

RESEARCH ARTICLE

Expression of microRNA-218 and its Clinicopathological and Prognostic Significance in Human Glioma Cases

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Abstract

Background: MicroRNAs are a class of noncoding RNAs which regulate multiple cellular processes during tumor development. The purpose of this report is to investigate the clinicopathological and prognostic significance of miR-218 in human gliomas. **Materials and Methods:** Quantitative RT-PCR (qRT-PCR) was conducted to detect the expression of miR-218 in primary normal human astrocytes, three glioma cell lines and 98 paired glioma and adjacent normal brain tissues. Associations of miR-218 with clinicopathological variables of glioma patients were statistically analyzed. Finally, a survival analysis was performed using the Kaplan-Meier method and Cox's proportional hazards model. **Results:** The expression level of miR-218 in primary normal human astrocytes was significantly higher than that in glioma cell lines ($p < 0.01$). Also, the expression level of miR-218 in glioma tissues was significantly downregulated in comparison with that in the adjacent normal brain tissues ($p < 0.001$). Statistical analyses demonstrated that low miR-218 expression was closely associated with advanced WHO grade ($p = 0.002$) and low Karnofsky performance score ($p = 0.010$) of glioma patients. Kaplan-Meier analysis with the log-rank test showed that patients with low-miR-218 expression had poorer disease-free survival and overall survival ($p = 0.0045$ and 0.0124 , respectively). Multivariate analysis revealed that miR-218 expression was independently associated with the disease-free survival ($p = 0.009$) and overall survival ($p = 0.004$) of glioma patients. **Conclusions:** Our results indicate that miR-218 is downregulated in gliomas and that its status might be a potential valuable biomarker for glioma patients.

Keywords: Glioma - microRNA-218 - disease-free survival - overall survival

Asian Pac J Cancer Prev, 16 (5), 1839-1843

Introduction

Gliomas are the most common and malignant tumors in the brain of humans, which represent about 70% of all brain tumors (Jemal et al., 2011). The World Health Organization (WHO) classification scheme divides gliomas into grades I to IV, with increasing levels of malignancy (Louis et al., 2007). The WHO classification serves as a criterion to predict the patient clinical outcomes, but recent studies demonstrate that this criteria alone may not be sufficient to predict the prognosis of patients with glioma. Improvements made in neurosurgical techniques, development of new chemotherapeutic agents, and exploitation of accurate radiotherapy, but the extremely poor prognosis of glioma patients remains still poor during the last three decades (Taylor, 2010). Thus, it is important to understand the molecular mechanisms involved in glioma development which are of value in the development of novel molecular prognostic biomarkers for this deadly disease.

MicroRNAs (miRNAs) are a recently discovered class of short non-coding endogenous RNA molecules that

regulate gene expression at the posttranscriptional level and induce translational repression, mRNA cleavage, or destabilization by binding to the 3'-untranslated region (3'-UTR) of the target mRNAs (Bartel, 2004). It has been reported that miRNAs regulate various human physiological and pathological processes, such as cell proliferation, differentiation, development and tumorigenesis (Friedman JM and Jones PA, 2009; Tüfekci et al., 2014). In human cancers, miRNAs can function as tumor suppressors or oncogenes by targeting oncogenes or tumor suppressor genes (Shenouda SK and Alahari SK, 2009). Recently, microRNA-218 (miR-218) has been reported to serve as a tumor suppressor in numerous types of cancer by regulation of the expression of target genes. In glioma, miR-218 could inhibit glioma invasion, migration, proliferation, and cancer stem-like cell self-renewal by targeting the polycomb group gene Bmi1 (Tu et al., 2013). Also, miR-218 acts as a tumor suppressor by targeting multiple cancer phenotype-associated genes in medulloblastoma (Venkataraman et al., 2013). Meanwhile, Gao' et al showed that miR-218 could inhibit glioblastoma invasion, migration, proliferation and stemness (Gao and

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Jin, 2014). These data clearly demonstrate that miR-218 functions as a tumor suppressor and targeting it will be a promising strategy for the treatment of glioma. However, the clinicopathological and prognostic significance of miR-218 in glioma patients is unclear and remains to be further elucidated.

To address this problem, qRT-PCR assay was performed to detect the expression of miR-218 in 98 paired of glioma and the adjacent non-neoplastic brain tissues. Also, the association between miR-218 expression and clinicopathological variables of glioma patients was statistically analyzed. Furthermore, the Kaplan-Meier method and Cox's proportional hazards model were used to analyze prognostic potential.

Materials and Methods

Cell culture

A primary normal human astrocytes (NHA) and three glioma cell lines (U87, U118, T98) were obtained from Institute of Cell Biology (Shanghai, China). All cell lines were cultured in RPMI 1640 (Invitrogen, Inc.) supplemented with 10% FBS (Sigma, USA), 100 units/mL penicillin G, and 100 µg/mL streptomycin (Gibco, USA) at 37°C in a humidified 5%CO₂/95% air atmosphere.

Tissue samples

A total of 98 paired of primary glioma tissues and the adjacent normal brain tissues from glioma patients were collected at the Department of Pathology or Neurosurgery in Jingling or Jiangsu Province Hospital during the period from 2004 to 2008. All the slides of glioma tissues were re-evaluated according to WHO classifications. The clinicopathological information of the patients is shown in Table 1. The tumors were frozen at -80 °C in a guanidinium thiocyanate solution and RNA was extracted from the samples according to a standard Trizol RNA extraction method (Invitrogen, CA, USA). Written informed consent was obtained from all the patient. The Chinese Medical Association Society of Medicine's Ethics Committee approved all aspects of this study in accordance with the Helsinki Declaration.

qRT-PCR detection of miR-218 expression

Total RNA isolation from cells or tissues was performed using mirVana miRNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX, USA) following the manufacturer's protocol. RNA concentrations were measured using the SPECTRAmax microplate spectrophotometer (Molecular Devices Corp). Total miRNA from cells or tissues was extracted by using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) following the manufacturer's instructions. cDNA was synthesized from 5 ng of total RNA by using the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA), and the expression levels of miR-218 were quantified by using miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems). qRT-PCR was performed by using the Applied Biosystems 7500 Sequence Detection system. The expression of miRNA was defined based on the threshold cycle (Ct), and relative expression levels were

calculated as $2^{-[(Ct \text{ of miR-218}) - (Ct \text{ of RNU6B})]}$ after normalization with reference to expression of RNU6B small nuclear RNA.

Statistical analysis

All statistical analyses were performed using the SPSS 18.0 software package (SPSS, Chicago, IL, USA). The data were presented as the mean±SD. The Chi-squared test was used to determine the clinicopathological significance of miR-218 expression in glioma patients. Differences in patient survival were determined by the Kaplan-Meier method and log-rank test. A Cox proportional hazards regression analysis was used for multivariate analyses of prognostic values. A difference was considered statistically significant when $p < 0.05$.

Results

Expression of miR-218 was significantly reduced in human glioma cell lines and tissues

First, qRT-PCR was used to determine the expression of miR-218 in a primary normal human astrocytes (NHA) and three glioma cell lines (U87, U118, T98) normalized to RNU6B. It was observed that the expression level of miR-218 in NHA was significantly higher than that in glioma cell lines (Figure 1A). Then, the expression of miR-218 was detected in 98 paired of glioma and the adjacent normal brain tissues. As shown in Figure 1B, the expression level of miR-218 in glioma tissues (mean±SD, 2.12±0.86) was significantly lower than that in the adjacent normal brain tissues (mean±SD, 8.46±1.45) ($p < 0.001$). Furthermore, the expression level of miR-218 in high-grade glioma tissues (III+IV) was significantly lower than that in low-grade tissues (I+II) ($p < 0.001$; Figure 1C).

Association between miR-218 expression and clinicopathological variables of glioma patients

To better understand the clinicopathological

Table 1. Correlation between of miR-218 Expression with Clinicopathological Variables of Glioma Patients

Variables	Low miR-218 expression (n=58)		High miR-218 expression (n=40)		p-value
	N	%	N	%	
Gender					0.635
Male	32	55.2	24	60.0	
Female	26	44.8	16	40.0	
Age (year)					0.253
≤55	28	48.3	13	32.5	
>55	30	51.7	27	67.5	
Extension of resection					0.759
Subtotal	20	34.5	15	37.5	
Total	38	65.5	25	62.5	
WHO Grade					0.002*
I/II	22	37.9	28	70.0	
III/IV	36	62.1	12	30.0	
KPS					0.010*
≥80	21	36.2	25	62.5	
<80	37	63.8	15	37.5	

*N, number; *Statistically significant difference ($p < 0.05$). WHO, World Health Organization; KPS, Karnofsky performance score

Table 2. Multivariate Analysis of the Correlation of Prognosis with Various Clinicopathological Variables and miR-218 Expression in Glioma Patients

Variables	5-year DFS			5-year OS		
	HR	95%CI	p-value	HR	95%CI	p-value
Age (>55 vs ≤55 year)	1.142	0.764-2.071	0.364	1.546	0.543-1.823	0.186
Gender (Male vs Female)	2.567	0.818-3.258	0.092	1.702	0.673-2.056	0.255
Extent of resection (subtotal vs total)	1.088	0.692-1.788	0.106	2.077	0.882-2.786	0.088
WHO Grade (III+IV vs I+II)	2.441	1.892-3.012	0.026*	1.982	0.777-2.156	0.223
KPS (≥80 vs <80)	3.012	0.718-4.118	0.178	2.982	0.804-3.128	0.078
miR-218 expression (Low vs High)	2.855	1.562-3.666	0.009*	3.225	1.499-4.172	0.004*

*HR, hazard ratio; 95%CI, 95% confidence interval; KPS, Karnofsky performance score. *Statistically significant difference ($p < 0.05$)

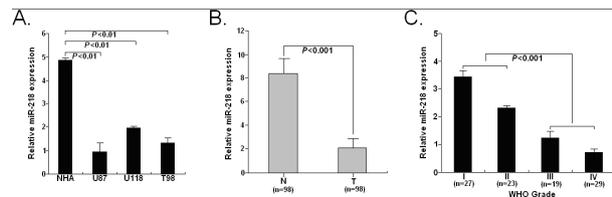


Figure 1. qRT-PCR Detection of miR-218 Expression in Glioma Cell Lines and Tissues. (A) qRT-PCR was performed to determine miR-218 expression in a primary normal human astrocytes (NHA) and three glioma cell lines (U87, U118, T98). (B) qRT-PCR was performed to determine miR-218 expression in 98 paired of glioma tissues and the adjacent normal brain tissues. (C) qRT-PCR was performed to determine miR-218 expression in glioma tissues with different pathological grades (WHO Grade I, II, III and IV). RNU6B was used as an internal control. Each experiment was performed at least in triplicate. Corresponding P values analyzed by t-tests are shown. T: glioma tissues; N: the adjacent normal brain tissues

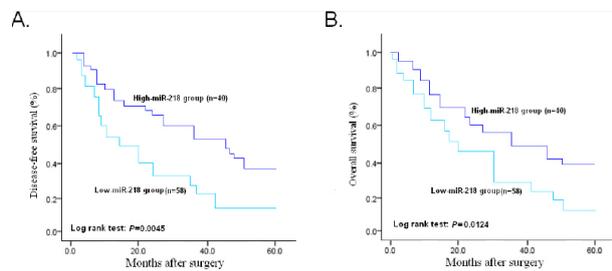


Figure 2. Kaplan-Meier Survival Curves of Glioma Patients According to the Expression of miR-218. (A) The 5-year DFS of glioma patients with high or low miR-218 expression. (B) The 5-year OS of glioma patients with high or low miR-218 expression. The P-value was calculated using the log-rank test between patients with high- and low-fold changes

significance of miR-218 expression in glioma, we divided the 98 glioma patients into two groups: patients who express miR-218 at levels less than the cutoff value (8.46) were assigned to the low-miR-218 group (n=58), and those with expression above the cutoff value were assigned to the high-miR-218 group (n=40). The correlations of miR-218 expression with clinicopathological variables of patients were statistically analyzed and summarized in Table 1. It was observed that low miR-218 expression was closely correlated with advanced pathological grade and low Karnofsky performance score (KPS) ($p=0.002$ and 0.010 , respectively). However, the expression of miR-218 was not found to be correlated with other factors of patients including gender, age at diagnosis, and extent of

resection ($p=0.856$, 0.138 and 0.586 , respectively).

Prognostic significance of miR-218 in glioma patients

The prognostic significance of miR-218 expression level was evaluated for the OS and DFS of patients in 98 glioma patients. In each analysis, patients were divided into the high and low miR-218 expression groups, as described above. During the follow-up time, 22 glioma patients (20.4%) were still alive, but 76 patients (77.6%) died (50 from low-miR-218 expression group, and 26 from high-miR-218 expression group). Kaplan-Meier analyses were performed to further investigate the correlations of miR-218 expression level with survival of glioma patients. As shown in Figure 2A, the 5-year DFS of low-miR-218 expression group was significantly shorter than that of high-miR-218 expression group ($p=0.0045$). Moreover, the 5-year OS of low-miR-218 expression group was also significantly shorter than that of high-miR-218 expression group ($p=0.0124$; Figure 2B). These findings suggest that low miR-218 expression in tissues could predict worse OS and DFS in glioma patients.

Then, in a multivariate analysis based on the Cox proportional hazards regression model, the independent predictive value for miR-218 expression as well as relevant clinicopathological variables (age, gender, WHO grade and KPS) was determined (Table 2). Multivariate analysis revealed that status of miR-218 expression (HR=2.855, 95% CI: 1.562-3.666; $p=0.009$) and WHO grade (HR=2.441, 95% CI: 1.892-3.012; $p=0.026$) were independently correlated with DFS of patients, and low miR-218 expression was an independent prognostic factor for poor OS of patients (HR=3.225, 95% CI: 1.499-4.172; $p=0.004$).

Discussion

In the present study, we first showed that miR-218 was significantly downregulated in human glioma cell lines or tissues and low miR-218 expression was observed to be closely correlated with advanced WHO grade and lower KPS. Additionally, glioma patients with low miR-218 expression showed poorer survival, and multivariate analysis indicated that low miR-218 expression was an independent prognostic factor for predicting the survival of patients. Taken together, these data demonstrated that downregulated miR-218 might play a critical role in glioma development and be a valuable prognostic factor for glioma patients.

It has been reported that miRNAs play important

roles in regulating a variety of biological processes of eukaryotic cells (Costa FF, 2005). It is estimated that more than 30% of all genes and the majority of genetic pathways are subject to regulation by multiple miRNAs (Sevignani et al., 2006). Thus, it is no doubt that dysregulated miRNAs may be involved in many aspects of glioma tumorigenesis and progression (Zhang et al., 2012). Recently, some miRNAs in human gliomas were identified to have potential tumor diagnostic and prognostic values. For example, Sun et al reported that overexpression of microRNA-155 predicts poor prognosis in glioma patients (Sun et al., 2014). Wu and his colleagues showed that miR-21 may be a candidate independent marker for gliomas, especially those with high pathological grade (Wu et al., 2013). Also, a prospective cohort study from Wang et al offers the convincing evidence for the first time that miR-214 and its target gene UBC9 may contribute to the development and the clinical outcome of glioma, and are valuable prognostic factors for glioma patients, suggesting that a combined detection of miR-214/UBC9 expression may benefit us in predicting the prognosis of patients with advanced gliomas (Wang et al., 2014). Additionally, some miRNAs were reported to be correlated with chemo- or radioresistance of glioma. Chen et al reported that miR-136 can target E2F1 to reverse cisplatin chemosensitivity in glioma cells (Chen et al., 2014). Wang et al reported that miR-181b independently predicted chemoresponse to temozolomide and enhanced temozolomide sensitivity via MEK1 downregulation (Wang et al., 2013). Interestingly, epigenetic regulation of miRNA-211 by MMP-9 governs glioma cell both chemosensitivity and radiosensitivity, so either rescuing miR-211 expression or downregulation of MMP-9 may have a new therapeutic application for GBM patients in the future (Asuthkar et al., 2012). Yang and his colleagues explored the potential of PU-PEI-miR145 as a novel therapeutic approach for malignant brain tumors, and showed that microRNA-145 with cationic polyurethane-short branch PEI could lead to inhibition of cancer stem cell-like properties and reduced chemoradioresistance of glioblastoma (Yang et al., 2012). These data clearly demonstrated that miRNAs are involved in glioma development and can be exploited as diagnostic or prognostic biomarkers and molecular therapeutic targets for glioma patients. In this study, we focus on miR-218 and our aim is to investigate its clinicopathological and prognostic values in glioma.

Recently, miR-218 is found to be downregulated in many human malignant tumors. In HCC, the low expression of miR-218 was reported to confer a poor 5-year survival in HCC patients and miR-218 may serve as a prognostic biomarker and induce apoptosis and growth arrest by downregulating BMI-1 in HCC (Tu et al., 2014). Likewise, reduced miR-218 in pancreatic ductal adenocarcinoma tissues was correlated with tumor progression, and might be an independent poor prognostic factor for patients (Zhu et al., 2014). Meanwhile, this miRNA inhibits cell invasion and migration of pancreatic cancer via regulating ROBO1 (He et al., 2014). Also, it was reported that silencing of miRNA-218 promotes migration and invasion of breast cancer via Slit2-Robo1 pathway (Yang et al., 2012). Interestingly, tumor-suppressive

microRNA-218 inhibits cancer cell migration and invasion via targeting of LASP1 in prostate cancer (Nishikawa et al., 2014). It was also found that miR-218 could inhibit migration and invasion in other human cancers by targeting multiple mRNAs, including glioma (Yamamoto et al., 2013; Yamasaki et al., 2013; Tu et al., 2013). The emerging role of tumor-suppressive microRNA-218 in targeting glioblastoma stemness is also reported (Gao X and Jin W, 2014). Mathew and his colleagues identified an miR-218-RTK-HIF2 α signaling axis which promotes GBM cell survival and tumor angiogenesis, particularly in necrotic mesenchymal tumors (Mathew et al., 2014). Importantly, microRNA-218 acts as a tumor suppressor by targeting multiple cancer phenotype-associated genes in medulloblastoma, including CDK6, RICTOR, and CTSB (cathepsin B) (Venkataraman et al., 2013). However, the correlations of miR-218 expression with clinicopathological factors or prognosis of glioma patients are unknown. To the best of our knowledge, this is the first report to investigate the prognostic value of miR-218 expression in human glioma. In this study, we first detected the expression of miR-218 in primary normal human astrocytes and glioma cell lines, and showed that the expression level of miR-218 in glioma cell lines was lower than that in normal human astrocytes. Then, we further analyzed the expression of miR-218 in 98 paired of glioma and the adjacent normal brain tissues, and showed that the expression level of miR-218 in glioma tissues was significantly lower than that in the adjacent normal brain tissues. In addition, statistical analyses indicated that low miR-218 expression was observed to be significantly associated with advanced WHO grade and low KPS. These results demonstrated that downregulation of miR-218 might contribute to glioma progression. Then, we analyzed the correlation of miR-218 expression with prognosis of glioma patients. It was observed that patients with high-miR-218 expression showed poorer DFS and OS than those with low-miR-218 expression. Furthermore, multivariate analysis indicated that low miR-218 expression was an independent prognostic factor for patients. Thus, the positive linkage between miR-218 downregulation and poor prognosis may be helpful to identify glioma patients with poorer prognosis in clinic.

In summary, this study demonstrated that miR-218 was frequently downregulated in glioma cells and tissues, and correlated with grade and KPS of glioma patients. This study also demonstrated for the first time that status of miR-218 expression was an independent prognostic biomarker for glioma patients.

Acknowledgements

The authors thank Feng Zhang for pathological diagnosis and sample collection. Thanks to the Department of Pathology of the Jiangsu Province Hospital for their technique support.

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