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Original Article

High miR-196a and low miR-367 cooperatively correlate with unfavorable prognosis of high-grade glioma

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Abstract: Identification of microRNAs (miRNAs) could be beneficial for the diagnosis and prognosis of glioma. Therefore, we attempted to identify and develop specific miRNAs as prognostic and predictive markers for glioma patients. We compared the expression profiles of 365 miRNAs between 4 glioblastomas (GBMs, WHO grade IV) and 4 anaplastic astrocytomas (AAs, WHO grade III) using miRNA qPCR Array. MiR-196a ($P = 0.004$, fold change = 289.86) and miR-367 ($P = 0.044$, fold change = 0.03) were identified as the most up-regulated and down-regulated miRNAs in GBMs compared with AAs, respectively. We subsequently examined miR-196a and miR-367 expression levels in an independent series of 63 gliomas including 50 GBMs and 13 AAs, as well as 10 non-neoplastic brain tissues, and statistically analyzed the associations between miRNA expression and clinicopathological characteristics and survivals of these glioma patients. MiR-196a and miR-367 showed significant increased and decreased expression in high-grade gliomas relative to non-neoplastic brains, as well as in GBMs versus AAs, respectively. Additionally, high-miR-196a and low-miR-367 expression, alone or in combination, statistically correlated with aggressive clinicopathological features of gliomas. Furthermore, overall survivals of glioma patients with high-miR-196a, low-miR-367 and high-miR-196a/low-miR-367 expression tended to be shorter than the corresponding control groups (all $P \leq 0.001$). Moreover, multivariate analysis indicated high-miR-196a/low-miR-367 as an independent prognostic indicator for glioma patients ($P = 0.005$, risk ratio = 1.8). Our results suggested that both high-miR-196a and low-miR-367 expression may be associated with aggressive progression and unfavorable clinical outcome in glioma patients. And combination of high-miR-196a and low-miR-367 expression may be a novel biomarker in identifying a poor prognosis group of high-grade glioma.

Keywords: microRNA, miR-196a, miR-367, high-grade glioma, glioblastoma, prognosis

Introduction

Gliomas are the most common and aggressive type of primary brain tumor. These tumors can be classified into four different grades according to the World Health Organization (WHO) classification system [1]. Despite the introductions of modern therapeutic approaches including surgical resection, radiation therapy and chemotherapy, the response and prognosis of patients with aggressive form of these tumors remain very poor [1]. The grade IV glioma, also known as glioblastoma multiforme (GBM), is the most common and aggressive form of glioma, with a median survival of only 12-15 months as compared to 2-5 years for patients with grade III gliomas and 6-8 years for low grade (I and II) gliomas [1]. Because of the poor survival of high-grade glioma patients, it is very important to identify new biomarkers that can be used in clinicopathologic determinants of prognosis and in the choice of better therapeutic strategies.

The introduction of molecular biomarkers in the management of patients with cancer may improve their clinical outcomes. Along with the recent development of molecular biological technologies, such as microarray-based high-throughput gene expression and genetic profiling, many biomarker candidates have been generated for human cancers [2]. Recent molecular and genetic profiling studies have identified several markers and unique signatures as prognostic and predictive factors of malignant glioma [3, 4]. On the other hand, pre-

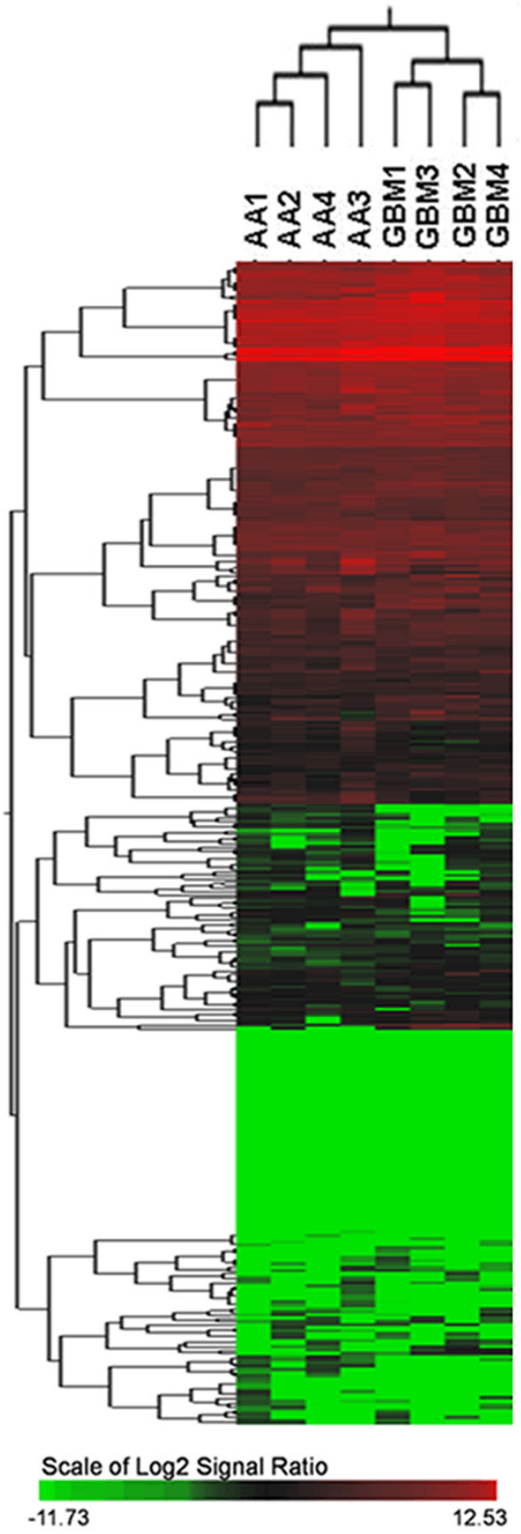

Figure 1. Hierarchical clustering analysis of 365 miRNA expression profiles in 8 high-grade gliomas (4 GBMs and 4 AAs), detected by miRNA qPCR array analysis. Normalized ΔCT (CT_{RNU6B}- CT_{miRNA}) values corresponding to the expression level of each miRNA are color-coded. Red, higher miRNA expression; green, lower miRNA expression. Gliomas were distinctly divided into 2 main clusters including GBMs and AAs respectively.

vious studies have suggested that expression-based clustering using microRNAs may yield more accurate histological and prognostic sample classification than clustering based on mRNA expression [5, 6]. Additionally, several advantages such as much smaller number and relative less to degradation have been demonstrated for miRNAs over messenger RNAs (mRNAs) as biomarkers. Therefore, it is more workable to identify reliable miRNA biomarkers from genome-wide miRNA expression data than from genome-wide gene expression data. Recent studies have indicated that expression profiles of miRNAs are associated with patients' survival and are able to function as prognostic and predictive indicators in glioma, based on public database entries [7-11] or independent tissue cohorts [12-15]. Most of these identified miRNAs have also been proved to be involved in the tumorigenesis and function as oncogenes or tumor suppressors in human gliomas, such as miR-21 [16, 17], miR-155 [18-20], miR-196 [21, 22], miR-221/222 [23] and miR-326 [24, 25]. Thus, the identification of the miRNA expression signature for malignant gliomas, in particular glioblastoma, is of great significance not only for predicting clinical outcomes, but also for understanding the molecular mechanisms of tumorigenesis and developing novel therapeutics of these malignancies.

In the attempt to identify molecular determinants associated with aggressive behavior in high-grade gliomas, we compared the expression profiles of 365 miRNAs between 4 GBMs (grade IV) and 4 AAs (grade III) with the worst and a relative favorable clinical outcome, respectively. We identified 34 miRNAs that were significantly differentially expressed in GBMs as compared with AAs and picked up the most up- and down-regulated miRNA, miR-196a and miR-367, respectively. Expression levels of these two miRNAs were subsequently examined in an independent series of 63 high-grade gliomas (50 GBMs and 13 AAs) and the significant differences of expression between GBMs and AAs were validated for both of miR-196a and miR-367. Furthermore, we analyzed the associations with clinicopathological characteristics and prognosis for these two miRNAs in these glioma patients and found that high-miR-196a and low-miR-367 significantly corre-

Table 1. miRNAs remarkably up- or down-regulated in GBMs versus AAs

Up-regulated microRNA	*P* value (Student's *t* test)	Fold Change (GBM vs. AA)
hsa-miR-196a	0.0035	289.86
hsa-miR-615	0.0473	61.37
hsa-miR-363	0.0378	24.85
hsa-miR-196b	0.0449	10.52
hsa-miR-155	< 0.0001	10.35
hsa-miR-135b	0.0172	9.06
hsa-miR-15b	0.0023	6.26
hsa-miR-429	0.0279	6.01
hsa-miR-21	0.0169	5.72
hsa-miR-432*	0.0373	5.40
hsa-miR-214	0.0320	5.19
hsa-miR-449b	0.0414	4.71
hsa-miR-551b	0.0039	4.53
hsa-miR-130b	0.0117	4.51
hsa-miR-34a	0.0060	3.85
hsa-miR-148a	0.0076	3.83
hsa-miR-199a	0.0228	3.58
hsa-miR-378	0.0113	3.55
hsa-miR-296	0.0206	3.15
hsa-miR-335	0.0033	3.09
hsa-miR-93	0.0322	3.08
hsa-miR-126	0.0423	3.08
hsa-miR-382	0.0396	3.05
Down-regulated microRNA	***P* value (Student's *t* test)**	**Fold Change**
hsa-miR-367	0.0436	0.03
hsa-miR-105	0.0115	0.04
hsa-miR-504	0.0009	0.11
hsa-miR-517c	0.0144	0.14
hsa-miR-184	0.0052	0.14
hsa-miR-575	0.0278	0.18
hsa-miR-601	0.0070	0.19
hsa-miR-128b	0.0125	0.21
hsa-miR-326	0.0055	0.25
hsa-miR-383	0.0153	0.25
hsa-miR-101	0.0167	0.32

Abbreviations: AA, anaplastic astrocytoma; GBM, glioblastoma.

lated with aggressive clinicopathological features and poor prognosis of high-grade glioma.

Materials and methods

Glioma specimens and patients

Glioma specimens were obtained from patients during surgery at First Affiliated Hospital of China Medical University. A portion of the tumor tissue was saved and made into paraffin sections for histopathologic diagnosis in strict accordance with World Health Organization (WHO) criteria by two experienced neuropathologists, with differences resolved by careful discussion. And the remaining tissue was snap-frozen in liquid nitrogen then stored at -80°C for RNA extraction and other biological molecular experiments. Before the RNA extraction from frozen tissues, the adjacent tumor tissues were subjected to frozen sections and reviewed by a pathologist to ensure that a minimum of 80% tumor cells were included in the sample. To profile the global miRNAs expression of malignant glioma, 8 high-pathological gliomas including 4 GBMs and 4 AAs were selected for the global miRNA expression screening by TanMan real-time quantitative PCR array. Subsequently, expression levels of miR-196a and 367 were examined on an independent series of 63 gliomas including 50 GBMs and 13 AAs, as well as 10 non-neoplastic brain tissues for calibration purpose, by conventional miRNA real-time PCR assay. These non-neoplastic brain tissues used as controls were obtained by collecting donations with consents from individuals who died in traffic accidents and were confirmed to be free of any prior pathological lesions. For glioma patients, none of them had received chemotherapy or radiotherapy prior to surgery, and all patients were well followed up. Overall survival time was calculated from the date of the initial surgical operation to death. Patients, who died of diseases not directly related to their gliomas or due to unexpected events, were excluded from this study. The present study was approved by the Ethics Committee of China Medical University.

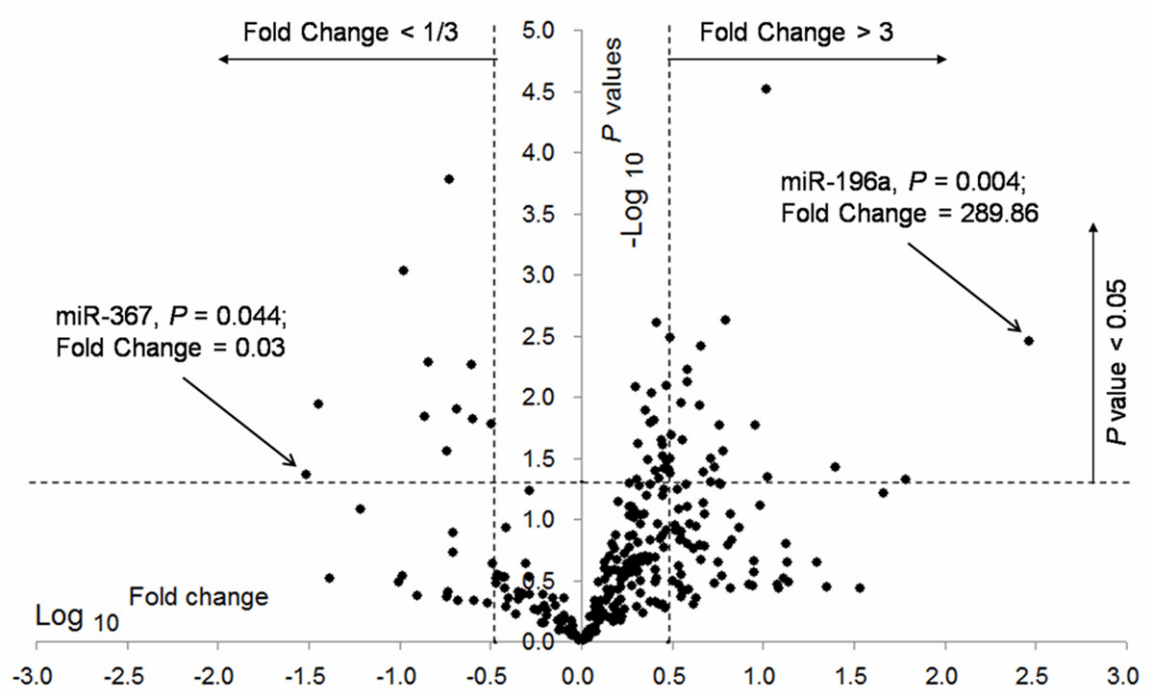

Figure 2. A volcano plot showing the fold change in the expression 365 miRNAs between 4 GBMs and 4 AAs, detected by miRNA qPCR array analysis. The volcano plot shows the distribution of these 365 miRNAs according to their *P* values. The horizontal dotted line, left and right vertical dotted lines indicate *P* value = 0.05, fold change < 1/3 and > 3, respectively. A set of 34 miRNAs were identified to be remarkably increased (fold change > 3 and *P* < 0.05) or decreased (fold change < 1/3 and *P* < 0.05) in GBMs versus AAs. Among these 34 miRNAs, the most up- and down-regulated in GBMs versus AAs were miR-196a (right arrow; fold change = 289.86 and *P* = 0.004) and miR-367 (left arrow; fold change = 0.03 and *P* = 0.044), respectively.

RNA extraction, reverse transcription and real-time PCR quantification for miRNA detection

Total RNA was extracted from frozen tissues of glioma and non-neoplastic brain using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacture's instruction. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA quality was measured using a denaturing 15% polyacrylamide gel.

To compare the global miRNAs expression levels between GBMs and AAs, PCR array assays were applied by using TaqMan Human MiRNA Arrays v1.0 (Applied Biosystems, Foster City, CA, USA). In brief, 365 human miRNAs and endogenous controls RNU6B 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 perform real-time PCR amplification using Applied Biosystems 7900HT Fast Real-Time PCR System. Relative quantification of miRNA expression was calculated with the $2^{-\Delta\Delta}$Ct method.

To examine the expression levels of miR-196a and miR-367 in glioma tissues and cell lines, cDNA synthesis and subsequent quantitative real-time PCR were performed using a TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems) and individual TaqMan miRNA assay (Applied Biosystems), and Applied Biosystems 7500HT Fast Real-Time PCR System (Applied Biosystems), as previously described [26]. RNU6B were used as endogenous controls, non-neoplastic brain tissues and human astrocyte were used for calibrations. Relative quantification of miR-196a and miR-367 expression was calculated with the $2^{-\Delta\Delta}$Ct method.

Statistical analysis

All computations were carried out using the software of SPSS version19.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD).

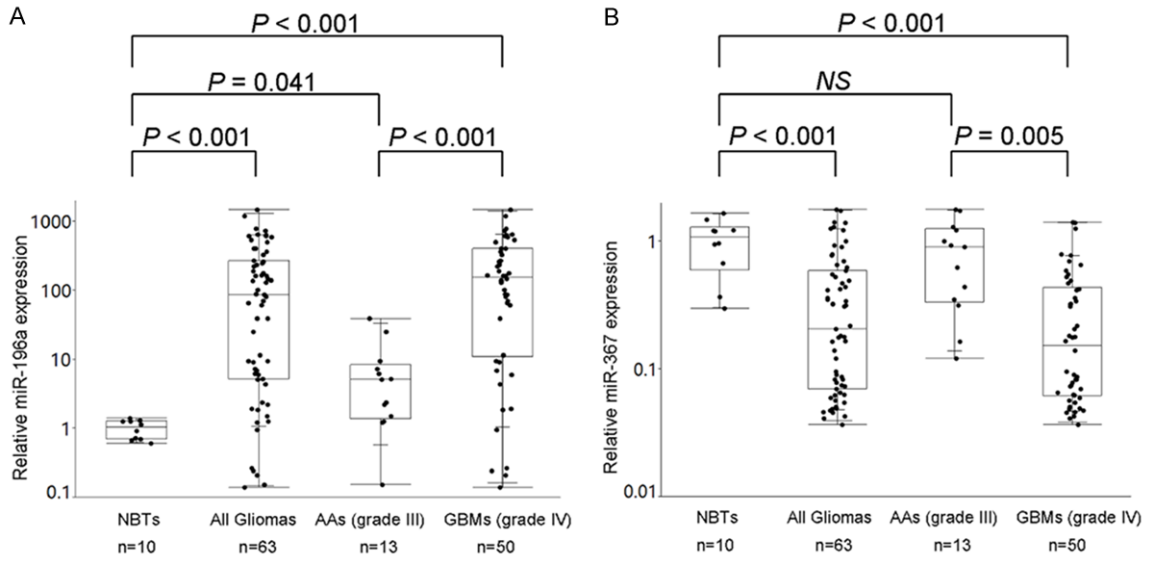

Figure 3. miR-196a (A) and miR-367 (B) expression in 63 high-grade glioma tissues (50 GBMs and 13 AAs), and 10 non-neoplastic brain tissues, detected by quantitative reverse transcriptive real-time polymerase chain reaction (qRT-PCR) analysis. Dots indicate log2 of the relative quantification (RQ) values of miRNA expression levels, normalized by RNU6B. A. The expression level of miR-196a were found to be remarkably increased in glioma tissues compared to normal brain tissues ($P \leq 0.001$). Both GBMs and AAs showed significantly higher expression of miR-196a than normal brains tissues ($P \leq 0.001$ and = 0.041, respectively). Statistically significantly higher expression of miR-196a ($P \leq 0.001$) was observed in GBMs versus AAs. B. miR-367 were remarkably decreased in glioma tissues compared to normal brain tissues ($P \leq 0.001$). Significantly lower miR-367 expression was observed in GBMs ($P \leq 0.001$) but not in AAs as compared with normal brains. Statistically significantly lower expression of miR-367 ($P = 0.005$) was observed in GBMs versus AAs.

Student's t-test was used to compare the expression levels of miRNAs between GBMs and AAs, as well as between gliomas and non-neoplastic brains. A hierarchical clustering analysis was performed to evaluate the miRNA expression profiles in high-grade gliomas. The X^2 test was used to analyze the relationship between miRNA expression and the clinicopathological characteristics. A life table was calculated according to the Kaplan-Meier method. Risk ratios for the time-to-event endpoint were estimated using the multivariate Cox regression analysis in a forward stepwise method to evaluate the effect of multiple independent prognostic factors on overall survival outcome. Differences were considered statistically significant when P was less than 0.05.

Results

Comparison of miRNA expression profiles between GBMs and AAs

To analyze the associations of miRNA expression profiles with tumor grade and prognosis in high-grade glioma, we examined the expression levels of 365 human miRNAs in 8 high-grade gliomas including 4 GBMs (WHO grade IV) and 4 AAs (WHO grade III), by TaqMan microRNA PCR array assays. An unsupervised hierarchical clustering analysis was performed on the gliomas using normalized ΔCT (CT_{RNU6B}-CT_{miRNA}) values corresponding to the expression levels of 365 miRNAs. As shown in **Figure 1**, gliomas were distinctly divided into 2 main clusters including 4 GBMs and 4 AAs respectively. Subsequently, we statistically compared miRNA expression levels between GBMs and AAs to identify the crucial miRNAs for distinguishing these 2 subtypes of glioma, by student's *t*-test. In total, 43 miRNAs showed significantly different expression between GBMs and AAs ($P < 0.05$). Among these, 34 (**Table 1**) miRNAs were remarkably up or down-regulated which were defined as fold changes of mean expression level in GBMs versus AAs were > 3 or < 1/3, respectively (**Figure 2**). Furthermore, among these 34 miRNAs, miR-196a (fold change = 289.86) and miR-367 (fold change = 0.03) showed the most increased and decreased

Table 2. Correlation of miR-196a and/or miR-367 expression with clinicopathological characteristics of high-grade gliomas

Clinico-pathological features	No. of cases (n, %)	High-miR-196a		Low-miR-367		miR-196a and miR-367 expression (n, %)			
		(n, %)	*P* value	(n, %)	*P* value	a	b	c	*P* value
WHO grade			< 0.001		0.005				< 0.001
III	13 (20.6%)	0 (0.0%)		2 (15.4%)		0 (0.0%)	0 (0.0%)	13 (100%)	
IV	50 (79.4%)	31 (62.0%)		30 (60.0%)		19 (38.0%)	12 (24.0%)	19 (38.0%)	
Age			0.159		0.338				< 0.001
> 50	48 (76.2%)	26 (54.2%)		26 (54.2%)		16 (33.3%)	10 (20.8%)	22 (45.8%)	
≤ 50	15 (23.8%)	5 (33.3%)		6 (40.0%)		3 (20.0%)	2 (13.3%)	10 (66.7%)	
Gender			0.101		0.707				< 0.001
Male	32 (50.8%)	19 (59.4%)		17 (53.1%)		10 (31.3%)	9 (28.1%)	13 (40.6%)	
Female	31 (49.2%)	12 (38.7%)		15 (48.4%)		9 (29.0%)	3 (9.7%)	19 (61.3%)	
KPS			0.135		0.535				< 0.001
< 90	41 (65.1%)	23 (56.1%)		22 (53.7%)		14 (34.1%)	9 (22.0%)	18 (43.9%)	
≥ 90	22 (34.9%)	8 (36.4%)		10 (45.5%)		5 (22.7%)	3 (13.6%)	14 (63.6%)	

Abbreviations: KPS, Karnofsky performance scale; 'a' refers to high-miR-196a/low-miR-367 group; 'b' refers to high-miR-196a/high-miR-367 & low-miR-196a/low-miR-367 group; 'c' refers to low-miR-196a/high-miR-367 group.

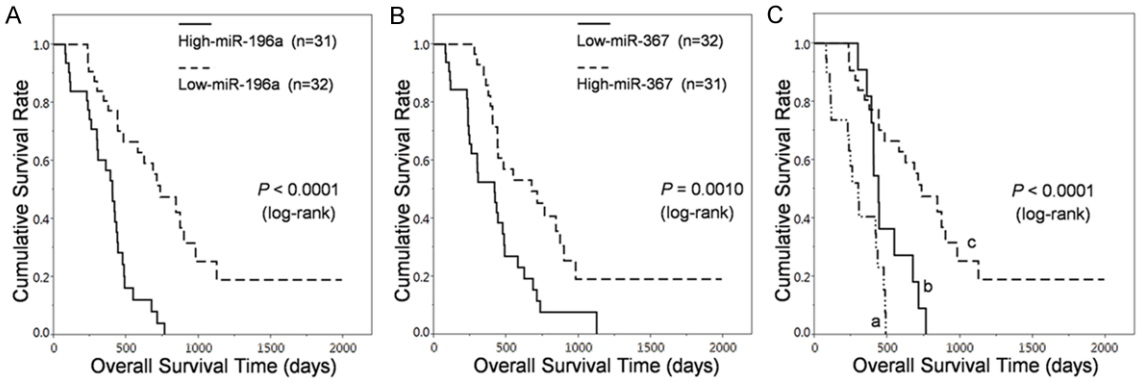

Figure 4. Prognostic performance of miR-196a and miR-367 expression patterns in high-grade gliomas. A. Among the 63 high-grade glioma patients (50 GBMs and 13 AAs), those with high miR-196a expression (left, solid line, n = 31) had significantly shorter survival periods than did patients with low miR-196a expression (right, dotted line, n = 32; $P \leq 0.0001$); B. Patients with low miR-367 expression (left, solid line, n = 32) had significantly shorter survival periods than patients with high miR-367 expression (right, dotted line, n = 31; $P = 0.001$); C. Glioma patients were divided into 3 groups according to expression pattern of miR-196a and miR-367. 'a' refers to high-miR-196a/low-miR-367 group (n = 19); 'b' refers to low-miR-196a/low-miR-367 & high-miR-196a/high-miR-367 group (n = 12); 'c' refers to low-miR-196a/high-miR-367 group (n = 32). Patients with high-miR-196a/low-miR-367 had the worst overall survival time (n = 19; $P \leq 0.0001$).

expression in GBMs as compared with AAs, respectively (**Figure 2** and **Table 1**). Therefore, we selected these two miRNAs for further analyses in another panel of gliomas.

Up-regulation of miR-196a and down-regulation of miR-367 in glioma tissues

To further evaluate the dysregulation of miR-196a and miR-367 in malignant gliomas, we examined their expression levels in an independent panel of 63 high-grade gliomas including 50 GBMs and 13 AAs, as well as 10 non-neoplastic brain tissues for control purpose, by qRT-PCR. As shown in **Figure 3A**, expression of miR-196a were remarkably increased in glioma tissues compared with non-neoplastic brain tissues (fold change = 209.40; $P \leq 0.001$, Student's *t*-test). Grade III and IV gliomas both showed significantly higher expression of miR-196a as compared with non-neoplastic brains ($P = 0.041$ and $P \leq 0.001$, respectively, **Figure 3A**). In addition, expression of miR-196a showed a distinctly upward tendency along with

Table 3. Univarite and multivariate Cox regression analysis for overall survival in glioma patients

Univariate analysis					Multivariate analysis			
Variate	No. of case (%)	Mean OS	95% CI	*P* (log-rank)	Variate	RR	95% CI	*P*
WHO grade				≤ 0.001	WHO grade			≤ 0.001
III	13 (20.6%)	1330	936-1724		IV vs. III	16.9	3.5-82.0	
IV	50 (79.4%)	444	378-509					
Age				0.066	Age			0.177
> 50	48 (76.2%)	547	409-685		> 50 vs. ≤ 50	0.5	0.2-1.4	
≤ 50	15 (23.8%)	801	509-1094					
Gender				0.631	Gender			0.197
Male	32 (50.8%)	629	425-832		Male vs. Female	1.5	0.8-2.8	
Female	31 (49.2%)	559	467-651					
KPS				0.021	KPS			0.951
< 90	41 (65.1%)	487	389-585		< 90 vs. ≥ 90	1.0	0.5-2.1	
≥ 90	22 (34.9%)	841	557-1125					
Surgical resection				0.165	Surgery			0.195
GTR	31 (49.2%)	679	498-860		PR vs. GTR	1.5	0.8-2.7	
PR	32 (50.8%)	569	387-750					
miR-196a/miR-367 expression				≤ 0.001	miR-196a/miR-367 expression			0.005
a	19 (30.2%)	298	229-367		a vs. b vs. c	1.8	1.2-2.8	
b	12 (19.0%)	494	401-587					
c	32 (50.8%)	890	649-1131					

Abbreviations: KPS, Karnofsky performance scale; GTR, gross total resection; PR, partial resection; OS, overall survival; RR, risk ratio. 'a' refers to high-miR-196a/low-miR-367 group; 'b' refers to high-miR-196a/high-miR-367 & low-miR-196a/low-miR-367 group; 'c' refers to low-miR-196a/high-miR-367 group.

the increasing malignancy degree of gliomas (fold changes and *P* values of grade IV vs. III were 32.02 and ≤ 0.001, respectively, **Figure 3A**). On the contrary, miR-367 was remarkably down-regulated in high-grade gliomas as compared with normal brains (fold change = 0.41; *P* ≤ 0.001) and statistically significant difference was also validated between expression levels of GBMs and AAs (fold changes and *P* values of grade IV vs. III were 0.36 and 0.005, respectively, **Figure 3B**). GBMs had statistically significantly lower miR-367 expression as compared with non-neoplastic brains (*P* ≤ 0.001, **Figure 3B**). No significant difference was between AAs and non-neoplastic brains.

miR-196a up-regulation and miR-367 down-regulation correlate with aggressive clinicopathological features of glioma

Subsequently, the associations of miR-196a and miR-367 expression with several clinicopathological features including tumor grade, patients' age at diagnosis, gender and preoperative Karnofsky performance scale (KPS) of these high-glioma patients were evaluated by X^2 test as summarized in **Table 2**. We assigned gliomas to high-miR-196a group and low-miR-196a group that were tumors with miRNA expression above and under the median value of miR-196a expression in all of the 63 gliomas, respectively (n = 31 and 32 for high-miR-196a and low-miR-196a groups, respectively). Similarly, gliomas were also assigned to high-miR-367 (n = 31) and low-miR-367 (n = 32) groups for miR-367. As shown in **Table 2**, high-miR-196a and low-miR-367 expression were both significantly associated with advanced tumor grade (*P* ≤ 0.001 for high-miR-196a and *P* = 0.005 for low-miR-367, respectively, X^2 test). However, no statistically significant correlation was observed between high-miR-196a and low-miR-367 expression and other clinicopathological factors (**Table 2**). In addition, we evaluated correlations of the combination of high-miR-196a and low-miR-367 with clinicopathological features mentioned above. As summarized in **Table 2**, according to the expression patterns of these two miRNAs, gliomas were divided into high-

miR-196a/low-miR-367 group (n = 19), high-miR-196a/high-miR-367 & low-miR-196a/low-miR-367 group (n = 12) and low-miR-196a/high-miR-367 group (n = 32). As shown in **Table 2**, we found that patients belonged to the high-miR-196a/low-miR-367 group were more frequently with advanced tumor grade ($P \leq 0.001$), with advanced age ($P \leq 0.001$), male ($P \leq 0.001$) and with low pre-operative KPS ($P \leq 0.001$) than those belonged to other groups with different expression patterns for these two miRNAs.

High miR-196a and low miR-367 predicts poor survival in high-grade glioma patients

Furthermore, to evaluate the prognosis performance of miR-196a and miR-367 in glioma patients, we analyzed association between expression patterns of these two miRNAs and clinical information in these glioma patients mentioned above, by performing log rank test and Kaplan-Meier analysis. We observed that both miR-196a and miR-367 displayed significant correlations with glioma patients' overall survival. As shown in **Figure 4A**, patients in high-miR-196a group (n = 31) had significantly poor overall survival compared to patients in low-miR-196a group (n = 32) ($P \leq 0.0001$, log rank test). Similarly, low-miR-367 was also significantly associated with poor overall survival (n = 32 for low-miR-367 group and n = 31 for high-miR-367 group, $P = 0.001$, **Figure 4B**). In addition, we evaluated the correlation of combination of high-miR-196a and low-miR-367 with patients' survival. As shown in **Figure 4C**, according to the expression patterns of these two miRNAs, gliomas were classified into high-miR-196a/low-miR-367 group (Group a, n = 19), high-miR-196a/high-miR-367 & low-miR-196a/low-miR-367 group (Group b, n = 12) and low-miR-196a/high-miR-367 group (Group c, n = 32). Of note, patients with high-miR-196a/low-miR-367 had the worst overall survival (mean overall survival times for Group a, b and c were 298, 494 and 890 days, respectively, $P \leq 0.0001$, **Figure 4C**). Moreover, univariate and multivariate analysis using the Cox proportional hazard regression model was performed to determine whether high-miR-196a/low-miR-367 and other clinical parameters are independent factors for prognostic prediction in glioma patients. Our result showed that both high-miR-196a/low-miR-367 ($P = 0.005$; risk ratio 1.8, multivariate Cox regression analysis) and high pathological grade ($P \leq 0.001$; risk ratio 16.9, multivariate Cox regression analysis) were independent predictors of poor prognosis in glioma patients (**Table 3**).

Discussion

Predicting the clinical outcomes of cancer patients is very important and forms the foundation of personalized cancer therapy. The overall survival of patients with high-grade gliomas varies from 1 week to a few years, suggesting that the clinical prognostic factors reach their limit in identifying prognostic subgroups for personalized treatment. Based on the recent development of microarray-based high-throughput miRNA expression profiling, several candidate miRNAs that are associated with patients' survival and involved in tumorigenesis of human gliomas have been identified [7-15]. However, these studies mentioned above were mainly performed on GBM cohorts. MiRNAs that can discriminate between grade III and IV gliomas, which are clinically and pathologically difficult to distinguish, have been little identified.

In the present study, we explored the comparison of expression profiles of 365 miRNAs between grade IV and III gliomas by miRNA qPCR-array method and identified the most up- and down-regulated miRNAs, miR-196a and miR-367, respectively. We also examined miR-196a and miR-367 expression in an independent panel of 63 high-grade gliomas and analyzed the associations between expression of these two miRNAs and several clinicopathological characteristics of these glioma patients. As results of our analysis, we found that: (i), expression profile of large scale miRNAs could accurately classify high-grade gliomas into subgroups with relative favorable and worse clinical outcomes; (ii), a total of. Among the 34 identified candidate miRNAs that were remarkably increased or decreased in GBMs as compared with AAs, miR-196a and miR-367 showed the most up-and down-regulation, respectively; (iii), High-miR-196a and low-miR-367 expression, alone or in combination, statistically correlated with aggressive clinicopathological features and shorter survival of gliomas. High-miR-196a/low-miR-367 was an independent indicator for poor prognosis high-grade glioma patients. Our data suggest that miR-196a and miR-367 could be valuable markers for prognosis predicting of glioma.

Recent studies have proved that expression-based clustering using miRNA profile can yield more accurate histological and prognostic cancer classification than clustering based on mRNA expression profile [5, 6]. A pioneered research study about miRNA-based glioma classification, by Kim et al. in 2011 identified five GBM subclasses, each of which was determined to be clinically and genetically distinct, based on the significant differences they displayed in terms of patient race, age, treatment response, and survival, using expression profile of 121 strictly selected miRNAs [8]. Based on expression profile of 365 miRNAs, we in the present study classified high-grade gliomas into AA-group and GBM-group that clinically show distinctly different prognosis with each other (**Figure 1**). Taken together with Kim et al.'s findings, miRNAs might be useful for subclassifying gliomas in a manner that allows for more accurate prognosis and for the development of molecular-based treatment decisions. The use of such a classification system may aid in prognosis and in the selection of subclass-specific therapies that will improve clinical outcome for glioma patients.

Furthermore, we demonstrated the prognosis significance of expression pattern of miR-196a and miR-367, which showed the foremost up- and down-regulation in GBMs versus AAs among the 34 candidate miRNAs identified in in our screening data (**Figure 2** and **Table 1**). miR-196a is located in HOX gene clusters and potentially targets HOXB8, HOXC8, HOXD8 and HOXA7, which have also been proved to play important roles in tumorigenesis of several human cancers including glioma [27-29]. Previous studies have showed the overexpression of miR-196a in glioblastoma cell lines and tissues and indicated the prognostic significance of this miRNA in glioma patients [12, 21]. A recent study showed evidence that miR-196a exerted its oncogenic effect in glioblastoma by inhibition of IκBα [22]. These findings suggest that miR-196a function as an oncogenic miRNA in tumorigenesis and are a valuable prognostic predictor of glioma. In addition, accumulating studies have indicated the implication of this miRNA in other human cancers; however, its role may differ among various cancer types. It has been found to be up-regulated in pancreatic cancer, non-small cell lung cancer, head and neck squamous cell carcinomas, cervical cancer, colorectal cancer, oral cancer and osteosarcomas, but down-regulated in breast cancer and melanoma [30-38]. Functionally, miR-196a has been found to promote cancer cell proliferation, migration and invasion of non-small cell lung cancer, head and neck squamous cell carcinoma, cervical cancer and oral cancer cell lines [30-33]; It can be used to correctly differentiate pancreatic cancer from benign pancreatic tissue, and high expression of miR-196a is found to predict poor survival [34]; Strong expression of miR-196a is also associated with a poor prognosis in patients with osteosarcoma [35]; High miR-196a levels correlate with aggressive progression and unfavorable prognosis in patients with colorectal cancer [36]. In contrast, miR-196a can suppress invasion and metastasis of breast cancer cells [37], and also inhibit the invasive behavior of melanoma cells [38]. Our finding confirmed the oncogenic role and prognostic value of miR-196a in human malignancies, however, the detailed biological mechanism of involvement in glioma tumorigenesis for this miRNA deserves further analysis. On the other hand, miR-367 is a member of miR-302-367 cluster (miR-302s), which are exclusively expressed at high levels in embryonic stem cells (ECSs) indicating their essentiality of self-renew and pluripotency maintenance in stem cells [39]. Recently, miR-302s have been implicated in reprogramming and tumorigenesis [40, 41]. The involvement of miR-302s in glioma stem cells (GSCs) have also been demonstrated by several groups [42, 43]. Fareh et al. in 2012 found that miR-302s drastically affected self-renewal and infiltration properties of glioma-initiating cells through CXCR4 repression and consequent disruption of the sonic hedgehog (SHH)-GLI-NANOG network [42]; Rafiee et al. recently reported that serum deprivation led to the generation of tumorospheres, enrichment of miR-302 positive cells and upregulation of a number of pluripotency genes, in glioma cell lines [43]. In addition, the prognositc significance of miR-367 has been reported by several groups [44-46]. Chae et al. found a functional polymorphism in miR-367 as prognostic factor for colonic cancers [44]; Campayo et al. has demonstrated that low miR-145 and high miR-367 are associated with unfavorable prognosis in resected non-small cell lung cancer [45]; In particular, Costa et al. has identified miR-367 as a potential prognostic marker in ependymoma, a common pediatric central nervous sys-

tem tumor originates from ependymal cells located in the lining of ventricular surfaces in the brain [46]. However, the associations between miR-367 expression and clinical outcome of high-grade glioma remain unclear. We in the present study reported the significant correlation of low-miR-367 with poor survival of gliomas, suggesting the tumor suppressive role of this miRNA in these malignant brain tumors. To our knowledge, this is the first study to evaluate the prognosis performance of miR-367 expression in large panel of glioma patients. The detailed biological mechanism of miR-367 down-regulation in glioma tumorigenesis and progression deserves further study.

Moreover, several other miRNAs among the 34 candidates have also been reported to be correlated with patients' survival and/or be involved in tumorigenesis or progression of glioma, by functioning as oncogenes or tumor suppressors. For example, miR-21 has been reported to play important roles in preventing apoptosis and target a network of key tumor-suppressive pathways in glioblastoma cells, as well as have prognostic significance in glioma patients [14, 16, 17]; as a novel anti-apoptotic miRNA, miR-363 was recently indicated to be overexpressed and promote glioblastoma stem cell survival via direct inhibition of Caspase 3, Caspase 9, and BIM [47]; in our data, miR-155 showed the most significantly differential expression among the up-regulated candidate miRNAs ($P < 0.0001$, fold change = 10.35, **Table 1**). This miRNA has been previously indicated to promote the cell proliferation and contribute to progression of glioma [18, 20], in addition, its overexpression predicts poor prognosis in glioma patients [19]; miR-128 is down-regulated in glioblastomas and functions as a tumor suppressor through the direct repression of the Bmi-1 oncogene [48]; the tumor-suppressive miRNA, miR-326, has been revealed to inhibit glioma cell survival by directly regulating expression of its target genes, Notch-1 and PKM2 [24, 25], furthermore, low miR-326 expression was reported to be associated with unfavorable outcome of glioma patients [15]. These miRNAs should be included into the molecular-based glioma classification system as key miRNAs and synthetically analyzed in the future studies. On the other hand, further investigations are necessary to evaluate the prognosis performance and tumorigenesis involvement in malignant gliomas for miRNAs identified in the present study but have not been previously reported.

In conclusion, we compared expression profiles of 365 miRNAs between WHO grade IV and III gliomas, and identified the most up- and down-regulated miRNAs, miR-196a and miR-367, respectively. Our survival analysis revealed that both high-miR-196a and low-miR-367 may correlate with aggressive progression and unfavorable clinical outcome in high-grade glioma patients. The combination of high-miR-196a and low-miR-367 may be a valuable biomarker in identifying a poor prognosis group of high-grade glioma. Such findings would lead to new approaches of diagnostic and therapeutic for patients with this malignancy.

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Disclosure of conflict of interest

None.

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