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Original Article

Expression of miR-9 and Wnt/β-catenin in Uyghur and Han patients with high-grade gliomas in Xinjiang

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Abstract: Purpose: To study miR-9 gene and Wnt/β-catenin mRNA expression in high-grade gliomas from Uyghur and Han patients in Xinjiang and to correlate abnormal microRNAs with clinicopathological features. Methods: This study included 49 previously untreated ethnic Chinese (25 Han and 24 Uyghur) patients who underwent surgical excision of high-grade gliomas at the First Affiliated Hospital of Xinjiang Medical University from 2011 to 2014. Samples from two patients with epilepsy were used as the normal brain tissue control group. The expression of miR-9 and β-catenin was examined using reverse transcription-polymerase chain reaction in formalin-fixed paraffin-embedded primary tissue specimens. The relationships between the expression of miR-9 and β-catenin, the clinicopathologic features, and the survival rate of patients were investigated. The relationship between miR-9 and β-catenin RNA expression and the prognosis of patients were evaluated using the chi-square test, Cox's regression model and GraphPad Prism survival curve analysis. Results and conclusions: The rates of positive miR-9 and β-catenin mRNA expression in high-grade gliomas were significantly higher than those in control (P<0.05). The expression of miR-9 was positively correlated with that of β-catenin (P=0.002). The expression of miR-9 and β-catenin showed no correlation with patient ethnicity, sex and tumor location (P>0.05). Single factor analysis determined that high mRNA expressions of miR-9, β-catenin were associated with the prognosis of high-grade gliomas (P<0.05). The high expression of miR-9 and β-catenin plays a role in the progression, suggesting that miR-9 and β-catenin may be suitable prognostic markers for high-grade gliomas.

Keywords: miR-9, Wnt/β-catenin, high-grade gliomas

Introduction

High-grade gliomas are among the most common malignant neoplasms of the central nervous system (CNS). According to the World Health Organization (WHO) classification of CNS tumors, gliomas can be divided into grades I-IV. Grades I and II are low-grade gliomas that occur mostly in children, are generally benign, and can be treated by surgery. Grades III and IV are high-grade gliomas that are malignant and more aggressive, and confer a poor prognosis. The prevalence rate of high-grade gliomas is about 29.5/10 million [1]. Currently, the average survival time of patients with high-grade gliomas is about 12-14.3 months [2]. The high probability of metastasis and recurrence is the major reason for poor prognosis. However, the precise molecular mechanism of metastatic dissemination remains unclear. Therefore, understanding the factors involved in the metastasis of high-grade gliomas is required for the identification of new prognostic biomarkers and therapeutic targets.

MicroRNAs (miRNAs), a diverse class of gene repressors consisting of 18-24 nucleotides, play important roles in gene regulation by pairing with the 3'-untranslated region (3'-UTR) of target mRNAs to direct their posttranscriptional repression [3]. MiR-9, a subtype of miRNA that is expressed in a variety of tumor cells, has a close relationship with tumor proliferation and metastasis [4-7]. MiR-9 functions as a transcriptional regulatory factor that regulates target genes in the signaling pathway, affecting cell growth, proliferation, apoptosis and other basic biological behaviors. The Wnt/β-catenin signaling pathway plays a significant role in the occurrence and development of high-grade gli-

Table 1. Sequences of primers used for PCR-amplification

Primer	Sequence
mir-9-F	GCG GCG GTC TTT GGT TAT
mir-9-R	ATC CAG TGC AGG GTC CGA
Has-U6-F	CTC GCT TCG GCA GCA CA
Has-U6-R	AAC GCT TCA CGA ATT TGC
β-catenin-F	TGG TGA CAG GGA AGA CAT
β-catenin-R	CCA TAG TGA AGG CGA ACT
β-actin-F	TGG CAC CCA GCA CAA TGA
β-actin-R	CTA AGT CAT AGT CCG CCT
Mir-9-RT	GTC CTA TCC AGT GCA GGG
Has-U6-RT	AAC GCT TCA CGA ATT TGC

Note: RT-PCR: Reverse Transcription-Polymerase Chain Reaction. F: Forward primer; R: Reverse primer; RT: reverse transcription primer.

omas [8]. However, the roles of miR-9 and Wnt/β-catenin in the development and progression of high-grade gliomas remain unclear. This study investigated the mRNA expression levels of miR-9 and β-catenin and correlated the clinicopathological features and clinical prognosis in Uyghur and Han patients with high-grade gliomas.

Materials and methods

Tissue specimens

This study examined formalin-fixed and paraffin-embedded samples of high-grade gliomas from 49 previously untreated ethnic Chinese (25 Han and 24 Uyghur) patients who underwent surgical excision of high-grade gliomas at the First Affiliated Hospital of Xinjiang Medical University from January 2011 to December 2014. Samples from two patients with epilepsy were used as the normal brain tissue control group. Ethical approval was obtained from the ethics committee of the First Affiliated Hospital of Xinjiang Medical University. The histological diagnoses were WHO III and IV high-grade gliomas, according to the WHO standard pathology classification system. None of the patients had received preoperative radiotherapy or chemotherapy. The study included 25 male and 24 female patients with a median age of 49.52 years (range: 12-72 years). The median ages of the Uyghur and Han patients were 45.32 and 53.51 years, respectively. Among the 49 tumors, 31 were located in the frontal-temple and 18 were located in other regions. All specimens were evaluated independently by two experienced neuropathologists who were blinded to the clinical outcome of the patients, according to the 2007 WHO classification of CNS tumors.

Reverse transcription-polymerase chain reaction (RT-PCR)

Reagents and instruments: Total RNA was prepared using the RNeasy FFPR kit (Qiagen, Valencia, CA, USA). The RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA). PCR was performed using SYBR green qPCR Master mix (Thermo Scientific) and PCR primers (Shanghai OE Biotech. Co., Ltd., Shanghai, China). The instruments used were the Quawell ultra micro ultraviolet spectrophotometer Q5000 (San Jose, CA, USA), the C1000 PCR amplification instrument (Bio-Rad, Hercules, CA, USA) and the iQTM5 multiplex real-time fluorescence quantitative PCR instrument (Bio-Rad). miR-9 and β-catenin primer sequences are listed in **Table 1**.

Total RNA extraction and RT-PCR: Five slices (thickness of 5~10 μm) from each sample were cut from the paraffin-embedded tissues. Total RNA was isolated using the RNeasy FFPR Kit, and the purity and concentration was detected using an ultraviolet spectrophotometer. Total RNA was reverse transcribed using the First Strand cDNA synthesis kit according to the manufacturer's protocol. The products were separated by electrophoresis through an agarose gel (20 g/L), followed by quantification. qPCR was performed on a MyiQ cycler (Bio-Rad) using the SYBR Green qPCR MasterMix. The cycle parameters were set as follows: an initial 3-min incubation at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Data were evaluated using the DDCt method with U-6 and β-actin as reference mRNAs. Error bars were obtained from triplicate PCR samples using the $2^{-\Delta\Delta Ct}$ standard error of the mean through the exponential term, as described previously.

Survival data

Overall survival (OS) was defined as the period from the first operation to death or last follow-up. Patients who survived to the last follow-up

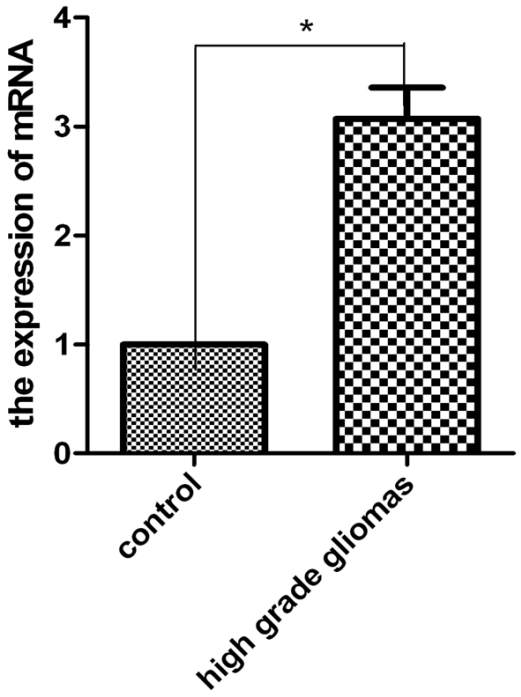

Figure 1. Relative quantities of miR-9 mRNAs in high-grade glioma and control samples (*P<0.05).

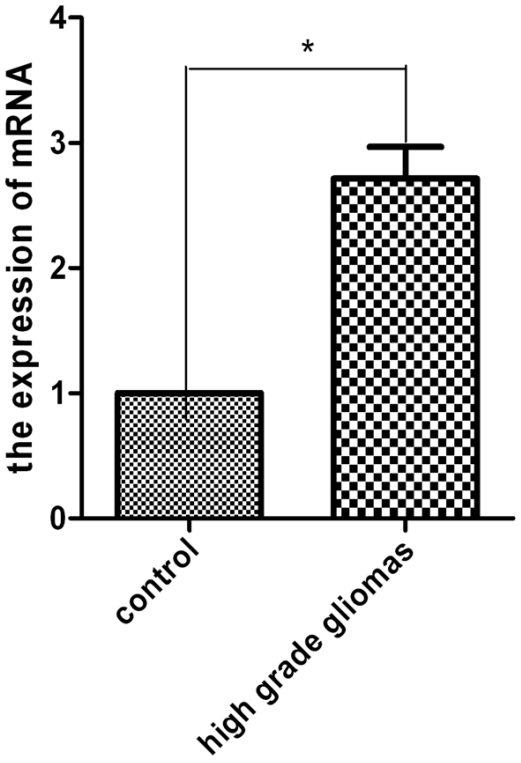

Figure 2. Relative quantities of β-catenin mRNAs in high-grade glioma and control samples (*P<0.05).

Table 2. The relationship between miR-9 and β-catenin mRNA expression in high-gradegliomas

miR-9	β-catenin		Total	χ^2	P
	+	-			
+	35	5	40	12.382	0.002
-	3	6	9		
Total	38	11	49		

Note: +: upregulation compared with control; -: down-regulation compared with control; statistical analysis was conducted using the χ^2 test in SPSS software (ver. 17.0).

were considered as a censored event in the analysis. Among the 49 patients with tumors, 41 cases were followed for an average of 2.5 years. The median survival time was 12.0 months.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism (ver. 5.0; GraphPad Software Inc., San Diego, CA, USA) and SPSS (ver.17.0; SPSS Inc., Chicago, IL, USA) software. For group comparisons of proportions, the χ^2 and/or Fisher's exact test were conducted. For analysis of the survival data, the survival curve function in GraphPad Prism was used and the log-rank test was used to test for survival differences among groups. P<0.05 was considered to indicate statistical significance.

Results

Upregulated expression of miR-9 was detected in 40/49 high-grade gliomas (81.63%) and that of β-catenin was detected in 38/49 (77.55%) high-grade glioma tumors, compared with corresponding nontumorous tissues. The average expression was significantly higher in tumor tissues than in their normal counterparts (P<0.05). The relative expression quantities of miR-9 and β-catenin mRNAs were 3.07 ± 0.52 and 2.71 ± 0.49, respectively (**Figures 1**, **2**). The miR-9 relative expression quantities in Han and Uyghur patients were 3.13 ± 0.55 and 3.01 ± 0.51, respectively. The β-catenin mRNA relative expression quantities in Han and Uyghur patients were 2.87 ± 0.48 and 2.55 ± 0.54, respectively. Correlation analysis of the relative quantitative expression of miR-9 and β-catenin showed a strong positive correlation between miR-9 and β-catenin (P=0.002) (**Table 2**): the higher

Table 3. Correlation between miR-9/β-catenin expression and high-grade glioma clinicopathological features in 49 patients

Variable	n%	miR-9 expression level			*P*	β-catenin expression level			*P*
		High expression	Low expression	Ratio Low/High		High expression	Low expression	Ratio Low/High	
Age, years					*0.049*				*0.033*
<45	17 (34.69)	11	6	0.545		10	7	0.700	
≥45	32 (65.31)	29	3	0.103		28	4	0.143	
Sex					*0.463*				*0.675*
Male	25 (51.02)	19	6	0.316		20	5	0.250	
Female	24 (48.98)	21	3	0.143		18	6	0.333	
Ethnicity					*0.725*				*0.496*
Han	25 (51.02)	21	4	0.190		18	7	0.389	
Uyghur	24 (48.98)	19	5	0.263		20	4	0.200	
Tumor location					*0.708*				*0.507*
Frontal-temple	31 (63.27)	26	5	0.192		23	8	0.348	
Other locations	18 (36.73)	14	4	0.286		15	3	0.200	

Note: Statistical analyses were conducted using the χ^2 test in SPSS software (ver. 17.0).

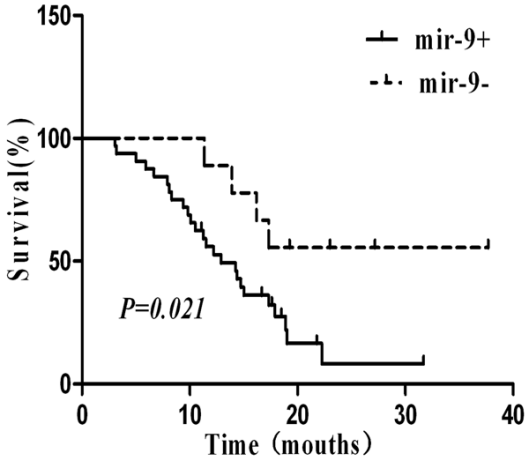

Figure 3. The survival curves of patients with high expression and low expression of miR-9 were significantly different (*P*=0.021).

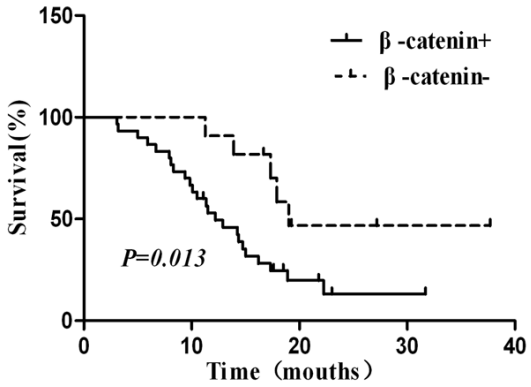

Figure 4. The survival curves of patients with high expression and low expression of β-catenin were significantly different (*P*=0.013).

the abnormal expression of miR-9, the higher the positive rate of β-catenin.

Expression of miR-9 and β-catenin and correlation with clinicopathological features

The prevalence of abnormal expression of miR-9 and β-catenin mRNAs in patients aged ≥45 years was higher than that in patients aged <45 years (P<0.05). The abnormal expression of miR-9 and β-catenin was not significantly associated with patient sex, ethnicity, or tumor location (P>0.05; **Table 3**).

Upregulation of miR-9 and β-catenin is associated with poor prognosis

Forty-one patients with high-grade gliomas were analyzed at the last follow-up visit. Univariate analysis determined that abnormal expressions of miR-9 (*P*=0.021) and β-catenin (*P*=0.013), age of onset (*P*=0.039), and use of chemotherapy (*P*=0.003), radiotherapy (*P*=0.016), and chemo-radiotherapy (*P*=0.006) were associated with the survival time of patients with high-grade gliomas (**Figures 3-6**). The higher the expression of miR-9, the worse the prognosis; and the younger the age of onset, the better the prognosis. The patients' ethnicity (*P*=0.732), sex (*P*=0.214), and tumor location (*P*=0.307) showed no significant relationships.

Discussion

High-grade gliomas represent some of the most common malignant tumors of the CNS in adults.

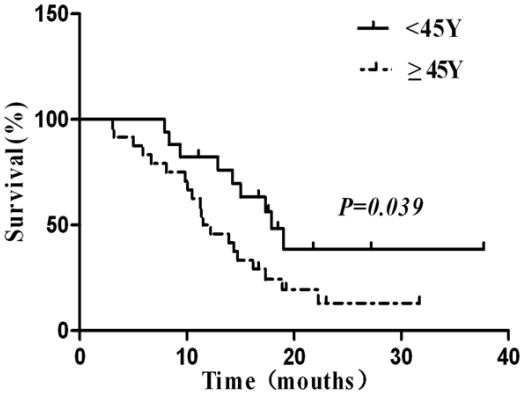

Figure 5. The survival curves of patients ≤45 and >45 years of age were significantly different (P=0.039).

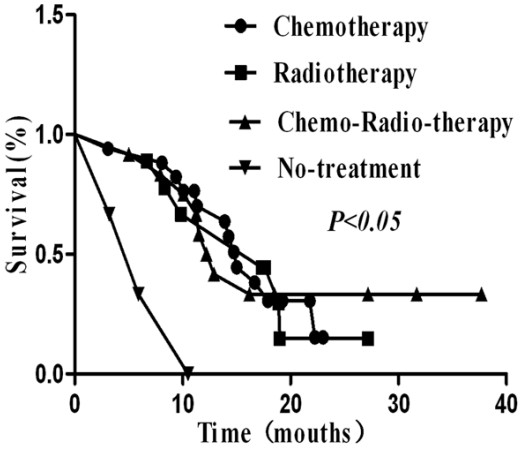

Figure 6. The survival curves of patients according to treatment type showed significant differences ($P_{chemo-therapy}$=0.003, $P_{radiotherapy}$=0.016, $P_{chemo-radiotherapy}$=0.006).

Of these, 72% are reported in the frontotemporal, with the remaining 28% located in other regions. With recent advances in molecular biology, great progress has been made in understanding the molecular biology of gliomas. However, the origin of tumor cells in high-grade gliomas lack effective targeted treatment measures. Therefore, it is critical to explore the molecular pathological mechanisms of high-grade glioma to provide a more accurate assessment of prognosis and to develop targeted clinical therapies in patients.

miRNAs are evolutionarily conserved, non-coding small RNAs (18-24 nucleotides) involved in post-transcriptional gene regulation. They function in a variety of biological behaviors such as differentiation, proliferation and apoptosis of tumor cells [8, 9]. MiR-9, a highly conserved miRNA, is expressed predominantly in the CNS of the developing embryo and plays a role in pro-differentiation [10]. In addition to its involvement in neurogenesis, miR-9 is upregulated in primary brain tumors [5]. Upregulation of miR-9 has been reported in breast cancer [11], colorectal cancer [12] and melanoma [13]. Interestingly, downregulation of miR-9 has been reported in gastric [14] and ovarian cancers [15], suggesting a diverse role of miR-9 in various cancers. Using RT-PCR, this study found that the RNA expression of miR-9 in high-grade gliomas was significantly higher than that in the control group, consistent with previous reports. This result suggests that miR-9 is an oncogene, and that high expression can cause the occurrence of tumors. Therefore, miR-9 expression may be used not only to distinguish between benign and malignant tumors, but also in the early detection of tumors.

Many studies [16-18] have shown that abnormal activation of the Wnt signaling pathway is involved in the occurrence of a variety of human tumors. β-catenin, encoded by 16 exons located on chromosome 3p21, is an important member of the Wnt pathway. β-catenin, a cytoskeletal component involved in tissue structure and morphology, is an important regulator of the cell-cell adhesion system [19]. In addition, β-catenin is a critical signaling molecule in the classical Wnt signal transduction pathway. Previous studies [20] have shown that members of the Wnt gene family are overexpressed in gliomas. The overexpression of β-catenin influences the biological behaviors of cell proliferation, invasion, apoptosis and other malignant phenotypes. Consistent with these studies, our RT-PCR analysis demonstrated that β-catenin was significantly overexpressed in high-grade gliomas, suggesting that β-catenin may participate in tumor metastasis of high-grade gliomas.

Many studies [21, 22] have shown that miR-9 is closely related to the signaling pathway. Ma et al. [11] reported that miR-9 could target E-cadherin and allow liberation of β-catenin, which then activates vascular endothelial growth factor (VEGF) to promote metastasis in breast cancer. Song et al. [23] showed miR-9 overexpression activated β-catenin and induced the β-catenin nuclear shift in esophageal

squamous cell carcinoma (ESCC), thereby promoting the proliferation and metastasis of ESCC. Daniel et al. [24] reported that miR-9 was highly expressed in glioblastoma stem cells, and may be involved in the occurrence and development of stem cells. However, the role of miR-9 in the development and progression of high-grade gliomas remains unclear. Using RT-PCR, the present study showed that the RNA expressions of miR-9 and β-catenin were significantly higher in high-grade gliomas. Moreover, we showed a strong positive correlation between miR-9 and β-catenin expression (P= 0.002): the higher the abnormal expression of miR-9 mRNA, the higher the positive rate of β-catenin expression. Both miR-9 and β-catenin play an important role in the biological behavior of malignant high-grade gliomas. miR-9 may be related to the activation of the classical Wnt signaling pathway through regulation of the expression of the β-catenin signaling pathway and contributing to the induction of tumor cells.

Our results confirmed that the abnormal expression of miR-9 and β-catenin is related to the metastasis and invasion of tumor cells #. We found that patients over 45 years of age had higher rates of abnormal expression in their high-grade tumors than patients aged under 45 years. Additionally, we observed that the median age of onset was lower in Uyghur patients compared to Han patients. This observed trend may be related to the poorer lifestyle of the Uyghur patients, characterized by living in a remote area, lower economic status, consanguineous marriage, harsher labor activities, and diets rich in high fat, pickled foods, with fewer vegetables. Further studies will be required to investigate this trend between the two ethnic groups, as it did not reach statistical significance. However, no significant group differences were observed in ethnicity, sex or tumor location.

This study showed that the median survival time of the patients was 12 months. High expression of miR-9 is a common feature in high-grade gliomas, and high expression of miR-9 is closely related to poor prognosis and poor outcome after treatment. Song et al. [23] found that miR-9 could increase cell migration and metastasis, implying that miR-9 is a metastamiR in ESCC, and could be used as a prognostic biomarker in ESCC. According to the Cox regression analysis, expression of miR-9 is an independent prognostic factor in patients with high-grade gliomas, and can be used as a prognostic factor in patients with poor prognosis. According to the GraphPad survival curve analysis, the survival rate of patients with high expression of miR-9 was significantly lower than that of patients with low expression. Recent studies suggested that abnormal expression of β-catenin is a common event in malignant tumors. This study showed that the prognosis of patients with high expression of β-catenin was significantly lower than that of those with low expression, suggesting that β-catenin may be one of the predictors for the prognosis of high-grade gliomas. Furthermore, patient age and treatment modalities were associated with prognosis: the younger the age, the better the prognosis. Ethnicity (P= 0.732), sex (P=0.214), and tumor location (P=0.307) showed no significant differences in the clinical prognosis.

Conclusion

This study showed that miR-9 and β-catenin were overexpressed in high-grade gliomas. There was a positive correlation between miR-9 and β-catenin expression in high-grade gliomas. The high expression of miR-9 may promote the proliferation, invasion, and metastasis of tumor cells, and may decrease the sensitivity of tumor cells to chemotherapy and radiotherapy. Overexpression of miR-9 and β-catenin was associated with poor prognosis in patients. MiR-9 analysis offers a new approach toward developing an effective molecular evaluation and prognostic strategy for the diagnosis of high-grade gliomas. MiR-9 and β-catenin are promising new targets for the treatment of high-grade gliomas. Combined analysis of miR-9 and β-catenin may be more accurate and available for the clinical diagnosis and treatment. Thus, the determination of miR-9 and β-catenin mRNA expression in high-grade gliomas will improve the accuracy of clinical diagnosis, which could guide treatment and lead to improved prognosis.

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Disclosure of conflict of interest

None.

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