

MiR-508-5p is a prognostic marker and inhibits cell proliferation and migration in glioma

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Abstract. – OBJECTIVE: Increasing evidence has informed that dysregulation of microRNAs (miRNAs) may contribute to carcinogenesis in human. The aim of the present study was to determine the role of miR-508-5p in glioma.

PATIENTS AND METHODS: Quantitative real-time PCR was performed to detect the expression levels of miR-508-5p in glioma and normal control tissues. *In vitro*, migration assays and a wound-healing assay were performed to determine the effects of miR-508-5p. Associations between miR-508-5p expressions and various clinicopathological characteristics were analyzed. Survival rate was determined with Kaplan-Meier. Univariate and multivariate analyses were performed to estimate the effects of the expression of miR-508-5p on survival.

RESULTS: The expression of miR-508-5p was downregulated in glioma tissues and cell lines. Low miR-508-5p expression was related to WHO grade ($p = 0.005$) and KPS score ($p = 0.013$). Moreover, Kaplan-Meier survival analysis showed that low miR-508-5p expression was significantly associated with short overall survival ($p = 0.0059$). Furthermore, multivariate analysis revealed that miR-508-5p was an independent prognostic factor for the overall survival in glioma ($p = 0.002$). Finally, forced expression of miR-508-5p could inhibit glioma cell growth and metastasis *in vitro*.

CONCLUSIONS: Taken together, our study suggested that miR-508-5p may be served as a novel prognostic factor and may lead to new treatment strategies for glioma.

Key Words:

miR-508-5p, Glioma, Prognosis, Proliferation, Migration.

significant mortality and morbidity worldwide². Despite great efforts to improve therapeutics, includes maximal surgical resection and concurrent chemo-radiotherapy, glioma has the worse prognosis among cancers with a median survival of 14 months^{3,4}. A major reason for treatment failure is tumor invasion. Thus, identifying novel biomarkers and clarifying the molecular mechanisms of the metastasis is urgently needed to improve therapeutic strategies and predict clinical outcome.

The discovery of small noncoding RNAs has revolutionized our understanding of complex gene networks. MicroRNAs (miRNAs) are small noncoding RNA molecules, 21-22 nucleotides in length⁵. Increasing evidence shows that miRNAs play essential roles in a variety of biological and pathological processes, including proliferation and metastasis^{6,7}. More importantly, downregulation of tumor suppressor miRNAs or oncogenic miRNA overexpression plays pivotal roles in tumorigenesis^{8,9}. Bao et al¹⁰ first showed that miR-508-5p may function as a tumor suppressor in glioma. However, its clinical significance and effect of metastasis for glioma is largely unknown.

In this study, we observed that miR-508-5p was downregulated in glioma tissues and cell lines. High expression level of miR-508-5 was correlated with reduced survival of glioma patients. Further cells experiment revealed that overexpression of miR-508-5 suppresses glioma cell proliferation and migration. Our study may hopefully provide a better understanding of the progression of glioma.

Introduction

Human gliomas, which originate from neural stromal cells, are the most common and malignant brain tumor in human¹. It accounts for 81% of all malignant brain tumors in adults, leading to

Patients and Methods

Patients

Human glioma tumor tissue samples were obtained from patients undergoing surgical resection at the Department of Neurosurgery, Liyin

People's Hospital. The patients' information is summarized in Table I. The present study was approved by the Ethics Committee of Liyin People's Hospital and Yanzhou People's Hospital, and each patient had written informed consent.

Cell Lines and Cell Culture

The human glioma cell lines U87, U373, U251, and A172 were obtained from the American Type Culture Collection. These cell lines were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Normal human astrocytes (NHAs) were obtained from the type culture collection of the Chinese Academy of sciences (Pudong, Shanghai, China). The complete media were refreshed every 3 days to maintain the adherent cells.

miRNA Transfection

For RNA transfection, the cells were seeded into each well of 24-well plates, and then transfected with mature miR-508-5p mimics or negative control (miR-NC) using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Extraction and Quantitative Real-Time PCR

Total RNA and miRNA detection was performed from glioma tissues as previously de-

scribed¹¹. MiRNA detection was performed using commercial assays (Applied Biosystems, Foster City, MA, USA).

CCK8 Assay for Cell Proliferation

The proliferation of ovarian cancer cells was evaluated using cell-counting Kit-8 according to the manufacturer's instruction. The absorbance of each well was measured with a microplate reader set at 450 nmol/L and 630 nmol/L. All experiments were performed in triplicate.

Wound-Healing Assay

Cells were cultured in 6-well plates and grown in RPMI 1640 with 10%FCS until. Cell monolayer approached confluence then a scratch was made using a plastic. Pipette tip to produce a wound in each well. The distance between the two sides of the wound was measured with a graduated ruler, and relative scratch breadth was determined by a ratio of average breadth.

Tumor Cell Migration Assay

Transwell 24-well plates coated with diluted Matrigel were used to determine cell invasion. The medium was supplemented with 1% heat-inactivated FBS in the upper chamber, and the lower chamber was filled with 20% FBS as a chemoattractant. The lower chamber was added with 0.6 ml of the medium with 10% FBS. After 48 h of incubation, those on the upper surface of Millicell chambers-noninvasive cells were

Table I. Associations of miR-508-5p expression with clinicopathological features of 155 glioma patients.

Clinical characteristics	miR-508-5p expression		p-value
	High, n (%)	Low, n (%)	
Age			0.694
> 45	30 (39)	28 (36)	
≤ 45	47 (61)	50 (64)	
Gender			0.274
Male	51 (66)	45 (58)	
Female	26 (34)	33 (42)	
WHO grade			0.005
I-II	30 (39)	48 (62)	
III-IV	47 (61)	30 (38)	
KPS score			0.013
> 80	48 (62)	33 (42)	
≤ 80	29 (38)	45 (58)	
Extent of resection			0.674
> 98%	31 (40)	34 (44)	
≤ 98%	46 (60)	44 (56)	
Tumor size			0.297
> 5 cm	41 (53)	48 (62)	
≤ 5 cm	36 (47)	30 (38)	

scraped with a cotton swab. Cells, which had migrated to the lower membrane, were counted using five-spot-sampling method with a microscope.

Statistical Analysis

Differences between groups were examined for statistical significance by the Student *t*-test or the χ^2 test. Survival curves were estimated by the Kaplan-Meier method. Significant variables in univariate models were further analyzed by multivariate Cox proportional hazards regression models to identify independent prognostic factors. The statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ indicated significant difference.

Results

Expression of miR-508-5p is Downregulated in Human Glioma Tissues and Cell Lines

To determine whether miR-508-5p is involved in regulation of human NPC tumorigenesis, we firstly detected miR-508-5p expression in glioma tissues and cell lines. As shown in Figure 1, we found that the expression level of miR-508-5p in glioma tissues decreased significantly compared to their matched non-tumor tissues ($p < 0.05$). Furthermore, our data showed that the glioma cell lines, including U87, U373, U251, and A172, had significantly lower levels of miR-508-5p than the control cell line (all, $p < 0.05$).

The Associations of miR-508-5p Expression with Clinicopathological Features

Table I presents the results of the correlation analysis between the relative miR-508-5p levels and clinicopathological features of glioma. The results showed that there were significant differences in WHO grade and KPS score between the high miR-508-5p expression group and the low miR-508-5p expression ($p = 0.005$, and $p = 0.013$, respectively). However, there was no significant difference in miR-508-5p; the expression was observed with age, gender, extent of resection and tumor size (shown in Table I). Our results suggested that miR-508-5p might be closely related to the development of glioma.

Impact of miR-508-5p Expression on Prognosis

To further explore the prognostic value of miR-508-5p expression in glioma, we performed Kaplan-Meier survival analysis to analyze the association between miR-508-5p expression and overall survival. Our data showed that low miR-508-5p was correlated with poor overall survival ($p = 0.0059$, Figure 2). To determine the possibility of serum miR-508-5p as an independent risk factor for poor prognosis, we performed Cox regression analysis to evaluate clinicopathological factors and the level of serum miR-508-5p expression. The results showed that miR-508-5p expression, WHO grade and KPS score were independent prognostic markers for overall survival of glioma patients (Table II).

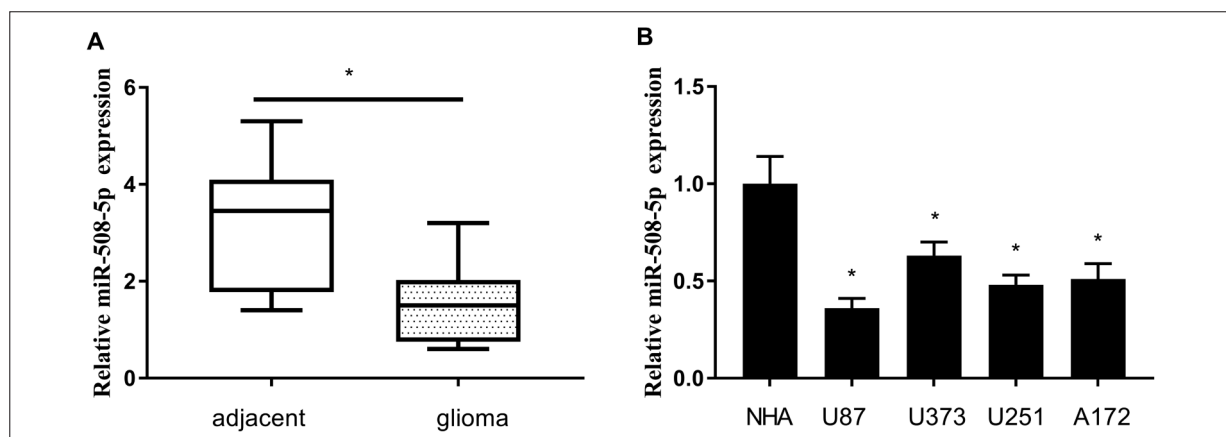


Figure 1. miR-508-5p is commonly down-regulated in glioma tissues and glioma cell lines. **A**, Levels of miR-508-5p in glioma tissues compared with their adjacent normal tissues. **B**, The relative expression levels of miR-508-5p were determined in the human glioma cell lines (U87, U373, U251, and A172) as well as in the normal human astrocyte cell line (NHA); * $p < 0.05$ compared with respective control.

Table II. Univariate and multivariate analyses of prognostic factors in glioma patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
Age	1.23	0.56-1.85	0.27	1.14	0.83-1.54	0.43
Gender	1.93	0.84-2.66	0.19	1.73	0.96-2.24	0.28
WHO grade	1.73	1.33-2.63	0.003	1.51	1.12-4.77	0.006
KPS score	2.34	1.64-4.38	0.005	2.04	1.19-5.61	0.003
Extent of resection	1.57	0.73-2.47	0.22	1.19	0.55-2.23	0.36
Tumor size	1.03	0.88-1.35	0.14	1.41	0.73-2.05	0.19
miR-508-5p	1.45	1.16-3.05	0.001	1.31	1.02-4.47	0.002

miR-508-5p Inhibited Tumor Growth and Progression

As we showed that miR-508-5p was significantly downregulated in both glioma tissues and cell lines, we further studied the functional role of miR-508-5p in glioma. We transfected glioma cell lines U251 with miR-508-5p mimics. RT-PCR showed that miR-508-5p was significantly overexpressed in U251 cell lines after transient transfection (Figure 3A). Next, we found miR-508-5p overexpression results in decreased cell growth by CKK-8 (Figure 3B). Then, wound healing assay and transwell assay were performed to examine the biological significance of miR-508-5p in glioma metastasis. As shown in Figure 3C, we found that cell invasion capacity was significantly decreased when the glioma cells were transfected with miR-508-5p mimics. In transwell assay, the cells were observed after transfection with miR-508-5p or miR-NC, we found that miR-508-5p overexpression inhibited glioma cell migration (Figure

3D). The results indicated that miR-508-5p suppressed the migration and invasion of glioma U251 cells.

Discussion

Gliomas are among the most frequent and aggressive cerebral tumors¹². Identification of possible diagnostic and prognostic biomarkers may play pivotal roles in the treatment and improvement of prognosis. In recent years, more researchers believed that dysregulation in miRNAs was involved in tumor development in many different tumor types and could be used to develop as biomarkers and prognosis factors^{13,14}. In the present study, our attention focused on miR-508-5p.

MiR-508-5p is a newly found miRNA. Up to date, there are only a few researches on its function. Liu et al¹⁵ reported that high miR-508-5p levels could be independent indicators for poor survival of patients with chronic heart failure. Wu et al¹⁶ showed that miR-508-5p was downregulated in hepatocellular carcinoma tissues when compared to the non-tumorous tissues and miR-508-5p could suppress cell proliferation and tumor growth by directly downregulating MESDC1 expression in hepatocellular carcinoma progression. Also, Shang et al¹⁷ found that miR-508-5p could directly suppress the expression of ABCB1 and ZNRD1, resulting in enhancement of cancer cell resistance to multiple chemotherapeutics *in vitro* and *in vivo*. They also identified that low miR-508-5p expression predicted poorer overall survival in patients with gastric cancer. Previously, Bao et al¹⁰ reported that miR-508-5p was significantly downregulated in glioma tissues, and that ectopic expression of miR-508-5p inhibits glioma cell proliferation and tumor

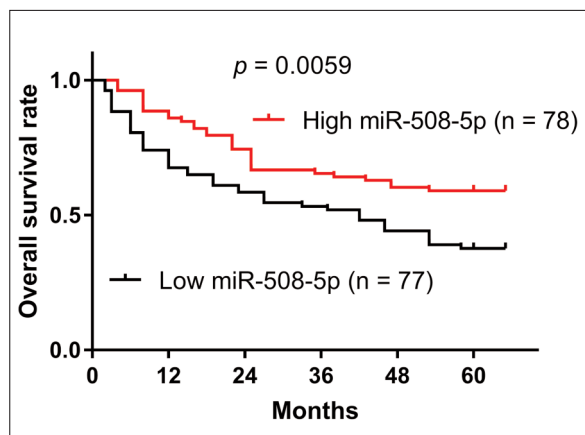


Figure 2. Kaplan-Meier curves for overall survival and miR-508-5p expression in group of 155 glioma patients.

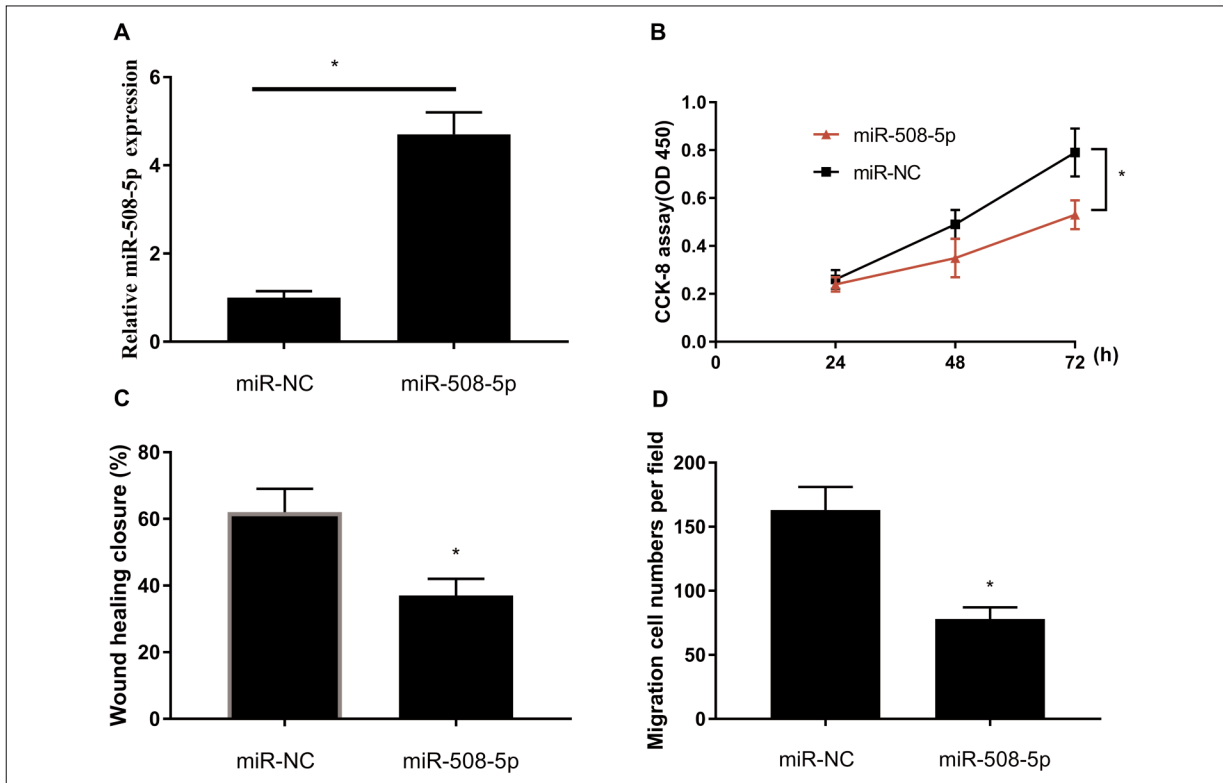


Figure 3. Overexpression of miR-508-5p suppresses glioma cell growth and metastasis. **A**, MiR-508-5p expression was significantly higher in U251 cells infected with pre-miR-508-5p than in cells infected with control constructs. **B**, CCK-8 assay was performed to examine proliferation of U251 cells at different time points. **C**, **D**, Migratory ability of miR-NC- and pre-miR-508-5p-transfected U251 cells. * $p < 0.05$.

growth by targeting GPNMB. The above results revealed that miR-508-5p may serve as a tumor suppressor in glioma. However, the association between miR-508-5p expression in gliomas and prognosis has not been reported to our knowledge.

In the present study, we demonstrated that miR-508-5p acted as a tumor suppressor in glioma, and that miR-508-5p expression was frequently downregulated in glioma tissues and cell lines. Then, we analyzed its clinicopathologic and prognostic significance. Our results showed that low miR-508-5p expression was related to WHO grade and KPS score. To explore the potential prognostic value of miR-508-5p, we performed survival analysis and log-rank test. The results suggested that low expression of miR-508-5p can be related to shorter overall survival than high expression. Furthermore, multivariate cox regression analysis acknowledged our hypothesis that decreased miR-508-5p expression was independently associated with poor survival in glioma. Finally, we assessed the effect of miR-

508-5p on proliferation, migration and invasion. Results showed that overexpression of miR-508-5p significantly inhibited proliferation, metastasis and invasion of glioma *in vitro*. The finding of the anti-invasive effect of miR-508-5p suggested that it has an important tumor-suppressive function in glioma, which partially defined the poor prognosis of the glioma patients with low miR-508-5p expression.

Conclusions

The expression levels of miR-508-5p were positively associated with overall survival, suggesting that miR-508-5p could serve as a new prognostic biomarker in glioma.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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