LABORATORY INVESTIGATION

Expression level of human miR-34a correlates with glioma grade and prognosis

Haifeng Gao · Hongyang Zhao · Wei Xiang

Abstract The aim of this study is to investigate the expression level of microRNA-34a (miR-34a) in glioma patients and its significance for predicting the prognosis of glioma. In this study, we examined the expression of miR-34a in glioma tissues of various World Health Organization (WHO) grades and explored the association between miR-34a expression and clinical and pathological parameters of glioma patients. We found that the tissues from high-grade gliomas (grade III and IV) had much lower miR-34a expression compared to normal brain tissues. The results of a 72-month follow-up in 146 glioma patients further demonstrated that miR-34a expression levels positively correlated with tumor WHO grades. Additionally, in the patients with grade III and IV gliomas, lower miR-34a expression correlated with worse progression-free survival and overall survival. Univariate and multivariate analysis revealed that miR-34a was an independent prognostic indicator for glioma. Additionally, we explored the correlation between miR-34a expression and p53 status and Bcl-2 expression in grade III and IV glioma tissues. Wild-type p53 tumors displayed significantly higher miR-34a expression level than mutant p53 tumors. In addition, glioma tissues with high miR-34a expression had dramatically lower Bcl-2 expression levels than tissues with low miR-34a expression. These findings indicate the role of miR-34a in tumor progression may be closely associated with p53 mutation and inversely correlated to Bcl-2 expression. In conclusion, our work presents comprehensive evidence for miR-34a expression as a novel and potentially useful signature for predicting prognosis of glioma.

Keywords MicroRNA-34a · Glioma · p53 · Bcl-2 · Prognosis · Oncology

Introduction

Gliomas are the most common primary central nervous system (CNS) neoplasms. In recent years, although significant progress has been made in diagnosis, surgery, radiation therapy, and chemotherapy for gliomas, the prognosis of patients with malignant gliomas remains poor [1]. Improved understanding of the biology and molecular factors involved in the development, progression, metastasis, and drug resistance of gliomas is imperative. Additionally, novel molecular signatures that can predict the prognosis and response to treatment of this disease with reliable clinical significance are also needed. Currently, however, few molecular signatures have been validated and widely accepted as prognostic indicators in clinical practice.

MicroRNAs (miRNAs), as a type of small RNA molecules encoded by eukaryotic nuclear DNA, play an important role in the post-transcriptional regulation of gene expression. Studies on bioinformatics and microarray analysis estimated that approximately 30 % of all genes are regulated by various miRNAs [2]. MicroRNA-34a (miR-34a) belongs to one of evolutionarily conserved miRNA families (miR-34 family). It is located on chromosome 1p36 and acts as a potent tumor suppressor gene in a p53-dependent or -independent manner [3]. Previous studies

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have reported that several key molecules were identified as targets of miR-34a, including Bcl-2 [4], transcriptional repressor protein YY1 [5], Notch1 [6], and Notch2 [6]. In medulloblastoma cells, miR-34a was proven to be able to confer chemosensitivity through modulation of melanoma-associated antigen-A (MAGE-A) and p53 [7]. Silber et al. [8] demonstrated that the repression of miR-34a malignant gliomas could up-regulate the expression of platelet-derived growth factor receptor alpha (PDGFRA) and consequently promote tumorigenesis.

Although extensive studies have been performed to explore the role of miR-34a in the cellular processes of glioma cells, little is known about the relationship between the expression level of miR-34a in glioma tissues and the progression of disease. Whether miR-34a could be a potential indicator for predicting the prognosis of glioma patients is still lack of solid evidence. Therefore, in this study, we examined the expression level of miR-34a in glioma tissues of various World Health Organization (WHO) grades and explored the association between miR-34a expression and the clinical and pathological parameters of glioma patients. The results indicated that miR-34a may be regarded as a potential prognostic indicator for glioma patients.

Materials and methods

Acquisition of tissue specimens

The study protocol and acquisition of tissue specimens were approved by the Biomedical Research Ethics Committee of Tongji Medical College, Wuhan, China. A total of 151 glioma tissue specimens were obtained from patients who received surgical treatment at the Department of Neurosurgery, Union Hospital, Wuhan, China between April 2005 and December 2008. Normal brain tissues were collected from 15 patients with severe traumatic brain injury for whom partial resection of the brain was required during decompression treatment. Before collection, written informed consent was obtained from each patient. All specimens were collected in the operating room right after tumor resection. The collected samples were quickly frozen in liquid nitrogen and stored at −70 °C.

The selection criteria for glioma patients enrolled in this study were as follows: (1) primary diagnosis of glioma without a history of other tumors; (2) having complete clinical and pathological data including sex, age, WHO pathological grade, Karnofsky performance status (KPS) score, extent of resection, mean tumor diameter (MTD), clinical features (primary vs. secondary), adjuvant therapy (radiotherapy or chemotherapy); (3) having complete follow-up data for evaluating tumor relapse or progression after surgery; (4) obtained tissue specimen was in sufficient quantity for experimental use.

In the first year after surgery, patients received follow-up every 2 months. After the first postoperative year, follow-up was every 3 months until June 2012. The median duration of follow up was 23 months (range 3–72 months). Progression-free survival (PFS) was defined as the time duration from surgery to tumor progression diagnosed by magnetic resonance imaging (MRI) or death. Overall survival (OS) was defined as the time duration from surgery to death. The patients alive at the end of follow-up were censored.

RNA isolation and quantitative real-time PCR

Total miRNA was extracted from the frozen normal brain and glioma tissue specimens using miR-Vana RNA Isolation Kit (Ambion, Austin, TX). Specific primers for miR-34a and endogenous control RNU6B were purchased from Applied Biosystems. Reverse transcription was performed with TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using the Applied Biosystems 7900 qRT-PCR System (Applied Biosystems, Foster City, CA). All analyses were repeated independently three times. Relative expression levels were calculated based on the cycle threshold (Ct) values with normalization to RNU6B according to Schmittgen and Livak [9]. A relative expression level ($2^{-\Delta Ct}$) above 1.0 was defined as "high expression of miR-34a" and a $2^{-\Delta Ct}$ below 1.0 was defined as "low expression of miR-34a". Additionally, glioma samples of WHO grades III and IV were used for detecting the mRNA expression Bcl-2. Briefly, 50 ng of total RNA extracted from these samples was used for qRT-PCR analysis with specific synthetic primers [10]. Beta-actin was used as endogenous control.

Determination of p53 status

High resolution melting (HRM) method was used to determine p53 status (wildtype vs. mutant) in glioma samples at WHO grade III and IV. Briefly, 50 mg glioma sample was used for genomic DNA extraction. The specific primers for detecting p53 mutations were designed according to the literature [11]. The following PCR was performed using a reaction mixture including 10 ng of genomic DNA, $1\times$ HRM PCR Master Mix (QIAGEN, Germany), 3.0 mM MgCl$_2$, 250 nM of each primer, and PCR grade water in a 20 µl volume. All PCR reactions were performed in triplicate. PCR cycling and HRM analysis was performed on the LightCycler® 480 High Resolution Melting Master system (Roche, Switzerland) according to protocols in the literature [12].

Western blotting analysis

Glioma samples of WHO grade III and IV were used for Western blotting analysis of the protein expression level of Bcl-2. Briefly, total protein was extracted with T-PER Tissue Protein Extraction Reagent (Pierce Biotech, Rockford, IL). Equal quantities of protein were then separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immediately transferred onto polyvinylidene difluoride (PVDF) membranes afterwards. The PVDF membranes were blocked with 10 % nonfat dry milk in Tris-buffered saline containing 0.1 % Tween 20 (TBST). Then the membranes were incubated with anti-Bcl-2 (Cell Signaling Tech, Danvers, MA) or anti-β-actin (Santa Cruz Biotech, Santa Cruz, CA) antibodies overnight at 4 °C. The membranes were then washed with TBST for at least three times and incubated in horseradish peroxidase-conjugated secondary IgG antibody-diluted solutions for 1 h at room temperature. Electrophoretic bands were detected using enhanced chemiluminescent (ECL) reagents (Pierce Biotech, Rockford, IL) and densitometrically analyzed using ImageJ software. All analyses were repeated independently three times.

Statistical analysis

SPSS 10.0 (SPSS Inc., Chicago, IL) was utilized for statistical analysis. For the results of qRT-PCR and Western blotting, statistical significance between groups was assessed by the nonparametric Kruskal–Wallis test for multiple comparisons. Chi square test was used to evaluate a relationship between two variables. Cumulative survival was calculated using the Kaplan–Meier method and analyzed by log-rank test. Cox regression model was used for univariate and multivariate analyses. In multivariate analysis, the models adjusted for sex, age, KPS score, radiotherapy, chemotherapy, resection, MTD, and primary versus secondary were used to assess the effect of miR-34a expression. A p value <0.05 was considered sufficient for statistical significance.

Results

Of the 151 glioma samples collected for this study, five were pilocytic astrocytoma (WHO grade I). As this tumor is biologically distinct from fibrillary grade II–IV tumors [13], we excluded these samples from further analysis. The tumor grade and pathological classification of the remaining 146 samples are listed in Table 1.

Of the donors of the remaining 146 samples, three (one with grade III anaplastic astrocytoma and two with grade IV glioblastoma multiforme) were lost to follow-up after

disease progression during the follow-up period. Therefore, these patients were censored for OS analysis. By the end of follow-up, 112 out of 143 patients (78.3 %) died from glioma and 31 of 143 patients (21.7 %) were alive. The median PFS and OS for overall patients were 19.7 (95 % CI, 15.3–23.6 months) and 26.8 months (95 % CI, 21.8–29.8 months), respectively. The OS rates for overall patients were 85 % at 1 year, 54 % at 2 years, and 35 % at 3 years, respectively.

Expression of miR-34a in human glioma tissues

The expression level of miR-34a in normal brain tissue and all glioma tissues with WHO grade II–IV is shown in Fig. 1. The results showed that the highest expression of miR-34a was achieved in normal brain tissues (median $2^{-\Delta Ct}$ was 3.55); while a significant decrease in miR-34a expression was observed in glioma tissues when compared with normal brain tissue ($p = 0.0378$ for normal brain tissue versus grade II glioma tissue, $p < 0.0001$ for normal brain tissue versus grade III glioma tissue, and $p < 0.0001$ for normal brain tissue versus grade IV glioma tissue, respectively). Furthermore, grade IV glioma tissue displayed the lowest expression level (median $2^{-\Delta Ct}$ was 0.66), indicating a correlation between miR-34a expression level and glioma WHO grade.

Expression of miR-34a correlates with glioma grade

Correlation between miR-34a expression level and various clinical and pathological variables in 146 glioma patients is summarized in Table 2. The results indicate that a large percentage of patients with grade III and IV gliomas (53/82) also had low miR-34a expression, while most of patients with grade II gliomas (48/64) had high miR-34a expression ($p < 0.0001$). This finding was consistent with the RT-PCR results that we obtained, which demonstrated that there was a significant correlation between miR-34a expression level and glioma grades. Nevertheless, there was no significant correlation between miR-34a expression and other variables including sex, age, KPS score, radiotherapy, chemotherapy, resection, MTD, and primary versus secondary ($p > 0.05$).

Correlation between the expression of miR-34a and prognosis in glioma patients

In grade II glioma patients, miR-34a expression did not significantly correlate with shorter PFS and OS ($p > 0.05$) (data not shown). This was most likely due to the fact that low-grade tumors have a lower risk of recurrence and death as opposed to high-grade tumors, thus concealing the prognostic effect of miR-34a. Therefore, it is more

Table 1 Tumor grade and pathological classification of glioma samples collected for this study

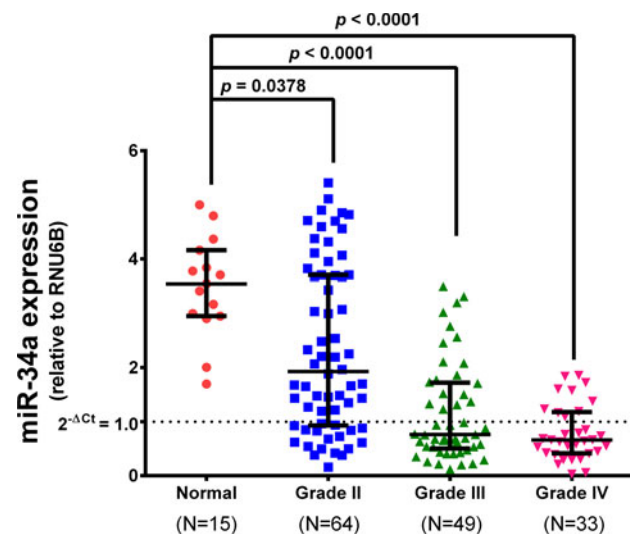| Tumor grade	Pathological classification	Number and proportion
N = 146		
WHO grade II	Diffuse astrocytoma	43 (29.4 %)
	Oligodendroglioma	21 (14.4 %)
WHO grade III	Anaplastic astrocytoma	35 (24.0 %)
	Anaplastic oligodendroglioma	14 (9.6 %)
WHO grade IV	Glioblastoma multiforme	33 (22.6 %)

Fig. 1 Quantitative RT-PCR analysis of miR-34a expression level in 15 normal brain tissues and 146 glioma tissues with WHO grades II–IV. Relative miR-34a expression level (value of $2^{-\Delta Ct}$) in each sample was calculated based on comparative Ct method with normalization to RNU6B. Each *data point* represents the mean of $2^{-\Delta Ct}$ values from three independent assays. *Bars* represent median, 25th and 75th percentiles. *N* represents the *number* of tissue samples. Statistical differences between groups were assessed by nonparametric Kruskal–Wallis test for multiple comparisons

Table 2 Correlation between miR-34a expression level and clinical and pathological variables

Variables	miR-34a		p Value
	High expression	Low expression	
Number of patients	77	69	
WHO grade			<0.0001
II	48	16	
III–IV	29	53	
Age			0.8610
≥65	25	24	
<65	52	45	
Sex			0.4042
Male	41	42	
Female	36	27	
KPS score			1.0000
≥70	54	49	
<70	23	20	
Radiotherapy			0.6159
Yes	47	39	
No	30	30	
Chemotherapy			0.4503
Yes	56	54	
No	21	15	
Resection			0.8529
>98 %	55	51	
<98 %	22	18	
MTD			0.8681
≥5 cm	43	37	
<5 cm	34	32	
Primary versus secondary			0.3961
Primary	65	54	
Secondary	12	15	

KPS score Karnofsky performance status score, *MTD* mean tumor diameter

meaningful and accurate to assess the prognostic value of miR-34a in the patients with high-grade gliomas (i.e. WHO grade III and IV gliomas).

The results of Kaplan–Meier survival analysis in the patients with grade III and IV gliomas are shown in Fig. 2. The expression levels of miR-34a displayed prognostic significance for PFS and OS of these cases. The median values of PFS and OS of the patients with high miR-34a expression were 14.1 months (95 % CI, 9.9–15.4 months) and 20.2 months (95 % CI, 15.5–23.3 months), respectively, which were significantly longer than those in the patients with low miR-34a expression who had a median PFS value of 9.1 months (95 % CI, 7.2–12.4 months) and a median OS value of 13.1 months (95 % CI, 11.4–16.9 months)

($p = 0.0254$ for PFS and $p = 0.0074$ for OS, respectively; Fig. 2a, b). By the end of follow-up, all patients with low miR-34a expression died from glioma, while two patients with high miR-34a expression were still alive.

Table 3 summarizes the results of Cox regression analysis of PFS and OS from the causes associated with miR-34a expression in grade III and IV glioma patients. Univariate analysis demonstrated that miR-34 expression was a potential prognostic factor for progression and survival of glioma patients [relative hazard ratio (HR) was 1.649 and 1.855, respectively]. Multivariate analysis with two different models further confirmed the findings achieved in univariate analysis, indicating low miR-34a expression level was an independent risk factor of poor
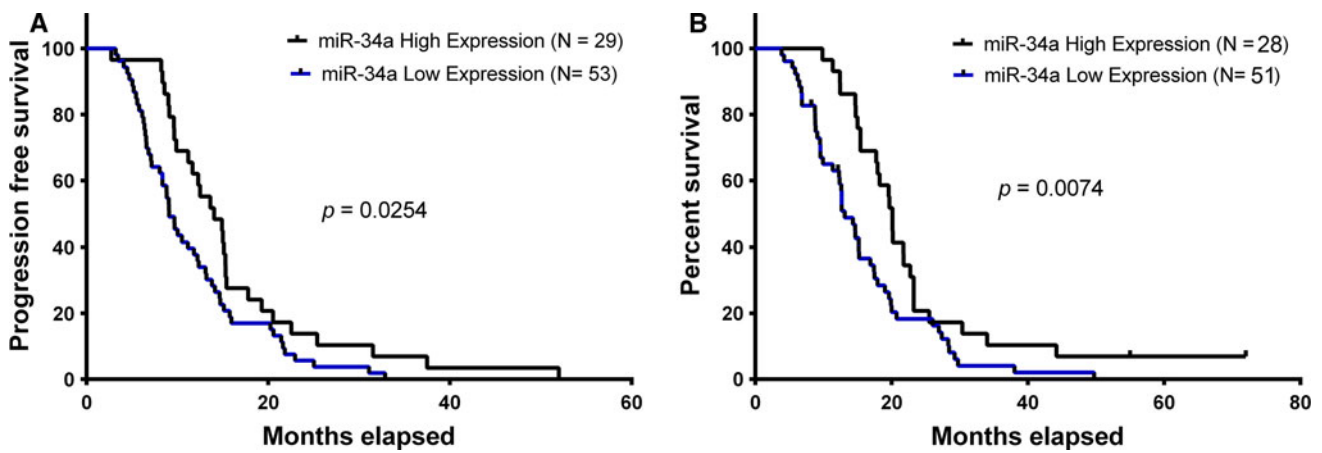
Fig. 2 Low expression levels of miR-34a correlate with shorter PFS and OS in high-grade glioma patients: the Kaplan–Meier PFS (**a**) and OS curves (**b**) for the patients with grade III and IV gliomas who were stratified by miR-34a expression. Three patients were lost to follow-up after disease progression during the follow-up period. Therefore, they were censored from the OS analysis. Statistical difference between two survival curves was assessed by the log-rank test

prognosis in glioma patients (relative HR = 2.132 and 2.002 for PFS, and relative HR = 2.357 and 2.129 for OS, respectively).

Expression of miR-34a correlates with p53 status and Bcl-2 expression

Given the close relationship between miR-34a and p53 signaling pathway, we also explored the correlation between miR-34a expression and p53 status and Bcl-2 expression in grade III and IV glioma tissues. The results are shown in Fig. 3. When these tissues were stratified with p53 status (wildtype vs. mutant), we found that miR-34a expression in wild-type p53 tumors was significantly higher than that in mutant p53 tumors (Fig. 3a) ($p < 0.0001$). In addition, when stratified with miR-34a level, quantitative RT-PCR showed

that glioma tissues with high miR-34a expression had dramatically lower mRNA expression levels of Bcl-2 when compared to the tissues with low miR-34a expression ($p = 0.0011$) (Fig. 3b). Consistent results were also obtained in western blotting analysis, namely Bcl-2 protein expression was lower in the tissues with high miR-34a expression (Fig. 3c).

Discussion

Although surgery, radiation therapy, and chemotherapy have been able to prolong survival, the prognosis is still typically poor for glioma patients [1, 14]. Lack of a powerful prognostic indicator has been a big hurdle for the treatment of glioma in clinic over the past few decades.

Table 3 Cox regression analysis of PFS and OS from the causes associated with miR-34a expression in patients with grade III and IV gliomas

miR-34a		Hazard ratio (95 % CI)					
		Univariate analysis	*p* Value	Multivariate analysis—Model 1[a]	*p* Value	Multivariate analysis—Model 2[b]	*p* Value
PFS	High expression	1	–	1	–	1	–
	Low expression	1.694 (1.061–2.705)	0.027	2.132 (1.295–3.511)	0.003	2.002 (1.183–3.387)	0.01
OS	High expression	1	–	1	–	1	–
	Low expression	1.855 (1.156–2.977)	0.01	2.357 (1.411–3.937)	0.001	2.129 (1.236–3.667)	0.006

PFS progression-free survival, *OS* overall survival, *CI* confidence interval, *KPS score* Karnofsky performance status score, *MTD* mean tumor diameter

[a] Adjusted for sex, age, and KPS score

[b] Adjusted for all covariates including sex, age, KPS score, radiotherapy, chemotherapy, resection, MTD, and primary versus secondary
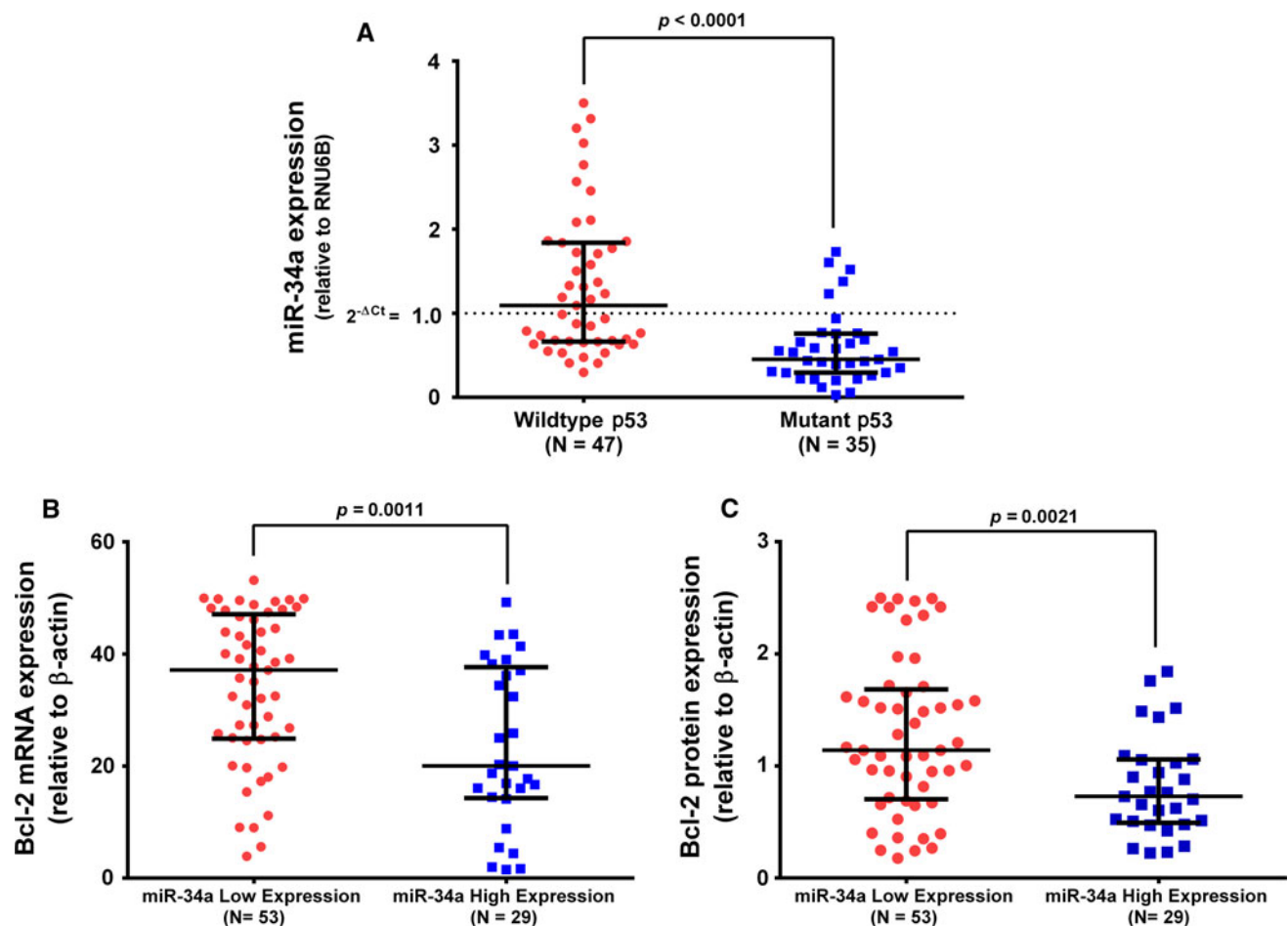
Fig. 3 Correlation between miR-34a expression and p53 status or Bcl-2 expression: **a** the p53 status (wildtype or mutant) of the glioma samples with WHO grades III and IV were determined using HRM method. Relative miR-34a expression level (value of $2^{-\Delta Ct}$) in each sample was calculated based on comparative Ct method with normalization to RNU6B. Each *data symbol* represents the mean of $2^{-\Delta Ct}$ values from three independent assays. The results show that miR-34a expression in wild-type p53 tumors is significantly higher than that in mutant p53 tumors. **b** and **c** Bcl-2 mRNA and protein expression level of the glioma samples with WHO grades III and IV were measured by quantitative RT-PCR (**b**) and western blotting (**c**) with normalization to β-actin. These samples were stratified by miR-34a expression. Each *data symbol* represents the mean of $2^{-\Delta Ct}$ values or relative integral optical density (IOD) values from three independent assays. The results show that there is a negative correlation between the expression of miR-34a and Bcl-2. *Bars* represent median, 25th and 75th percentiles. Statistical difference between groups was assessed by the nonparametric Kruskal–Wallis test for multiple comparisons

In this study, we examined the miR-34a expression in glioma tissues at various WHO grades. We found that the tissues from high-grade glioma (grade III and IV) had much lower miR-34a expression level than low-grade glioma (grade II) and normal brain tissues. The results of a 72-month follow-up in 146 glioma patients confirmed this finding and also demonstrated that miR-34a expression significantly correlated with survival of glioma patients. Therefore, we present evidence here for miR-34a as a potential prognostic indicator for human gliomas.

MicroRNA-34a has been implicated in regulating various cellular processes that are deregulated in tumors, such as growth, proliferation, differentiation, and apoptosis [4, 15–17]. As a candidate tumor suppressor gene and a downstream transcriptional target of p53, miR-34a has been shown to be down-regulated in human tumor cells including glioma cell lines [15, 18, 19]. Although there exist a few reports that miR-34a expression decreased in human glioblastoma and had a correlation with prognosis [6, 20], more comprehensive studies are needed to confirm the significance of miR-34a expression level for the prognosis of glioma patients. In the current study, we found that a large percentage of patients with grade III and IV gliomas (53/82) had low level of miR-34a expression, while the opposite was the case in the patients with grade II glioma (48/64 displayed high miR-34a expression). This result indicated that the down-regulated miR-34a expression was highly associated with the malignant phenotype of gliomas. However, there was no significant correlation of miR-34a expression with other clinical and pathological

variables, most likely indicating its independent prognostic significance.

Currently, glioma classification depends largely on traditional approaches including histological type, clinical stage, etc. [1]. Insufficient and inaccurate information for predicting patient outcome is often present from these approaches, especially for patients at the early stages of disease. Recently, some researchers have reported that the expression of molecules such as Bcl-2, bone sialoprotein (BSP), and annexin A2 (ANXA2) may be useful as potential prognostic indicators [21–23]. In our survival analysis, when the patients with grade III and IV gliomas were stratified by miR-34a expression, the patients with high miR-34a expression showed significantly longer PFS and OS than those with low miR-34a expression, demonstrating miR-34a may be an independent indicator for predicting survival of glioma patients.

In tumor cells, miR-34a has been identified as a target of p53 and is able to induce apoptosis in p53-dependent manner [3, 18]. P53 mutation was also shown to directly regulate the expression of miR-34a [3, 24]. For example, Mraz et al. [25] reported that in chronic lymphocytic leukemia (CLL) the expression of miR-34a was decreased and this down-regulation associated with p53 mutations. In our study, when the glioma tissues at grade III and IV were stratified by p53 status, wild-type p53 tumors displayed significantly higher miR-34a expression levels than mutant p53 tumors. This finding was consistent with that reported by Li et al. [6]. Therefore, the low expression of miR-34a in high-grade glioma patients may be at least partly due to the p53 mutation in these patients. Additionally, other factors such as the frequent deletion of chromosome 1p36 and CpG methylation of the miR-34a promoter in glioma tissues could have close correlation with the reduced expression of miR-34a [3]. Further study on identifying potential key factors affecting miR-34a expression in glioma patients is still in progress.

Bcl-2 is a key molecule involved in p53 pathway and has been reported to be directly regulated by miR-34a [26–28]. Many studies have demonstrated the p53-miR-34a-Bcl-2-dependent manner is one of the most important mechanisms for tumor apoptosis and miR-34a exerts its tumor-suppressing function through inhibiting the expression of Bcl-2 [3]. Conversely, decreased expression of miR-34a could result in over-expression of Bcl-2, which may protect tumor from apoptosis and confer resistance to chemotherapy and radiotherapy causing an acceleration of the malignant progression and metastasis [24]. Fels et al. [21] demonstrated that the over-expression of Bcl-2 was common in the patients with high-grade gliomas. In our study, we found that glioma tissues with high miR-34a expression had significantly lower Bcl-2 expression level compared with tissues having low miR-34a expression,

showing a potential association with the role of miR-34a in tumor progression.

In conclusion, we demonstrated that the decreased expression of miR-34a is closely correlated with the increased glioma grade and poorer PFS and OS. This raises the possibility for miR-34a levels to be used as an independent indicator for predicting the prognosis of glioma patients. The small sample size and retrospective nature of this study suggest that these findings need to be investigated further using a prospective study with a larger patient population.

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