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
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% CO₂ cell incubator.

MTT assay
U251 cells were seeded in 96-well plates (3×10³ 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 “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 iCycler thermal cycler (Bio-Rad). The expression of miR-323-5p was assessed using the 2-ΔΔCt method and normalized to the U6 RNA (Ribobio) and to β-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-expression 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

| Table 1. Primers for Clones Construction and Real Time PCR |
|-----------------------------------------------------------|
| S l i G F - 3 U T R - S                                      |
| 5' - AGA ACT A TG T G C C A G C A G CT C A C A C T G C - 3' |
| I G F - 3 U T R - AS                                       |
| 5' - A TA A G C T T G G C C A G G A G G C C A G - 3'       |
| I G F - 3 U T R m - S                                      |
| 5' - G A A C T T G A T C A A G C C A T G G G C C C A G - 3' |
| I G F - 3 U T R m - AS                                    |
| 5' - C C T G G G G C C A T C T G G C T T G A T C A A G T T C - 3' |
| I G F - F                                                 |
| 5' - C C C A A G C A C A A G G A A - 3'                   |
| I G F - R                                                 |
| 5' - A C T C G T G C A G C A A A G G A T - 3'              |
| G A P D H - F                                             |
| 5' - C T T C A A C G A C C A C T T G T - 3'                |
| G A P D H - R                                             |
| 5' - T G T C C A G G G G T C T T A C T - 3'                |
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**MiR-323-5p 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-323-5p 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

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**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-503 inhibited the propagation of U251 cells by MTT assay. *p<0.05, **p<0.01, ***p<0.001

**Figure 3.** Overexpression of 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 transduced 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
belongs to a family of tyrosine kinase receptors, which glioma cell. U251 and inhibit the proliferation and migration of the ability of U251, which altogether suggested that the over-

assay revealed that miR-323-5p could limit the migration miR-NC group at 48 hours. In addition, cell wound healing could increase the apoptosis rate of U251 compared to consistent with flow cytometry, showing that miR-323-5p the proliferation of U251 after 48 hours, which was also shown that miR-323-5p could markedly suppress the level of IGF-1R 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.

References
Chan JA, Krichevsky AM, Kosik KS, et al (2005). MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res, 65, 6029-33. Chen G, Shen ZL, Wang L, et al (2013). Hsa-miR-181a-5p expression and effects on cell proliferation in gastric cancer. Asian Pac J Cancer Prev, 14, 3871-5. Esquela-Kerscher A, Slack FJ (2006). Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer, 6, 259-69. Furnari FB, Fenton T, Bachoo RM, et al (2007). Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev, 21, 2683-710. Ge YF, Sun J, Jin CJ, et al (2013). AntagomiR-27a targets FOXO3a in glioblastoma and suppresses U87 cell growth in vitro and in vivo. Asian Pac J Cancer Prev, 14, 963-8. Gu S, Jin L, Zhang F, et al (2009). Biological basis for restriction of microRNA targets to the 3’untranslated region in mammalian miRNAs. Nat Struct Mol Biol, 16, 144-50. Gualberto A, Pollak M (2009). Clinical development of inhibitors of the insulin-like growth factor receptor in oncology. Curr Drug Targets, 10, 923-36. Huang F, Lin C, Shi YH, et al (2013). MicroRNA-101 inhibits cell proliferation, invasion, and promotes apoptosis by regulating cyclooxygenase-2 in Hela cervical carcinoma cells. Asian Pac J Cancer Prev, 14, 5915-20. Jansen M, Yip S, Louis DN (2010). Molecular pathology in adult gliomas: diagnostic, prognostic, and predictive markers. Lancet Neurol, 9, 717-26. Jiang QQ, Liu B, Yuan T (2013). MicroRNA-16 inhibits bladder cancer proliferation by targeting cyclin D1. Asian Pac J Cancer Prev, 14, 4127-30. Jovanovic M, Hengartner MO (2006). miRNAs and apoptosis: RNAs to die for. Oncogene, 25, 6176-87. Kent OA, Mendell JT (2006). A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. Oncogene, 25, 6188-96.
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Lavon I, Zrihan D, Granit A, et al (2010). Gliomas display a microRNA expression profile reminiscent of neural precursor cells. *Neuro Oncol*, 12, 422-33.

LeRoith D, Helman L (2004). The new kid on the block (ade) of the IGF-1 receptor. *Cancer Cell*, 5, 201-2.

Lynam-Lennon N, Maher SG, Reynolds JV (2009). The roles of microRNA in cancer and apoptosis. *Biol Rev Camb Philos Soc*, 84, 55-71.

Meng F, Henson R, Weheb-Janek H, et al (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterol*, 133, 647-58.

Motoyama K, Inoue H, Takatsuno Y, et al (2009). Over- and under-expressed microRNAs in human colorectal cancer. *Int J Oncol*, 34, 1069-75.

Nam EJ, Yoon H, Kim SW, et al (2008). MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res*, 14, 2690-5.

Purow B, Schiff D (2009). Advances in the genetics of glioblastoma: are we reaching critical mass? *Nat Rev Neurol*, 5, 419-26.

Qiu S, Lin S, Hu D, et al (2013). Interactions of miR-323/miR-326/miR-329 and miR-130a/miR-155/miR-210 as prognostic indicators for clinical outcome of glioblastoma patients. *J Transl Med*, 9, 11-10.

Resnicoff M, Abraham D, Yutanawiboonchaw W, et al (1995). The insulin-like growth factor I receptor protects tumor cells from apoptosis in vivo. *Cancer Res*, 55, 2463-9.

Ryan BM, Robles AI, Harris CC (2010). Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer*, 10, 389-402.

Siva AC, Nelson LJ, Fleischer CL, et al (2009). Molecular assays for the detection of microRNAs in prostate cancer. *Mol Cancer*, 6, 8-17.

Takamizawa J, Konishi H, Yanagisawa K, et al (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res*, 64, 3753-6.

Wen PY, Kesari S (2008). Malignant gliomas in adults. *N Engl J Med*, 359, 492-507.

Xu Y, Luo S, Liu Y, et al (2013). Integrated gene network analysis and text mining revealing PIK3R1 regulated by miR-127 in human bladder cancer. *Eur J Med Res*, 18-29.

Zhang B, Pan X, Cobb GP, et al (2007). MicroRNAs as oncogenes and tumor suppressors. *Dev Biol*, 302, 1-12.