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Diagnostic, grading and prognostic role of a restricted miRNAs signature in primary and metastatic brain tumours. Discussion on their therapeutic perspectives

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Abstract

At present, brain tumours remain one of the "hard-to-treat" malignancies with minimal improvement in patients' survival. Recently, miRNAs have been shown to correlate with oncogenesis and metastasis and have been investigated as potential biomarkers for diagnosis, prognosis and therapy prediction in different brain malignancies. The aim of the current study was to select an accurate and affordable brain tumour detection and grading approach. In the present study, we analysed the applicability of a restricted miRNA signature that could differentiate among patients with primary as well as metastatic brain tumours. Fresh tumour tissues were collected from Bulgarian patients ($n = 38$), including high-grade gliomas ($n = 23$), low-grade gliomas ($n = 10$) and brain metastases ($n = 5$) from lung cancer. Total RNAs enriched with microRNAs were isolated and differentially expressed miRNAs were analyzed by RT-qPCR using TaqMan Advanced miRNA assay. We selected a signature of miR-21, miR-10b, miR-7, miR-491 that showed good diagnostic potential in high-grade gliomas, low-grade gliomas and brain metastases compared with normal brain tissues. Our results showed that miR-10b could reliably differentiate brain metastases from high-grade gliomas, while miR-491 could distinguish low-grade from high-grade gliomas and brain metastases from low-grade gliomas. We observed that miR-21 and miR-7 correlated with disease recurrence, survival status and the Karnofsky Performance Status. The selected signature of miR-7, miR-21, miR-10b and miR-491 could be used as a highly accurate diagnostic, grading and prognostic biomarker in differentiating various types of brain tumours. Our data suggest that the 4-miRNAs signature could be further analysed for predicting treatment response and for future miRs-based targeted therapy. The ongoing studies on miRs-based targeted therapy related to our selected miRNA signature are also reviewed.

Keywords miR · High-grade glioma · Low-grade glioma · Metastases · Diagnosis · Prognosis

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Abbreviations

AKT	A serine/threonine protein kinase
AUC	Area under the curve
BAX	Bcl-2-associated X-protein
BM	Brain metastases
CDKN1A/2A	Cyclin-dependent kinase inhibitor 1A/2A
CXCR4	X-C Chemokine Receptor 4
DEMs	Differentially expressed microRNAs
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
FC	Fold change
GBM	Glioblastoma multiforme
HGG	High-grade gliomas
HOXD10	Homeobox D10
IDH	Isocitrate dehydrogenase
KLF4	Kruppel like factor 4

KPS	Karnofsky performance status
LGG	Low-grade gliomas
LNA	Locked nucleic acids
MAPK	Mitogen-activated protein kinase
MGMT	O⁶-methylguanine—DNA methyltransferase
mTOR	Mammalian target of rapamycin
miRNA	Micro ribonucleic acid
mRNA	Messenger RNAs
NF1	Neurofibromin 1
NF-kB	Nuclear factor kappa B
NSCLC	Non-small cell lung cancer
PDCD4	Programmed Cell Death 4
PI3K	Phosphatidylinositol 3-kinases
PTEN	Phosphatase and tensin homolog
RAS	Rat sarcoma virus
ROC	Receiver-operating characteristics
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SPRY2	Sprouty homolog 2
TMZ	Temozolomide
TP53	Tumour protein p53
WHO	World Health Organization

Introduction

Gliomas are brain tumours, categorized according to the World Health Organization (WHO) as low-grade gliomas (LGG) and high-grade gliomas (HGG) based on the malignancy level, histopathological and molecular data (Louis et al. 2016). HGG (WHO grade III and grade IV) are characterized by a poor clinical outcome. Glioblastoma multiforme (GBM; WHO grade IV) is the most aggressive HGG accounting for 54% of all clinically diagnosed gliomas (Dolecek et al. 2012). The 5-year survival rate of patients with GBM is less than 5% (Olar and Aldape 2012). Some of the putative risk factors for brain tumours are exposure to ionizing radiation (Bondy et al. 2008) and probably some infectious agents (Alibek et al. 2013; Nikolova et al. 2020). Despite the available treatment for patients diagnosed with HGG, as a maximal surgical resection, radiotherapy and chemotherapy, the median survival rate has hardly been changed in the last decades (Poon et al. 2020). Nowadays, molecular biomarkers, such as *IDH, 1p/19q* co-deletion, *MGMT* promoter methylation among others, are strongly recommended to be used for more accurate tumour classification (Feldman et al. 2020). LGG (WHO grade I and II) have a better prognosis compared to the HGG patients. However, up to 70% of LGG progress to HGG within a decade, due to their infiltrative nature and therapy resistance (Jooma et al. 2019).

Brain metastases (BM) or secondary brain tumours are the most frequently occurring intracranial neoplasms (Nayak et al. 2012). BM from lung cancer is the most commonly reported (up to 88%) (Nayak et al. 2012; Villano et al. 2015) and represent a main cause of high patient mortality and morbidity (Barnholtz-Sloan et al. 2004). The rate of BM will continue to increase in the future due to the targeted therapies and increased patients' survival with primary tumours (Disibio and French 2008). Given the frequent tumour relapse and high mortality rate, the identification of novel metastases-related biomarkers could improve patient monitoring and survival.

Many studies have shown that microRNAs (miRNAs) are tissue specific and their normal expression is disturbed in different types of cancers (Calin and Croce 2006). MiRNAs are a class of non-coding RNAs of 18–25 nucleotides that post-transcriptionally suppress mRNA expression (Bartel 2009). One miRNA regulates multiple messenger RNAs (mRNA) due to the imperfect complementary binding to mRNA 3′UTR (3′-untranslated regions) (Thomson et al. 2011). It is estimated that more than 60% of protein-coding genes are controlled by miRNAs (Friedman et al. 2009). In recent years, a growing number of studies have shown that differentially expressed miRNAs (DEMs) possess a potential for distinguishing tumour tissue from normal tissue (Ventura and Jacks 2009), differentiating glioma grades (Kim et al. 2011), as well as differentiating primary from metastatic brain tumours (Nass et al. 2009). Additionally, the potential of DEMs in the identification of metastatic tumour origin and histological subtyping has been demonstrated (Søkilde et al. 2014; Tang et al. 2018).

Many authors have verified individually deregulated miRNA in brain tumours, but only few studies have investigated the biomarker potential of miRNA signature in both primary and metastatic brain tumours. Hence, the aim of the present study was to determine whether a minimal number of miRNAs representing a miRNA signature could discriminate between different stages of primary brain tumours and secondary (metastatic) brain tumours. Additionally, we wanted to select a miRNA signature that simultaneously combines good biomarker potential, practicability and cost-effectiveness. It was also of interest whether combining these individual miRNAs in a restricted signature could further increase their diagnostic, prognostic and grading power in tumour tissues collected from Bulgarian patients with primary and metastatic brain malignancies. To this end, we conducted a literature search in order to select experimentally verified miRNAs with a disturbed expression in different brain malignancies. Literature databases—PubMed, Google Scholar and Web of Science were searched for relevant articles between January 2000 and June 2021, using the following keywords: "miRNA", "miR", "glioma", "glioblastoma", "astrocytoma", "high-grade glioma", "low-grade

glioma", "brain metastases", "primary brain tumours", "secondary brain tumours", "diagnosis", "prognosis", "therapy and biomarker". Based on the previous literature, miR-7, miR-21, miR-10b, miR-34a and miR-491 were selected for our subsequent analysis. This is the first study, analysing the combination of expression profiles of miR-7, miR-21, miR-10b, miR-34a and miR-491 in brain tumour tissues. The ability of the selected four-miRNA signature of miR-7, miR-21, miR-10b and miR-491, to accurately diagnose and stratify patients with primary and metastatic brain tumours was demonstrated. In addition, using more than one DEM as a signature could increase diagnostic specificity and sensitivity as well as their biomarker power. Our miRNA signature showed good diagnostic, grading and prognostic potential, can be easily implemented in clinical practice. We believe that accurate and affordable brain tumour detection and grading may help to improve the treatment and the patient's quality of life. Finally, the proposed 4-miRNA signature could be used in a larger cohort of patients with brain tumours and could be analysed for future miRs-based targeted therapies.

Materials and methods

Tumour sampling

Fresh tumour tissues ($n = 38$) were sampled in the period January 2019—December 2020. The research protocol was approved by the Ethics Committee of the Medical University of Sofia and all patients have signed an informed consent before the sample collection. In this study we included 10 LGG samples (WHO grade I-II) (5 females, 5 males; mean age: 36.9 ± 25.35), 23 HGG samples (WHO grade III-IV) (16 females, 7 males; mean age: 58.55 ± 14.35) and 5 BM from lung cancer (2 females, 3 males; mean age: 59.4 ± 12.99). For reference controls we used commercial RNA—total RNAs—human adults normal brain tissues Catalogue number: R1234035-50 (Biochain, USA) and adjacent normal brain tissues. Samples were placed in a RNA latter (Qiagen, Germany) for 24 h and stored at − 80 °C until RNA extraction. The clinical characteristics of the participants are summarised in Table 1.

RNA isolation

Total RNA was isolated using the miRNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. The protocol is based on phenol:chloroform and silica column isolation of total RNA with miRNA recovery. RNA was eluted in 50 μL RNase-free water and stored at − 80 °C until further analysis. RNA concentrations and quality were measured spectrophotometrically by NanoDrop (Thermo Scientific, USA).

RT-qPCR

TaqMan Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007, Thermo Fisher Scientific, USA) was used with pre-amplification step and 10 ng of total RNA was reverse transcribed into cDNA according to manufacturer's instructions. The resulting complementary DNA (cDNA) was used as a template for miRNA expression analyses using the TaqMan Advanced miRNA Assay (Cat No A25576, Thermo Fisher Scientific, USA). Predesigned TaqMan assay IDs for the selected miRNAs (Table 2) and TaqMan Fast Advanced Master Mix (Cat No 4444557, Thermo Fisher Scientific, USA) were used. RT-qPCR was performed using a DTprime Real-time thermocycler (DNA-Technology, Russia) with the following thermal settings: enzyme activation—20 s at 95 °C, 40 cycles of denaturation 3 s at 95 °C and annealing—30 s at 60 °C.

Statistical analysis

The Cp was used to calculate relative miRNA expression levels using the formula ($2^{-\Delta\Delta Cp}$). MiRNA-877 was used as an endogenous control. Data are expressed as the mean $\pm$ standard deviation. MiRNA expression levels in tumour tissues were analyzed using two-tailed, non-parametric Kruskal–Wallis Analysis of Variance (ANOVA) that was chosen in order to improve the accuracy of the results and increase the statistical power of the tests (Van Hecke 2012), as our data was not normally distributed (Shapiro–Wilk $p < 0.001$). Significant Kruskal–Wallis ANOVA was followed by a Post hoc test for different groups calculations. Bonferroni corrections were used for multiple comparisons adjustment. Differences in clinicopathological characteristics between different groups of patients were evaluated by the Fisher's exact test and the non-parametric Spearman's correlation. To evaluate the diagnostic and predicting value of tissue miRNAs in different grade tumours, the receiver operating characteristics (ROC) curves were constructed and the area under the curve (AUC) was calculated. All experiments were performed in duplicate. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 26 (IBM Corporation, Armonk, NY, USA). For all statistical analyses, missing cases were excluded using pairwise (analysis by analysis) for the sake of optimal data use and reduction of information loss. Owing to the robustness of the used statistical analyses and the nature of the non-parametric tests, the effect of the missing data is minimized.

Table 1 Clinicopathological characteristics of patients with LGG, HGG and BM

Variable	Group		
	HGG	LGG	BM
N	23	10	5
Age (mean ± SD)	58.55 ± 14.35	36.90 ± 25.35	59.40 ± 12.99
Gender			
Male	7 (30.4%)	5 (50%)	3 (60%)
Female	16 (69.6%)	5 (50%)	2 (40%)
KPS Score			
≥ 70	14 (60.87%)	6 (60%)	3 (60%)
< 70	8 (34.78%)	4 (40%)	2 (40%)
No information available	1 (4.35%)		
Therapy			
Chemotherapy	1 (4.35%)	1 (10%)	0 (0%)
Chemotherapy and radiation therapy	16 (69.56%)	2 (20%)	4 (80%)
No therapy	1 (4.35%)	6 (60%)	1 (20%)
No information available	5 (21.74%)	1 (10%)	
Recurrence of tumor			
Yes	3 (13.04%)	2 (20%)	0 (0%)
No	20 (86.96%)	8 (80%)	5 (100%)
Additional diseases			
No	6 (26.09%)	2 (20%)	1 (20%)
Arterial Hypertension	6 (26.09%)	3 (30%)	1 (20%)
Diabetes	0 (0%)	1 (10%)	0 (0%)
Arterial hypertension and diabetes	6 (26.09%)	1 (10%)	2 (40%)
Other	3 (13.04%)	3 (30%)	1 (20%)
No information available	2 (8.69%)		
Survival status			
Alive	5 (21.74%)	5 (50%)	0 (0%)
Dead	13 (56.52%)	4 (40%)	5 (100%)
No information available	5 (21.74%)	1 (10%)	

HGG high-grade glioma; *LGG* low-grade glioma; *BM* brain metastases; *KPS* Karnofsky Performance Status

Table 2 ID numbers and names of miRNAs used in the experiment

MiRNA name	TaqMan advanced miRNA assay ID
hsa-miR-21-5p	477975_mir
hsa-miR-10b-5p	478494_mir
hsa-miR-7-5p	483061_mir
hsa-miR-491-5p	478132_mir
hsa-miR-34a-5p	478048_mir
hsa-miR-877-5p	478206_mir
hsa-miR-191-5p	477952_mir

Results

Four microRNAs (miR-21, miR-7, miR-491 and miR-10b) showed statistically significant deregulation ($p < 0.05$) between HGG, LGG, BM and controls (Fig. 1).

Expression analyses showed a significant increase of miR-21 between HGG compared to normal control group $p = 0.004$. Overexpression of miR-21 was observed also in LGG and BM compared to normal brain tissues but it did not pass the significance level after Bonferroni correction $p > 0.05$ (Table 3). MiR-7 showed a significant decrease ($p = 0.016$) in the group of HGG compared to the control group. MiR-491 was down-regulated in HGG compared to LGG, $p = 0.025$. MiR-491 was significantly deregulated in HGG and BM compared to controls with $p = 0.002$ and $p = 0.009$, respectively. The expression of miR-10b was significantly elevated in HGG compared to normal brain tissues $p = 0.001$. Overexpression of miR-10b was
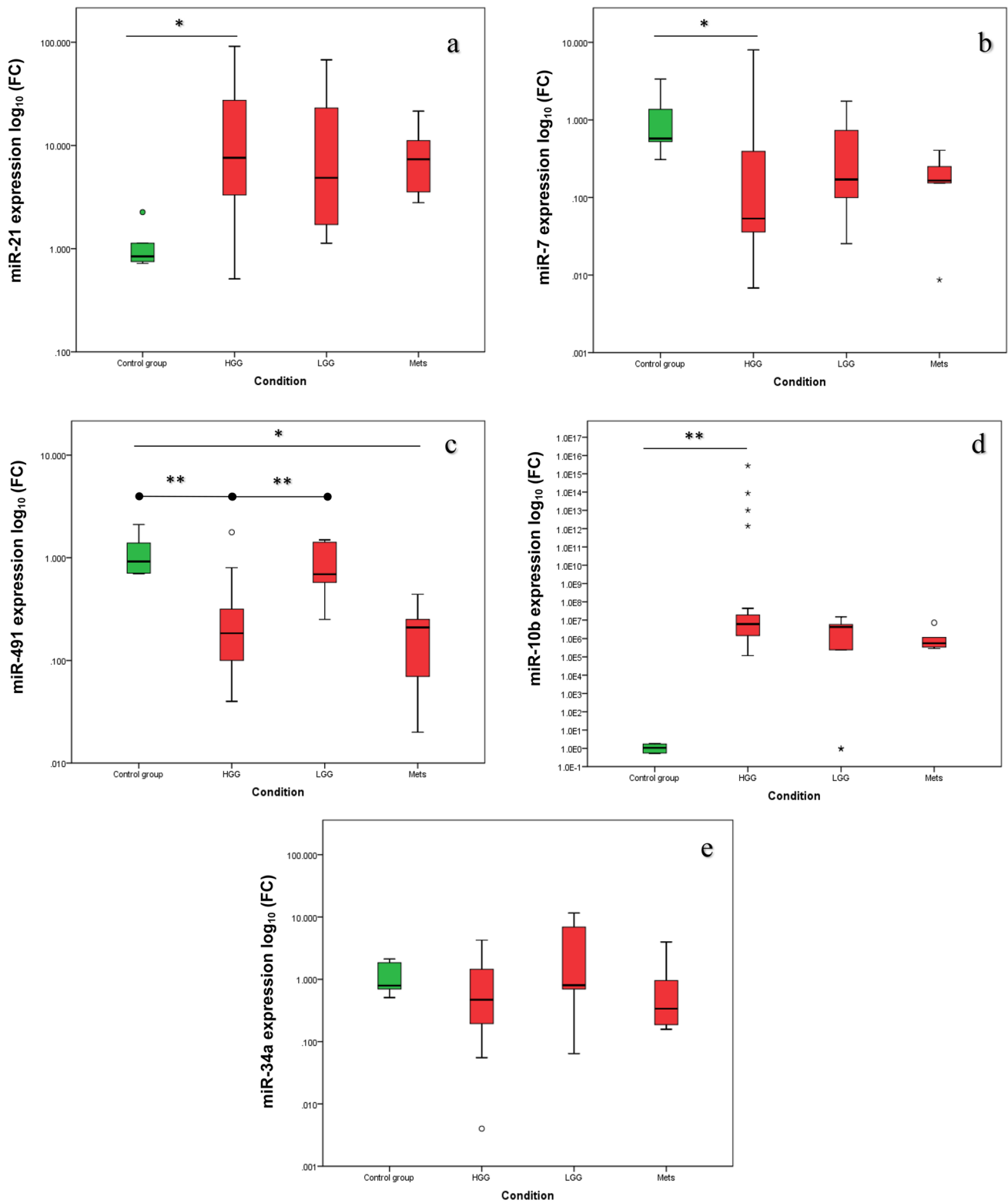
Fig. 1 Box plots of DEMs in patients with HGG, LGG and BM compared to the control group analysed by RT-qPCR. Expression profile of **a** miR-21, **b** miR-7, **c** miR-491, **d** miR-10b and **e** miR-34a vs controls. *Note*. Error bars present standard error of the mean (SEM). $*p < 0.05$, $**p < 0.01$, assessed using Kruskal–Wallis test followed by Bonferroni correction for multiple tests. *FC* fold change, Mets—BM

Table 3 Significantly DEMs in gliomas

miRNA ID	miR-21		miR-7		miR-491		miR-10b	
Comparison of Condition	p	Adj. p	p	Adj. p	p	Adj. p	p	Adj. p
Control-LGG	0.021*	0.126	0.139	0.834	0.357	1.00	0.037*	0.220
Control-HGG	0.001**	**0.004****	0.003**	**0.016***	<0.001***	**0.002****	<0.001***	**0.001****
Control-Mets	0.015*	0.092	0.045*	1.000	0.002**	**0.009****	0.099	0.594
LGG-HGG	0.608	1.00	0.133	0.800	0.004**	**0.025***	0.065	0.388

Significant p values are in bold ($p < 0.05$)

Data calculated by Kruskal–Wallis test, followed by a Post-hoc test. p value; Adj.p—significance values have been adjusted by the Bonferroni correction for multiple tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

observed in LGG and BM but below the significance level ($p < 0.05$) after Bonferroni corrections. In our study miR-34a did not show a statistical significant decrease between different brain tumours. Data analysis of up-regulated miRs (miR-21and miR-10b) and down-regulated miRs (miR-7 and miR-491) in brain malignancies compared to controls are shown in Table 3 before and after Bonferroni correction for adjustment of multiple comparisons.

The data were normalised against commercially available RNA isolated from normal adult brain tissue (Biochain, USA) that we preferred to add due to HGG diffuse nature and invasion into the nearby "normal looking" brain tissues and adjacent normal brain. MiRNA fold change was normalised against the same species' short-noncoding RNA. We analysed miR-191-5p and miR-877 for stably expressed endogenous reference controls. GeNorm (Vandesompele et al. 2002), Bestkeeper (Pfaffl et al. 2004) and RefFinder (Xie et al. 2012) suggested miR-877 as the most stably expressed miRNA.

Roc analysis and AUC

Receiver-operating characteristic curves were constructed and AUC was calculated to evaluate the diagnostic efficiency of the selected miRNAs in patients with different brain tumours.

High-grade glioma

ROC curve analyses showed that miR-21 and miR-10b are both up-regulated, while miR-7 and miR-491are down-regulated in HGG and possess the potential to distinguish HGG from controls (Fig. 2).

MiR-491 was down-regulated in HGG compared to LGG. The ROC curve analysis showed that miR-491 can

serve as a grading biomarker and can differentiate HGG and LGG (Fig. 3).

Low-grade glioma

ROC analysis showed that miR-21, miR-10b and miR-7 can differentiate LGG from controls with AUC = 0.908 ($p = 0.008$, 95% CI 0.742–1.0), AUC = 0.867 ($p = 0.028$, 95% CI 0.669–1.0) and AUC = 0.861 ($p = 0.045$, 95% CI 0.65–1), respectively (data not shown). While miR-491 showed a good diagnostic potential to distinguish HGG from LGG AUC = 0.835 ($p = 0.003$, 95% CI 0.698–0.971) and could be applied as a grading biomarker between LGG and HGG (Fig. 3).

Brain metastases

MiR-491 can differentiate LGG from BM, while miR-10b can distinguish GBM from BM (Fig. 4). In addition, miR-21 and miR-10b that are up-regulated in BM compared to controls showed AUC = 1, $p = 0.014$ while miR-7 and miR-491 were down-regulated with AUC = 0.971, ($p = 0.007$, 95% CI 0.886–1.0) and AUC = 1 ($p = 0.004$, 95% CI 1.0), respectively (data not shown).

With respect to the continuous clinicopathological variables, Spearman analysis showed a negative correlation between patients' KPS and miR-21 $\rho = -0.430$, $p = 0.008$. Additionally, negative correlations were found between patients' age and OS ($\rho = -0.762$, $p < 0.001$), PFS ($\rho = -0.672$, $p < 0.001$); and KPS ($\rho = -0.528$, $p = 0.001$). We found a positive correlation between miR-491 and miR-7, $\rho = 0.482$, $p = 0.002$; between miR-21 and miR-34a, $\rho = 0.689$, $p < 0.001$; between miR-21 and miR-10b, $\rho = 0.336$, $p = 0.042$.

Subsequently, mRNA expression was recoded into categories (low, normal and high) and Fisher's exact test was performed to evaluate the relationship between categorical clinicopathological data and DEMs. We observed that
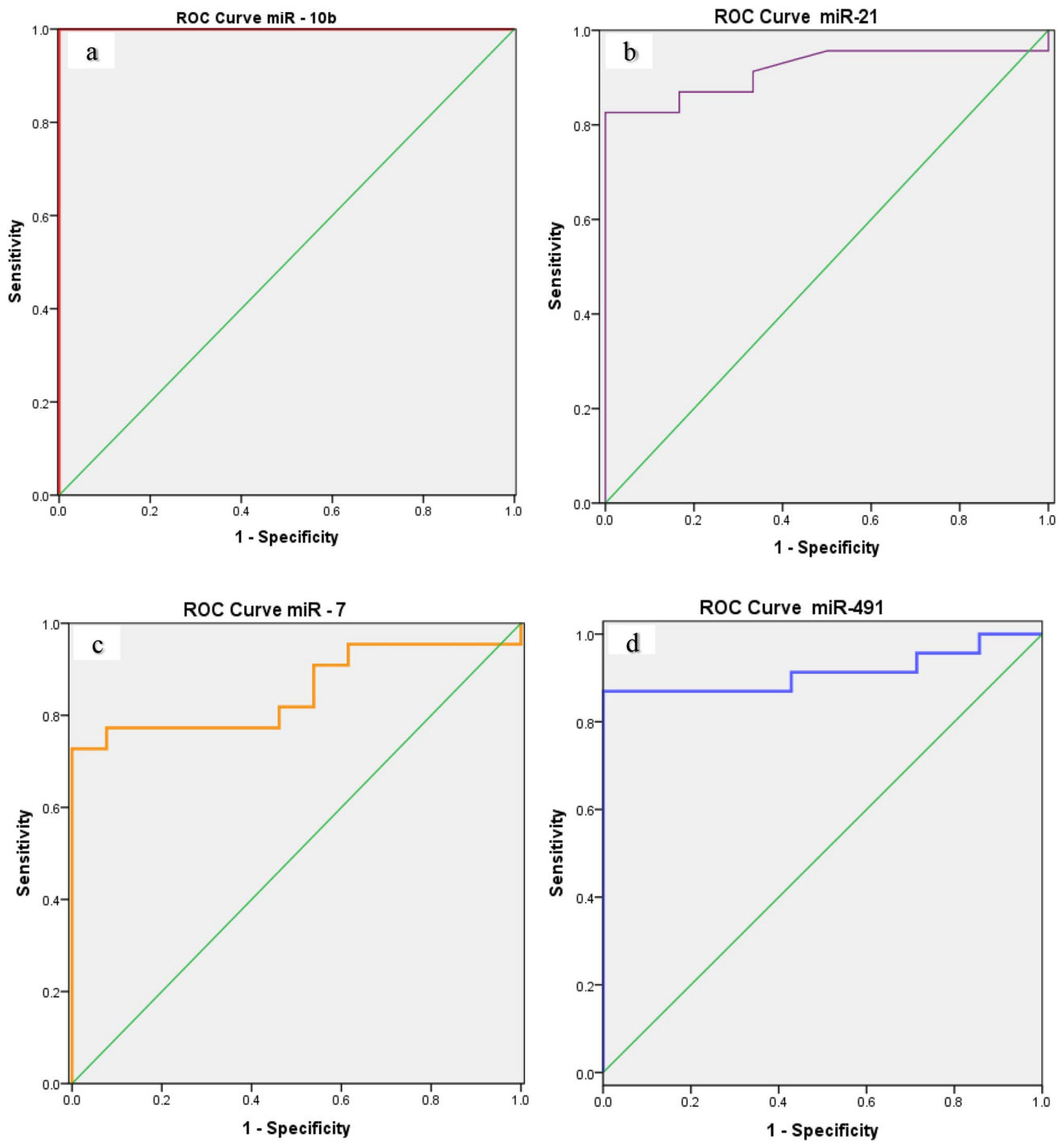
Fig. 2 ROC curve analyses and AUC evaluated diagnostic efficiency between HGG and controls. **a** miR-10b is up-regulated in HGG with AUC=1 (p=0.001, 95% CI 1.0–1.0), **b** miR-21 is up-regulated in HGG with AUC=0.917 (p=0.002, 95% CI 0.813–1), **c** miR-7 is down-regulated in HGG, AUC=0.832, (p=0.009, 95% CI 0.688–0.976), **d** miR-491 is down-regulated in HGG, AUC=0.913, (p=0.001, 95% CI 0.809–1)
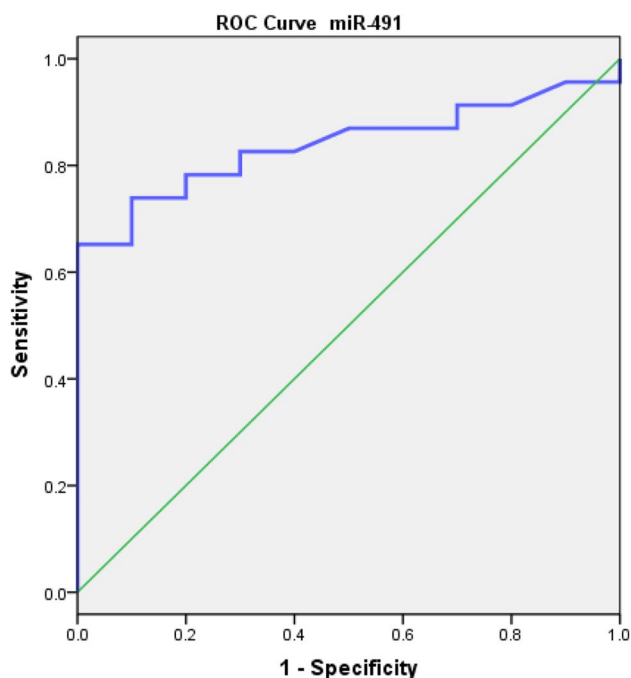
Fig. 3 ROC curve analysis and AUC of miR-491. MiR-491 possesses a good diagnostic potential to distinguish HGG from LGG AUC $= 0.835$ ($p = 0.003$, 95% CI 0.698–0.971)

miR-7 and miR-21 were associated with disease recurrence ($p = 0.008$ and $p = 0.043$, respectively). MiR-21, miR-7 and miR-491 correlated with patient survival status (dead or alive; $p = 0.013$, $p = 0.004$ and $p = 0.044$, respectively). Interestingly, miR-491 correlated with patient therapy ($p = 0.003$; Table 4).

Discussion

Despite the most recent medical achievements, the prognosis of brain tumour patients remains poor due to late manifestation of the disease, heterogeneous nature and frequent recurrence. High relapse rate and progression have been observed in HGG and LGG, despite the advances in surgery, radiotherapy and chemotherapy. Given that gliomas with similar histology have different molecular profiles, effective diagnostic, prognostic and predictive biomarkers are still in a huge demand. Nowadays, several molecular biomarkers have been approved together with a histopathology assessment to aid in the glioma classification and more precise brain tumour grading and therapy (Lima et al. 2012). Moreover, managing cancer metastases is still very challenging. Development of biomarkers to identify patients who are at high risk of metastatic disease, is of main clinical importance. Numerous oncogenic and tumour-suppressor miRNAs have been identified to play critical role in primary and metastatic brain tumours development and progression (Kefas et al.
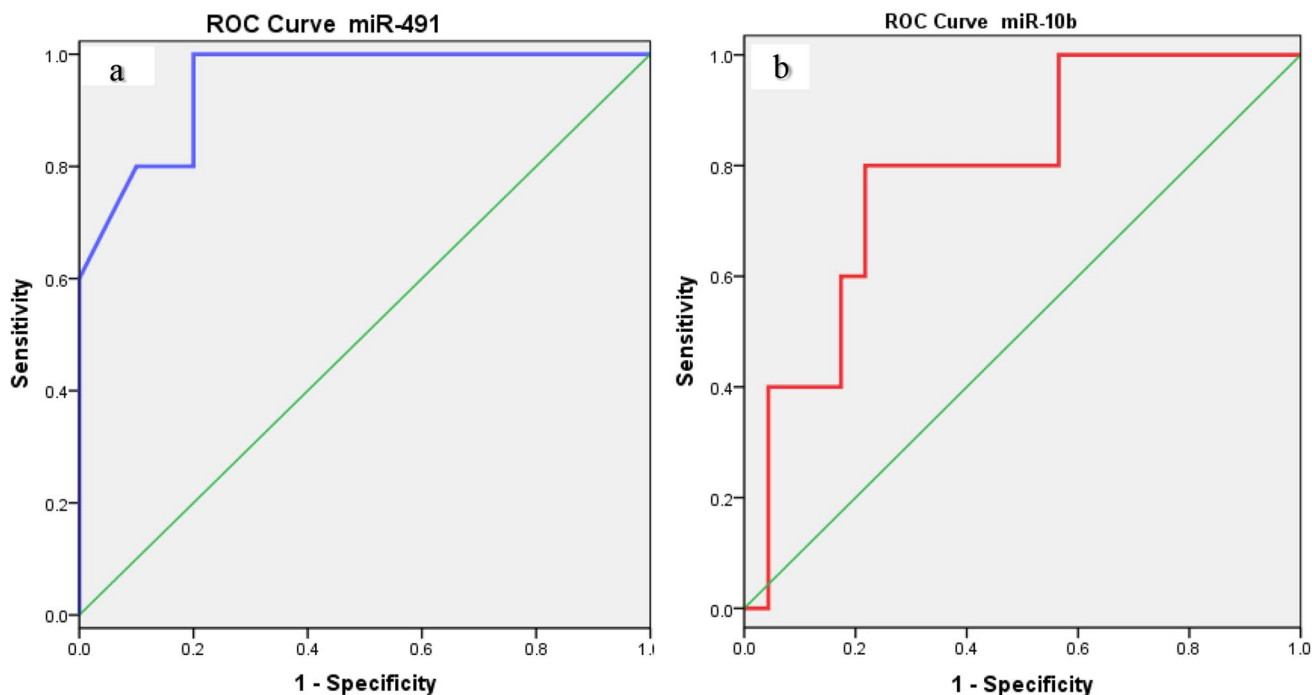

Fig. 4 ROC curve analysis and AUC of **a** miR-491 between LGG and BM AUC $= 0.95$ ($p = 0.006$, 95% CI 0.844–1.0) and **b** miR-10b between HGG and BM AUC $= 0.791$ ($p = 0.044$, 95% CI 0.588–0.995)

Table 4 Associations between clinicopathological data and DEMs

Patients' parameters	miR—21	miR—7	miR—491
	P value	*P* value	*P* value
Survival status (dead or alive)	**0.013***	**0.004***	**0.044***
Disease recurrence	> 0.05	**0.008***	**0.043***
Therapy	> 0.05	> 0.05	**0.003***

Significant *p* values are in bold ($p < 0.05$)

2008; Li et al. 2009a, b; Papagiannakopoulos et al. 2008; Sheedy and Medarova 2018; Zhen et al. 2016). Significant efforts are underway to use miRNAs as molecular biomarkers with clinical applications. A lot of studies have described deregulated miRNAs but there are far less studies focused on DEMs signature in primary and metastatic brain tumour tissues. In the present study, using RT-qPCR, we selected a DEMs signature of only four miRs (miR-21, miR-10b, miR-7, miR491) in tissue samples collected from Bulgarian patients with HGG, LGG and BM from lung cancer. The signature showed significant differential expression ($p < 0.05$) and potential to differentiate between primary and metastatic brain tumours, normal tissues and correctly classify brain tumour subtypes. The performance of the 4-miRNAs in the analysed cohort suggests that our signature is a novel and reliable biomarker for brain malignancies management.

Deregulated miRNA in HGGs

We observed deregulation of miR-21, miR-7, miR-10b and miR-491 in patients with HGG and glioblastoma multiforme ($p < 0.05$). In concordance with previous data, in our study miR-21 was significantly up-regulated in HGG as compared to normal brain tissues ($p = 0.004$) and could be used as a diagnostic biomarker ($p = 0.002$). In brain tumours, miR-21 activates EGFR/AKT and RAS/MAPK pathways (Melnik, 2015; Hatley et al. 2010) and as a result, it positively regulates angiogenesis (Sun et al. 2017) and inhibits apoptosis (Chan et al. 2005). Moreover, the observed up-regulation of miR-21 in HGG is probably related to the disease invasiveness, enhanced by necrotic cells, since miR-21 has been highly expressed in the bordering necrotic areas in tumours. These data support that miR-21 is an important player in HGG ability to self-renew, facilitated by hypoxia-induced vascularization (Hermansen et al. 2016). Including miR-21 in our signature could be valuable for stratifying patients at high risk of progression after surgery (Lakomy et al. 2011).

Another up-regulated miRNA, with diagnostic potential in HGG observed in our study was miR-10b ($p = 0.001$). Interestingly, we found miR-10 expressed only in malignant tissues but not in the normal brains, highlighting its role as a pro-oncogenic miRNA and its potential application as a brain tumour specific biomarker. Furthermore, we showed

that miR-10b can distinguish between HGG and brain metastasis from lung cancer, in the analysed samples. The observed data in our cohort support the possible involvement of miR-10b in cancer cell migration. The discrimination of primary HGG from BM is very important because of their substantially different clinical management. In support of this, miR-10b by activating AKT expression, decreases radiation-induced apoptosis (Zhen et al. 2016) and its inhibition in vivo results in glioma growth reduction (Gabriely et al. 2011). Hence, including miR-10b in a biomarker signature could not only improve brain tumour diagnosis and grading but also could be used as a therapy predictive biomarker (Shi et al. 2010). In addition, it is known that both miR-21 and miR-10b increase HGG proliferation, aggressiveness and invasiveness by targeting tumour suppressor genes (Papagiannakopoulos et al. 2008; Schramedei et al. 2011; Sheedy and Medarova 2018) and also correlate with the WHO brain tumour grades (Sasayama et al. 2009), degree of invasion, patient's prognosis and overall survival (Zhang et al. 2016). The elevated expression of both miRNAs is associated with decreased radiation and chemotherapy sensitivity of GBM and therapy resistance (Shi et al. 2010; Wang et al. 2017; Zhen et al. 2016). Given the oncogenic potential of miR-21 and miR-10b in brain malignancies, our study supported the applicability of both up-regulated miRNAs as potential diagnostic, prognostic and predictive biomarkers.

Two miRNAs showed significant down-regulation in HGG as compared to the control tissues—miR-7 ($p = 0.009$) and miR-491 ($p = 0.001$). The down-regulation of miR-7 and miR-491 in HGG is explained by their tumour suppressor role (Fang et al. 2012; Chang et al. 2015; Liu et al. 2014). MiR-7 and miR-491 regulate EGFR and AKT signalling pathways inhibiting cell proliferation, invasion and metastases in normal tissues (Kefas et al. 2008; Liu et al. 2015). Interestingly, down-regulation of miR-491 could partly be related to its deletion together with the tumour suppressor *CDKN2A* that has been frequently disturbed in HGG (Liu et al. 2015). Moreover, our analysis showed that miR-491 can serve as a grading biomarker, differentiating HGG and LGG. We observed that miR-491 was more downregulated in HGG compared to LGG and correlated with poor patient outcome (Liu et al. 2015). Furthermore, we found that miR-7 and miR-21 correlated with the disease recurrence and can be used as prognostic biomarkers.

In the previous studies, the oncogenic and tumour suppressor actions of the 4 miRNAs included in our signature were analysed predominantly individually (Chang et al. 2015; Gabriely et al. 2011; Krichevsky and Gabriely 2009; Liu et al. 2014; Liu et al. 2015, Zhou et al. 2010). Furthermore, given the above data, combining 4-miRNAs in the proposed signature will increase the test sensitivity and can be applied as a potential novel strategy for HGG treatment.

Deregulated miRNA in LGG

In our study, miR-21 ($p = 0.008$), miR-10b ($p = 0.028$) and miR-7 ($p = 0.045$) showed diagnostic potential to differentiate LGG from controls. MiR-21 is one of the first widely investigated miRNA and its overexpression in low-grade brain cancers (Zhi et al. 2010) could facilitate their diagnosis, patient prognosis and glioma classification (Conti et al. 2009). Using biomarkers that can classify glioma molecular profiles together with histopathology assessment will aid brain tumour grading (Khani et al. 2019). In concordance with previous studies, we observed that miR-10b was up-regulated in LGG (Gabriely et al. 2011). All of the selected Bulgarian patients with LGG showed detectable levels of miR-10b, while its expression was missing in non-neoplastic controls. Interestingly, we observed no expression or normal levels of miR-10b in two of our patients and they showed the highest OS in the group of LGG probably related its action as an inhibitor of tumour suppressor genes and metastasis suppressor genes (Sheedy and Medarova 2018). It has been reported that miR-10b correlated to WHO brain tumour grades (Sasayama et al. 2009) and poor patient's prognosis in LGG (Zhang et al. 2016). Therefore, including miR-10b in our signature will increase the diagnostic and grading potential and could be used as a sensitive biomarker for detecting underestimated high-grade primary brain cancers.

Additionally, our data showed that down-regulated miR-491 can distinguish HGG from LGG ($p = 0.001$) indicating its grading biomarker power. Moreover, miR-491 showed high differentiating potential between LGG and BM ($p = 0.006$). We believe that using miR-491 in our profile increased the level of accuracy and correctly classified LGG as compared to HGG and BM. We found that miR-491 expression correlated with therapy ($p = 0.003$). We did not find a correlation between miR-491 and the presence of additional diseases, although, in a 5-year prospective analysis, Sidorkiewicz et al., have revealed that miR-491 has been able to predict the development of type II diabetes in prediabetes patients (Sidorkiewicz et al. 2020). In our study, miR-7 showed down-regulation in LGG and a diagnostic potential for differentiation of LGG from normal brain ($p = 0.045$). Given that miR-7 is a tumour suppressor and inhibits EGFR and AKT signalling pathways (Fang et al. 2012; Kefas et al. 2008) it is expected to be more downregulated in HGG than in LGG, as we observed. The received data could improve the LGG diagnosis and facilitate the clinical decision in underestimated cases or borderline histology results between LGG and HGG. Therefore, this will improve patient's therapy and outcome.

We think that miRNAs analysis of miR-21, miR-10b, miR-7 and miR-491 in LGG could improve the molecular profiling, patient stratification and the histopathology assessment, helping the precise glioma classification.

Deregulated miRNA in brain metastases from lung cancer

Identifying patients who are at high risk of metastatic disease is of main clinical importance but still challenging. Using DEMs, involved in cell invasion and migration, as potential biomarkers could facilitate monitoring and managing of the most common brain metastases from lung cancer.

Our approach of selecting miR-21, miR-7, miR-10b and miR-491 succeeded in the diagnosis of primary and metastatic brain tumours. MiR-491 and miR-7 were down-regulated while miR-21 and miR-10b were up-regulated significantly in BM and can serve as diagnostic biomarkers differentiating BM from the control group and between BM and LGG. The upregulated miR-21 and miR-10b of our selected signature, have been associated with cell migration and invasion (Gabriely et al. 2008; Grunder et al. 2011; Sheedy and Medarova 2018). Moreover, miR-21 elevated expression not only correlated with brain metastases but also with more advanced clinical stages (Krichevsky and Gabriely 2009) and has been observed to be able to distinguish non-small-cell lung carcinoma—NSCLC with BM from NSCLC without BM (Dong et al. 2016). Liu et al. have observed that miR-21, by targeting PTEN, increases proliferation, metastases and therapy resistance in NSCLC (Liu et al. 2013) indicating the putative therapy predicting role of miR-21 in patients with lung cancer brain metastases.

According to the ROC curve analysis, differentiating BM from LGG and HGG is facilitated by adding miR-491 and miR-10b, respectively in our signature. As one of the first metastasis-related DEMs, the elevated expression of miR-10b in our patients is possibly related to increased tumour migration and metastases, as has been observed in various types of cancers (Bai et al. 2017; Heidary et al. 2015; Ma et al. 2007; Zhang et al. 2019). Pro-metastatic activity of miR-10b is related to its targets—metastasis suppressor genes—*PTEN, NF1, HOXD10, CDKN1A, CDKN2A, TP53, KLF4* and analysis of miR-10b expression could be informative for tumour stage (Sheedy and Medarova 2018). Moreover, the discriminating ability of our profile could be very valuable in some rare cases of problematic clinical decision or if the primary tumour is unknown due to insufficient histopathology and immunohistochemistry data.

Downregulated miR-7 showed a potential to differentiate BM from normal brain tissue ($p = 0.007$) in our cohort and both with down-regulated miR491 are connected to increased cell proliferation, epithelial-mesenchymal transition (EMT) and cell migration in BM (Xiao 2019; Guo

et al. 2021). Restoration of miR-491 expression has observed to suppress proliferation, EMT, invasion and metastases in vitro and in vivo by targeting Wnt3a/β-catenin (Meng et al. 2019) or by regulating the AKT pathway (Guo et al. 2021).

We could conclude that our signature of miR-21, miR-491, miR-7 and miR-10b is able to correctly diagnose the most common BM disseminated from lung cancer and correctly classify the primary and metastatic brain tumour from lung cancer.

Correlation analyses showed that up-regulation of miR-21 and down-regulation of miR-7 and miR-491 correlated with poor outcome of brain tumour patients. Moreover, we found that miR-21 correlated with the KPS of the patients. In concordance with previous studies, we observed a correlation between miR-7 and miR-21 with glioma disease recurrence. The observed correlation indicated their prognostic and monitoring application for patients with brain tumours. Interestingly, miR-491 correlated with patient therapy. MiRNA regulation network understanding is still in its infancy, but probably some compensatory mechanisms or involvement of competing endogenous RNAs can be related to the observed correlation.

In the present study, we did not observe significant deregulation of miR-34a in different brain tumours. Contrary to the reported data that miR-34a has been decreased in GBM (Li et al. 2009a, b; Guessous et al. 2010), especially in the proneural GBM subtype (Genovese et al. 2012; Gao et al. 2013) and it regulates tumour invasion (Lopez et al. 2018) and is related to EMT and metastasis (Gregory et al. 2008), we did not show a statistically significant decrease in miR-34a. There might be several reasons as to why we didn't identify significant deregulation in the expression of miR-34a. Probably, it could be due to the heterogeneous nature of brain tumours, different analysis methods, small patient cohorts or endogenous controls used for data normalization. In our experiments we used miR-877 as a stably expressed endogenous reference control. We decided to normalise our results against the same species short-noncoding RNA because other RNA species such as mRNA, rRNAs, small nuclear or nucleolar RNAs might differ in size, expression, extraction efficiency and reverse transcription.

Some limitations in our study are small sample size, especially for LGG and BM, brain tumour heterogeneity and some missing clinicopathological data. According to us, due to the robustness of the used statistical analyses and the nature of the non-parametric tests the effect of above mentioned limitations is minimized regarding the generalizability of the results.

MiRNA and therapy

Remarkably, our deregulated 4-miRNAs signature of miR-21, miR-10b miR-7, miR-491 showed high level of diagnostic accuracy in HGG, LGG and BM. Recent research data have shown that restoration of DEMs could be an effective strategy for personalized anti-cancer therapy (Salarinia et al. 2016). Therefore, the selected miRNAs in our study have the potential to become targets for future miRNA-based therapy development. The current standard of treatment of HGG and GBM are maximal surgical resection, radiotherapy and Temozolomide (TMZ) chemotherapy, although the recurrence rate and poor patients outcome remain high. By now, treatment of HGG and metastatic cancer patients represents an important clinical challenge.

The up-regulated miR-21 observed in our cohort, has been found to protect glioma cells from Temozolomide-induced apoptosis by reducing the pro-apoptotic protein BAX and the activity of caspase-3 (Li et al. 2009a, b; Shi et al. 2010). Additionally, miR-21 has enhanced the resistance to carmustine by decreasing the expression of Spry2 protein (Wang et al. 2017). Experiments with inhibition of miR-21 have shown cell cycle arrest and restoration of p53-mediated apoptosis in HGG (Papagiannakopoulos et al. 2008). Previous studies have reported that inhibition of miR-21 in *PTEN* mutant cell lines suppresses AKT and EGFR pathways and activates cell caspases (Ren et al. 2010). Using RNP-based nanoparticles for suppressing upregulated miR-21 in GBM cells, has resulted in tumour apoptosis, PTEN and PDCD4 restoration, tumour growth regression and increased overall survival (Lee et al. 2017). Inhibition of miR-21 and C-X-C Chemokine Receptor 4 (CXCR4) in malignant glioma has suppressed AKT and Raf pathways and has led to reduced proliferation, invasiveness and migration in vitro and in vivo (Liu and Yang 2020). Therefore, miR-21 silencing could increase TMZ treatment efficiency and might be used as an adjuvant therapy in TMZ-resistant glioblastoma cases.

MiR-10b inhibition suppresses glioma growth, proliferation and survival without affecting normal brain cells *in vitro* and *in vivo* (Gabriely et al. 2011; Teplyuk et al. 2016). In glioblastoma cell cultures, inhibition of miR-21 and miR-10b, preceding TMZ treatment, has led to cell cycle arrest and decreased tumour viability (Ananta et al. 2016; Dong et al. 2012). Moreover, miR-10b has been observed to decrease the radiation sensitivity of GBM cells by activation of AKT expression, migration and decreased radiation-induced apoptosis (Zhen et al. 2016). Inhibition of miR-10b by magnetic nanoparticles conjugated to LNA-based miR-10b antagomirs, has led to elimination of distant metastases in vivo. The authors have observed accumulation of the nanodrugs in metastases probably due to their addiction to miR-10b activities. Moreover, anti-miR-10b therapy combined with doxorubicin resulted in a significant proliferation

reduction, complete regression of distant metastases and reduced cancer mortality (Yoo et al. 2015, 2018).

The deregulated miR-7 reported in our study has also been observed in Temozolomide resistant gliomas (Jia et al. 2019). Using lentiviral vectors for restoring the miR-7 expression in GBM has led to suppression of oncogenic EGFR/PI3K/AKT pathways, while in NSCLC normalization of miR-7 induces apoptosis, inhibits cell proliferation and migration by suppression of BCL-2 (Bhere et al. 2018; Xiao et al. 2019). Although, we found no correlation between miR-7 and diseases, such as hypertension and diabetes (Wan et al. 2017), data have shown that Metformin, used for type II diabetes treatment, restores the miR-7 expression (Dong et al. 2020). Interestingly, Metformin has downregulated miR-21 expression, inhibiting cancer cell growth (Pulito et al. 2017). Both Metformin and miR-7 mimic therapy inhibited AKT/mTOR, MAPK/Erk and NF-kB signalling pathways (Dong et al. 2020).

In the present study, we could not find a significant deregulation of miR-34a, although the liposomal miR-34 mimic is one of the first molecules that recently entered phase I clinical trials for treatment of solid tumours (Hong et al. 2020).

Although miRNA related therapy approaches are still a relatively new area and the majority of them are at their preclinical stage, the above data clearly showed their therapeutic potential. Our result indicated that the 4-MRNA selected signature could be analysed as future therapeutic targets for improving the clinical management of brain malignancies. Moreover, we believe that combining the above-mentioned miRNAs in our signature might have an advantage for miRNA-based targeted therapy in primary and metastatic brain tumours.

In the present study, aiming to select accurate, affordable and applicable biomarker, based on the prior knowledge of disturbed miRNA expression and functional analysis in normal and malignant brain tissues, we observed a signature of only four deregulated miRNAs. Our signature could facilitate the clinical decision in underestimated or borderline histology cases and showed diagnostic, prognostic and grading biomarker potential in patients with primary and metastatic brain tumours. The miRNA signature proposed in this study needs to be evaluated in a larger patient cohort as it could improve patient monitoring, therapy and outcome.

Conclusion

In conclusion, a miRNA signature that will be used in the clinical practice should be accurate, affordable and should include a limited number of miRNAs. In this study, we selected a signature of miR-21, miR-10b, miR-7 and miR-491 that showed high level of accuracy as a diagnostic,

grading and prognostic biomarker in primary and metastatic brain tumours. In addition, we observed a correlation of our signature with patient outcome, disease recurrence and KPS. To the best of our knowledge, this is the first study reporting that the signature of miR-21, miR-7, miR-10b and miR-491 could accurately separate between the normal and malignant brain tissues, as well as primary and BM from lung cancer. The received data indicated the clinical potential of our signature as adjuvant biomarkers and miRNA-based future targeted therapies for patients with brain malignancies.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval Approval was obtained from the ethics committee of the Medical University of Sofia (KENIMUS-2019). The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication Patients signed informed consent regarding publishing their data.

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