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 clinicopathological 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 sulfate-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. 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). 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. 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. 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. MiRNAs are involved in a broad of biological pathways, such as cell proliferation, apoptosis, cell cycle, differentiation, metabolism, etc. 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. 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. Recently, let-7b has been reported to act as a tumor suppressor in several human cancers, including gastric cancer, papillary thyroid carcinoma, etc. In glioma, the study carried out by Song et al 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
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^ΔΔCt method. Each test was repeated 3 times. The primer sequences were as follows: let-7b forward, 5’-GGGTGAGTGTTTGTGTTG-3’ and reverse 5’-CAGGGAAGGCTAGTGTTGTG-3’; U6 forward, 5’-CTCGCTTCGGAGCAC-3’ and reverse 5’-AACGCTTCAGAATTTCGT-3’.

2.3. Western blot

The protein of tissues was extracted and separated using 10% sodium dodecyl sulfate-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 interval.

### Table 1

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

| Factors     | Cases (n=127) | Low (n=67) | High (n=60) | x²  | P values |
|-------------|---------------|------------|-------------|-----|----------|
| 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  |               |            |             |     |          |
| ≤5cm        | 77            | 41         | 36          | 0.019 | .891     |
| >5cm        | 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.
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 let-7b expression was significantly reduced in glioma tissues compared with 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
The expression patterns of miRNAs are specific, and take part in various physiological and pathological glioma.\textsuperscript{[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.\textsuperscript{[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 miRNAs in glioma.

**Table 2**

| Factors       | HR (95% CI)       | P values | HR (95% CI)       | P values |
|---------------|-------------------|----------|-------------------|----------|
| Age, y        | 0.951 (0.618–1.464) | .220     | –                 | –        |
| 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 (0.738–1.933) | .424     | –                 | –        |
| Let-7b expression | 2.518 (1.601–3.962) | <.001    | 2.415 (1.531–3.808) | <.001    |

Cl=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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