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Original Article

Down-regulation of *let-7f* predicts a poor prognosis in human glioma

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Abstract: Background: *Let-7f* has been confirmed to express in different malignant human tumors, it usually participates in tumor progression and development by different target factors. However, the studies about the association between *let-7f* and glioma are limited. The aim of this study was to evaluate the potential value of *let-7f* as a biomarker in the prognosis of glioma. Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of *let-7f* in 136 glioma tissues and 25 normal specimens. Peristin expression was evaluated by western blotting. The relationship between *let-7f* expression and clinical factors was analyzed by chi-square test. And Kaplan-Meier and Cox regression analysis was used to estimate the prognostic value of *let-7f*. Results: The *let-7f* expression was remarkably down-regulated in glioma tissues compared with normal brain tissues ($P<0.001$), and *let-7f* expression in patients with low WHO grade was significantly higher than that with high grade. The expression of periostin was decreased in patients with glioma compared to normal controls. And the expression of *let-7f* was significantly related to tumor size and WHO grade. Kaplan-Meier analysis suggested that patients with low *let-7f* expression had a worse overall survival than those with high *let-7f* expression ($P<0.001$). Multivariate analysis indicated *let-7f* could act as an independent prognostic biomarker for patients with glioma. Conclusions: *Let-7f* acted as a tumor suppressor and participated in the development and progression of glioma. Besides, it could be a valuable prognostic bio-marker in patients with glioma.

Keywords: Glioma, *Let-7f*, periostin, prognosis

Introduction

Glioma, stemming from glial cells, is the most common malignant tumor occurring in central nervous system [1, 2]. It accounts for approximately 40% of intracranial tumors and 70% of adult malignant primary brain tumors [3, 4]. Glioma is divided into four pathologic grades by World Health Organization (WHO) classification: pilocytic astrocytoma (PA, WHO grade I), diffuse astrocytoma (DA, WHO grade II), anaplastic astrocytoma (AA, WHO grade III), and glioblastoma (GBM, WHO grade IV) [5]. Despite important advances in the managements and treatment of glioma had obtained, the prognosis of glioma remains poor due to its high proliferation, diffuse invasion, chemical and radiation therapy resistance [6-9]. Consequently, it is indispensable to further explore sensitive and specific molecular markers associated with prognosis of glioma.

MicroRNAs (miRNAs) are a class of short non-coding RNAs with a length of 18-25 nucleotides. They were considered to play a vital role in the regulation of cell proliferation, apoptosis, metabolism, differentiation, and stem cell maintenance [10-12]. Moreover, microRNAs can improve mRNA degradation, prevent mRNA from being translated, and thus regulate post-transcriptional expression of target genes by binding to the complementary target mRNA [13]. Evidences continuously confirm that microRNAs are involved in tumor angiogenesis and act as tumor suppressors or oncogenes [14]. *Let-7f,* a number of *let-7* miRNAs family, is

Table 1. The relationship between *let-7f* expression and the clinicopathological parameters in glioma patients

Parameters	Cases (n)	*Let-7f* expression		*P* value
		High	Low	
Age				0.204
≥50	65	40	25	
<50	71	36	35	
Gender				0.619
male	69	40	29	
female	67	36	31	
Tumor size				0.006
≥5 cm	68	46	22	
<5 cm	68	30	38	
Smoking history				0.131
Yes	62	39	23	
No	74	37	37	
WHO grade				0.026
I, II	67	31	36	
III, IV	69	45	24	
Extent of resection				0.911
Total	65	36	29	
Portion	71	40	31	

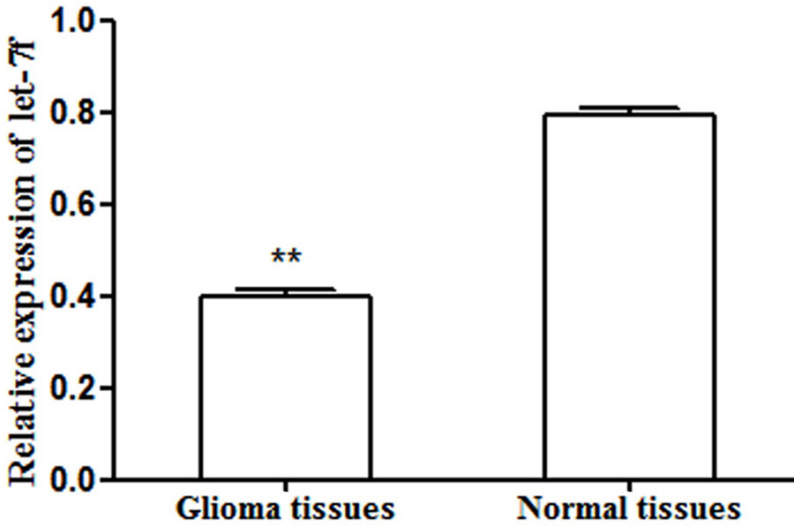

Figure 1. Relative expression of *let-7f* in glioma patients and normal controls. The expression of *let-7f* in glioma was significantly decreased compared with normal control specimens ($P<0.001$).

located at 9q22.3 [15]. And it had been verified to aberrant expression in several of cancers. More importantly, recent studies have indicated that *let-7f* is decreased in glioma tissue specimens compared with normal brain tissue [16-18]. However, the prognostic value of *let-7f* in glioma still remains unclear.

In this study, we detected the expression of *let-7f* in glioma tissues and normal brain tissues, investigated the relationship between its expression and clinical factors. Besides, we firstly estimated the prognostic value of *let-7f* in glioma.

Materials and methods

Patients and specimens

A total of 136 patients who were diagnosed as glioma and without preoperative treatment (chemotherapy or radiotherapy) from The Hulun Buir People's Hospital were collected in our study. In addition, 25 patients without glioma in this hospital were taken as controls. The study protocol was approved by the Ethics Committee of The Hulun Buir People's Hospital. All participants had signed written inform consents in advance.

Tumor tissues and healthy tissues were extracted from patients with glioma and healthy controls, respectively. Then all tissues samples were severally frozen by liquid nitrogen immediately and stored at -80°C until use. Clinicopathologic data of the patients were recorded in **Table 1**. Follow-up was conducted at least 5 years by telephone visit. Patients who died from unexpected events or other diseases were excluded from our study.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from the patients with glioma and controls using TRIzol reagent (Invitrogen). The first chain of cDNA was synthesized by reverse transcription with TaqMan Micro-RNA Reverse Transcription Kit according

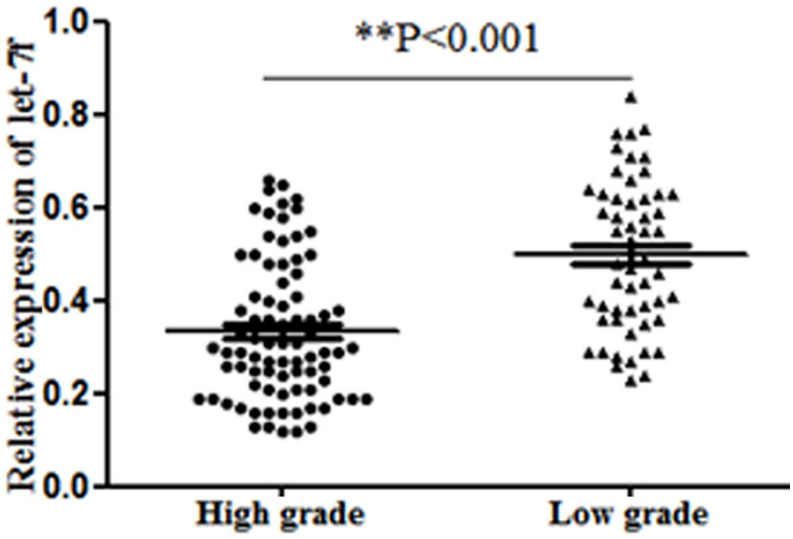

Figure 2. Relative expression of *let-7f* in glioma patients with different WHO grade. *Let-7f* expression in low WHO grade was higher than that in high grade (*P*<0.001).

to the manufacturer's protocol. RT-PCR reaction was conducted in an ABI 7500 Real-Time PCR instrument (Applied Biosystems, Inc). U6 was taken as internal control. The primer of *let-7f* and U6 were as followed: *let-7f*: reverse-5'-CTCAACTGGTGTCGTGGAGTCGGCAAGAGTCGGCAATTCAGTTGAGAACTATACAAT- 3'; forward-5'-ACACTCCAGCTGGGTGAGGTAGTAGATT-3'; U6: reverse-5'-AACGCTTCA-CGAATTTGCGT-3'; forward-5'-CTCGCTTCGGCAGCACA-3'. The relative quantities of *Let-7f* were calculated via $2^{-\Delta\Delta CT}$ method. Each experiment was in triplicate.

Western blot analysis

Total protein was isolated from all tissues samples using Cellytic MT Cell Lysis Reagent and separated on 10% sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane (PVDF; Millipore, USA). After blocked with 5% blocking buffer, the membranes were incubated with primary antibodies overnight at 4°C with primary periostin antibody (1:1,000 dilution). After washing with 19 PBST (PBS 0.1% Tween-20), membranes were incubated with HRP-conjugated secondary antibody (1:2,000 dilution) for 1 h at room temperature. Then, the proteins were visualized with ECL chemiluminescence reagents (Beyotime, China). β-actin was taken as internal controls.

Statistical analysis

The statistical analysis was performed with SPSS 13.0 software (SPSS, Chicago, IL). All data were expressed as the mean ± SD. The difference between two groups was analyzed by Student's t-test. Chi-square test was used to stimulate the relationship between *Let-7f* expression and clinicopathologic data. Survival analysis was made via Kaplan-Meier analysis while the prognostic value of *let-7f* was evaluated by multivariate analyses with Cox regression analysis. *P*<0.05 was considered to be statistically significant.

Results

Let-7f was significantly down-regulated in glioma tissues

The relative expression levels of *let-7f* (normalized to U6) were detected by qRT-PCR analysis in 136 glioma tissues and 25 normal brain samples. As shown in **Figure 1**, the expression levels of *Let-7f* (0.399±0.174) in glioma tissues which was significantly lower than that in normal brain tissues (0.792±0.077) (*P*<0.001). Moreover, compared to the patients of low-grade (I-II), the expression levels of *let-7f* (0.499±0.162) was significantly decreased in those with high-grade (III-IV) (0.333±0.150) (**Figure 2**, *P*<0.001). These results demonstrated *let-7f* might a tumor suppressor in glioma.

Expression of periostin in glioma

In previous study, periostin was considered to the targeted gene of *let-7f.* Therefore, we measured the expression of periostin in the tissues of glioma patients and normal controls. The results showed that the expression of periostin in glioma tissues was higher than that in normal specimens (0.306±0.024 vs. 0.106±0.027), and the difference between them was significant (*P*<0.001, **Figure 3A** and **3B**).

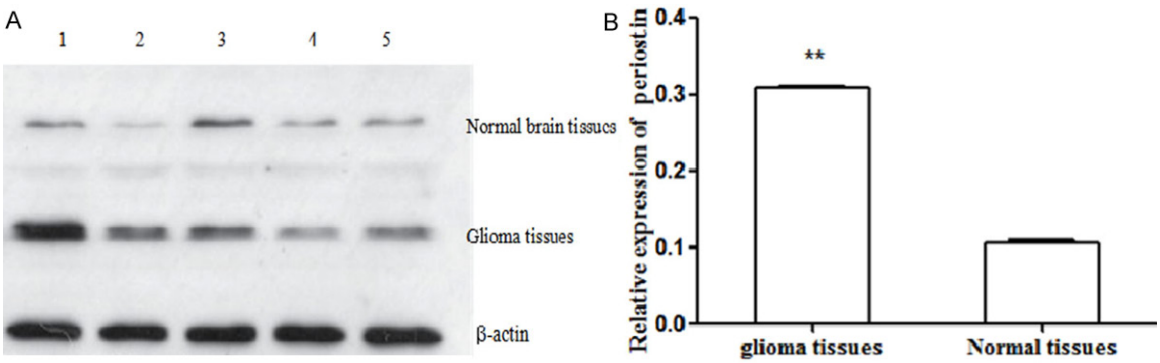

Figure 3. The expression of periostin in glioma patients and Normal controls. It was remarkably increased in glioma patients compared with normal control specimens (P<0.001).

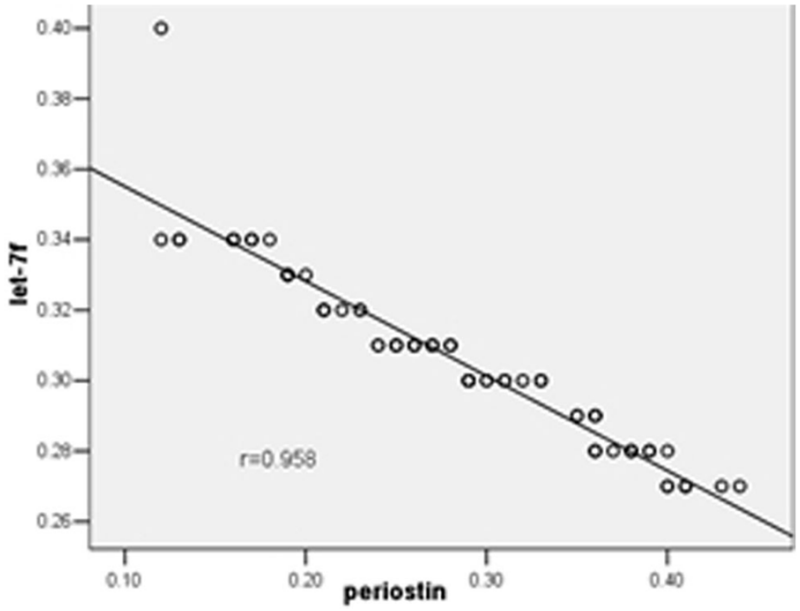

Figure 4. The correlation between *let-7f* expression and periostin expression. *Let-7f* expression was significantly negative correlated with periostin expression (r=-0.958).

Correlation between let-7f expression and periostin

Based on the detection of the expression of *let-7f* and periostin, we further explored whether there was a correlation between them in glioma. And we found as the increasing of *let-7f* expression, the expression of periostin was decreased which revealed they had a negative correlation (**Figure 4**).

Association between let-7f expression and clinicopathological parameters in glioma

We manually defined glioma specimens into two groups with the meda in expression of *let-7f* (0.399±0.174). 60 cases were in high *let-7f* expression group and 76 cases in low *let-7f* expression group. And then based on the classification, we further investigated the association between *let-7f* expression and clinicopathological characteristics of glioma patients. Results showed that *let-7f* expression was significantly associated with tumor size (P=0.006) and WHO grade (P=0.026). However, *let-7f* was not found to be associated with age, gender, smoking history and extent of resection (P>0.05) (**Table 1**).

Prognostic value of let-7f in glioma patients

Researches above revealed *let-7f* expression in glioma was significantly associated with tumor progression. Thus, with the aim to evaluate the prognostic value of *let-7f* expression in human glioma, we conducted the Kaplan-Meier and Cox regression analysis. Kaplan-Meier analysis with the log-rank test was utilized to evaluate the difference of overall survival between two groups (high and low expression of *let-7f*). Patients with low *let-7f* expression had a significantly worse overall survival compared to those with high *let-7f* expression (Log rank test, P<0.001; **Figure 5**). Univariate and multivariate analysis manifested WHO grade (HR=3.618; 95% CI 1.347-9.723; P=0.011) and low *let-7f*

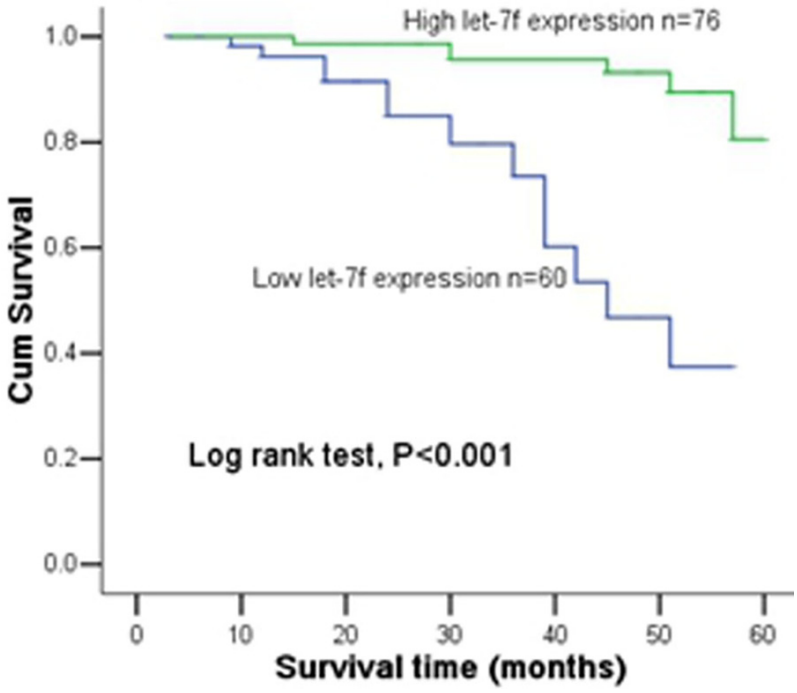

Figure 5. Kaplan-Meier analysis for the OS of patients with glioma according to the expression of *let-7f.* Patients with low *let-7f* expression had a shorter OS than those with high expression (Log rank test, *P*<0.001).

expression (HR=7.722, 95% CI=2.834-21.038; *P*=0.000) were tightly related to the prognosis of glioma (**Table 2**). And they might be independent prognostic factors in patients with glioma.

Discussion

As the most common intracranial tumor, it is difficult to evaluate the prognosis of patients with glioma in clinical practice because of the heterogeneity of patients. Moreover, glioma had the characteristics of high proliferation and invasion potentials which lead to a poor prognosis [19]. It was reported that the survival time of most patients diagnosed as glioblastoma was less than one year [20]. Therefore, identification and confirmation of appropriate biomarkers are still urgent for evaluating the prognosis and guiding tumor treatment of glioma.

In previous studies, few molecular signatures have been validated and widely accepted as prognostic indicators in clinical practice. For instance, Lai et al., found the high expression of miR-210 was not only serve as a diagnostic but a potential prognostic indicator in glioma [21]. MiR-155 was considered to be up-regulated in glioma patients and could be an independent prognostic marker according to the study of Sun et al [19]. Guan et al, detected the expression of miR-504 and showed its down-regulation could predict a poor prognosis [22]. Plasma miR-221/222 family was overexpression and could serve as an independent diagnostic and prognostic marker in glioma [23] However, the prognostic value of *let-7f* in glioma had never been reported.

Let-7f was reported to take effects in a variety of physiological and pathological processes including immunocyte differentiation, angiogenesis, replicative senescence, pulmonary arterial hypertension and carcinogenesis [15, 24-26]. Evidences from previous studies have indicated that *let-7f* have significantly abnormal expression in various cancers such as primary breast cancer, hepatocellular carcinoma, papillary thyroid cancer, ovarian carcinoma, gastric cancer and pancreatic cancer [27-32]. In current study, we detected the *let-7f* expression via qRT-PCR analysis and found its up-regulation in glioma. This was consistent with previous studies. Besides, we considered its expression was influenced by tumor size and WHO grade obviously. Then we tried to analyze its clinical significance in glioma. Kaplan-Meier analysis showed patients with high *let-7f* expression had a longer overall survival than those with low expression which indicated *let-7f* was correlated with the prognosis of glioma. Univariate and multivariate analysis further revealed that low *let-7f* expression could be an independent prognostic factor for glioma patients.

As a kind of bone adhesion molecule regulating osteoblast adhesion and differentiation, periostin is up-regulated in different malignant cancers, such as breast cancer, cholangiocarcino-

Table 2. Univariate and multivariate analysis for evaluating the prognostic value of *let-7f* in patients with glioma by Cox regression analysis

Parameters	Univariate analysis		Multivariate analysis	
	Risk ratio (95% CI)	*P*	Risk ratio (95% CI)	*P*
Age	1.048 (0.368-2.982)	0.930	-	-
Gender	2.331 (0.839-6.475)	0.104	-	-
Tumor size	1.092 (0.353-3.376)	0.879	-	-
Smoking history	2.058 (0.715-5.925)	0.181	-	-
WHO grade	5.573 (1.775-17.498)	0.003	3.618 (1.347-7.723)	0.011
Extent of resection	1.751 (0.617-4.975)	0.293	-	-
Let-7f level	11.320 (3.490-36.715)	0.000	7.722 (2.834-21.038)	0.000

ma and prostate cancer [33, 34]. Moreover, previous reports showed that periostin expression was higher in high-grade glioma patients and is an independent prognostic factor [35]. Related researches revealed periostin was a target gene which lead to *let-7f* could inhibit the proliferation, migration, and invasion of glioma cells [18]. Therefore, we measured the expression of periostin in glioma patients. Western blotting analysis showed that periostin was upregulated in glioma tissues, and the association between *let-7f* expression and periostin expression in glioma was significantly negative.

In conclusion, *let-7f* expression is decreased in gliomas. And its expression was not influenced by tumor size and WHO grade bur regulated by periostin. Moreover, *let-7f* could act as an independent prognostic indicator in glioma. This study was firstly provide evidences that *let-7f* is related to the prognosis of glioma. However, as the limitation of sample size as well as the unclear mechanism, some further studies are necessary.

Disclosure of conflict of interest

None.

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