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 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 molotherapy improved progression free survival with preservation of quality of life and reduction of corticosteroids use, but did not improved overall survival (OS). Therefore, another agents, such as cilegitude, 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 chemoradioresistance 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 cortices resected during surgery for intractable epilepsy of 10 patients were used as non-tumor control brain tis-

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.
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 mM 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-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 MDC12887, Life Technologies) or mirVana miRNA Mimic Negative Control (cat. no. 4464058, Life Technologies) oligonucleotides and equimolar concentration of Lipofectamine 2000 accordingly 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 (BIO-TEK) at 570 nm wavelength. All experiments were run in triplicates.

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^{ΔΔCT} 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 Kaplan-Meier survival curves. Cox proportional hazards regression analyses were performed to assess the independent contribution of the miRNA signature-based Risk Score and clinico-pathologic 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^-6 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 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 we 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 1). 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 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).

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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 all GBM samples for each of these miRNAs.

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

<table>
<thead>
<tr>
<th>Factors</th>
<th>Univariate Cox regression</th>
<th>Multivariate Cox regression</th>
<th>Long-rank test P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>P value</td>
</tr>
<tr>
<td><strong>Progression free survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk Score</td>
<td>2.00</td>
<td>1.37–2.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TMZ</td>
<td>0.48</td>
<td>0.28–0.82</td>
<td>0.007</td>
</tr>
<tr>
<td>PS</td>
<td>1.69</td>
<td>0.70–4.04</td>
<td>0.242</td>
</tr>
<tr>
<td>MGMT</td>
<td>0.54</td>
<td>0.23–0.96</td>
<td>0.035</td>
</tr>
<tr>
<td><strong>Overall survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk Score</td>
<td>2.72</td>
<td>1.84–4.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Extent of resection</td>
<td>1.00</td>
<td>0.49–2.04</td>
<td>0.991</td>
</tr>
<tr>
<td>TMZ</td>
<td>0.64</td>
<td>0.37–1.08</td>
<td>0.096</td>
</tr>
<tr>
<td>PS</td>
<td>1.79</td>
<td>0.76–4.22</td>
<td>0.186</td>
</tr>
<tr>
<td>MGMT</td>
<td>0.51</td>
<td>0.29–0.91</td>
<td>0.022</td>
</tr>
</tbody>
</table>

**Bolded values are factors significant in multivariate cox regression analysis. CI, confidence interval; HR, hazard ratio; MGMT, methylation status of O6-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 all GBM samples for each of these miRNAs.
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 downregulated 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 tissues.

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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