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Decreased *let-7b* is associated with poor prognosis in glioma

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Abstract

Abnormal expression of *let-7b* has been observed in many tumors, including glioma. However, the clinical significance of *let-7b* in glioma remained unclear. The aim of the study was to explore the correlation of *let-7b* expression with clinicopathological factors and prognosis in human glioma.

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to detect the relative expression of *let-7b* in glioma tissues. The association of *let-7b* expression with clinicopatholoigcal features of glioma patients was estimated using chi-square test. Overall survival curves were plotted using Kaplan–Meier method with log rank test. The prognosis analysis was performed using Cox regression model, and the results were shown as hazard ration (HR) with 95% confidence interval (CI).

The relative expression of *let-7b* was significantly lower in glioma tissues than that in normal brain tissues ($P < .001$). Furthermore, *let-7b* level was closely correlated with World Health Organization (WHO) grade ($P = .027$) and Karnofsky performance score (KPS) ($P = .018$). Survival analysis indicated that glioma patients with low *let-7b* expression had significantly shorter overall survival time than those with high expression (log rank test, $P < .001$). *Let-7b* might be an independent prognostic biomarker for glioma ($P < .001$, HR = 2.415; 95% CI: 1.531–3.808).

Let-7b may be a promising prognostic factor in glioma.

Abbreviations: 3′′-UTR = 3′-untranslated regions, CI = confidence interval, GBM = glioblastoma multiform, HR = hazard ratio, KPS = Karnofsky performance score, miRNAs = microRNAs, PVDF = polyvinylidene fluoride, qRT-PCR = quantitative real-time polymerase chain reaction, SD = Standard deviation, SDS-PAGE = sodium dodecyl sulfonate-polyacrylamide gel electrophoresis, WHO = World Health Organization.

Keywords: glioma, *let-7b*, prognosis, survival

1. Introduction

Human glioma, originating from astrocytes or astroglial precursors, is a frequently diagnosed human malignant central nervous system neoplasm.[1] Based on malignancy degree, World Health Organization (WHO) divides gliomas into 4 grades: pilocytic astrocytoma (WHO grade I), diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III), and glioblastoma multiform (GBM, WHO grade IV).[2] The commonly used therapeutic strategies for glioma include neurosurgery, chemotherapy, and radiotherapy, and these treatments have been significantly improved clinical outcomes of glioma cases over the last decades. However, the prognosis of glioma patients still remains dismal, especially those diagnosed with GBM.[3–5] It has been reported that the median survival time of GBM patients is rough 14 months, due to the aggressive tumor progression and treatment resistance.[6,7] Therefore, it is necessary to explore novel molecular biomarkers to guide treatments and predict clinical outcomes in glioma.

MicroRNAs (miRNAs), a class of small non-coding RNAs, play regulatory roles in gene expression network via binding to the 3′-untranslated regions (3′-UTR) of their target mRNAs.[8] MiRNAs are involved in a broad of biological pathways, such as cell proliferation, apoptosis, cell cycle, differentiation, metabolism, etc.[9,10] Abnormal expression of miRNAs may impair multiple cellular functions, thus resulting in diseases, like cancer. Accumulating evidences have reported that miRNAs as tumor suppressor or oncogene are involved in various cancers.[11] *Let-7b* is originally observed in nematode *Caenorhabditis elegans*, and belongs to the let-7 family which is a conserved family of miRNAs with 12 members.[12,13] Recently, *let-7b* has been reported to act as a tumor suppressor in several human cancers, including gastric cancer, papillary thyroid carcinoma, etc.[14,15] In glioma, the study carried out by Song et al[16] demonstrated that *let-7b* hold the capacity to inhibit malignant behaviors of glioma cells in vitro. However, the clinical significance of *let-7b* in glioma had been rarely reported in the relevant studies.

In present study, we detected the relative expression of *let-7b* in glioma tissues, and analyzed the correlation between *let-7b*

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expression and clinicopathological factors of glioma patients. Additionally, we also evaluated the prognostic significance of *let-7b* in glioma.

2. Methods and materials

2.1. Patients and tissue samples

A total of 127 glioma were recruited from the Department of Neurosurgery in Harrison International Peace Hospital between October 2009 and May 2011. The primary glioma diagnosis was reviewed histologically by 2 experienced neuropathologists. The glioma tissue and adjacent normal tissue samples were obtained from the patients, and immediately snap-frozen in liquid nitrogen, then stored at –80°C until RNA extraction. None of patients had received preoperative treatments, including chemotherapy or radiotherapy. The clinicopathological features of all the patients were summarized in Table 1.

All the glioma patients were enrolled in a 5-year follow up investigation. The glioma patients were followed up no >3 months intervals during the first 2 postoperative years, and no >6 months thereafter. Overall survival time was calculated from the date of the initial surgery to death. Patients who died from other diseases rather than gliomas or unexpected events were excluded from this study.

The study was completed with the approval of the Research Ethics Committee of Harrison International Peace Hospital. Each participant signed the written informed consent form before sample collection.

2.2. RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The RNA concentration and purity were measured using NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Houston, TX). Only the samples with A260/A280 ratio between 1.8 and 2.0 were utilized for the subsequent analysis.

The relative expression of *let-7b* was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The reaction was carried out using miRNA quantitative real-time PCR kit in an ABI Prism 7500 Sequence Detector System (Applied Biosystems, Foster City, CA). U6B small nuclear RNA (*U6*) was used as an internal control. The relative expression of *let-7b* was normalized to *U6*, and calculated using $2^{-\Delta\Delta Ct}$ method. Each test was repeated 3 times. The primer sequences were as follows: *let-7b* forward, 5′-GGGTGAGGTAGTAGGTTGTGTG-3′ and reverse 5′-CAGGGAAGGCAGTAGGTTGT-3′; *U6* forward, 5′-CTCGCTTCGGCAGCACA-3′ and reverse 5′-AACGCTTCACGAATTTGCGT-3′.

2.3. Western blot

The protein of tissues was extracted and separated using 10% sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche) by electroblotting. PVDF membrane was blocked by non-fat milk at room temperature for 24 hours or 4°C for overnight. The membrane was incubated by primary antibodies (1:1000) at 4°C for overnight, and then incubated with second antibody (1:2000, Abcam, China) for 1.5 hours at room temperature. The target band of protein was analyzed using ECL Western blotting kit (Millipore, Boston, MA).

2.4. Statistical analysis

All data were analyzed using SPSS 18.0 (SPSS Inc, Chicago, IL), and graphs were plotted by GraphPad Prism 5.0 (GraphPad Software Inc., CA). The data of *let-7b* expression values were expressed as mean ± standard deviation (SD), and the statistical differences between glioma tissues and adjacent normal brain tissues were determined by independent student's *t* test. Chi-squared test was performed to estimate the association of *let-7b* level with clinicopathological characteristics. The survival curves were graphed using Kaplan–Meier method with log-rank test. Additionally, Cox proportional hazards model was used to identify prognostic biomarkers for glioma patients. The results were estimated using hazard ratio (HR) with 95% confidence

Table 1

The relationships between *let-7b* expression and clinicopathological factors of glioma patients.

Factors	Cases (n = 127)	*Let-7b* expression		χ^2	*P* values
		Low (n = 67)	High (n = 60)		
Age, y					
≤50	68	35	33	0.097	.755
>50	59	32	27		
Gender					
Male	72	37	35	0.125	.724
Female	55	30	25		
Tumor size					
≤5 cm	77	41	36	0.019	.891
>5 cm	50	26	24		
WHO grade					
Low	85	39	46	4.872	.027
High	42	28	14		
KPS					
>90	91	42	49	5.614	.018
≤90	36	25	11		

High = WHO III and WHO IV, KPS = Karnofsky performance score, Low = WHO I and WHO II, WHO = World Healthy Organization.

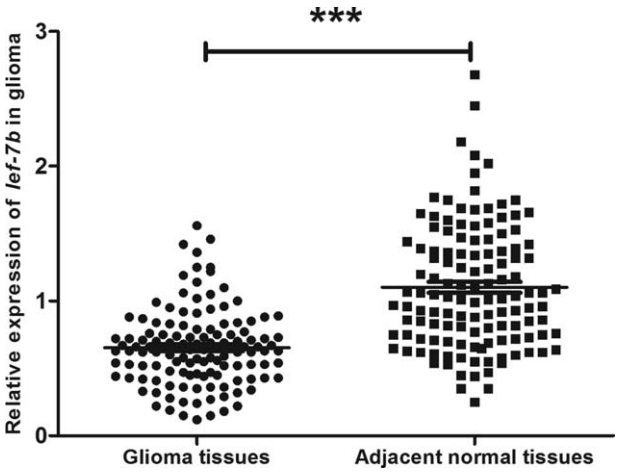

Figure 1. The relative expression of *let-7b* in glioma tissues was detected by quantitative real-time polymerase chain reaction (qRT-PCR). Results showed that *let-7b* expression was significantly reduced in glioma tissues compared with adjacent normal tissues (***: $P < .001$).

interval (CI). P values $<.05$ were considered as statistically significant.

3. Results

3.1. Down-regulation of let-7b level in glioma tissues

In this study, tissue specimens were collected from 127 glioma cases including 72 men and 55 women. The expression profile of *let-7b* was detected using qRT-PCR method. Results showed that the expression level of *let-7b* was significantly lower in glioma tissues than in adjacent normal tissues (0.65 vs 1.10, $P < .001$) (Fig. 1).

Meanwhile, we checked the expression of E2F transcription factor 2 (E2F2) and E-cadherin levels in glioma tissues and adjacent normal tissues by Western blot. The results found that E2F2 level was significantly higher in glioma tissues than that in adjacent normal tissues, while E-cadherin level was obviously decreased in glioma tissues ($P < .05$ for both) (Fig. 2).

3.2. Association between let-7b expression and clinicopathological features of glioma patients

According to their median level of *let-7b*, the glioma patients were classified into 2 groups, including low *let-7b* expression group (n = 67) and high *let-7b* expression group (n = 60). Chi-square test demonstrated that the down-regulation of *let-7b* showed obvious association with high WHO grade ($P = .027$) and low Karnofsky performance score (KPS) ($P = .018$). There were no statistical relationships between *let-7b* expression and age, sex, or tumor size (all $P > .05$) (Table 1).

3.3. Let-7b expression was correlated with overall survival of glioma patients

Kaplan–Meier method and log-rank test were performed to evaluate the overall survival of glioma patients according to their expression patterns of *let-7b*. The curves showed that glioma patients with low *let-7b* expression were more likely to undergo shorter survival time than those with high expression (log rank test, $P < .001$, Fig. 3).

Univariate and multivariate analyses were used to determine whether *let-7b* and various clinical features were prognostic factors among glioma cases. Analysis results demonstrated that *let-7b* expression ($P < .001$; HR = 2.415; 95% CI: 1.531–3.808), and WHO grade ($P = .018$; HR = 1.704; 95% CI: 1.094–2.655) were independent prognostic indicators for glioma patients (Table 2).

4. Discussion

Glioma, especially glioblastoma, is the most destructive brain tumor in adults. Despite of great improvements in early diagnosis and treatments, the clinical prognosis of glioma patients still remains dismal, due to tumor heterogeneous.[17] Currently, several clinicopathologic variables have been used to guide treatment and predict prognosis for glioma patients, such as histopathologic grades and KPS score. However, the clinical value of these biomarkers are limited.[18] In recent years, with the development of sequencing techniques, it is commonly accepted that various genetic alterations are involved in etiology of

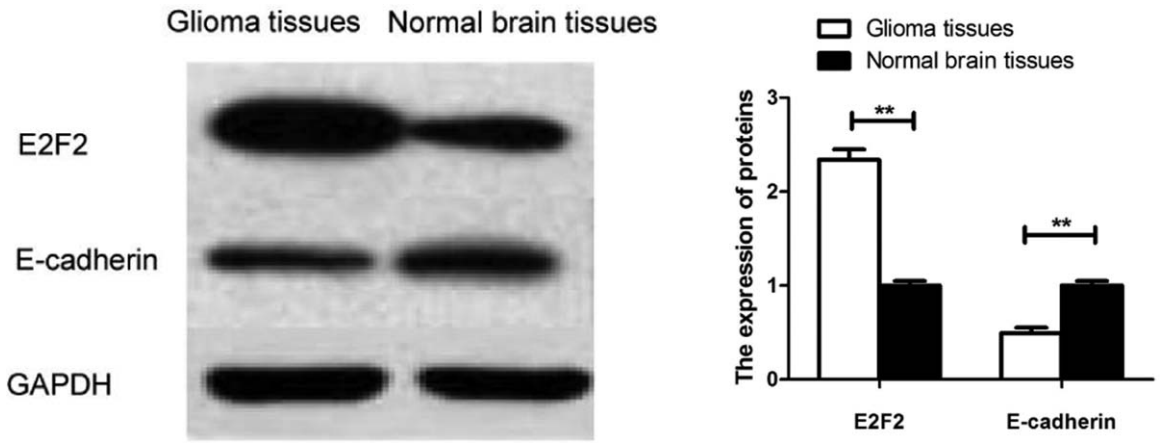

Figure 2. The expression of E2F2 and E-cadherin proteins in glioma tissues and normal brain tissues by Western blot. The result showed that in glioma tissues, the expression of E2F2 was significantly high, but E-cadherin expression level was obviously low, compared with normal brain tissues. **$P < .01$ represented the significant difference between the compared the 2.

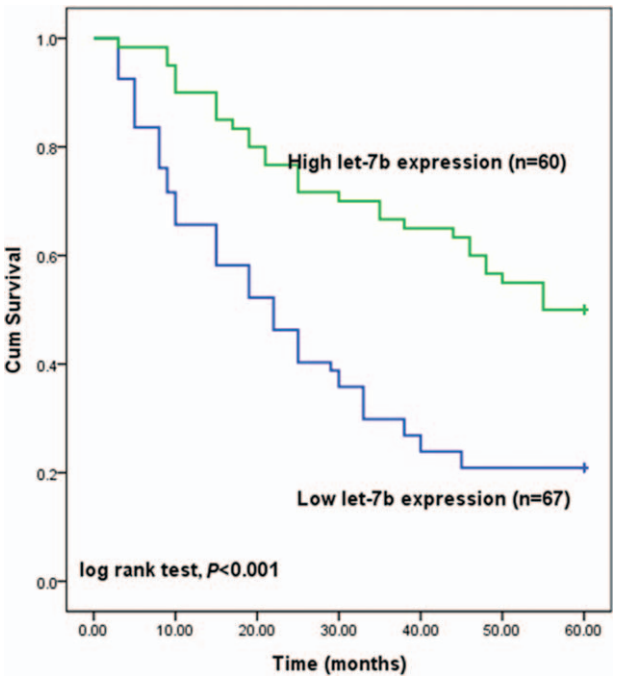

Figure 3. Kaplan–Meier curve for glioma patients according to their expression levels of *let-7b*. The glioma patients with low *let-7b* expression had a lower survival than those with high *let-7b* expression (log rank test, $P < .001$).

glioma.[19] These genetic alterations are involved in tumor development, progression, metastasis, and drug resistance that may be used for molecular biomarkers for tumor classification and treatment decision.[20] However, few molecular signatures have been confirmed and widely recognized as prognostic indicators in clinical application. Therefore, it is necessary to explore more and reliable molecular biomarkers for glioma. In current study, we explored the prognostic value of *let-7b* for glioma.

MiRNAs have the ability to regulate >30% mRNA expression, and take part in various physiological and pathological processes.[21] The expression patterns of miRNAs are specific to human diseases or cancers, suggesting their potential as cancer biomarkers.[22]*Let-7b* belongs to let-7 family, a widely accepted as a tumor suppressor miRNA family. The down-regulation of *let-7b* has also been observed in several human cancers, moreover, its expression levels show close association with tumor progression, revealing its potential as a prognostic biomarker. For examples, Schubert et al[23] found that the level of *let-7b* was significantly associated with clinical outcome parameters of prostate cancer patients that *let-7b* might be a potential independent biomarker for prostate cancer patients. Ma et al[24] reported that breast cancer patients with low *let-7b* expression had unfavorable prognosis, suggesting *let-7b* might serve as cancer suppressor gene in the development and progression of breast cancer. Based on these studies, we speculated that *let-7b* might be also a prognostic biomarker for glioma. However, the relevant studies had been rarely reported.

In current study, we investigated the correlation between the expression level of *let-7b* and overall survival of glioma patients. We found that *let-7b* expression level was significantly down-regulated in glioma tissues. In addition, the *let-7b* expression was strongly associated with WHO grade and KPS. The data revealed that *let-7b* as a tumor suppressor in glioma, and its down-regulation might contribute to malignant tumor progression. The study scheduled by Song et al[16] demonstrated that *let-7b* could inhibit malignant behaviors of glioma cells via suppressing the expression of E2F2. Tian et al[25] demonstrated that *let-7b* played anti-tumor action in glioma through directly knocking down inhibitor of nuclear factor kappa B kinase subunit epsilon expression and indirectly promoting the expression of E-cadherin. Xi et al[26] reported that *let-7b* might be a core miRNA in regulating the candidate genes involved in development of glioma. All these researches might guide our further investigations on the mechanisms of anti-tumor action of *let-7b* in glioma.

Additionally, we also investigated the prognostic significance of *let-7b* in glioma. We found that glioma patients with low *let-7b* expression had significantly shorter overall survival than those with high expression. Multivariate analysis suggested that low *let-7b* expression was an independent factor for poor overall survival in malignant glioma patients. *Let-7b* might be a valuable indicator for prognosis prediction in brain tumor patients. Despite of the encouraging results, there were still several limitations in current study. First, the sample size was relatively small. Second, the detailed biological mechanism by which *let-7b* was decreased in gliomas was still unclear. As we all know, miRNAs have no protein coding ability, and they take part in biological processes through their targets. According to the published articles, E2F2 and E-cadherin were confirmed as direct targets of *let-7b* in glioma.[16,25] In our study, we only proved that the expression of E2F2 was increased in glioma tissues, while E-cadherin showed decreased expression. Further bioinformatics analyses and luciferase reporter assay should be performed to address the targeted relationship between *let-7b* and E2F2 or E-cadherin. In addition, miRNAs are widely presented in cells

Table 2

The univariate and multivariate analyses for overall survival of factors in glioma patients.

	Univariate analysis		Multivariate analysis	
Factors	**HR (95% CI)**	***P* values**	**HR (95% CI)**	***P* values**
Age, y	0.951 (0.618–1.464)	.820	–	–
Gender	1.164 (0.751–1.802)	.497	–	–
Tumor size	1.105 (0.713–1.711)	.655	–	–
WHO grade	1.845 (1.187–2.868)	.006	1.704 (1.094–2.655)	.018
KPS	1.211 (0.758–1.935)	.424	–	–
Let-7b expression	2.518 (1.601–3.962)	<.001	2.415 (1.531–3.808)	<.001

CI = confidence interval, HR = hazard ratio, KPS = Karnofsky performance score; -: indicated no related data, WHO = World Healthy Organization.

and extracellular, and its function in human body is mediated by its targeted genes. The study carried out by Winkler et al[27] suggested that the function of *let-7b* in biological processes could be influenced by the localization of its target genes in different types of cells and subcell. Therefore, to explore the mechanisms of *let-7b* in glioma, the localization analysis was necessary for *let-7b* as well as its targets. Further well-designed study with larger sample size will be required to address the above issues.

In conclusion, the expression of *let-7b* is decreased in human glioma tissues, and negatively correlates with WTO grade and KPS score. *Let-7b* may be a candidate prognostic biomarker for glioma patients.

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Visualization: Yi Xiang.
Writing – original draft: Yi Xiang, Zhaosheng Sun.
Writing – review & editing: Yi Xiang, Zhaosheng Sun.

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