

## RESEARCH ARTICLE

# MiR-323-5p acts as a Tumor Suppressor by Targeting the Insulin-like Growth Factor 1 Receptor in Human Glioma Cells

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## Abstract

**Background:** MicroRNAs, small noncoding RNA molecules, can regulate mammalian cell growth, apoptosis and differentiation by controlling the expression of target genes. The aim of this study was to investigate the function of miR-323-5p in the glioma cell line, U251. **Materials and Methods:** After over-expression of miR-323-5p using miR-323-5p mimics, cell growth, apoptosis and migration were tested by MTT, flow cytometry and cell wound healing assay, respectively. We also assessed the influence of miR-323-5p on the mRNA expression of IGF-1R by quantitative real-time reverse transcriptase PCR (qRT-PCR), and on the protein levels by Western blot analysis. In addition, dual-luciferase reporter assays were performed to determine the target site of miR-323-5p to IGF-1R 3'UTR. **Results:** Our findings showed that over-expression of miR-323-5p could promote apoptosis of U251 and inhibit the proliferation and migration of the glioma cells. **Conclusions:** This study demonstrated that increased expression of miR-323-5p might be related to glioma progression, which indicates a potential role of miR-323-5p for clinical therapy.

**Keywords:** miR-323-5p - glioma - proliferation - apoptosis - IGF-1R

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## Introduction

Gliomas are the most common primary brain tumors (Jansen et al., 2010), posing a disproportionately high mortality rate of more than 70% of cases in two years after diagnosis (Furnari et al., 2007). Also, GBM, classified as grade IV by the World Health Organization, is the most frequent and aggressive type with a median survival of 14 months, which is difficult to be treated and thus recurrences could arise nearly in each case (Wen et al., 2008). Despite novel insights and advances have been devoted to therapy, the prognosis of GBM patients still remains poor (Purow et al., 2009). The high recurrence rate and mortality of GBM make it urgent to investigate molecular mechanisms involved in GBM progression so as to develop the effective clinical therapies in GBM.

IGF-1R is a transmembrane protein that contains two extracellular subunits with the ligand-binding site and two transmembrane subunits with intracellular tyrosine kinase activity (LeRoith et al., 2004). The up-regulation of IGF-1R has been found in many types of cancers including glioma, and it could protect glioma cells from apoptosis (Resnicoff et al., 1995), suggesting that the inhibition of IGF-1R could be an effective way for glioma treatment.

MicroRNAs (miRNAs) are the class of short, endogenous, non-coding RNA molecules that regulate the gene expression by binding with incomplete

complementary strand of the 3'-untranslated regions (3'-UTRs), resulting in either the inhibition of mRNA translation or the induction of mRNA degradation (Gu et al., 2009).

Interestingly, miRNAs are abnormally expressed in a wide variety of human cancers including GBM, playing the critical roles in tumorigenesis (Jovanovic et al., 2006; Kent et al., 2006; Ryan et al., 2010), but also these miRNAs have been implicated in tumor growth and carcinogenesis (Ge et al., 2013; Jiang et al., 2013). So we may characterize them as an important type of oncogenes or tumor suppressors (Esquela-Kerscher et al., 2006). Recent studies have revealed that miR-323 was one of the down-regulated miRNAs in miRNA expressional profiles of glioma samples associated with clinical outcome of glioblastoma patients (Lavon et al., 2010; Qiu et al., 2013). However, the function and potential regulatory mechanisms of miR-323 in glioma are still unknown.

In this study, we present the evidence that miR-323 acts as a tumor suppressor via reducing cell proliferation and migration, and inducing apoptosis of glioma cells. We further show that IGF-1R is a direct functional target of miR-323-5p, suggesting that the function of miR-323-5p on glioma cells may result from posttranscriptional inhibition of IGF-1R. Future research will be focused on the clinical application of miR-323-5p mediated antitumor effects on glioma.

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## Materials and Methods

### Cell culture

U251 (from ATCC) cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100u/ml penicillin and 100u/ml streptomycin at 37°C in 5% CO<sub>2</sub> cell incubator.

### MTT assay

U251 cells were seeded in 96-well plates (3×10<sup>3</sup> cells/well). After transfected with NC mimics or miR-323 mimics. The U251 cells were performed MTT assay at 24, 48 or 72 hours. MTT solution (5.0 mg/ml in phosphate-buffered saline) was 15μl/well, and cells were incubated for another 4 hours at 37°C. DMSO (150μl/well) was added and incubated for 30 minutes in 37°C to dissolve the purple formazan crystals. The plates were finally read on an ELISA plate reader at 570nm. Assays were performed in triplicate in three independent experiments.

### Plasmids construction

3'-UTR from the predicted miR-323 target gene IGF-1R was amplified from U251 cell genome by PCR and cloned into the multiple clone sites in the pmiR-Reporter vector. To produce mutant plasmid, the predicted binding sequence "CCAC" was replaced with by "TGGG" by overlapping PCR. The primers can be found in Table 1.

### Luciferase assay

U251 cells were seeded into a 24-well plate 12 hours before transfection. 50ng pIGF-3UTR or mutant plasmid was transfected with NC mimics or miR-323 mimics, and 5 ng pRL-TK was co-transfected as internal control. 48 hours after transfection, the firefly and renilla luciferase activities were assayed using the Dual-Glo Luciferase assay system from Promega according to the manufacturer's protocol.

### Real time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen), and 2ug cDNA synthesis was performed by a RevertAid First Strand cDNA Synthesis Kit (Fermentas). The SYBR Green-based real-time PCR analysis in the

**Table 1. Primers for Clones Construction and Real Time PCR**

SIGF-3UTR-S	5'-AGAAGCTAGTGCCAGCAGCTCACACTGC-3'
IGF-3UTR-AS	5'-AATAAGCTTGGCCAGGAGAGGCCAG-3'
IGF-3UTRm-S	5'-GAACCTTGATCAAGACCCAGATGGGCCAGG-3'
IGF-3UTRm-AS	5'-CCTGGGCCATCTGGGTCTTGATCAAGTTC-3'
IGF-F	5'-CCCAAGACCCAGAAGGAA-3'
IGF-R	5'-ACTCGTGAGAGCAAAGGAT-3'
GAPDH-F	5'-CTTCAACGACCACTTTGT-3'
GAPDH-R	5'-TGGTCCAGGGTCTTACT-3'

iCycler thermal cycler (Bio-Rad). The expression of miR-323-5p was assessed using the 2- $\Delta\Delta$ Ct method and normalized to the U6 RNA (Ribobio) and to  $\beta$ -actin for the IGF-1R. The primers were in Table 1.

### Western blot

Cells were harvested and lysed by RIPA for 10 min at 4°C, and then the supernatants were collected. Proteins were separated by 8% SDS-PAGE and then transferred to PVDF membranes. After blocking with 5% nonfat milk in TBST, membrane was incubated with the primary antibodies, followed by HRP-conjugated goat anti-rabbit or anti-mouse IgG, and then visualized with an ECL detection system.

### Apoptosis assay

The annexin V-FITC Apoptosis Detection Kit I was used to detect and quantify apoptosis by flow cytometry. The transfected U251 cells were harvested in cold PBS and collected by centrifugation for 5 minutes at 800rpm. Cells were resuspended in the binding buffer, stained with FITC-labeled annexin V for 5min and immediately analyzed by FACScan Flow Cytometer. Data was analyzed by Cell Quest software.

### Cell motility assays

U251 cells were seeded onto 6-well plate, and then a linear wound was made by a 10μl pipette tip across the confluent cell monolayer. Cells were grown in DMEM supplemented with 5% FBS for additional 48 hours. The cell motility was measured by photographing at five random fields at the time of wounding (time 0) and at 24 and 48 hours after wounding.

### Statistical Analysis

The data are presented as the mean+standard deviation from at least 3 separate experiments. The differences between two groups were analyzed using a Student's t test. Differences were considered to be statistically significant at \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.

## Results

### MiR-323-5p over-expression inhibited proliferation in glioma

Despite miR-323-5p had been found to be down-regulated in glioma tissues by microarray, we first detected the expression of miR-323-5p using mimics and examined its effect on cell proliferation using MTT assay. As shown in Figure 1, miR-323-5p over-regulation significantly inhibited the proliferation of U251 glioma cell line compared with miR-NC. Also, there was no significant difference between these two groups, but there were significant differences between them after 48 hours.

### Over-expression of miR-323-5p promoted glioma cell apoptosis

Based on the result of MTT assay, we further analyzed the effect of miR-323-5p on apoptosis in U251 by performing Annexin V and PI double staining at 48 hours. The early-phase and late-phase apoptotic cells were both

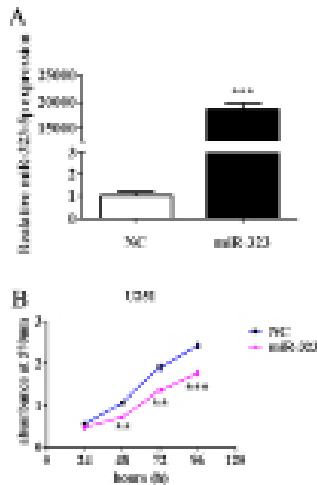
increased in U251 cell line after transfected with miR-323-5p mimics for 48 hours compared with miR-NC cells (Figure 2A). The apoptosis rate in miR-323-5p group and miR-NC group were 9.4% and 1.8%, respectively (Figure 2B). Annexin-V-FITC/PI double staining assay also showed that miR-323-5p induced apoptosis of U251 cell line, suggesting miR-323-5p could enhance the apoptosis of glioma cells.

**MiR-323-5p suppressed glioma cells migration**

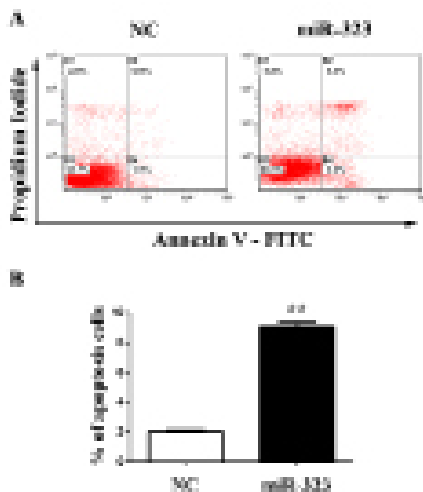
To evaluate the impact of miR-323-5p on the migration rate of U251 cells, we performed cell wound healing assay. The migration rate of miR-323-5p transfected cells was significantly reduced compared to miR-NC transfected cells both at 24 hours and 48 hours (Figure 3A, B). These results indicated that miR-323-5p could inhibit the migration of U251 cells.

**MiR-323-5p directly targeted IGF-1R in U251 cells**

As shown in Figure 4A, the binding of miR-323-

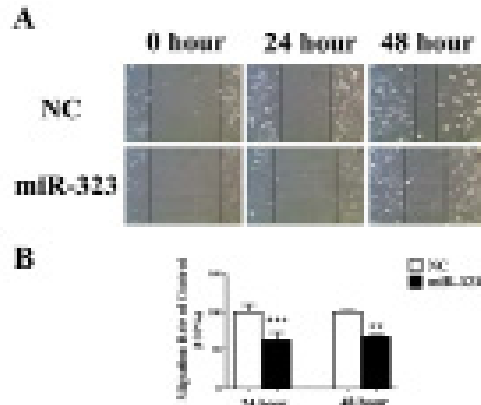


**Figure 1. The miR-323-5p expression was significantly up-regulated in U251 cells transfected with miR-323 mimics compared with NC mimics.** 48 hours after transfected with miR-323 mimics or NC mimics, the U251 cells were collected and detected miR-323 expression by qPCR

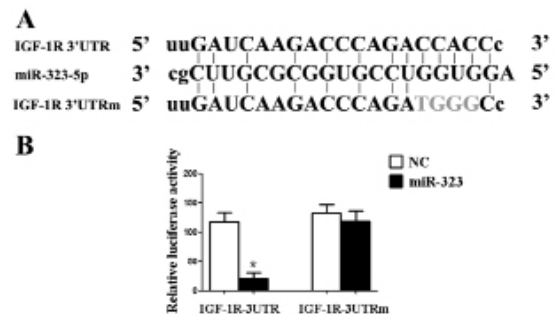


**Figure 2. Overexpression of miR-323 inhibited the propagation of U251 cells by MTT assay.** \*p<0.05, \*\*p<0.01, \*\*\*p<0.001

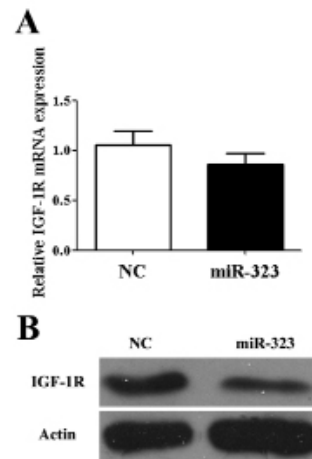
5p to IGF-1R 3'UTR was analyzed by "miRanda", an effective miRNA target prediction software. To further examine whether IGF-1R is a direct target of miR-323-5p, we sub-cloned the IGF-1R 3'UTR fragment which contained the miR-323-5p binding site ligation with



**Figure 3. MiR-323 induced apoptosis of U251 cells measured by Annexin V-FITC/propidium iodide (PI) staining.** Histograms showed that total apoptosis rate of U251 cells, containing early and late apoptosis rates, was significantly increased after transfection with miR-323 mimics. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001



**Figure 4. MiR-323 inhibited migration U251 cells.** The wound healing assay showed delayed closure in miR-323 transfected cells compared with NC at 24 hours and 48 hours time points in U251 cells. The mean percentage of wound closure at 24 hours and 48 hours after wounding were significantly decreased in U251 cells transfected with miR-323. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001



**Figure 5. MiR-323 directly targeted IGF-1R mRNA 3'-UTR.** Luciferase reporter assays showed that miR-323 suppressed the luciferase activity of wild-type IGF-1R 3'-UTR

pMIR dual luciferase reporter vectors. As shown in Figure 4B, over-expressing miR-323-5p decreased luciferase activity of IGF-1R 3'UTR, but no any effect on IGF-1R mutant 3'UTR. The results suggested that miR-323-5p specifically affected the 3'UTR of IGF-1R. Moreover, the protein level of IGF-1R was significantly down-regulated after transfected with miR-323-5p for 48 hours using western blotting analysis, but there was no significant differences of mRNA levels in all groups (Figure 5A, B). Collectively, these results demonstrated that IGF-1R was a direct target of miR-323-5p.

## Discussion

It is well known that miRNAs can act as oncogenes or tumor suppressors, of which dysregulation is a common feature in human cancers, including glioma (Lynam-Lennon et al., 2009; Chen et al., 2013; Huang et al., 2013). Also, it has been predicted that the expression of more than one-third of human protein-coding genes was thought to be regulated by miRNAs (Zhang et al., 2007).

The current studies have identified the direct link between the relative quantitative miRNA expression and its function by detecting the expression of miRNAs in clinical samples (Motoyama et al., 2009), but also the expressional profiles of miRNAs indicated that the miRNAs were obviously down-regulated or up-regulated in tumor samples compared to normal samples (Siva et al., 2009).

Significantly, MiR-21 has been demonstrated to be over-expressed in a wide variety of tumors, including human glioma, hepatocellular cancer and ovarian cancer (Chan et al., 2005; Meng et al., 2007; Nam et al., 2008). On the contrary, let-7 has been markedly reduced in lung cancers and miR-127 has been down-regulated in human bladder cancers (Takamizawa et al., 2004; Xu et al., 2013). Recently, it has also been indicated that miR-323 was markedly reduced in glioma tissues in contrast to normal tissues by miRNA array analysis (Lavon et al., 2010). Furthermore, survival analysis showed that the expression of miR-323 was increased in patients with no-recurrence or long-time progression, indicating that miR-323 may act as an important role in glioma (Qiu et al., 2013).

In present study, we have determined the role of miR-323-5p in proliferation, apoptosis and migration of glioma cells, and thus uncovered the regulatory mechanism of miR-323-5p in glioma cells. By the constructive cell models of over-expressing miR-323-5p in U251 and screened expressional efficiency of miR-323-5p, it has been shown that miR-323-5p could markedly suppress the proliferation of U251 after 48 hours, which was also consistent with flow cytometry, showing that miR-323-5p could increase the apoptosis rate of U251 compared to miR-NC group at 48 hours. In addition, cell wound healing assay revealed that miR-323-5p could limit the migration ability of U251, which altogether suggested that the over-expression of miR-323-5p could promote apoptosis of U251 and inhibit the proliferation and migration of the glioma cell.

To date, various evidences indicated that IGF-1R belongs to a family of tyrosine kinase receptors, which

plays important roles in signal transduction pathways, and thus IGF-1R and its ligand may be involved in the initiation and progression of human cancers (Gualberto et al., 2009), which also stimulate us that inhibition of the expression of IGF-1R may inhibit tumor growth and pro-apoptotic of glioma cells, and this pathway may provide new therapeutic strategies for glioma prevention and treatment by targeting IGF-1R.

So, in this study, we predicted the hundreds of targets of miR-323-5p using software, and considered IGF-1R to be a well-known oncogene, also an effective regulation target of miR-323-5p. Moreover, in according with our prediction, our present study demonstrated that miR-323-5p could significantly inhibit the activity of IGF-1R 3'UTR that containing the seed site of miR-323-5p, but also, miR-323-5p mimics could down-regulate the protein level of IGF-1R, emphasized that IGF-1R is an effective regulative target of miR-323-5p.

In summary, our results confirmed that miR-323-5p acted as a tumor suppressor gene, including inhibition of tumor cell growth and migration, promotion of cell apoptosis in glioma cells, but also targeted to the IGF-1R, proposing that IGF-1R may be a main anti-tumor target associated with miR-323-5p, and thus specific and stable over-expression of miR-323-5p in glioma tissues may be a new therapeutic strategies for glioma.

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