Imaging, Diagnosis, Prognosis

MiRNA-196 Is Upregulated in Glioblastoma But Not in Anaplastic Astrocytoma and Has Prognostic Significance

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Abstract

Purpose: MicroRNAs (miRNA) are short noncoding RNAs that can play critical roles in diverse biological processes. They are implicated in tumorigenesis and function both as tumor suppressors and as oncogenes. The clinical significance of miRNA expression profiles in malignant gliomas remains unclear.

Experimental Design: In this study, we examined the expression levels of 365 mature human miRNAs in 12 malignant gliomas, including 8 glioblastomas and 4 anaplastic astrocytomas, using TaqMan real-time quantitative PCR arrays. A validation study was done to corroborate a subset of the results, including expression levels of miR-196a, -196b, -21, and -15b, by analyzing 92 malignant gliomas by conventional real-time PCR. We modeled the relationship between the expression levels of these miRNAs and the survival rate of 39 glioblastoma patients by Kaplan-Meier method and multivariate analysis.

Results: Expression profiles in glioblastomas and anaplastic astrocytomas suggested that 16 miRNAs were candidate markers associated with the malignant progression of gliomas. Among them, miR-196a showed the most significant difference (P = 0.0038), with miR-196b also having a high significance (P = 0.0371). Both miRNAs showed increased expression levels in glioblastomas relative to both anaplastic astrocytomas and normal brains in the validation study. Furthermore, patients with high miR-196(expression levels showed significantly poorer survival by the Kaplan-Meier method (P = 0.0073). Multivariate analysis showed that miR-196 expression levels were an independent predictor of overall survival in all 39 glioblastoma patients (P = 0.021; hazard ratio, 2.81).

Conclusions: Our results suggest that miR-196 may play a role in the malignant progression of gliomas and may be a prognostic predictor in glioblastomas.

MicroRNAs (miRNA) are small noncoding RNA molecules, 19 to 24 nucleotides in length, that contribute to the regulation of crucial processes, such as cell proliferation, apoptosis, development, and differentiation (1, 2). Recent studies have shown that miRNAs are implicated in tumorigenesis and function as tumor suppressor genes or oncogenes (3–5). Characterization of the miRNA expression patterns in cancer cells is thought to have substantial value for diagnostic and prognostic determinations as well as for eventual therapeutic interventions (6–13). Several miRNAs, such as miR-7, -21, -128, -221, and -222, have recently been reported to act as a tumor suppressors or oncogenes in glioblastomas, the highest grade glioma (14–18). Recently, the number of newly identified miRNAs has exploded, but the biological role of these miRNAs has not yet been elucidated. In addition, the clinical significance of miRNAs in glioma patients is still unclear, although several groups have reported the alteration of miRNA expression in glioma (14–18).

With hundreds of human miRNAs reported thus far and many more awaiting experimental validation, these molecules represent one of the largest classes of gene regulators. As the number of newly discovered miRNAs is still increasing, large-scale screening is necessary to profile the global miRNA expression. MiRNA microarrays, the most commonly used tool for the large-scale screening of miRNA expression, do not amplify miRNA, and thus the sensitivity is often compromised in these assays (19). In contrast, the stem-loop reverse transcription-PCR (RT-PCR) can profile miRNA expression with superior sensitivity and specificity. However, RT-PCR is not practical for
Translational Relevance

In the present study, we quantitatively examined the microRNA (miRNA) expression profiles of malignant gliomas on the whole-genome scale and identified several miRNAs associated with the malignant progression of glioma. Furthermore, we indicated the clinical implications of miR-196 in malignant glioma patients. Our work provides evidence that the alteration in miRNA expression might be involved in the malignant progression of glioma and may have an effect on the prognosis of glioblastoma patients. In particular, miR-196, the most malignancy grade correlated miRNA indicated here, should play an important role in the pathogenesis of glioblastomas. Furthermore, the identification of miR-196 might have substantial value for diagnostic and prognostic determinations as well as for eventual therapeutic interventions of this most malignant tumor in the human central nervous system.

Materials and Methods

Glioma samples and patients

Glioma specimens were obtained from patients during surgery at the Kyushu University Hospital and five affiliated hospitals. A portion of the tumor tissue was saved and made into paraffin sections for histologic diagnosis in strict accordance with World Health Organization (WHO) criteria by two established neuropathologists (SOS and TI), and the remaining tissue was snap-frozen in liquid nitrogen then stored at -80°C. A total of 92 glioma specimens were collected, including 74 glioblastomas and 18 anaplastic astrocytomas. Among those collected, specimens from 12 malignant gliomas (4 anaplastic astrocytomas and 8 glioblastomas) were selected according to the pathologic diagnosis and pivotal genetic alterations [1p, 10p, 10q, and 19q loss of heterozygosity (LOH), and epidermal growth factor receptor (EGFR) gene amplification and expression; see Supplementary Table S1 for details] for the global miRNA expression examination by TaqMan real-time quantitative PCR array. Prior to RNA extraction from these 12 frozen samples, the adjacent tumor tissues were subjected to frozen sections and reviewed by a pathologist (SOS) to ensure that a minimum of 80% tumor cells were included in the sample. Subsequently, a validation study was done on all of the 92 malignant gliomas as well as 3 normal brain tissues (for calibration purpose) by conventional real-time PCR. Of these three normal brain tissues, one was purchased from Ambion as total RNA (FirstChoice Human Brain Reference RNA), whereas the other two were obtained from two patients who underwent treatment at Kyushu University Hospital, one being an epileptic brain and the other a nonneoplastic portion of the autopsy brain. Finally, we picked up 46 consecutive malignant glioma cases (7 anaplastic astrocytomas and 39 glioblastomas), who underwent primary surgery at Kyushu University Hospital from 2002 to 2007, for the further clinical analysis. All of the cases were treated with the same protocol, which was radiotherapy and chemotherapy (ACNU or Temozolomide), and were well followed up. The present study was approved by the Ethics Committee of Kyushu University.

RNA extraction, reverse transcription, and real-time PCR quantification

Total RNA was extracted from the frozen samples using a mirVana miRNA Isolation Kit (Ambion). PCR array assays were conducted using TaqMan Human MiRNA Arrays v1.0 (PE Applied Biosystems). In brief, all 365 human mature miRNAs and 2 endogenous controls, RNU44 and RNU48, were reverse transcribed using 8 predefined reverse transcription primer pools containing up to 48 reverse transcription primers each. The reverse transcription products were subsequently loaded onto the TaqMan Array to do real-time PCR amplification using Applied Biosystems 7900HT Fast Real-Time PCR System. Relative quantification of miRNA expression was calculated with the 2-ΔΔCt method.

To corroborate and validate the expression level measurements of miR-196a, -196b, -21, and -15b, cDNA synthesis and subsequent real-time PCR were done using a TaqMan MiRNA Reverse Transcription kit (Applied Biosystems), individual TaqMan MiRNA assay (Applied Biosystems), and Applied Biosystems 7500HT Fast Real-Time PCR System (Applied Biosystems), as previously described (22). Both RNU44 and RNU48 were used as endogenous controls, and nonneoplastic brain tissues were used for calibration.
LOH analysis by microsatellite markers
Tumor and nontumor DNA were evaluated by a PCR-based LOH assay (23) using 21 microsatellite markers located on chromosomes 1p, 10, and 19q, as shown in Supplementary Table S2. The allelic status was assessed using the criteria established in a previous study (23).

EGFR gene amplification, EGFR wild-type and VIII expression
For EGFR gene copy number, real-time PCR was done with the use of the ABI PRISM 7500 Fast Real-time PCR System (Applied Biosystems). All measurements were made in triplicate. LINE1 and blood DNA were used as endogenous control and calibrator, respectively. A tumor with EGFR amplification was defined when its copy number of EGFR was >5. EGFRvIII expression was detected by RT-PCR as described previously (24). Briefly, total RNA was extracted from frozen tumor samples. cDNA was synthesized and amplified with the use of primers designed specifically to amplify EGFR (1,044-bp product) and EGFRvIII (243-bp product). Wild-type EGFR transcripts were quantified by real-time RT-PCR using the ABI PRISM 7500 Fast Real-time PCR System (Applied Biosystems) with SYBRGreen. Expression of the housekeeping gene HPRT1 was used as endogenous control. The threshold value of overexpression was defined as 2.5-fold of mean expression level of three normal brain tissues. The primer sequences are provided in Supplementary Table S3.

Statistical analysis
Student's t-test was used to compare the expression levels of miRNAs in different glioma subtypes. A hierarchical clustering analysis was done to evaluate the miRNA expression profiles in anaplastic astrocytoma and glioblastoma. The log-rank test was used to make univariate assessments of survival rates. With the use of the Cox proportional hazards regression model, multivariate analysis was carried out to identify predictive factors for the survival of glioblastoma patients. Statistical analysis was done using JMP 7.0.

Results

Expression profiles of miRNAs correlated to the malignant progression of gliomas
To test the correlation between miRNAs and the malignant progression of gliomas, we analyzed the expression levels of 365 mature human miRNAs in the total RNA extracted from frozen tissues of four anaplastic astrocytomas and eight glioblastomas, using TaqMan real-time quantitative PCR array assays. Experimentally normalized ΔCt values corresponding to the expression levels of 365 miRNAs were used to cluster anaplastic astrocytomas and glioblastomas by unsupervised hierarchical clustering. As shown in Fig. 1A, all four anaplastic astrocytomas and two of the eight glioblastomas were found to cluster in one main branch, with the other branch containing the remaining glioblastomas. Thus, anaplastic astrocytomas were clearly distinguished from the majority of the glioblastomas. To correlate the crucial miRNA expression levels with glioma progression, Student's t-test was used to calculate the significant differences between miRNA expression levels in anaplastic astrocytomas and glioblastomas. In total, 16 miRNAs (Table 1) were found to be differentially expressed in the two histologic subtypes, based on the statistical criteria P < 0.05. With ΔCt values used for these 16 miRNAs only, a clustering analysis was conducted again to classify the 12 gliomas (Fig. 1B). This time, the four anaplastic astrocytomas and eight glioblastomas were distinctly segregated into two clusters, with the exception of one glioblastoma sample, GBM1294. However, GBM1294, which was included in the branch containing the anaplastic astrocytomas, showed a clear segregation and a relatively long interval from anaplastic astrocytomas (Fig. 1B). These finding suggest that miRNA expression patterns reflect biological and pathologic characteristics of malignant gliomas. Among these 16 miRNAs, miR-196a showed the most significant differential expression (P = 0.0038). Furthermore, this miRNA was expressed approximately 100-fold higher in the glioblastomas than in the anaplastic astrocytomas. MiR-196b, which differs by only one base from miR-196a, was also included in the analysis. Recent studies have reported the upregulation of miR-196a and/or -196b in a variety of tumors, including glioblastoma cell lines (25), prostate (26), or pancreatic cancer (10, 27). In addition, miR-21, which was included among the 16 miRNAs listed above, has been previously implicated in glioblastomas (14). MiR-15b, a member of the miR-15/16 family, also showed significantly different expression levels in glioblastomas and anaplastic astrocytomas in our results (P = 0.007). We concluded that expression levels of these miRNAs are significantly associated with WHO grade and therefore they could be useful biological markers for the malignant progression of glioma.

Upregulation of miR-196a and -196b in glioblastomas
To evaluate the correlation between these candidates and malignant progression, we examined the expression of miR-196a, -196b, -15b, and -21 in a panel of 92 malignant glioma tissues, including 74 glioblastomas and 18 anaplastic astrocytomas, by individual real-time PCR. As shown in Fig. 2, all of these miRNAs were significantly upregulated in glioblastoma relative to normal brain tissues (Student's t-test, P < 0.001), with the exception of miR-15b when compared with the RNU48 control. The miRNA expression levels of miR-196a, -196b, and -15b were shown to be significantly higher in glioblastomas than in anaplastic astrocytomas (Student's t-test, P ≤ 0.001). A similar result was observed in miR-21 when RNU48 was used as an endogenous control (Student's t-test, P ≤ 0.05). Taken together, our results determined that expression levels of miR-196a and -196b were definitively higher in glioblastomas compared with either anaplastic astrocytomas or normal brains, regardless of
the endogenous control used, suggesting that they could be relevant to the malignant progression of gliomas.

**High levels of miR-196 expression correlated with poor survival in malignant glioma patients**

MiRNA expression profiles are reported to have diagnostic and prognostic value in the context of cancer treatment (6–13). To evaluate the clinical implications of the miRNAs identified in this study, we analyzed the clinical data from 46 malignant glioma patients (7 anaplastic astrocytomas and 39 glioblastomas) whose samples were tested in the validation experiment. The correlation between expression levels of miRNAs (determined in the validation experiment) and overall survival was measured through Kaplan-Meier survival curve analysis with a log-rank comparison using a binomial variable of high or low expression relative to the average expression levels of the miRNAs. No significant correlation was found between overall survival and the expression levels of the miR-21 ($P = 0.5832$) and miR-15b ($P = 0.2478$). In contrast, highly statistically significant correlation was observed between the overall survival and the expression levels of miR-196a (Fig. 3A, left; $P = 0.0052$) and miR-196b (Fig. 3A, middle; $P = 0.0149$). However, when the analysis was carried out on the subset of 39 glioblastoma patients, the correlation was not statistically significant (Fig. 3B, left and middle; $P = 0.0519$ for miR-196a and $P = 0.0619$ for miR-196b). MiR-196a and -196b differ by only one nucleic acid and share the majority of predicted target sequences (69.5% for miR-196a and 70.6% for miR-196b, http://miRNA.sanger.uk/cgi-bin/target/v5). Thus, to reasonably evaluate the value of these two miRNAs for prognostic prediction, we divided the glioblastoma patients into two groups: a low-expression group containing samples in which expression of both miR-196a and -196b was lower than the average, and a high-expression group, in which expression of either miR-196a or -196b was higher than the average. Based on this grouping, patients with the high expression levels of miR-196 showed a shorter overall survival ($P = 0.0003$; Fig. 3A, right) compared with patients in the low-expression group, even among glioblastoma patients ($P = 0.0073$; Fig. 3B, right). In addition, multivariate analysis using the Cox proportional hazard regression model showed that a high level of miR-196 was an independent and significant ($P = 0.021$, hazard ratio, 3.37) predictor of short overall survival in glioblastoma patients (Table 2).
Discussion

Recent studies have implicated miRNAs in a variety of human cancers, and their expression signatures can provide insight into the diagnosis and prognosis of human cancers (6–13). This study provides the first report of quantitative global miRNA expression profiles, including expression data for 365 human miRNAs in malignant gliomas from clinical tissue samples. Subsets of the miRNA expression profiles showed significant correlations with the WHO grades of malignant gliomas. Furthermore, we showed that miR-196 is strongly upregulated in glioblastomas, and the high level of miR-196 expression is significantly correlated with the shorter survival of glioblastoma patients.

It has been reported that miRNA expression profiles reflect cellular differentiation and distinguish tumors of different developmental origin (4, 25). Several recent studies characterized the miRNA expression profiles associated with specific tumor subtypes according to histotype, malignant grade, and clinical features (28–32). Our study revealed significant differences between the expression levels of 16 miRNAs in the sets of 4 anaplastic astrocytomas and 8 glioblastomas. Furthermore, classification of these 12 malignant gliomas via the expression patterns of the 16 miRNAs showed significant correlations with the WHO grade of the tumor. These 16 miRNAs may be involved in tumorigenesis and the progression of malignancy in gliomas, and they may be useful for distinguishing glioblastomas from anaplastic astrocytomas.

Interestingly, in our data, two glioblastomas (GBM1266 and 1294) showed more similar miRNA expression profiles to anaplastic astrocytomas than to other glioblas-

tomas (Fig. 1A). Even clustered via the 16 candidate miRNAs, one of them (GBM1294) was still classified into the anaplastic astrocytoma group (Fig. 1B). To exclude the possibility of misdiagnosis, we reviewed the histologic sections and confirmed the accuracy of the diagnosis (photomicrographs are shown as Supplementary Fig. S1). In addition, it forcefully supported our diagnosis that both of the two tumors showed total chromosome 10 LOH (Supplementary Table S1). However, glioblastoma is a histopathologically and genetically heterogeneous tumor, and histopathologic diagnosis by rule represents the most malignant part of the tumor. The sampling bias may thus occur even when samples are taken from the same tumor tissue. This is also the common limitation of such studies using frozen sample; however, given that most of the glioblastoma cases fell into the same cluster, we believe our study clearly showed the relevance of miRNA expression to these histotypes. Nevertheless, it is also possible that there are some subpopulations of glioblastomas with different miRNA expression patterns even among those meeting the same histopathologic criteria of glioblastoma. Further studies on a larger series, which would include investigating the relationship between miRNA expression patterns and clinical courses, will be needed.

Among the 16 miRNAs identified, miR-196a was upregulated by a factor of 100 in glioblastomas relative to anaplastic astrocytomas. A similar trend was observed for miR-196b, which differs by only one base from miR-196a. Our data are consistent with a recent study that identified mir-196a and mir-196b as candidate oncogenes for central nervous system (CNS) tumor cells due to the fact that both genes are expressed at higher levels in CNS tumor-derived cell lines compared with normal brain cells (25). Increased miR-196 expression levels, by factors of 10 to 100 in some kinds of human cancers, relative to their matched normal tissues, has also been reported in two recent studies (27, 33), further supporting the relevance of miR-196 to gliomas. These findings suggest that upregulation of these two miRNAs could contribute to tumorigenesis and malignant progression, and may have diagnostic and prognostic value for glioblastomas. Several previous reports have shown that miR-21 is overexpressed in a variety of cancers (34–39). In particular, this miRNA has been suggested to play an important role in preventing apoptosis (14). MiR-21 is also a component in a network of key tumor-suppressive pathways in glioblastoma cells (40). Consistent with these reports, we observed increased expression levels of miR-21 in glioblastomas relative to normal brain tissues. Glioblastomas were observed to express significantly higher levels of this miRNA in our data. This observation supports a potential role for miR-21 in the malignant progression of gliomas.

MiR-15b, a member of miR-15/16 family, was also differentially expressed in glioblastoma and anaplastic astrocytoma tumors. Interestingly, miR-15b and its family members have been reported to be downregulated and function as tumor suppressor genes in several types of

### Table 1. Differential expression of miRNAs in glioblastoma versus anaplastic astrocytoma

<table>
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<tr>
<th>GeneName</th>
<th>P</th>
<th>Fold change</th>
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<td>hsa-miR-15b</td>
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<td>3.3</td>
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<td>0.101</td>
</tr>
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</tr>
<tr>
<td>hsa-miR-504</td>
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</tr>
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</tr>
<tr>
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<tr>
<td>hsa-miR-135b</td>
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</table>
cancer (41, 42). On the other hand, it has been suggested that miR-15b regulates cell cycle progression and is overexpressed in glioma cells (43), which is consistent with our results.

Several other miRNAs were identified in the screening study; for instance, our result showed that miR-128 expression levels were different by a factor of 0.15 in glioblastoma and anaplastic astrocytoma samples (Table 1). In addition, this miRNA was significantly downregulated in both anaplastic astrocytomas and oligodendrogial tumors when compared with normal brain tissues (our unpublished data). Consistent with our findings, it has been reported that miR-128 is downregulated in glioblastomas (16) and functions as a tumor suppressor through the direct repression of the Bmi-1 oncogene (18). Consistently lower expression levels of the miR-302-367 cluster were observed in glioblastoma relative to anaplastic astrocytoma. The human miR-302-367 gene structure has been recently described, characterized, and functionally validated in human stem cells, and it was determined to be a potential stemness regulator in embryonic stem cells (44). This miRNA cluster was shown to reprogram tumor cells into an embryonic stem cell–like state (45). These miRNAs could be significant in glioma progression and may be used as markers to distinguish glioblastoma from anaplastic astrocytoma. These miRNAs may also be involved in the mechanism of malignant transformation in gliomas.

The significance of miRNAs in diagnostic and prognostic determination has been shown in a variety of human cancers, such as chronic lymphocytic leukemia (6), lung cancer (7, 8), pancreatic cancer (9, 10), neuroblastomas (11), breast cancers (12), and colon cancers (13). However, investigations into the correlation between miRNA expression patterns and patient survival of glioblastoma, the most frequent and malignant CNS tumor, have not been previously reported. Here, we have provided the first quantitative description of the relationship between deregulated miRNA expression and survival in malignant glioma patients. According to our validation

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**Fig. 2.** Dots indicate log2 of the relative quantification (RQ) values of miRNA expression levels, normalized by RNU48 (top) and RNU44 (bottom). Bar, log2 of the average RQ values for each group. MiRNAs (top) and histologic subtypes (bottom) examined are shown. NBT, normal brain tissue.
results, miR-21, -196a, -196b, and -15b were found to be expressed at high levels in glioblastomas, and higher WHO grade gliomas tended to have higher expression levels of these miRNAs. We surveyed the relationship between the expression levels of these miRNAs and the overall survival rate in malignant glioma patients. In contrast with previous studies, which showed evidence that miR-21 overexpression was associated with poor survival and advanced tumor stage (9, 13), there was no significant correlation between glioma patient survival and expression levels of miR-21 or miR-15b. In comparison, high miR-196 expression levels were significantly associated with poor survival in WHO grade III and IV glioma patients and even among glioblastoma patients, as determined by Kaplan-Meier analysis. Multivariate analysis showed that high expression level of miR-196 is an independent and significant predictor of poor prognosis in glioblastoma patients.

Our data suggest that a high miR-196 expression level is a valuable marker for pathologic diagnosis and prognosis prediction. Although miR-196 upregulation has been reported in several types of human cancer cells, including glioblastoma cell lines (25, 27, 33), and its value for prognostic prediction has also been indicated in pancreatic cancer (10), little is known about the function of miR-196 in tumorigenesis. Luthra et al. provided evidence that increased levels of miR-196a stimulated cell proliferation and suppressed apoptosis in other cancer cell lines by repressing Annexin A1 (ANXA1), a well-documented mediator of apoptosis and an inhibitor of cell proliferation (33). In contrast, Johnson et al. have shown the increased ANXA1 expression in gliomas (46) suggesting that miR-196 may affect glioma cell behavior by another pathway. It should be noted that miR-196 is encoded at three paralogous locations in the A, B, and C mammalian HOX clusters and has extensive, evolutionarily conserved complementarity to messages of HOXB8, HOXC8, and HOXD8. HOX genes are a large family of regulatory genes implicated in the control of developmental processes, and they are involved in the malignant transformation and progression of several types of tumors (47), including gliomas (48). It has been reported that the miR-196 miRNA downregulates HOXB8, HOXC8, HOXD8, and HOXA7 due to extensive complementarity at sites in the 3′ untranslated regions of the HOX genes representing each cluster (49). Therefore, it will be important to test the effects of miR-196 knockdown on these HOX genes in glioma cell lines. Furthermore, functional analysis using gain- or loss-of-function strategies should be done to validate the role of miR-196 in tumorigenesis and the malignant progression of glioblastomas.

In conclusion, we have provided large-scale miRNA expression profiles of malignant gliomas. This study identified candidate miRNAs that may correlate with tumorigenesis and the malignant progression of gliomas, and may be used to distinguish glioblastomas and anaplastic astrocytomas. We also showed that miRNA expression patterns reflect the biological and pathologic characteristics

Fig. 3. A, among 46 AA and GBM patients, those with high levels (higher than the average RQ values in GBM patients) of miR-196a (left, dotted line, n = 15) had significantly shorter survival periods than did patients with average levels of miR-196a expression (left, solid line, n = 31; P = 0.0052). Similar results were observed for miR-196b (middle; P = 0.0149) and miR-196a or miR-196b (right; P = 0.0003) expression levels. B, among 39 GBM patients, those with high levels (higher than the average) of miR-196a or -196b (right, dotted line, n = 19) had significantly shorter survival periods than did patients without (right, solid line, n = 20; P = 0.0073). No significance was found between the overall survival rate and the expression levels of either miR-196a (left; P = 0.0519) or miR-196b (middle; P = 0.0619).
of gliomas. Our results suggest that high miR-196 expression levels may play a role in the malignant progression of gliomas and may be a predictor of poor survival in glioblastomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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