal adenocarcinoma cells, both basally and in response to radiation. Ectopic re-expression of miR-31 led to expression alteration of 13 genes involved in DNA repair and significant re-sensitizing of radioresistant cells to radiation (13). Also miR-452 was one of four miRNAs, that have been identified by the comprehensive miRNA expression profiling to be differentially expressed in radioresistant laryngeal squamous cell carcinoma (14). Finally, in nasopharyngeal carcinoma cells, miR-204 is involved in radioresistance and epithelial-mesenchymal transition. Lu *et al.* described that both processes are regulated by the lncRNA NEAT1 through miR-204/ZEB1 axis (15). Interestingly, the same processes

Table I. *MiRNAs with significantly different expression levels in radioresistant and parental control GBM cell lines (p<0.05).*

miRNA	logFC	AveExpr	p-Value	miRNA	logFC	AveExpr	p-Value
miR-663	0.98	7.21	<0.01	miR-4734	0.86	7.59	0.02
miR-10b-star	1.64	3.61	<0.01	miR-4689	0.57	5.67	0.02
miR-4498	1.40	2.89	<0.01	miR-3178	0.86	8.30	0.03
miR-572	1.05	1.99	<0.01	miR-4521	-0.90	8.60	0.03
miR-4505	0.95	5.63	<0.01	miR-146b-5p	0.68	3.33	0.03
miR-4486	0.97	4.38	<0.01	miR-532-5p	-0.51	5.46	0.03
miR-4725-3p	1.20	1.61	<0.01	miR-582-5p	-0.64	0.93	0.03
miR-1301	0.94	2.17	<0.01	miR-4251	-0.51	0.50	0.03
miR-10a-star	1.33	2.29	<0.01	miR-4500	0.70	1.36	0.03
miR-3185	1.07	6.78	<0.01	miR-4640-5p	0.78	3.41	0.03
miR-4707-5p	0.88	6.96	<0.01	miR-3136-5p	-0.71	1.19	0.03
miR-29b	-1.26	6.58	<0.01	miR-3940-5p	0.53	8.41	0.03
miR-1908	0.81	7.98	<0.01	miR-150-star	1.01	2.23	0.03
miR-218	0.80	2.63	<0.01	miR-518e	-0.53	0.69	0.03
miR-3065-5p	-1.52	3.73	<0.01	miR-212	0.92	1.28	0.03
miR-3910	-0.79	1.49	<0.01	miR-143	2.19	2.43	0.03
let-7a-2-star	-0.78	4.46	<0.01	miR-2276	-0.59	2.09	0.03
miR-145	3.23	2.72	<0.01	miR-31	-0.51	7.08	0.03
miR-4497	1.01	10.03	<0.01	miR-548c-5p	-0.50	0.70	0.03
miR-4721	1.29	3.28	<0.01	miR-3621	0.62	5.39	0.03
miR-4697-3p	1.20	1.39	<0.01	miR-4313	-0.53	1.25	0.03
miR-30b-star	0.96	1.65	0.01	miR-4430	0.77	3.06	0.03
miR-1231	1.23	4.68	0.01	miR-422a	-0.73	6.10	0.04
miR-4778-5p	1.07	1.65	0.01	let-7e-star	1.02	1.95	0.04
miR-378e	-0.84	2.61	0.01	miR-4745-5p	0.75	8.05	0.04
miR-4651	0.68	6.78	0.01	miR-4466	0.56	9.55	0.04
miR-4440	-0.82	1.15	0.01	miR-4518	-0.52	0.60	0.04
miR-204	1.26	3.15	0.01	miR-520g	-0.50	0.63	0.04
miR-217	-0.65	0.95	0.01	miR-4649-5p	0.67	4.19	0.04
miR-1914-star	0.76	1.80	0.01	miR-185-star	-0.85	0.99	0.04
miR-126	-0.70	3.89	0.01	miR-302a	-0.53	0.82	0.04
miR-302d	0.82	1.29	0.01	miR-1909-star	0.56	1.54	0.04
miR-224	0.70	5.15	0.01	miR-4781-3p	-0.69	0.86	0.04
miR-2467-3p	-1.05	1.18	0.02	miR-138-2-star	0.47	0.96	0.04
miR-431	0.94	1.97	0.02	miR-4439	0.49	0.99	0.04
miR-4750	1.28	3.85	0.02	miR-648	-0.45	0.94	0.04
miR-4783-3p	0.63	2.65	0.02	miR-452	0.74	4.00	0.04
miR-1271	-2.86	2.23	0.02	miR-570	-0.90	0.86	0.04
miR-664-star	1.06	1.27	0.02	miR-3682-3p	0.78	1.74	0.04
miR-935	1.08	3.23	0.02	miR-4462	0.73	1.54	0.04
miR-22	-0.67	10.29	0.02	miR-887	0.66	1.49	0.04
miR-4443	0.85	5.14	0.02	miR-331-3p	0.84	3.60	0.04
miR-3197	0.91	2.64	0.02	miR-767-3p	-0.57	1.21	0.04
miR-617	-0.61	0.83	0.02	miR-3679-5p	0.48	4.00	0.04
miR-4634	1.25	3.96	0.02	miR-4749-5p	0.80	4.38	0.04
miR-3619-5p	0.73	3.82	0.02	miR-744-star	0.47	1.30	0.05
miR-4324	0.91	2.14	0.02	miR-4726-5p	-0.73	1.58	0.05
miR-1323	-0.91	1.36	0.02	miR-3188	1.13	3.28	0.05
miR-4682	0.64	1.13	0.02	miR-4746-3p	0.49	0.81	0.05
miR-378f	-0.76	5.59	0.02	miR-759	-0.52	0.56	0.05
miR-186-star	-0.72	0.94	0.02	miR-3591-3p	-0.62	0.78	0.05
miR-199a-5p	0.59	3.26	0.02	miR-615-3p	0.47	2.42	0.05
miR-4741	0.77	6.11	0.02	miR-762	0.48	8.48	0.05
miR-3617	1.61	1.79	0.02	miR-4454	-0.50	11.04	0.05
miR-4507	0.55	5.79	0.02	miR-652	0.54	4.56	0.05
miR-1290	-1.27	5.83	0.02	miR-3187-3p	0.83	3.25	0.05
miR-378	-0.58	6.43	0.02				

FC, Fold Change; AveExpr, average expression (signal on Affymetrix GeneChip).

are regulated by the double-negative feedback loop between lincRNA TUG1 and miR-145 in bladder cancer cells (16). MiR-145 plays a crucial role in modulation of radiosensitivity also in prostate cancer and HR-HPV⁺ cervical cancer where another lincRNA MALAT1 acts as sponge for miR-145 and mediates its functioning (17, 18). In addition to miR-145, sensitivity of cervical cancer to the ionizing radiation is affected also by miR-218 *via* promoting radiation-induced apoptosis (19). Yang *et al.* have described that lincRNA-p21, regulator of cell proliferation, apoptosis and DNA damage response, negatively regulated the expression and activity of β -catenin in glioma stem cells (GSCs) and down-regulation of lincRNA-p21 resulted from up-regulation of Hu antigen R (HuR) expression caused by miR-146b-5p down-regulation. Expectedly, miR-146b-5p overexpression increased apoptosis and radiosensitivity, decreased cell viability, neurosphere formation capacity and stem cell marker expression, and induced differentiation in GSCs (20). In breast cancer, miR-302a was down-regulated in irradiated cells. Additionally, the expression levels of miR-302a were inversely correlated with those of AKT1 and RAD52, two critical regulators of radioresistance. More promisingly, miR-302a sensitized radioresistant breast cancer cells to radiation therapy *in vitro* and *in vivo* and reduced the expression of AKT1 and RAD52 (21). Finally, miR-29 and miR-126 were both identified to be significantly down-regulated in the serum of the lung cancer patients after radiation treatment (22).

Altogether, our study revealed miRNAs differentially expressed in radioresistant GBM cell lines that may play a role in the molecular process of tumor cell response to radiotherapy in GBM patients.

Conflicts of Interest

The Authors have no conflict of interest.

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PŘÍLOHA 16

Význam mikroRNA u glioblastomových kmenových buněk

The Relevance of MicroRNAs in Glioblastoma Stem Cells

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Souhrn

Multiformní glioblastom je nejčastější intrakraniální malignitou astrocytárního původu dospělé populace. I přes absolvování komplexní terapie skládající se z maximální možné chirurgické resekce, adjuvantní konkomitantní chemoradioterapie s temozolomidem a následného podání temozolomidu v monoterapii se medián přežití pacientů pohybuje pouze mezi 12 a 15 měsíci od stanovení diagnózy. Za touto špatnou prognózou stojí jednak velmi často nemožnost dosažení dostatečně radikální chirurgické resekce a dále značná rezistence nádoru k adjuvantní léčbě, jež je v současné době velmi často dávána do souvislosti s přítomností tzv. glioblastomových kmenových buněk. Tyto buňky, stejně jako normální kmenové buňky, disponují několika unikátními vlastnostmi, jako jsou schopnost sebeobnovy, diferenciace a neomezeného, avšak pomalého buněčného dělení. Jejich rezistence ke konvenční terapii pak zcela jistě souvisí i se zvýšenou expresí DNA reparačních enzymů, antiapoptotických faktorů a mnohočetných lékových transportérů. Cílené ovlivnění těchto unikátních vlastností by proto mohlo být novým slibným terapeutickým přístupem vedoucím k zefektivnění léčby a zlepšení prognózy pacientů s multiformním glioblastomem. Jednou z možností, jak úspěšně ovlivňovat zmíněné vlastnosti, je cílená regulace mikroRNA (miRNA). Tyto krátké nekódující molekuly RNA posttranskripčně reprimují expresi více než 2/3 celkového počtu lidských genů, jež jsou mimo jiné součástí signálních drah spojovaných právě s kmenovými vlastnostmi buněk. Mimoto pozmeněné hladiny některých miRNA byly pozorovány u mnoha nádorových onemocnění, včetně multiformního glioblastomu.

Klíčová slova

nádorové kmenové buňky – multiformní glioblastom – mikroRNA

Summary

Glioblastoma multiforme is the most common intracranial malignity of astrocyte origin in adults. Despite complex therapy consisting of maximal surgical resection, adjuvant concomitant chemoradiotherapy with temozolomide followed by temozolomide in monotherapy, the median of survival ranges between 12 and 15 months from diagnosis. This infaust prognosis is very often caused by both impossibility of achieving of sufficient radical surgical resection and tumor resistance to adjuvant therapy, which relates to the presence of glioblastoma stem cells. Similarly to normal stem cells, glioblastoma stem cells are capable of self-renewal, differentiation, and unlimited slow proliferation. Their resistance to conventional therapy is also due to higher expressions of DNA repair enzymes, antiapoptotic factors and multidrug transporters. Therefore, targeting these unique properties could be a novel promising therapeutic approach leading to more effective therapy and better prognosis of glioblastoma multiforme patients. One of the approaches how to successfully regulate above-mentioned properties is targeted regulation of microRNAs (miRNAs). These small non-coding RNA molecules post-transcriptionally regulate expression of more than 2/3 of all human genes that are also involved in stem cell associated signaling pathways. Moreover, deregulated expression of some miRNAs has been observed in many cancers, including glioblastoma multiforme.

Key words

cancer stem cells – glioblastoma multiforme – microRNA

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Úvod

Multiformní glioblastom (glioblastoma multiforme – GBM) patří do skupiny high-grade astrocytomů a s incidencí 3,55 nových případů na 100 000 lidí ročně se řadí mezi nejčastěji se vyskytující intrakraniální malignity [1]. Tento nádor je tvořen rychle rostoucí masou málo diferencovaných astrocytů, která často prorůstá do okolní tkáně. Bývá velmi silně vaskularizován, špatně ohraničen a často inklinuje k nekrotizaci. Léčba je založena na maximální možné chirurgické resekci tumoru následované konkomitantní chemoradioterapií s temozolomidem (TMZ) a adjuvantním TMZ v monoterapii [2]. I přes tuto vysoce intenzivní léčbu je prognóza pacientů infaustní s mediánem celkového přežití (overall survival – OS) přibližně 12–15 měsíců od stanovení diagnózy [3].

Podle současných studií je jedním z důvodů špatné prognózy mnoha nádorových onemocnění malá populace nádorových buněk vyznačující se unikátními vlastnostmi, jako jsou schopnost sebeobnovy, diferenciace a neomezené proliferace. Tyto buňky disponují rovněž zvýšenou rezistencí k podávané léčbě, a stojí tak pravděpodobně za vznikem časných recidiv. Pro svoji podobnost se zdravými kmenovými buňkami bývají označovány jako nádorové kmenové buňky (carcinoma stem cells – CSCs) nebo také tumor iniciující buňky (tumor-initiating cells – TICs) [4]. Cílená regulace CSCs by tedy na základě současných poznatků mohla významně zefektivnit terapii mimo jiné i u GBM, kde byly tyto buňky mnohokrát pozorovány [5].

Jedna z možností, jak efektivně zasahovat do biologie CSCs, je skrze cílenou regulaci mikroRNA (miRNA), krátkých nekódujících RNA, které jsou schopné vazbou na cílovou molekulu mediátorové RNA (mRNA) posttranskripčně regulovat genovou expresi. Bylo prokázáno, že jsou tyto 18–25 nukleotidů dlouhé molekuly zapojené v regulaci většiny důležitých buněčných procesů, jako je proliferace, diferenciace, apoptóza anebo řízení buněčného cyklu. Není proto divu, že změna v expresi několika miRNA často vede na úrovni buňky k fatálním následkům ústícím až v její ma-

ligní transformaci [6]. MiRNA hrají klíčovou roli rovněž v biologii CSCs, kde se podílejí na udržování jejich jedinečných vlastností. Předpokládá se tedy, že by cílené ovlivnění konkrétních miRNA v CSCs mohlo vést ke ztrátě „stem-like“ fenotypu těchto buněk, což by v klinickém důsledku mohlo zefektivnit terapii, prodloužit čas do progresu onemocnění, a tedy i OS pacientů s prognosticky nepříznivými nádorovými onemocněními, mezi které se zcela jistě řadí i GBM [7].

Historie a biologie CSCs

Teorie CSCs je poměrně novým pohledem na nádorovou transformaci. Její kořeny však sahají až do 1. poloviny 20. století, kdy bylo zjištěno, že nádorová tkáň vykazuje značnou buněčnou heterogenitu a že dokonce i jednotlivé buňky jsou schopné iniciovat vznik nového nádoru [8]. Další výzkumy pak ukázaly, že některé nádorové buňky jsou multipotentní, a tedy schopné diferenciace do více buněčných podtypů [9].

Přímé důkazy o existenci CSCs však přinesly až na začátku 90. let 20. století nové technologie, jako je metoda FACS (fluorescence-activated cell sorting) a přístupy založené na transplantaci buněk do imunodeficientních myší. V roce 1994 Lapidot et al vyzolovali z akutní myeloidní leukemie na základě exprese markerů hematopoetických buněk (CD34⁺CD38⁻) buněčnou populaci, která vykazovala vysoce tumorogenní vlastnosti. Tyto buňky dnes označujeme jako nádorové kmenové (CSCs) nebo tumor iniciující (TICs) buňky [10]. Ovšem až v roce 2003 se podařilo poprvé vyzolovat CSCs i ze solidního nádoru, konkrétně z karcinomu prsu. Tyto buňky exprimující stejně jako normální kmenové buňky povrchové markery CD44⁺CD24^{-/low} byly po implantaci do imunodeficientních myší schopné iniciovat růst nového nádoru [11]. Poté následovaly studie, které potvrdily existenci CSCs i v dalších typech nádorových onemocnění: kolorektálním karcinomu, melanomu a u nádorů mozku včetně GBM [12–15].

Teorie CSCs předpokládá, že jsou buňky nádoru, stejně jako v případě nor-

mální zdravé tkáně hierarchicky uspořádány, přičemž na vrcholu stojí malá populace buněk dnes známá jako CSCs. Jejich původ je však doposud zahalen mnoha nejasnostmi. Nejčastěji diskutovanou teorií je, že vznikají ze somatických kmenových buněk tkáně, ze které se nádor vyvinul. Tuto hypotézu podporuje fakt, že somatické kmenové buňky jsou jedny z nejdéle žijících buněk. Jsou tedy vystaveny delšímu působení genotoxických vlivů než ostatní buňky a díky tomu se u nich mohou nakumulovat mutace vedoucí až k nádorové transformaci. Podle jiných autorů za vznikem CSCs stojí dediferenciace progenitorových nebo více diferencovaných nádorových buněk, kdy vlivem mutací získávají tyto buňky vlastnosti buněk kmenových [16,17].

Mezi tyto unikátní vlastnosti patří i schopnost sebeobnovy a diferenciace. CSCs jsou tedy stejně jako jejich nenádorové analogy schopny asymetrického dělení, při kterém vzniká jedna buňka mateřského fenotypu a jedna buňka diferencovaná. Mechanizmy zajišťující správnou funkci těchto vlastností podléhají v normálních kmenových buňkách přísné regulaci. Právě mutace, které do těchto schopností a signalizačních drah s nimi souvisejících zasahují, jsou jedním z předpokladů neoplastické transformace [17,18]. V tomto kontextu jsou nejčastěji zmiňovány signální dráhy Wnt, Notch a Hedgehog [19–22].

Přítomnost CSCs do značné míry vysvětluje nízkou efektivitu konvenční terapie u některých nádorových onemocnění. Tato terapie je totiž zaměřena především na rychle se dělící buňky nádoru. Avšak jak již bylo zmíněno, CSCs se většinu času vyskytují v tzv. klidovém stadiu buněčného cyklu, a tedy rychlost jejich proliferace není tak rapidní. To poskytuje buňce dostatečný čas, aby oprávil poškozenou DNA, v čemž jí navíc významně pomáhají zvýšené hladiny DNA reparačních enzymů a ABC transportérů (transportéry lékové rezistence) [17,18]. Dokonce bylo zjištěno, že CSCs, kterým se podařilo uniknout chemoterapii, byly více rezistentní a tuto vlastnost následně předávaly i svým dceřiným buňkám. Tato skutečnost tak koresponduje s faktem, že rekurentní tumory bývají

často k léčbě méně citlivé než primární nádor [23].

Glioblastomové kmenové buňky

Glioblastomové kmenové buňky (glioblastoma stem cells – GSCs) pocházejí pravděpodobně z neurálních kmenových buněk. Nasvědčuje tomu i skutečnost, že poprvé byly z GBM tkáně vyizolovány na základě exprese povrchového glykoproteinu CD133, jehož přítomnost je typická pro normální neurální kmenové buňky (neural stem cells – NSCs). GSCs, stejně jako NSCs rostou v bezsérovém mediu jako suspenzní sférické buněčné kolonie neboli neurosféry a vyznačují se expresí stejných identifikujících markerů [12,15,24]. Historicky nejvíce používaným markerem GSCs je již zmiňovaný CD133, který se však na základě posledních studií jeví jako nepřilíživě specifický [14,25,26]. Mnohem větší specifita je v současné době přisuzována molekule CD15 [27] a cytoskeletálnímu proteinu nestinu, jehož exprese se mimo jiné u gliomů ukázala být negativním prognostickým faktorem. V dnešní době se ale nabízí i další možnosti izolace GSCs nevyžadující použití povrchových markerů. Zajímavým způsobem identifikace této buněčné populace je kupříkladu již zmiňovaný fakt, že GSCs jsou pomalu se dělící buňky. Fluorescenční barvivo PKH-26 se naváže na povrch cytoplazmatické membrány buněk a s každým následným buněčným dělením se jeho množství na buňce sníží o polovinu. Dceřiné buňky proto vykazují nižší fluorescenční aktivitu než buňka mateřská. Pomocí FACS lze tedy následně identifikovat GSCs jako nejvíce fluorescenčně aktivní buňky v suspenzi [28]. Jiný způsob izolace GSCs je založen na schopnosti buněk aktivně se zbavovat škodlivých látek. Jde o metodu izolace tzv. side population, u které byla detekována zvýšená hladina ABC transportérů a jež rovněž disponuje kmenovými vlastnostmi [29].

Pro GSCs je typická perivaskulární lokalizace a jejich výskyt byl také objeven v okolí nekrotických ložisek, což dokládá fakt, že je stem-like fenotyp těchto buněk značnou mírou závislý na okolním mikroprostředí. Často diskutován je vliv hypoxie na chování GSCs [30].

Hypoxie indukuje expresi HIF (hypoxia inducible factor), který zvyšuje schopnost sebeobnovy jak GSCs, tak i non-GSCs. Zatímco HIF1 α je exprimován i neurálními kmenovými buňkami, HIF2 α je typický pouze pro GSCs, což z něj v podstatě činí ideální terapeutický cíl [31].

Správná identifikace a izolace GSCs je tedy klíčovým krokem pro další výzkum. Jejich eliminace, případně diferenciaci by mohla mít velký terapeutický význam a mohla by přinést novou naději pacientům postiženým GBM.

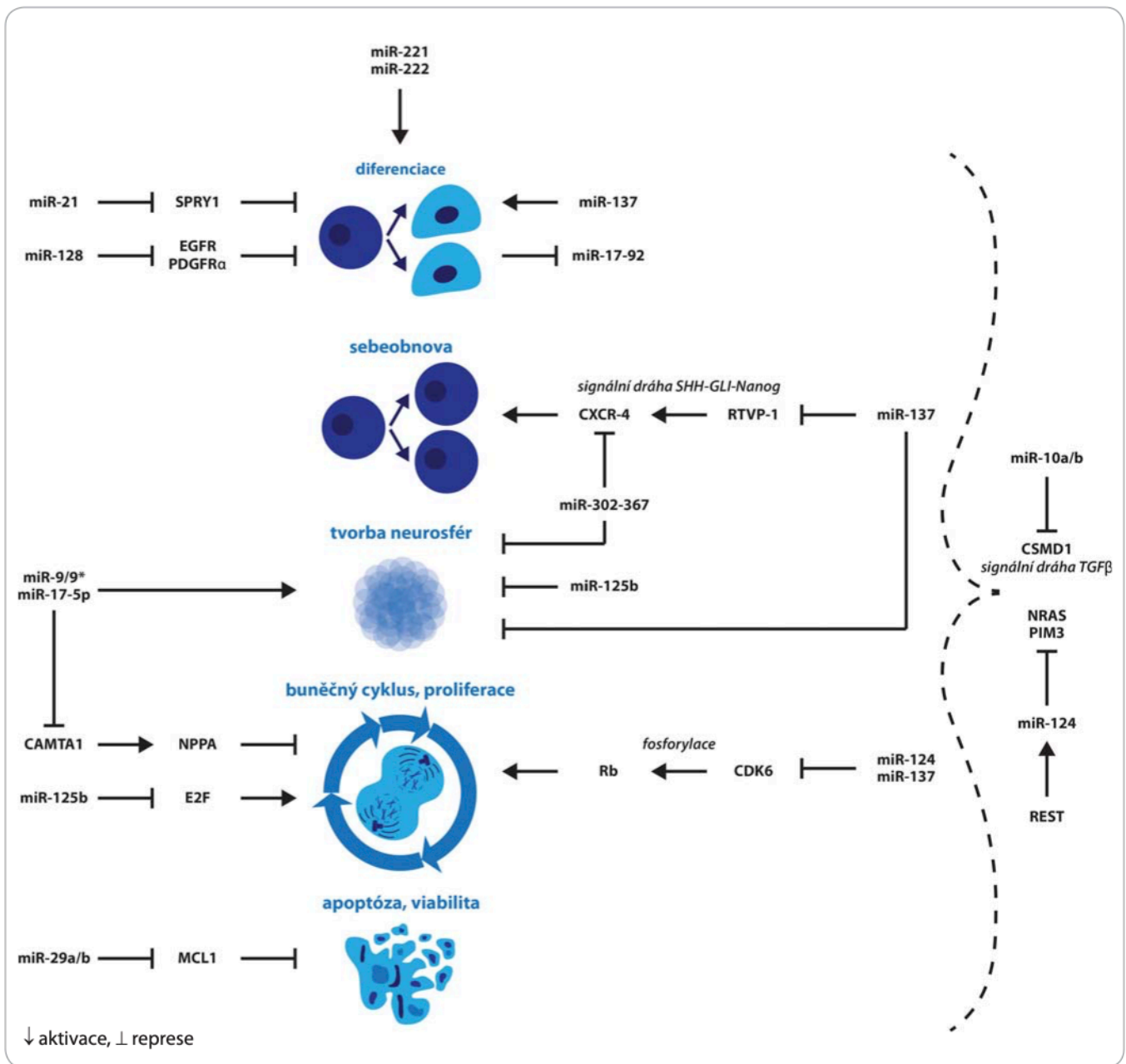
Význam miRNA v regulaci kmenových vlastností GSCs

MiRNA jsou krátké molekuly RNA se značným potenciálem regulovat genovou expresi. Podle současných výzkumů tvoří geny pro miRNA asi 3 % lidského genomu, ale jsou schopny ovlivňovat expresi až 60 % kódujících lidských genů [32,33]. Jejich regulační efekt se tedy samozřejmě uplatňuje ve většině důležitých buněčných procesů, jako je proliferace, diferenciaci, apoptóza, buněčný cyklus a stejně tak udržování kmenových vlastností buněk [6]. Regulační schopnosti miRNA jsou tak zapojeny i do biologie GSCs, což činí z těchto molekul ohnisko zájmu mnoha výzkumných kolektivů. Cílená regulace miRNA, které jsou zapojeny do řízení kmenových vlastností GSCs, by totiž mohla být využita jako nový efektní přístup léčby nádorových chorob, včetně GBM [7].

Tato strategie však v sobě skrývá mnohá úskalí. GSCs jsou velmi blízké NSCs a mechanismy udržující jejich kmenovost jsou často stejné či podobné u obou těchto buněčných populací. Důležité tedy je, aby léčba zaměřující se na GSCs cílila opravdu jenom na tyto buňky. Lang et al proto za použití metody hlubokého sekvenování identifikovali miRNA, které jsou rozdílně exprimované mezi těmito buněčnými populacemi, a tím získali sadu potenciálních terapeutických cílů pro cílenou léčbu, která cílí pouze na GSCs, a nikoli na NSCs. Signifikantně zvýšenou hladinu v GSCs vykazovaly miR-10a, miR-10b a miR-140-5p. Naopak snížená exprese byla detekována u miR-874 a miR-124. Jako přímý cíl miR-10a a miR-10b byl identifikován nádorový supresor CSMD1 (CUB and

SUSHI multiple domain protein 1), jehož ztráta nebo snížená hladina byly zaznamenány i u jiných typů nádorů [34,35]. Tento transmembránový protein je podle některých studií zapojen do signalizace TGF- β (transforming growth factor β), jež patří mezi hlavní regulátory kmenovosti a rovněž se podílí na mechanismech buněčného růstu a apoptózy [36,37]. Regulační vliv této miRNA byl popsán také pro další geny zapojené v nádorové transformaci, jako je PTEN (phosphatase and tensin homolog), STAT3 (signal transducer and activator of transcription 3), SDC-1 (syndecan 1), TIAM1 (T-cell lymphoma invasion and metastasis 1), NF-1 (neurofibromin 1). Inhibice miR-10b v GSC a GBM buňkách měla za následek výrazné snížení proliferace, migrace, invazivity a buněčného růstu, a to zejména v případě GSC [38].

Mezi přímé cíle nádorově supresorové miR-124 pak patří jednak známý onkogen NRAS (neuroblastoma RAS viral (v-ras) oncogene homolog), jenž je zapojen do procesu proliferace, diferenciaci a schopnosti přežívání buněk, a dále serin/treonin kináza PIM3 (Pim-3 proto-oncogene, serine/threonine kinase) modující buněčný cyklus [34]. MiR-124 byla rovněž identifikována jako cíl transkripčního faktoru REST (RE1-silencing transcription factor), jednoho z hlavních represorů neurální diferenciaci. Mezi její další cílové molekuly totiž patří fosfatáza SCP-1 (sarcolemmal calcium-binding protein 1), která výrazně potlačuje neurální diferenciaci [39,40]. Silber et al rovněž potvrdili zapojení miR-124 spolu s miR-137 do procesu diferenciaci, a to jak v NSCs, tak i v mozkových CSCs. Obě tyto miRNA ovlivňují proliferaci GBM buněčných linií prostřednictvím přímé inhibice cyklin dependentní kinázy 6 (CDK6), která se skrze fosforylaci proteinu Rb (retinoblastoma) podílí na řízení buněčného cyklu [41]. Bylo rovněž zjištěno, že supresorová miR-137 vykazuje v GBM z důvodu hypermetilace svého promotoru sníženou hladinu. Exprese této molekuly pak narůstá s mírou diferenciaci NSCs i GSCs. Po transfekci pre-miR-137 do GSCs klesá u těchto buněk schopnost sebeobnovy, tvoření neurosfér a rovněž exprese markerů kmenových buněk



Obr. 1. Zapojení miRNA v regulaci biologických vlastností GSCs.

Oct4 (POU class 5 homeobox 1), Nanog (Nanog homeobox), Sox2 (SRY (sex determining region Y)-box 2) a Shh (Sonic hedgehog). MiR-137 reguluje kmenové vlastnosti GSCs skrze svůj přímý cíl RTVP-1 (GLI pathogenesis-related 1 – GLIPR-1), jehož exprese přímo koreluje se stupněm malignity astrocytomů. RTVP-1 zvyšuje expresi CXCR-4 (chemokine (C-X-C motif) receptor 4), který je zapojen v signalizaci SHH-GLI-Nanog, a tím přispívá ke zvýšení schopnosti sebeobnovy buněk [42]. Tumorogenní

a invazivní marker CXCR-4 je přímým cílem klastru miR-302-367, a proto se i tato skupina miRNA pravděpodobně podílí na inhibici sebeobnovy, invazivity a infiltrace GBM iniciujících buněk. Mechanismus tohoto působení je pak rovněž připisován ovlivnění signální dráhy SHH-GLI-Nanog, ve které, jak už bylo uvedeno, je CXCR-4 zapojen. Po zvýšení hladiny miR-302-367 v GSCs došlo k poklesu schopnosti tvořit neurosféry a naopak ke zvýšení exprese astrocytárního markeru GFAP (glial fibrillary acidic

protein), což dokládá zapojení tohoto klastru i do procesu diferenciace [43]. Další miRNA zapojená do procesu diferenciace je miR-128. Po jejím zvýšení dochází k indukci diferenciace, a tudíž poklesu markerů kmenovosti, jako je Nestin nebo Sox2. Jejimi přímými cíli jsou známé mitogenní tyrozinkinázy EGFR (epidermal growth factor receptor) a PDGFRα (platelet-derived growth factor receptor, a polypeptide), u nichž už v předešlých studiích byl prokázán inhibiční vliv na neurální diferenciaci [44–46].

Aldaz et al se zaměřili na identifikaci molekul miRNA, jejichž exprese se během diferenciace GSCs mění, a tudíž se dá předpokládat jejich přímé zapojení do tohoto procesu. Míra exprese miR-93 a miR-106 se během diferenciace snižovala, naopak u miR-21, miR-29a, miR-29b, miR-221 a miR-222 se zvyšovala. Inhibice miR-221/222 v diferencujících GSCs měla za následek nárůst exprese proteinu nestin, ale pokles astrocytárních (GFAP) a neurálních markerů (neuron-specific class III beta-tubulin – TUJ1). Tento trend nebyl pozorován v případě miR-29a/b. Jejich zvýšení v GSCs nevyvolalo žádné změny v expresi markerů kmenovosti ani v diferenciaci, ale vedlo k prokazatelnému snížení viability a zvýšení apoptózy. MiR-29a/b jsou do procesu apoptózy zapojeny skrze svůj přímý cíl, antiapoptický protein Mcl-1 patřící do rodiny apoptických regulátorů Bcl-2. Překvapením studie bylo zjištění výrazné prodiferenční role miR-21, mnohými studiemi postulovanou jako miRNA se silně onkogenními vlastnostmi. Po zvýšení této miRNA v GSCs opět došlo ke snížení exprese nestinu a nárůstu TUJ1 a GFAP. Jejím přímým cílem byl určen SPRY1 (sprouty homolog 1, antagonist of FGF signaling (*Drosophila*)), který byl již dříve popsán jako inhibitor neurální diferenciace v myších embryonálních kmenových buňkách [47]. MiR-221/222 i miR-21 jsou všeobecně známé pro své onkogenní vlastnosti [48,49]. Ve výše popsané studii však autoři polemizují, že pokud by léčba cílila na tyto miRNA, neměla by požadovaný efekt zabráňující vzniku recidiv právě kvůli zapojení těchto miRNA do procesu diferenciace [47]. Schraivogel et al se zaměřili na rozdíly v expresním profilu miRNA mezi CD133⁺ a CD133⁻ buňkami. CD133, jak již bylo uvedeno, je jedním z nejpoužívanějších markerů pro identifikaci a izolaci GSCs. MiR-9/9*, miR-15b, miR-17-5p, miR-106 vykazovaly nejvyšší expresi u CD133⁺ buněk, miR-221/222, miR-27, miR-21 zase u CD133⁻ buněk. Cílená inhibice miR-9/9* a miR-17-5p vedla k redukci CD133⁺ buněk a jejich schopnosti tvořit neurosféry. Jako možný cíl těchto dvou miRNA byl identifikován transkripční faktor CAMTA1 (calmodu-

lin binding transcription activator 1), který indukuje expresi krátkého sekretovaného proteinu NPPA (natriuretic peptide A) a jeho receptoru, jež se podílí na inhibici proliferace [50]. Jiná výzkumná skupina zase uvedla, že k nejvíce sníženým miRNA v CD133⁺ oproti CD133⁻ buňkám patří miR-125b, jež je zapojena do řízení proliferace. Jejím přímým cílem je totiž člen rodiny E2F (E2F transcription factor), což jsou významní regulátoři buněčného cyklu. Po zvýšení její hladiny v CD133⁺ buňkách došlo k repressi exprese nestinu a CD133 a schopnosti těchto buněk tvořit neurosféry [51]. Exprese miR-17-92 se během diferenciace snižuje, což dokládá jejich význam v udržování kmenových vlastností GSCs. Tento klastř je zapojen v řízení proliferace a apoptózy skrze své cíle CDKN1A (cyclin-dependent kinase inhibitor 1A), E2F1 a PTEN. Jako přímý cíl této molekuly byl rovněž určen růstový faktor pojivové tkáně CTGF (connective tissue growth factor), který váže VEGFA (vascular endothelial growth factor A) a tím přispívá k inhibici nádorové angiogeneze [52]. Zapojení miRNA v regulaci biologických vlastností a exprese vybraných markerů u GSCs je shrnuto na obr. 1, resp. tab. 1.

Význam miRNA v chemoradiorezistenci GBM

Léčba GBM je založena na maximální možné chirurgické resekci tumoru následované konkomitantní chemoradioterapií s TMZ. Tato adjuvantní terapie však téměř ve všech případech selhává a u pacientů dochází k časnému relapsu onemocnění způsobeného pravděpodobně přítomností rezistentních GSCs. Právě molekuly miRNA byly mnohokrát popsány jako regulátory chemoradiorezistence u mnoha nádorových onemocnění včetně GBM. Z tohoto pohledu skrývá ve svém působení značný terapeutický potenciál miR-211, která výrazně zvyšuje senzitivitu GBM buněk k chemoradioterapii. Tato miRNA mimo jiné cílí MMP-9 (matrix metalloproteinase 9), čímž výrazně přispívá ke snížení invazivity a migrace GSCs. Samotná radioterapie způsobila nárůst MMP-9, a tím i invazivity a migrace. V kombinaci s miR-211 a inhibitorem MMP-9 však došlo k jejich výraznému snížení a rov-

Tab. 1. Zapojení miRNA v regulaci exprese vybraných markerů u GSCs.

	Regulovaná miRNA	Sledovaný marker
navýšení exprese miRNA	miR-137	↓ Oct-4 ↓ Nanog ↓ Sox2 ↓ Shh
	miR-302-367	↑ GFAP
	miR-128	↓ Nestin ↓ Sox2
	miR-21	↓ Nestin ↑ GFAP ↑ TUJ1
	miR-125b	↓ Nestin ↓ CD133
	miR-221/222	↑ Nestin ↓ GFAP ↓ TUJ1
snížení exprese miRNA	miR-9/9*	↓ CD133
	miR-17-5p	↓ CD133

↑ zvýšení exprese sledovaného markeru, ↓ snížení exprese sledovaného markeru
GSCs – glioblastoma stem cells

něž ke zvýšení apoptózy. Inhibice MMP-9 vede i k indukci apoptózy skrze iniciační kaspázu 9. Především studiemi bylo zjištěno, že samotné podání TMZ vede k navýšení mnohočetných lékových transportérů v plazmatické membráně, které jsou schopny aktivní exkrece škodlivých látek ven z buňky. Buňky vystavené společnému působení TMZ, miR-211 a MMP-9 inhibitoru toto zvýšení nevykazovaly, což dokládá značné ambice této miRNA v budoucí cílené léčbě [53]. Další miRNA, která by se mohla podílet na regulaci mnohočetných lékových transportérů a tím i chemorezistence GSCs je miR-328 [54]. Jako cíl této miRNA byl totiž určen ABCG2 (ATP-binding cassette, sub-family G), který rovněž patří do rodiny mnohočetných lékových transportérů a významnou mírou přispívá k lékové rezistenci buněk [55]. Přítomnost to-

hoto transportéru je charakteristická pro tzv. side-population buňky, jež disponují zvýšenou tumor iniciační kapacitou, schopností sebeobnovy a multipotence, tedy vlastnostmi typickými pro kmenové buňky [54]. Zvýšení senzitivity GSCs k chemoterapii způsobuje také inhibice miR-21. Tato miRNA se účastní regulace apoptózy skrze Bax (Bcl-2-associated X protein) a Bcl-2 (B-cell CLL/lymphoma 2). Její inhibice způsobuje nárůst proapoptického Bax na úkor antiapoptického Bcl-2, což má za následek celkové zvýšení apoptózy GSCs. Zhang et al zjistili, že tento efekt se v kombinaci inhibice miR-21 a podání TMZ ještě násobuje [56].

Závěr

Přestože je GBM nádorové onemocnění s velmi nízkou incidencí, jedná se o velmi agresivní malignitu do značné míry rezistentní ke konvenční terapii, a tedy s infaustní prognózou. Z tohoto důvodu je mu v poslední době vědeckými pracovníky věnována stále větší pozornost ve snaze objevit nové terapeutické přístupy umožňující překlenout zmiňovanou necitlivost k léčbě a prodloužit tak OS pacientů s tímto onemocněním. Nadějným terapeutickým cílem se zdají být GSCs, jež jsou pravděpodobně jednou z příčin biologicky nepříznivého chování tumoru. Jejich cílené ovlivnění prostřednictvím miRNA, přirozených regulátorů genové exprese, by pak mohlo vést k zvýšení senzitivity GBM k adjuvantní terapii, významnému prodloužení času do progresu onemocnění, a tedy i zlepšení celkové prognózy pacientů.

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Identification of microRNAs differentially expressed in glioblastoma stem-like cells and their association with patient survival

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Glioblastoma stem-like cells (GSCs) are critical for the aggressiveness and progression of glioblastoma (GBM) and contribute to its resistance to adjuvant treatment. MicroRNAs (miRNAs) are small, non-coding RNAs controlling gene expression at the post-transcriptional level, which are known to be important regulators of the stem-like features. Moreover, miRNAs have been previously proved to be promising diagnostic biomarkers in several cancers including GBM. Using global expression analysis of miRNAs in 10 paired *in-vitro* as well as *in-vivo* characterized primary GSC and non-stem glioblastoma cultures, we identified a miRNA signature associated with the stem-like phenotype in GBM. 51 most deregulated miRNAs classified the cell cultures into GSC and non-stem cell clusters and identified a subgroup of GSC cultures with more pronounced stem-cell characteristics. The importance of the identified miRNA signature was further supported by demonstrating that a Risk Score based on the expression of seven miRNAs overexpressed in GSC predicted overall survival in GBM patients in the TCGA dataset independently of the IDH1 status. In summary, we identified miRNAs differentially expressed in GSCs and described their association with GBM patient survival. We propose that these miRNAs participate on GSC features and could represent helpful prognostic markers and potential therapeutic targets in GBM.

Glioblastoma multiforme (GBM) is the most frequently occurring primary brain tumor of astrocytic origin in adults. Despite complex therapy consisting of maximal surgical resection, adjuvant concomitant chemoradiotherapy with temozolomide followed by temozolomide in monotherapy, the prognosis remains dismal¹. The short survival of GBM patients is caused by both the impossibility of achieving “biologically” radical surgical resection and tumor resistance to adjuvant therapy. Glioblastoma stem-like cells (GSCs) are thought to be an important contributor to the poor response to the adjuvant therapy due to the higher expressions of the DNA repair enzymes, antiapoptotic factors, and multidrug transporters^{2,3}. These rather slow proliferating cells are also capable of self-renewal and multilineage differentiation, are highly invasive, modulate immune response and promote angiogenesis. GSCs form gliomaspheres in serum-free media *in vitro*^{4,5} and have strong tumorigenic potential in immunodeficient animals recapitulating the hallmarks of the original tumors⁶. GSCs express, although to a variable extent, specific stemness markers such as the transcription factor Sox-2, the cytoskeletal protein nestin,

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and/or the cell surface glycoprotein CD133^{7,8}, which are generally used for their identification. According to some studies, the presence of GSCs as determined by functional assays as well as the expression of GSC markers is associated with the prognosis in GBM patients^{9–12}. Several studies have shown that microRNAs (miRNAs) are important molecular players closely related to the biological features of GSCs. MiRNAs are highly conserved, 18–25 nucleotide long non-coding RNAs that function as post-transcriptional regulators of gene expression by silencing their mRNA targets. It is estimated that miRNAs could regulate up to 60% of human genes including genes associated with the maintenance of the stem-like phenotype, differentiation, and chemo- and radioresistance^{13,14}. Thus, miRNAs play significant roles in the functions of various types of healthy as well as cancer stem-like cells including GSCs^{15–17}. Indeed, changes in miRNA expression were observed during the transition of GSCs to more differentiated phenotypes¹⁸ and e.g. the miR-302-367 cluster was shown to be able to abolish the stem cell characteristics of GSCs¹⁹. Our previous studies also demonstrated that miRNAs are able to predict the survival in GBM patients^{20,21}.

In this study, we identified a set of miRNAs that is closely associated with the stem-like phenotype of GBM cells. We further corroborated the importance of the most differentially expressed miRNAs by showing their potential to predict overall survival in GBM patients independently of the IDH1 mutation status. These miRNAs may thus play an important role in the pathogenesis of brain tumors and represent potential therapeutic targets affecting GSCs and overcoming the therapeutic resistance of GBM.

Results

Characterization of the paired glioblastoma cell cultures. We successfully derived paired primary cell cultures from several GBMs (8 men and 2 women; median age 64 years - min 52, max 78 years), which were propagated in both defined serum-free medium favoring the expansion of GSCs and in medium supplemented with 10% FBS (non-stem cells). The cells cultured in serum-free medium initially formed gliomaspheres (Fig. 1A) and were subsequently propagated on laminin or geltrex (Fig. 1B). The matched paired primary cell cultures propagated in serum containing media grew adherently (Fig. 1C). The majority of the GSC cultures exhibited CD133 expression as determined by flow cytometry (Fig. 1D) and could undergo differentiation into GFAP and beta III tubulin expressing cells when transferred into serum containing media (Fig. 1E). All of the paired GBM cell cultures were IDH1/2 wild-type, their characteristics are summarized in Supplementary Table S1.

Western blot (Fig. 1F) and qRT-PCR ($P = 0.002$; Wilcoxon paired test) (Fig. 1G) analyses revealed substantially higher Sox-2 expression in the cells cultured in serum-free conditions in comparison to the cells derived from the same patient sample propagated in serum containing medium. The expression of nestin, another stem cell marker, correlated with Sox-2 expression ($r = 0.7955$; $P < 0.0001$) and there was a trend for higher nestin expression in the GBM primary cells derived in serum-free conditions ($P = 0.065$; Wilcoxon paired test) (see Supplementary Figure S1).

The large majority of the cultures propagated in serum-free as well as serum containing media were tumorigenic in immunodeficient mice (7/8 and 6/8 cultures, respectively). Nevertheless, the paired GBM cell cultures formed xenograft tumors with distinct features. The tumors derived from the glioma primary cell cultures propagated in serum containing media were characteristically well demarcated and GFAP negative (Fig. 2C,D). In contrast, the paired cultures derived in serum-free conditions typically produced GFAP positive tumors with single cell infiltration into the surrounding brain tissue including the contralateral hemisphere, white matter tracts and tropism towards the periventricular regions (Fig. 2A,B).

In summary, we verified that the cell cultures derived in serum-free conditions have typical characteristics of glioma stem-like cells (GSCs).

MicroRNAs differentially expressed in the paired GSC and non-stem glioblastoma cell cultures. To identify a set of miRNAs characteristic for glioma stem-like cells we performed a genome-wide expression profiling of 2578 human miRNAs in the 10 paired GSC and non-stem GBM cell cultures derived in serum-free and serum supplemented medium, respectively. LIMMA analysis for paired samples revealed 431 significantly deregulated miRNAs in the GSCs in comparison with the non-stem GBM cells ($P < 0.05$) (see Supplementary Table S2). 51 miRNAs were deregulated at a significance level below 0.001 (25 miRNAs were upregulated and 26 miRNAs were downregulated). Importantly, among the 51 most deregulated miRNAs expression of 23 miRNAs correlated with Sox-2 expression at a significance level lower than 0.001, and 14 miRNAs correlated with both Sox-2 ($P < 0.001$) and nestin expression ($P < 0.05$) (Table 1). These data strongly suggest that several of the identified miRNAs are closely linked to the stemness of the glioma cell lines cultured in serum-free media.

Cluster analysis based on the 51 most differentially expressed miRNAs correctly classified all GSC and 80% of the non-stem cell cultures (Fig. 3A). This analysis also revealed that the main cluster I containing all GSC samples was divided into two subclusters. Subcluster IA was exclusively composed of GSC cultures and the pattern of miRNA expression was more distinct from serum cultures contained in cluster II. All analyzed cultures in subcluster IA were tumorigenic and exhibited pronounced multilineage differentiation. Subcluster IB, which contained the remaining four serum-free derived GSC cultures, also included two serum derived non-stem cell cultures; moreover, the serum-free derived cultures in this subcluster exhibited only little differentiation when exposed to 10% serum and one of them did not form tumors in immunodeficient mice. Collectively, these data suggest somewhat less pronounced stemness characteristics of GSC cultures in subcluster IB (Table 2, Supplementary Table S1). Statistical analysis comparing only cell cultures from subcluster IA, which exhibited more pronounced stemness characteristics, and cluster II samples containing the non-stem cell cultures revealed nine miRNAs (miR-9-3p, miR-93-3p, miR-93-5p, miR-106b-5p, miR-124-3p, miR-153-3p, miR-301a-3p, miR-345-5p, and miR-652-3p), which were all upregulated in GSCs at a significance level below 0.0001 (Fig. 3B).

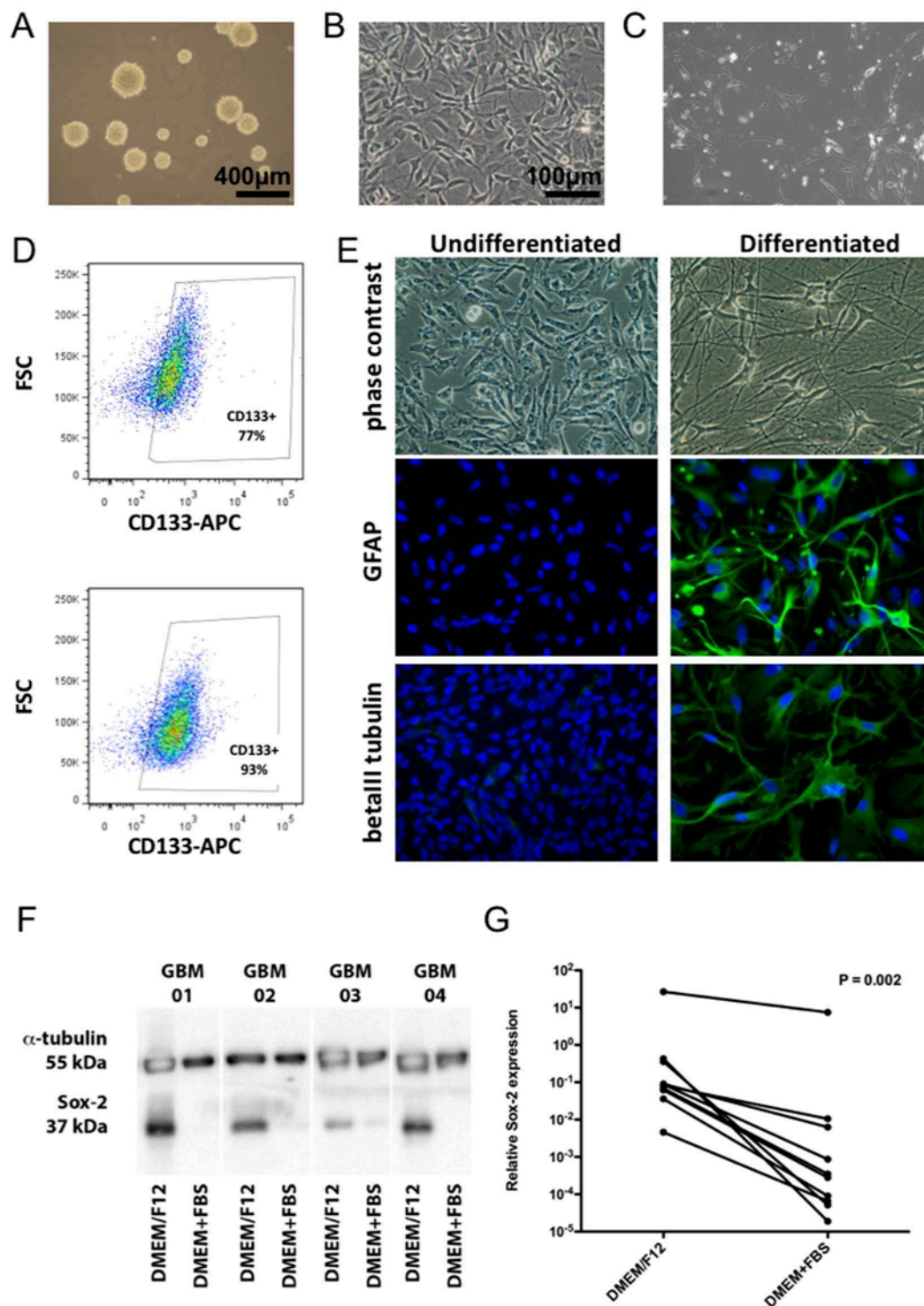


Figure 1. Characterization of the primary GBM cell cultures propagated in serum-free and serum containing media. (A) Cells growing in serum-free medium as gliospheres, (B) cells growing in serum-free medium on laminin, (C) adherent cell growing in serum containing medium, (D) detection of CD133 in two independent serum-free medium cultures, (E) differentiation of serum-free medium cultured cells induced by 10% fetal calf serum, (F) western blot analysis of Sox-2 and α -tubulin and (G) qRT-PCR of Sox-2 expression in GBM cells propagated in serum-free (DMEM/F12) and serum containing (DMEM + FBS) media. The P value signifies the statistical significance of the difference between the paired primary cell lines as assessed by the Wilcoxon paired test.

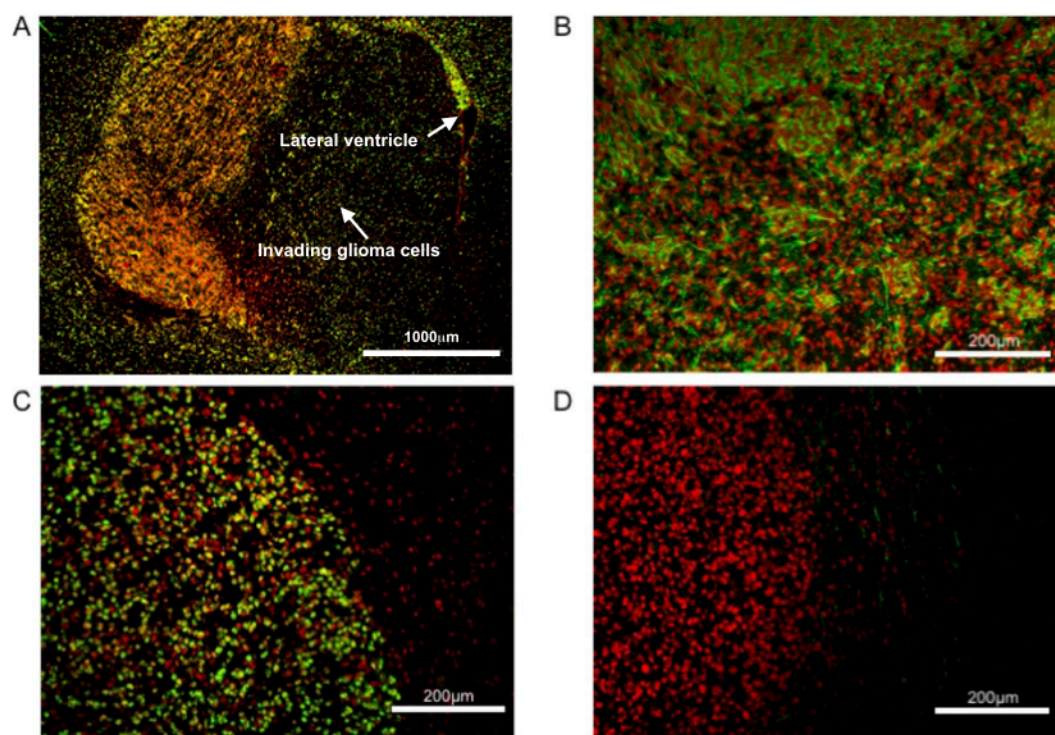


Figure 2. Characteristics of orthotopic xenotransplants derived from primary GBM cell cultures propagated in serum-free (A,B) and serum containing (C,D) media. (A and C) Detection of human glioma cells by an antibody against human nuclei (in green). (B and D) Detection of GFAP expression (in green). ToPro3 was used for nuclear counterstaining (red).

	miRNA	Sox-2		Nestin	
		Spearman r	P value	Spearman r	P value
Negative correlation	miR-3195	-0.85	<10 ⁻⁵	-0.60	0.006
	miR-3141	-0.83	<10 ⁻⁵	-0.47	0.036
	miR-4656	-0.81	<10 ⁻⁴	-0.51	0.023
	miR-100-5p	-0.79	<10 ⁻⁴	-0.39	NS
	miR-4739	-0.77	<10 ⁻³	-0.42	NS
	miR-3180	-0.75	<10 ⁻³	-0.55	0.013
	miR-1260b	-0.75	<10 ⁻³	-0.46	0.043
	miR-1233-5p	-0.74	<10 ⁻³	-0.49	0.029
	miR-4674	-0.73	<10 ⁻³	-0.54	0.015
	miR-328-5p	-0.73	<10 ⁻³	-0.48	0.032
	miR-378h	-0.72	<10 ⁻³	-0.48	0.034
	miR-4505	-0.71	<10 ⁻³	-0.46	0.045
	miR-5787	-0.71	<10 ⁻³	-0.47	0.036
	miR-1207-5p	-0.70	<10 ⁻³	-0.37	NS
Positive correlation	miR-345-5p	0.82	<10 ⁻⁵	0.57	0.011
	miR-1180-3p	0.78	<10 ⁻⁴	0.45	0.048
	miR-9-3p	0.76	<10 ⁻³	0.40	NS
	miR-124-3p	0.75	<10 ⁻³	0.34	NS
	miR-106b-3p	0.73	<10 ⁻³	0.42	NS
	miR-1301-3p	0.73	<10 ⁻³	0.41	NS
	miR-130b-3p	0.71	<10 ⁻³	0.49	0.029
	miR-93-3p	0.70	<10 ⁻³	0.37	NS
miR-106b-5p	0.70	<10 ⁻³	0.32	NS	

Table 1. MiRNAs correlating with Sox-2 and nestin expression in paired primary GBM cell cultures.

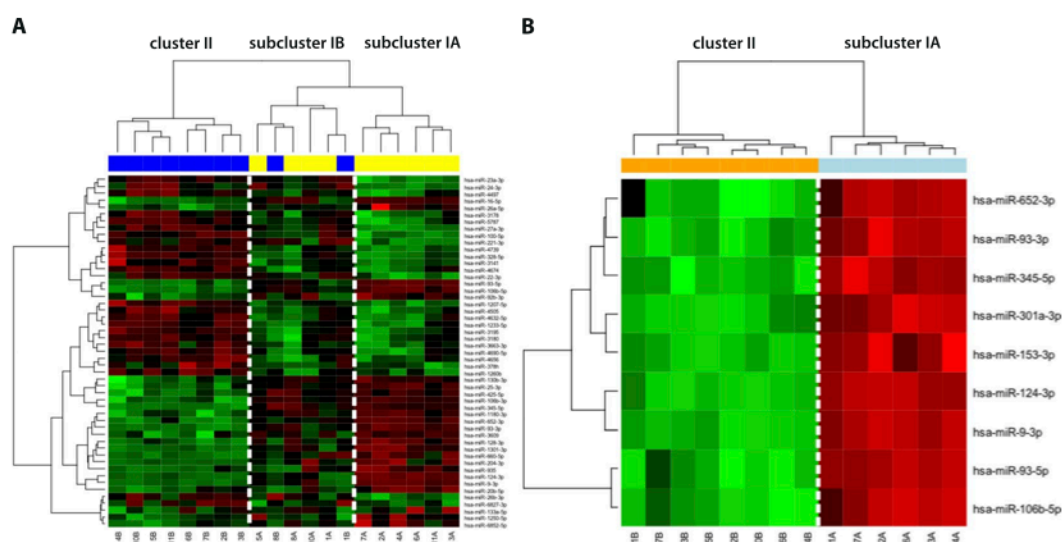


Figure 3. Hierarchical clustergram discriminating paired primary GSC (yellow) and non-stem glioblastoma cell cultures (blue) propagated in serum-free and serum containing medium, respectively. **(A)** Based on 51 differentially ($P < 0.001$) expressed miRNAs, **(B)** Based on 9 miRNAs differentially ($P < 0.0001$) expressed in GSC and non-stem cell cultures contained in subclusters IA and II, respectively. A gradient of green and red colors is used in the heatmap (green color indicates lower expression whereas red color indicates higher expression of individual miRNAs in analyzed samples).

Cluster	No. of GSC cultures	No. of non-stem cell cultures	Multilineage differentiation [†]	Tumorigenicity
IA	6	0	4/4**	5/5
IB	4	2	0/3	2/3 (GSC) 1/2 (non-stem cells)
II	0	8	n.d.	5/6

Table 2. Characteristics of the clusters identified based on the 51 most differentially expressed miRNAs.

[†]Number of cell cultures exhibiting pronounced differentiation in serum containing media/number of analyzed cell cultures, ** $P < 0.05$, Pearson's chi-squared test compared to cluster IB, n.d. - not determined.

Expression of all these miRNAs positively and statistically significantly correlated with Sox2 expression suggesting their close association with the stem cell-like phenotype of the GSCs.

MiRNAs differentially expressed in GSCs are associated with survival of GBM patients. To further support the potential importance of these miRNAs in GBM, we analyzed their relation to overall survival (OS) using the TCGA dataset comprising 485 GBM patients for whom OS and miRNA expression profiles were available. Seven out of nine of the miRNAs most differentially expressed between the GSC cluster IA and non-stem cell cluster II (Fig. 3B, miR-9-3p, miR-93-5p, miR-106b-5p, miR-153-3p, miR-301a-3p, miR-345-5p, and miR-652-3p) were represented in the TCGA dataset. First, we performed Z-score transformation on expression levels across all GBM samples for each of the aforementioned seven miRNAs; then, the seven-miRNA signature was used to calculate the Risk Score for each patient based on a linear combination of the miRNA expression level weighted by the regression coefficient derived from the multivariate Cox regression analysis^{22,23} as follows: risk score = $0.08270698 * \text{hsa-miR-652} + 0.15074626 * \text{hsa-miR-345} - 0.11310809 * \text{hsa-miR-301} - 0.15450420 * \text{hsa-miR-153} + 0.09238838 * \text{hsa-miR-9} - 0.18565873 * \text{hsa-miR-93} - 0.04894894 * \text{hsa-miR-106b}$. This composite miRNA Risk Score was a statistically significant prognostic factor in the univariate Cox regression analysis (HR = 2.718; 95% CI (1.814–4.073), $P < 1.26 * 10^{-6}$). Correspondingly, using the median value of the miRNA Risk Score as the threshold, GBM patients could be divided into a high-risk and a low-risk group. Kaplan–Meier analysis confirmed that OS of the high-risk patients was significantly lower in comparison with low-risk patients ($P < 3.26 * 10^{-5}$, log-rank test) (Fig. 4). We further tested the prognostic power of the seven-miRNA signature with respect to the IDH1 mutation status in a subset comprising 296 GBM patients for whom exome somatic mutation data were available. The univariate Cox regression analysis revealed that the seven-miRNA signature predicted OS in these patients with higher statistical significance ($P = 1.064 * 10^{-4}$; HR = 2.718; 95% CI (1.642–4.501)) in comparison with IDH1 status ($P = 3.3 * 10^{-3}$; HR = 0.3147; 95% CI (0.1393–0.7109)). These results were underscored using the multivariate Cox regression analysis ($P = 6.53 * 10^{-4}$; HR = 2.442; 95% CI (1.461–4.080) for the seven-miRNA signature and $P = 1.67 * 10^{-2}$; HR = 0.367; 95% CI (0.162–0.834) for IDH1 status). The whole model based on the seven-miRNA signature and IDH1 status predicted OS in GBM patients with a P

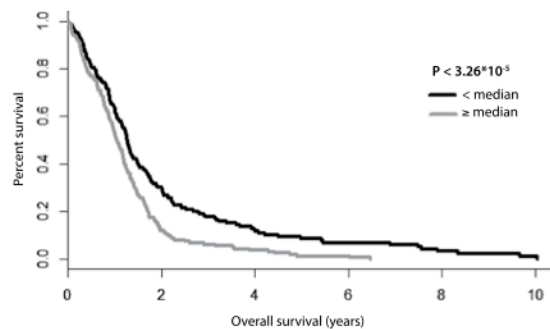


Figure 4. Kaplan-Meier survival curves estimating OS in GBM patients from the TCGA data set according to the 7-miRNA based Risk Score.

value 4.24×10^{-5} (see Supplementary Table S3). Importantly, the seven-miRNA signature was able to predict OS in both IDH1 wild-type ($n = 280$) and IDH1 mutated ($n = 16$) GBM patients ($P < 1.01 \times 10^{-3}$ and $P < 4.62 \times 10^{-2}$, respectively; log-rank test) (see Supplementary Figure S2).

Discussion

Glioblastoma multiforme (GBM), the most common malignant primary brain tumor arising from glial cells, is associated with fatal prognosis caused not only by its localization in the central nervous system, but especially by the high invasiveness and resistance to conventional therapies. This biological behavior is associated with the cellular and molecular heterogeneity, which is characteristic for this disease^{24,25}. Recent studies have suggested that GBM is driven and maintained by a subpopulation of clonogenic cells called glioblastoma stem-like cells (GSCs), which seem to play a crucial role in GBM biology^{26,27}. These cells also contribute to GBM chemoradioresistance through the activation of DNA damage checkpoint responses and the increase in DNA repair capacity^{28,29}. The small non-coding microRNAs (miRNAs) playing an important role in the posttranscriptional regulation of gene expression have been previously described in association with GBM initiation, progression, and resistance to therapy as well as with the maintenance of glioma stem-like cells³⁰.

In this study, we firstly successfully derived and characterized paired GBM cell cultures from several GBMs, which were propagated either under defined serum-free conditions, or in serum containing medium. These differing cell culture conditions most likely lead to the isolation of distinct cell subpopulations from the original tumor. Consistently with the literature, the paired cell lines displayed profound biological differences³¹. Glioma cells cultured in serum-free conditions frequently expressed CD133, although as described by others⁶ the quantity was variable in individual cell lines. The serum-free medium cultured cells also showed the potential to form gliomaspheres and differentiate into GFAP and beta III tubulin positive cells. In comparison with the paired glioma cultures derived in serum containing medium, the serum-free medium derived cells expressed significantly more Sox-2, the stemness marker crucial for the tumorigenicity of GSCs³², on both mRNA and protein levels. As previously reported³¹, the serum-free cultured cells characteristically formed highly infiltrative tumors when implanted into immunodeficient mice.

Having established that the cell lines cultured under serum-free conditions used in this study exhibit features typical of GSC, the paired GSC and non-stem cell cultures were utilized to uncover the miRNA expression pattern specific for GSC. Using global miRNA expression analysis, we revealed 51 most differentially expressed miRNAs. These miRNAs were able to classify the cell cultures into non-stem cell cluster II and two GSC subclusters IA and IB (Fig. 3A). Analysis of these two GSC subclusters showed that the first of them (IA) consisted of cultures with more pronounced GSC features compared to the second subcluster (IB) containing among others also two non-stem cell cultures. Subsequent analysis focusing on the miRNAs most differentially expressed between the GSC cluster IA and the non-stem cell cluster II highlighted nine miRNAs (miR-9-3p, miR-93-3p, miR-93-5p, miR-106b-5p, miR-124-3p, miR-153-3p, miR-301a-3p, miR-345-5p, and miR-652-3p) that were strongly upregulated in GSCs. Several of these miRNAs were previously described to be associated with the regulation of the stemness maintenance as well as with the biological behavior of GBM and survival of patients. MiR-9-3p (referred to as miR-9*) and its hairpin counterpart miR-9-5p (referred to as miR-9), which was also upregulated in GSCs though with lower statistical significance ($p < 3.3 \times 10^{-3}$; $\log_2 \text{FC} = 2.1$), seem to be specifically expressed in the brain^{33,34}, are evolutionary conserved from insects to human and are involved in vertebrate neural development³⁵⁻³⁷. Their activities are probably carried out through the effect on the Notch signaling pathway, especially by the targeting of Notch2 and the transcription factor Hes1, resulting in an enhanced differentiation and proliferation of neural stem cells (NSCs)^{38,39}. These data seem to be somewhat contradictory to our findings as we observed higher expression of both miRNAs in GSCs. The explanation may lie in the mutual regulation of miR-9/9* and Notch signaling. It was demonstrated that expression levels of miR-9/9* depend on the activation status of Notch signaling. While Notch inhibits differentiation of NSCs, it also induces miR-9/9*³⁸. Moreover, Hes1 expression oscillates with a period of 2–3 hours in NSCs and this oscillation is important not only for cell differentiation but also for proliferation as sustained Hes1 expression inhibits both processes^{39,40}. Thus, it can be assumed that miR-9/9* expression levels vary in time to allow cell proliferation. It also seems that the control mechanisms in GSCs are different from those in NSCs. In line with our findings, Schraivogel *et al.* reported that both miRNAs were highly abundant in CD133+ GSCs and their inhibition led to the reduced neurosphere formation and

stimulated cell differentiation⁴¹. Finally, the higher levels of miR-9/9* hairpin counterparts in GSCs could also contribute to the increased resistance of these cells to the conventional therapy. Munoz *et al.* recently showed that CD133+ GSCs expressed greater levels of miR-9 which led to the activation of the SHH/PTCH1/MDR1 axis. This axis has been shown to impart TMZ resistance. In the case of the CD133+ cells, the resistance is not acquired but seems to be inherent⁴².

Interestingly, miR-9 and miR-9* seem to be functionally linked with the miR-124, another most differentially expressed miRNA identified in our study. Staahl *et al.* published that mitotic exit in neurogenesis is *inter alia* partially driven by these three matured miRNAs⁴³. Another study described the synergistic effect of miR-9 and miR-124 on the strong suppression of the GTP-binding protein Rap2a and consequent promotion of neuronal differentiation of NSCs and dendritic branching of differentiated neurons⁴⁴. A very similar effect of the overexpression of miR-9/9* and miR-124 on the self-renewal and differentiation was observed by Roesse-Koerner *et al.* in neuroepithelial-like stem cells derived from human pluripotent stem cells⁴⁰.

MiR-106b-5p, miR-93-5p as well as miR-93-3p are members of the same miRNA gene cluster miR-106b~25 and it is thus not surprising that these miRNAs were jointly upregulated. Interestingly, this cluster seems to be linked to the biology of stem cells. Serum induced differentiation of GSCs was previously demonstrated to decrease the expression levels of miRNAs which belong to this cluster¹⁸ and Brett *et al.* showed that the expression of the entire miR-106b~25 cluster in adult mouse neuronal stem/progenitor cells increases their ability to generate new neurons⁴⁵. In the CD44+ gastric cancer stem-like cells, the entire cluster was significantly upregulated and inhibition of miR-106b led to a decreased self-renewal capacity and cell invasiveness through the suppression of the TGF- β /Smad signaling pathway⁴⁶.

Only few studies suggesting a direct link between the other miRNAs identified in our study and stem cell biology are available so far. Chang *et al.* described miR-345 to be enriched in mesenchymal stem cells found in Wharton's jelly matrix of human umbilical cord which were able to transdifferentiate into neuronal lineage cells⁴⁷. The miR-301 family has been recently shown to be the direct target of the SFRS2 splicing factor, an OCT4 regulated gene required for the pluripotency in human pluripotent stem cells⁴⁸. Stappert *et al.* demonstrated that miR-153 contributes to the shift of long-term self-renewing neuroepithelial-like stem cells from self-renewal to neuronal differentiation⁴⁹. Similarly, Tezcan *et al.* also demonstrated that miR-153 overexpression reduced tumorigenic capacity of GSCs by targeting the Nrf-2/GPx1/ROS pathway⁵⁰. In contrast with these data, we observed higher expression of both miR-153 and the members of the miR-301 family (miR-301a-3p, miR-130b-3p, and miR-130a-3p) in GSCs. Unfortunately, there are no studies which could help to explain these discrepancies. However, it seems that the stem cell-like state is dependent on many interacting molecules in feedback loops mutually balancing one another over time.

The Risk Score utilized in this study proved that the set of the identified miRNAs is associated with GBM prognosis independently of IDH1 mutation status, further suggesting their involvement in the disease pathogenesis. Higher tissue levels of miR-652, miR-345 and miR-9* positively contributed to increased values of the risk score and thus worse prognosis; nevertheless, miR-301, miR-153, miR-93 and miR-106b which were also upregulated in GSCs were in fact negative contributors. The explanation for these results is at present speculative but may involve the following aspects. Firstly, the relation between GSCs and survival is somewhat unclear as various studies failed to show a correlation between the GSC quantity assessed by CD133, nestin or CD15 staining and survival^{51,52}. Further, Pallini *et al.* observed that the percentage of CD133-positive cells somewhat paradoxically correlated with longer survival in recurrent glioblastoma, likely due to the higher presence of normal neural stem cells with possible antitumor properties, which may also apply to some newly diagnosed tumors⁵³. Another study described that the CD133-low GBMs showed more invasive growth and gene expression profiles characteristic of mesenchymal or proliferative subtypes, whereas the CD133-high GBMs showed features of cortical and well-demarcated tumors and gene expressions typical of proneuronal subtypes. Moreover, in contrast to various reports claiming that only CD133-positive GBM cells can initiate tumor formation *in vivo*, Joo *et al.* showed that CD133-negative cells also possess tumor-initiating potential⁵⁴. It should be further noted that CSCs are rather quiescent and slow-cycling, and some of the identified miRNAs may be regulators of this dormant state⁵⁵. Last but not least, non-transformed stromal cells may contribute to the tissue levels of miRNAs and the function of the particular miRNAs may be different in these cells compared to glioma cells.

Taken together, we identified a set of miRNAs that are differentially expressed in GSCs as compared to non-stem glioblastoma cells, several of which correlated with the expression of the stem cell markers Sox-2 and nestin. Our findings thus suggest that a complex set of miRNAs is involved in the regulation of the stem-like characteristics in glioblastoma. Moreover, a set of the most differentially expressed miRNAs correlated with the survival of GBM patients independently of the IDH1 status indicating that they might be prognostic markers and possibly new therapeutic targets.

Material and Methods

GBM samples and primary cell cultures. The 10 paired primary GBM cell cultures were derived from fresh tumor tissues samples obtained from GBM patients who underwent surgically resection at the Departments of Neurosurgery of the Hospital Na Homolce in Prague, University Hospital Brno, and University Hospital Ostrava. Study has been approved by the local Ethical Board at Hospital Na Homolce in Prague, University Hospital Brno, and University Hospital Ostrava. Written informed consent was obtained from all patients included in the study. All experiments were performed in accordance with relevant guidelines and regulations.

The fresh tissue sample was enzymatically dissociated with TrypLE (ThermoFisher Scientific) for 20 min at 37°C with agitation or using the Papain Dissociation System (Worthington) according to manufacturer's instructions. Single cell suspensions were seeded into 25 cm² tissue culture flasks (Techno Plastic Products AG) and cultured in either Dulbecco's modified essential medium supplemented with 10% FBS, 1% Glutamax (both ThermoFisher Scientific), 100 U/mL penicillin and 100 μ g/mL streptomycin, sodium pyruvate and non-essential

amino acids (all GE Healthcare), or in DMEM/F12 containing bFGF 20 ng/mL, EGF 20 ng/mL (both PeproTech), B27-supplement 1:50 (ThermoFisher Scientific), 1% Glutamax, 100 U/mL penicillin and 100 µg/mL streptomycin. After 1–3 weeks, adherent cells, which covered more than 2/3 of the culture flask in DMEM, were passaged using Trypsin-EDTA solution (Sigma-Aldrich). After approximately the same time, tumor spheres formed in DMEM/F12, and these were dissociated using Accutase (Sigma-Aldrich) and up and down pipetting and then passaged. Cells that initially formed spheres were dissociated and transferred to laminin (Sigma-Aldrich) or Geltrex (ThermoFisher Scientific) coated culture flasks and propagated as monolayer cultures^{56,57}. For the subsequent analyses, early passage cultures were used.

qRT-PCR quantification. Complementary DNA (cDNA) was synthesized from 1000 ng small RNA enriched total RNA using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) according to the manufacturer's protocol. qRT-PCR was performed using the LightCycler 480 Instrument II (Roche) in accordance with the standard TaqMan manufacturer's protocol using TaqMan Gene Expression Assays (GAPDH #Hs03929097_g1, SOX2 #Hs01053049_s1, NES #Hs00707120_s1; ThermoFisher Scientific). The data were evaluated using the second derivative maximum method with the arithmetic baseline adjustment. All qRT-PCR reactions were run in triplicate and average Cp and SD values were calculated. Relative expression levels were determined by the $2^{-\Delta C_p}$ method, where ΔC_p was calculated as follows: $\Delta C_p = C_p$ (gene of interest) – C_p (GAPDH).

Western blot analysis. Cell pellets were lysed with RIPA buffer (Sigma-Aldrich). Protein concentration was measured using a DC Protein Assay (Bio-Rad), samples were diluted with RIPA buffer to attain the same concentration of total protein and boiled for 10 min with the Laemmli sample buffer. Proteins (15 µg per well) were separated on 10% SDS-PAGE gels, and electrophoretically transferred to the polyvinylidene difluoride (PVDF) membrane (Merck Millipore). The membranes were blocked with 5% nonfat milk in PBS with 0.1% Tween 20 (PBS-T), then incubated either with an anti-Sox2 rabbit mAb or anti-alpha/beta-tubulin rabbit mAb (No. 3579 and 2148, respectively; both Cell Signaling Technology) diluted 1:1000 in blocking solution at 4 °C overnight. Subsequently, the membranes were incubated with anti-rabbit IgG antibody HRP conjugate (No. 7074, Cell Signaling Technology) diluted 1:2500 (60 min/RT). Each step was followed by washes in PBS-T. ECL-Plus detection was performed according to the manufacturer's instructions (Amersham).

Flow cytometry. Accutase (Sigma-Aldrich) was used to harvest adherent cells and dissociate gliomaspheres. The cell suspension was fixed with 2% paraformaldehyde for 1 hour at 4 °C, permeabilized (Intracellular Staining Permeabilization Wash Buffer, Biolegend) and stained using an anti-CD133 APC conjugated antibody (Miltenyi Biotec).

Differentiation of stem-like cell cultures, immunocytochemistry. To induce differentiation, 13×10^3 cells per cm^2 were plated on geltrex coated coverslips and cultured in differentiation medium (DMEM/F12, 10% FBS, 1% Glutamax, 100 µg/mL Streptomycin and 100 U/mL Penicillin G). The medium was exchanged every 2–3 days for 10–14 days. The coverslips were subsequently fixed with 4% paraformaldehyde (10 minutes at room temperature) and stained overnight at 4 °C using the antibodies against GFAP (GF-01, Exbio, 1:200) and beta III tubulin (Exbio, 1:250).

Orthotopic xenotransplantation glioma model, immunohistochemistry. The experimental use of animals was approved by The Commission for Animal Welfare of the First Faculty of Medicine of the Charles University in Prague and The Ministry of Education, Youth and Sports of the Czech Republic according to the animal protection laws. Generation of xenotransplants was performed as described previously⁵⁸. 5×10^5 cells in 5 µL of serum free medium were injected into 6–10 week old male NOD.129S7(B6)-Rag1tm1Mom/J mice (The Jackson Laboratory) with a Hamilton syringe 1.2 mm anterior from the bregma and 2.5 mm lateral from the midline to a depth of 3 mm using a stereotactic device (Stoelting Co.). Immunohistochemistry was performed on 10 µm thick frozen sections using antibodies against human nuclei (Chemicon, 1:500) and GFAP (GF-01, Exbio, 1:200) as described⁵⁹.

MiRNA microarray analysis. Small RNA enriched total RNA was isolated using the mirVana miRNA Isolation Kit (ThermoFisher Scientific). Nucleic acid concentrations and purities were controlled by UV spectrophotometry using Nanodrop ND-1000 (Thermo Scientific). To assess miRNA expression in Sox-2 high- and low-expressing GBM cells, the samples were analyzed with Affymetrix GeneChip miRNA 4.0 arrays (Affymetrix) containing 5607 probe sets for human small RNAs. Out of these probe sets the 2578 probe sets of human mature miRNAs were filtered. All steps of the procedure were performed according to the Affymetrix standardized protocol for miRNA 4.0 arrays. Intensity values for each probe cell (.cel file) were calculated using Affymetrix GeneChip Command Console (AGCC). Quality control of the microarray was performed with the Affymetrix miRNA QC Tool, version 1.1.1.0.

Microarray expression data analysis. All data were pre-processed and further analyzed by the software packages included in the R/Bioconductor⁶⁰. Pre-processing was performed by the RMA method with default parameters as implemented in the Bioconductor package oligo⁶¹. All data were log₂-transformed. To identify differentially expressed miRNAs, the LIMMA approach⁶² for paired samples was applied with additional Benjamini-Hochberg correction of P values. To determine the correlation between miRNA and Sox-2 or nestin expression, the Spearman rank correlation coefficient was used.

IDH1/2 mutation status analysis. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Fragments of 254 bp and 293 bp lengths spanning the sequences encoding the catalytic domains of IDH1 including codon 132 and IDH2 including codon 172, respectively were amplified using 12,5 pmol each of the primers: IDH1-F ACCAAATGGCACCATACGA, IDH1-R TTCATACCTTGCTTAATGGGTGT, IDH2-F GCTGCAGTGGGACCACTATT, and IDH2-R TGTGGCCTTGACTGCAGAG (primer sequences according to Hartmann *et al.*, 2009). PCR was performed using standard buffer conditions, 50–250 ng of DNA input and Taq DNA Polymerase (Invitrogen, USA). PCR consisted of 35 cycles with denaturing at 95 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min in a total volume of 25 µl. Two microliters of the PCR amplification product were subjected to sequencing using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, USA). Twenty-five cycles were performed employing 0,5 µl of 10 µM primer IDH1-F ACCAAATGGCACCATACGA or IDH2-R TGTGGCCTTGACTGCAGAG, with denaturing at 96 °C for 30 s, annealing at 50 °C for 15 s and extension at 60 °C for 4 min in a total volume of 10 µl. Sequences were determined using the sequencer (ABI 3500 Genetic Analyzer, Applied Biosystems) and the Mutation surveyor V4.0.9 software (SoftGenetics, USA).

Survival analysis. The relationship between overall survival and expression levels of the selected miRNAs was analyzed on The Cancer Genome Atlas (TCGA) data set (485 GBM patients)⁶³. To assess the miRNAs that were identified in this study for survival prediction, a Risk Score formula for predicting survival was developed based on a linear combination of the miRNA expression level weighted by the regression coefficient derived from the multivariate Cox regression analysis^{22,23}. Patients with high Risk Score are expected to have poor survival. Cox proportional hazards regression analysis was performed to assess the contribution of the miRNA signature to survival prediction⁶⁴. Patients were further stratified into a high-risk group and a low-risk group according to the Risk Score (cutoff value median) and survival was analyzed using the Kaplan-Meier method. Subsequently, both univariate and multivariate Cox regression analyses including seven-miRNA signature and IDH1 status were performed on the data subset comprising 296 GBM patients for whom exome somatic mutation data were available.

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Additional Information

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