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MicroRNA-181 family predicts response to concomitant chemoradiotherapy with temozolomide in glioblastoma patients

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MicroRNAs are endogenously expressed regulatory noncoding RNAs. Previous studies showed altered expression levels of several microRNAs in glioblastomas. In this study, we examined the expression levels of selected microRNAs in 22 primary glioblastomas and six specimens of adult brain tissue by real-time PCR method. In addition, we examined methylation status of MGMT promoter by methylation-specific real-time PCR, as this has been shown to be a predictive marker in glioblastomas. MGMT methylation status was not correlated with response to concomitant chemoradiotherapy with temozolomide (RT/TMZ). MiR-221 (p=0,016), miR-222 (p=0,038), miR-181b (p=0,036), miR-181c (p=0,043) and miR-128a (p=0,001) were significantly down-regulated in glioblastomas. The most significant change was observed for up-regulation in miR-21 expression in glioblastomas (p<0,001). MiR-181b and miR-181c were significantly down-regulated in patients who responded to RT/TMZ (p=0,016; p=0,047, respectively) in comparison to patients with progredient disease. Our data indicate for the first time that expression levels of miR-181b and miR-181c could serve as a predictive marker of response to RT/TMZ therapy in glioblastoma patients.

Key words: microRNA; MGMT methylation; glioblastoma; chemoradiotherapy; temozolomide;

Glioblastomas are the most common form of primary malignant tumors in the central nervous system of adults, accounting for 50–60% of primary brain tumors [1]. Despite a cytoreductive surgery and intense combined chemoradiotherapy, glioblastomas are the most lethal tumors with dismal prognosis. A marked and significant benefit of a 2-year overall survival (27% versus 11%) has been achieved in patients treated with a concomitant chemoradiotherapy with temozolomide (RT/TMZ) [2]. Because of an extremely short median survival time of glioblastoma patients and a diversity in therapy response, it is extremely important to find new biomarkers that can be used in prediction of clinical outcome in patients treated with RT/TMZ .

One of a number of novel approaches to molecular characterization of tumors is based on the expression profiling of microRNAs (miRNAs) which are short (18–25 nucleotides in length), noncoding RNA molecules that regulate gene expression post-transcriptionally. Bioinformatic tools predict that miRNAs are able to regulate approximately one-third of mammalian genes, including a significant number of oncogenes, tumor suppressor genes and genes associated with the invasion, dissemination and chemoresistance of tumors [3]. MiRNAs have been studied in association with a broad spectrum of oncological diseases [4]. Further, significant differences have been noticed in miRNA expression profiles of glioblastomas and normal brain tissues. Among others, miR-125b, miR-128a and miR-181a-c were found to be extremely down-regulated and miR-221/222 and miR-21 highly up-regulated in glioblastomas [5]. Following the discovery of specific glioblastoma miRNA expression profiles, several studies were undertaken that focused on the functional validation of each miRNA identified separately: miR-21 [6–10], miR-221/222 [11, 12], miR-181a/181b [13], miR-128 [14, 15],

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miR-124 and miR-137 [16]. From these the anti-apoptotic effects of miR-21 were repeatedly verified on established glioblastoma cell lines *in vitro* [6, 7, 8] and *in vivo* using the athymic nude mouse model [9] and clinical samples [9, 10].

Recently, the most frequently studied predictive marker in glioblastomas has been the ubiquitous DNA-repair enzyme, O^6-methylguanine–DNA methyltransferase (MGMT) [17]. Epigenetic silencing of the MGMT gene by a promoter methylation has been shown to be an independent predictor of response to alkylating chemotherapy and prognosis of patients with newly diagnosed glioblastomas treated with RT/TMZ [17–21].

The purpose of this study is, firstly, to identify the methylation status of an MGMT promoter and to quantify expression levels of selected miRNAs (miR-21, miR-221/222, miR-125b, miR-128a and miR-181a-c) as glioblastoma biomarkers through a quantitative real-time PCR, and, secondly, to evaluate their possible relationship in regards to the response to a standard RT/TMZ treatment in glioblastoma patients.

Patients and methods

Patients and treatment. A retrospective study was conducted by two centers (Masaryk Memorial Cancer Institute and University Hospital Brno, Czech Republic). The study included 22 patients with primary glioblastomas who underwent surgery at the Department of Neurosurgery of the University Hospital Brno. After surgery patients were treated at the Masaryk Memorial Cancer Institute under a standard protocol, receiving radiation therapy (2 Gy per fraction for 6 weeks; total dose of 60 Gy) plus concomitant chemotherapy with temozolomide (RT/TMZ) at 75 mg/m^2 daily, for 6 weeks. A response was evaluated at the first MRI scan performed 1 month after the concomitant RT/TMZ. A lesion enlargement was recorded in 11 (50%) patients (non-responders) while 11 (50%) patients indicated a response to the therapy. In the responding group of patients, the course followed an adjuvant treatment with temozolomide (150–200 mg/m^2 for 5 days in 4 week cycles). 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 Under a supervision of two experienced neuropathologists, 22 samples of tumor tissue were collected from, surgically

resected glioblastomas and six samples of an adult brain tissue were taken from areas surrounding arteriovenous malformations (AVM). The glioblastoma and brain formalin-fixed paraffin-embedded (FFPE) specimens (containing either >90% tumor or >90% normal tissue) were dissected and placed into nuclease-free microcentrifuge tubes. Total RNA isolation and small RNA enrichment procedures were performed with the mirVana miRNA Isolation Kit (Ambion, USA), according to the manufacturer's instructions. DNA was extracted using the Qiagen DNA Mini Kit (Qiagen, Germany), again following the manufacturer's instructions. Nucleic acid concentration and purity were controlled by UV spectrophotometry (A260:A280 > 2.0; A260:A230 > 1.8) using Nanodrop ND-1000 (Thermo Scientific, USA).

Methylation-specific polymerase chain reaction .Real-time quantitative, methylation-specific PCR (QMSP) was performed using a methodology previously described by Hattermann et al. [21]. Bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen, Germany), as described by the manufacturer. For each conversion reaction, 1 μg DNA was employed. CG Genome Universal Unmethylated DNA (S7822; Vial A; Millipore, Germany) and CG Genome Universal Methylated DNA (S7821; Millipore, Germany) were used for positive controls (100% values) and standard curves. The QuantiTect SYBR green PCR Kit (Qiagen, USA) and primers specific for fully methylated and fully unmethylated MGMT promoter sequences were used for QMSP. The primer pair corresponding to a specific ß-actin sequence was used as an internal standard to assess bisulfite conversion efficiency [21].

*Real-time quantification of miRNAs by stem-loop RT-PCR***.** Complementary DNA (cDNA) was synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA Assay protocol (PE Applied Biosystems, Foster City, Calif., USA). Reverse transcriptase (RT) reactions utilized 10 ng of RNA sample, 50 nM of stem-loop RT primer, 1 x RT buffer and 0.25 mM each of dNTPs, 3.33 U/μl MultiScribe RT and 0.25 U/μl RNase inhibitor (all from the TaqMan MicroRNA Reverse Transcription kit of Applied Biosystems; 4366597). Reaction mixtures (15 μl) were incubated in a TGradient thermal cycler (Biometra) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. Real-time PCR was performed using the Applied Biosystems 7000 Sequence Detection System. The 20-μl PCR reaction mixture included 1.3 μl of RT product, 1 x TaqMan (NoUmpErase UNG) Universal PCR Master Mix and 1 μl of primer and probe mix of the TaqMan MicroRNA Assay protocol (PE Applied Biosystems). Reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 10 min. 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.

*Statistical analysis***.** Expression data were normalized according to the expression of the RNU6B (Assay no. 4373381; Applied Biosystems). Statistical differences between miRNA levels in glioblastomas and adult brain tissue and differences in therapy response in relation to miRNA levels were evaluated using the nonparametric Mann-Whitney U test between 2 groups. Contingency tables were analyzed using Fisher's exact test. All calculations were performed using Statistica software version 6.0 (StatSoft Inc., USA).

Results

The MGMT promoter methylation status. The MGMT promoter was methylated in 10 cases (45%) and unmethylated in 12 cases (55%) and it was not related to any other clinical char-

Table 1. Patient characteristics and their relation to methylation status of MGMT[1] promotor.

	Total n = 22	%	Unmethylated n = 12 (55%)	%	Methylated n = 10 (45%)	%	Association with methylation status (Fischer's exact test)
Age							
≤50	7	32	4	34	3	30	P = 0.7059
>50	15	68	8	66	7	70	
Median = 55							
Range = 28-65							
Gender							
Male	14	64	9	75	5	50	P = 0.3467
Female	8	36	3	25	5	50	
Karnofsky score							
≥90	17	77	9	75	8	80	P = 0.8123
<90	5	33	3	25	2	20	
RT/TMZ response							
Responders	11	50	5	42	6	60	P = 0.4716
Non-responders	11	50	7	58	4	40	

[1]MGMT = O-6-methylguanine-DNA methyltransferase.

Table 2. Median fold changes of miRNAs in glioblastomas, their possible association with cancer and their putative targets.

microRNA	Fold change	Chromosome localization	Connection to cancer in previous reports	Putative targets[1]
miR-221	0.25	Xp11.3	↑ glioblastoma [11,12], ↑ thyroid cancer , ↑ hepatocellular carcinoma, ↑ prostate carcinoma, ↑ pancreatic cancer, ↑ breast cancer - tamoxifen resistance (see [4] for review)	CDKN1B (p27)*, CDKN1C (p57)*, KIT*, MYBL1, RECK, SOCS3, APAF1, TIMP3, TIMP2, TCF12
miR-222	0.22	Xp11.3		
miR-181a	0.4	1q31.3	↓ glioblastoma [5,13], ↓ chronic lymphocytic leukemia [22], ↑ associated with better response to S-1 in colorectal cancer [23]	HOXA11*, TCL1*, TGFBR1, TGFBI, TGFBRAP1, TGIF2, PLAU, MMP7, MMP14, MYBL1, SMAD2, SMAD7, MAPK1, KLK13, HLA-DR, CD14, SAP30, RAB38, HSPA9, CYTC, E2F5, MGMT
miR-181b	0.28	1q31.3		
miR-181c	0.29	19p13.12		
miR-125b	1.45	11q24.1	↓ glioblastoma [5], ↓ prostate cancer, ↑ ovarian cancer, ↓ associated with better survival in hepatocellular carcinoma (see [4] for review)	E2F3*, MCL1*, BCL2*, NTRK3*, ERBB2*, TP53INP1, SMAD2, RAB6B, CASP2, ETS1, ABL2, STAT3
miR-128a	0.03	2q21.3	↓ glioblastoma [5,14,15]	BMI1*, TGFBR1, TGIF2, SMAD2, RARA, NOVA1, BAG2, EGFR, ITGA5, GSPT1, VEGFB, CA7
miR-21	8.35	17q23.2	↑ glioblastoma [5-10], ↑ colorectal cancer [3], ↑ lung cancer, ↑ breast cancer and other solid cancers (see [4] for a review)	PDCD4*, TGFBI, TIMP3*, RHOB, RECK, PPARA, ASPN

[1] Human mRNAs displaying imperfect complementarity with particular miRNAs were selected according to the potential function in carcinogenesis using TargetScan, PicTar and miR-Base target prediction algorithms (3).
* The predicted target was verified by in-vitro functional analysis.

acteristic of glioblastoma, i.e. age, gender, performance status assessed by Karnofsky score or a response to a concomitant chemoradiotherapy with temozolomide (Table 1). The MGMT methylation proved to be independent of any of the miRNAs analyzed in this study.

Comparison of miRNA expression levels in glioblastomas and adult brain samples. The fold change of the miRNAs in glioblastoma samples, their possible relationship to cancer, and their putative and validated targets are all presented in Table 2. Both, the levels of significance and the medians of the relative expression values with their ranges defined by the 25th and 75th percentiles, are presented in Table 3. The Real-time PCR analysis indicated no significant difference in expression levels of miR-181a and miR-125b between glioblastomas and the normal brain tissue. In contrast, the expression level of miR-21 was significantly up-regulated (by nearly eight times) in the tumor compared with the normal brain tissue (Table 3). Conversely, miR-221, miR-222, miR-181b, miR-181c, were all significantly down-regulated in glioblastomas (Table 3). The most significant difference was observed in miR-128a with levels in glioblastomas approximately 40 times lower than in a normal brain tissue.

Correlation of patient response to RT/TMZ with miRNA expression levels. To evaluate the potential association of

Table 3. Comparison of normalized miRNA expression levels in glioblastomas and control brain tissue and their association with response to RT/TMZ.

	Glioblastoma vs. brain tissue[1]			RT/TMZ response[2]		
microRNA	**Glioblastoma n = 22**	**CBT[1] n = 6**	**P [3]**	**Responders n = 11**	**Non-responders n = 11**	**P [3]**
miR-221*	2.26 (1.43-6.90)	8.88 (8.21-21.76)	**0.016**	1.85 (1.60-3.40)	2.85 (1.15-9.86)	0.562
miR-222	4.81 (2.29-13.06)	22.01 (9.25-42.62)	**0.038**	4.62 (1.50-7.09)	10.34 (2.42-29.90)	0.243
miR-181a	1.97 (0.99-3.87)	4.89 (2.83-7.84)	0.073	1.52 (0.68-2.82)	2.88 (1.32-5.22)	0.116
miR-181b	6.28 (3.23-15.09)	22.62 (7.46-50.39)	**0.036**	5.17 (2.78-6.77)	12.59 (6.59-18.43)	**0.016**
miR-181c	0.57 (0.21-1.05)	1.95 (0.58-2.52)	**0.043**	0.33 (0.14-0.72)	0.91 (0.50-1.54)	**0.047**
miR-125b	50.36 (22.50-84.76)	34.76 (17.79-56.30)	0.502	45.5 (10.98-76.41)	55.2 (33.92-84.82)	0.519
miR-128a	0.02 (0.01-0.06)	0.76 (0.12-1.16)	**0.001**	0.02 (0.01-0.04)	0.04 (0.01-0.06)	0.652
miR-21	219.26 (75.24-562.87)	26.26 (18.68-30.84)	**<0.001**	266.87 (79.29-424.53)	165.04 (76.87-830.85)	0.747

*Medians of expression level related to RNU6B with 25th and 75th percentiles in parentheses.
[1]Control brain tissue from arteriovenous malformation surgeries; [2]Response to concomitant chemoradiotherapy with temozolomide; [3]Mann-Whitney U-test between 2 groups, bold indicate significance at the P = 0.05 level.

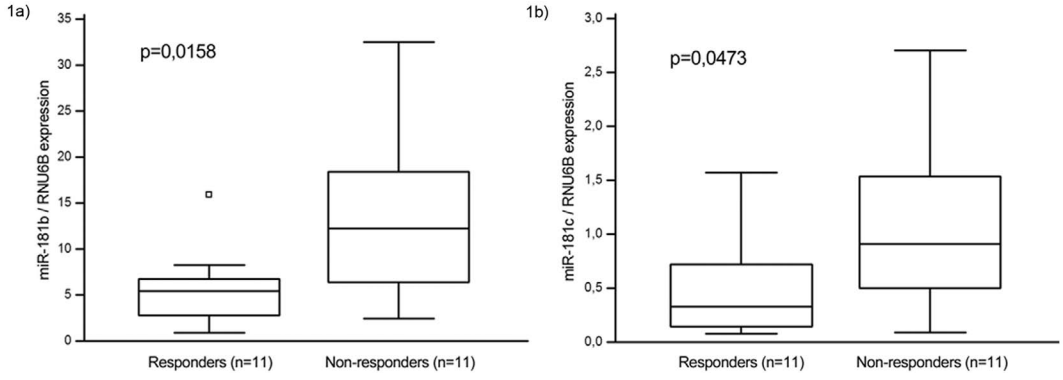

Figure 1. Response to concomitant chemoradiotherapy with temozolomide based on miR-181b (a) and miR-181c (b) expression levels (Mann-Whitney U test). Central box represents values from lower to upper quartile (25 to 75 percentile). Middle line represents the median. A line extends from minimum to maximum value, excluding outstanding values which are displayed as separate squares.

individual miRNAs with a patient response to RT/TMZ, the expression levels of each miRNA in the response group of patients were compared with their levels in the group of patients with the progressive disease. Patients who responded to RT/TMZ tended to have lower expression levels of members of the microRNA-181 family than those with the progressive disease. MiR-181b and miR-181c were significantly down-regulated in the glioblastomas of patients with response (Table 3, Figure 1a and 1b). On the other hand, no association was found between the treatment response and miR-221, miR-222, miR-181a, miR-125b, miR-128a, and miR-21 (Table 3).

Discussion

The most extensively studied predictive factor in glioblastoma patients is the epigenetic silencing of the MGMT gene by a promoter methylation. It has been associated with longer survival in patients who received alkylating agents. However, recent studies that have focused on the MGMT predictive potential have provided ambiguous results [17–20]. In this study, we used QMSP, the most sensitive method for the methylation analysis [21]. A positive methylation status of the MGMT promoter was detected in 45% of tumors. According to recent literature, the frequency of the MGMT promoter methylation in glioblastoma patients ranged from 45% to 65% [17–20]. Using this highly sensitive QMSP assay, we did not identify any relationship between the methylation status of the MGMT promoter and age, gender, performance status or response to RT/TMZ. Our observations are in accordance with data published by Sadones *et al.* [18] and Blanc *et al.* [19] and do not lead us to consider promoter methylation of the MGMT gene as a predictive factor of responsiveness to RT/TMZ in glioblastoma patients.

There are several advantages of using miRNAs instead of mRNAs as biomarkers. It is relatively easier to discover reliable biomarkers from approximately 1,000 miRNA candidates discovered to date than from over 40,000 genes. A further advantage is that, due to their small size and stem-loop structure, miRNAs are relatively more stable and less subjected to degradation during fixation and sample processing. This can also be of benefit for large retrospective studies based on archived FFPE samples. MiRNA-specific real-time PCR assays are very sensitive and require only a small amount (10–25 ng) of the total RNA from archived FFPE patient samples.

Our study confirmed the up-regulation of miR-21 in glioblastoma tissue that has been noted previously by others [6–10]. MiR-21 was the most up-regulated miRNA with an almost eight-fold expression increase in glioblastoma tissue. Chan *et al.* [6] have shown that inhibition of miR-21 expression led to a caspase activation and an associated apoptotic cell death in multiple glioblastoma cell lines, suggesting an anti-apoptotic function of miR-21. We did not observe higher levels of miR-221/222 in glioblastomas despite a previous study [11] showing that miR-221/222 were over-expressed in gliomas, with the tumor suppressor p27Kip1 proven to be their direct target [11, 12]. In contrast, we observed approximately four-fold lower levels of miR-221/222 in glioblastomas in comparison to the adult brain tissue (Table 3). The adult brain tissue was, for our purposes, collected during an AVM surgery. It is likely that the brain tissue, though excised from the margin of resection material, contained traces of micro-capillaries from around the AVM. It is generally known that miR-221/222 is found in the highest levels in endothelial cells. This could be responsible for the apparently low levels of miR-221/222 in the glioblastomas of our group of patients despite their absolute levels being comparable with those of previous reports [11, 12]. The down-regulation of MiR-125b noticed by Ciafre *et al.* [5] was not detected in glioblastomas in this study. However, the deregulation of miR-128a first identified by the same authors, was verified by our data. The miR-128a was, indeed, the most down-regulated miRNA in glioblastomas showing an approximately 40-fold decrease which is in concordance with observations of Godlewski et al. [14].

MiR-181a and miR-181b have been shown to function as tumor suppressors that trigger growth inhibition, induced apoptosis and inhibited invasion in glioma cells [13]. Accordingly, the expression levels of miR-181a-c in glioblastomas were, in our case, lower, whereas a down-regulation of miR-181b and miR-181c was statistically significant. Lower levels of miR-181b and miR-181c in glioblastomas, however, were positively correlated with response to RT/TMZ in glioblastoma patients. Nakajima *et al.* [23] were able to demonstrate similar results using the colorectal cancer model and its response to chemotherapy with fluoropyrimidine based drug S-1. Significantly lower levels of miR-181b ($P = 0.02$) were noticed in tumors of patients responding to S-1 treatment [23]. The potential of all microRNA-181 family members to regulate MGMT levels suggests a relationship with the chemosensitivity to alkylating agents such as temozolomide. The down-regulation of microRNA-181 family leads to an up-regulation of MGMT and, therefore, its association with a response to RT/TMZ through MGMT post-transcriptional regulation is implausible. Our data indicate that an alternative and efficient molecular mechanism exists by which microRNA-181 family sensitizes glioblastoma cells to a chemoradiotherapy. As a robust translational regulator, the microRNA-181 family can mediate a number of genes in response to an acute cellular stress caused by a drug treatment or radiation (Table 3).

To our knowledge, it is noted for the first time that the expression of miR-181b and miR-181c has shown a negative correlation with a response to RT/TMZ. Our findings also support the importance of miR-128a and miR-21 in pathogenesis of glioblastomas. Further studies and validations are needed but we suggest that microRNA-181 family might be used for prediction of RT/TMZ response in clinical practice. If validated, it would pave the way to better treatment decisions and, ultimately, an improvement in the survival rate of glioblastoma patients.

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