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Original Article

miR-506 inhibits the proliferation and invasion by targeting IGF2BP1 in glioblastoma

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Abstract: Increasing evidence has indicated that microRNAs (miRNAs) play an essential role in cancers. Deregulation of miR-506 was reported in several cancers. However, the expression and function of miR-506 in glioblastoma remain unclear. Our data showed that the level of miR-506 was downregulated in glioblastoma tissues and cell lines. Overexpression of miR-506 repressed cell growth, blocked G1/S transition, and suppressed cell invasion in glioblastoma cell. Moreover, IGF2BP1 was a direct target of miR-506 in glioblastoma cells. Knockdown of IGF2BP1 recapitulated the anti-proliferative and anti-invasive effects of miR-506, whereas IGF2BP1 overexpression antagonized the tumor-suppressive function of miR-506. Our data showed that miRNA-506 played a tumor suppressor gene role in human glioblastoma by regulating IGF2BP1 gene and might be a new therapeutic target of human glioblastoma.

Keywords: Glioblastoma, miRNA, miR-506, IGF2BP1

Introduction

Glioblastoma, arising from glial cells, is the most common brain tumor and represents one of the most aggressive and lethal human cancer types [1-5]. Surgery is the primary method for treatment of glioblastoma while chemotherapy is a common adjuvant therapeutic approach [6-8]. Despite the advancements in surgery, radiation and medical therapies for the treatment of glioblastoma, the average survival for glioblastoma patients is still only 14 months [5, 9-11]. Therefore, it is important to find the critical carcinogenic biomarkers and new and effective therapeutic strategies for glioblastoma [12, 13].

MicroRNAs (miRNAs) are 19 to 25 nucleotides long, single-stranded noncoding RNAs, which regulate gene expression by inhibiting translation or inducing mRNA degradation in general by binding to the 3'UTR of target mRNAs [14-19]. Accumulating reports suggest that miRNAs play important roles in cell growth, differentiation, invasion, apoptosis and migration [20-23]. Deviations from normal miRNA expression patterns play roles in the initiation and progression of many cancers, such as gastric cancer, hepatocellular carcinoma, breast cancer, osteosarcoma, lung cancer and bladder cancer [24-27]. miRNAs may function as oncogenes or tumor suppressor genes in the development of cancers [28, 29].

In the present study, the expression of miR-506 was downregulated in glioblastoma tissues and cell lines. Overexpression of miR-506 dramatically suppressed cell proliferation and invasion by regulating IGF2BP1 expression.

Materials and methods

Ethics statement

All patients written informed consent and are agreed to participate in this study. This study and consent was approved by the ethical board of the institute of The First affiliated Hospital of Zhengzhou University and complied with Declaration of Helsinki.

Tissue samples and cell lines

Glioblastoma samples and normal brain tissues were taken from our Hospital. The histological features of all specimens were diagnosis by pathologists according to the WHO criteria. None of these patients had received chemotherapy or radiotherapy before surgery. Human glioblastoma cell lines (U87, U251, LN229 and A172) were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Normal human astrocytes (NHAs) were get from Lonza and grown in the media supplemented with insulin, ascorbic acid, rhEGF, L-glutamine, GA-1000 and FBS.

RNA isolation and qRT-PCR

Total RNAs from cells or tissues were extracted using Trizol Agent (Invitrogen). The expression of miRNA and mRNA was quantified by real-time PCR on Applied Biosystems 7500 Real-Time PCR systems. Primers were designed as: IGF2BP1, forward: 5'-CCTGCTGGCTCAGTATGGT-3', reverse: 5'-GACATTCACCACTGCCGTCTC-3'; GAPDH, forward: 5'-TTGGTATCGTGGAAGGACTCA-3', reverse: 5'-TGTCATCATATTTGGCAGGTT-3'. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the individual U6 or GAPDH level.

Transient transfection

miR-506 mimics, negative control (scramble) were purchased from GenePharma (Shanghai, PR China). Cells transfected with oligonucleotides was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol at a final concentration of 30 nM.

Invasion assays

The infected cells were seeded in the top chamber with Matrigel-coated membrane, whereas the lower chamber was filled with 10% FBS as the chemoattractant and incubated for 72 h for the invasion assay. The number of invasive cells (lower side of the membrane) was fixed and stained with 0.1% crystal violet and was counted.

Cell growth and cycle assay

Cells were cultured on a 96-well plate at 3×10^4 cells/well. Viable cells were monitored by using a Cell Counting Kit-8 (Dojindo) after 1, 2 and 3 days. For the cell cycle assay, cells were trypsinized and then fixed with cold 75% ethanol overnight. The fixed cells were incubated with ribonuclease A and propidium iodide (PI) for 30 min, and then measured by flow cytometric analysis using FL2 histogram of a flow cytometer (FACSort; Becton-Dickinson, San Jose, CA, USA).

Western blotting analysis

Total protein extraction from tissues or cell. The cell lysates were prepared in lysis buffer and then separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel Electrophoresis) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk, and then incubated with the primary antibody. Antibody to IGF2BP1 was purchased from Abcam. The membranes were incubated with horseradish peroxidase-labeled corresponding immunoglobulin G (1:5,000) and analyzed using enhanced chemiluminescence-plus reagent (Pierce, Rockford, IL, USA).

Luciferase activity assay

The IGF2BP1 3'-UTR luciferase reporter construct was made by amplifying the IGF2BP1 mRNA 3'-UTR sequence. Cells were co-transfected pMIR/IGF2BP1 vector or pMIR/IGF2BP1/mut vector containing Firefly luciferase along with 0.05 μg of the pRL-TK vector (Promega) containing Renilla luciferase and 30 nM miR-506 mimic or scramble oligonucleotide. Luciferase activities were detected using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS, USA). Data were expressed as the mean ± SD and differences between groups were analyzed using Student's t test and the χ^2 test. Data were considered to be statistically significant when $P < 0.05$.

Results

The expression of miR-506 was downregulated in glioblastoma tissues and cell Lines

The expression of miR-506 was detected by qRT-PCR in 4 GBM cell lines (U87, U251, LN229

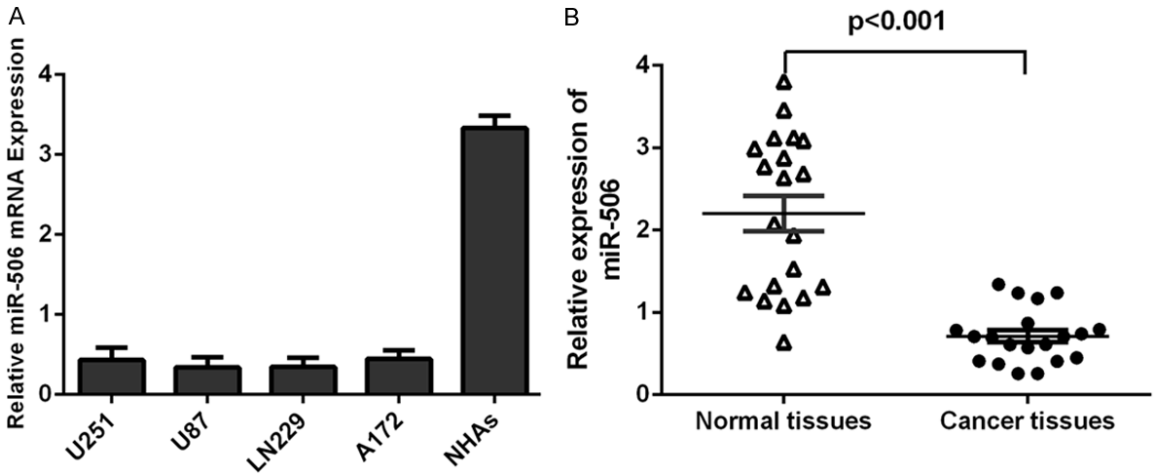

Figure 1. The expression of miR-506 was downregulated in glioblastoma tissues and cell Lines. A. Relative expression of miR-506 in glioblastoma cell lines (U87, U251, LN229 and A172) compared with the normal human astrocytes cell line (NHAs). B. Comparison of the average expression level of miR-506 between glioblastoma tissues and non-tumor tissues.

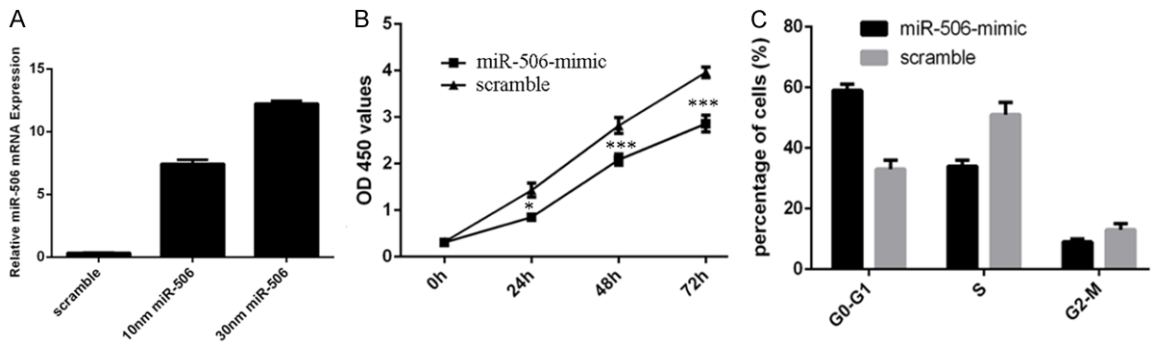

Figure 2. Overexpression of miR-506 repressed the glioblastoma cell proliferation. A. Relative expression of miR-506 in U87 cells transfected with miR-506 mimics or scramble. B. Cell proliferation was measured by CCK8 assay. U87 cells were transfected with miR-506 mimics or scramble. C. U87 cells were treated with the miRNA-506 mimics and cell cycle distributions were detected by flow cytometry 48 h later. Percentages of cells in different phases of the cell cycle are shown in the histogram. *$p < 0.05$ and ***$p < 0.001$.

and A172) and NHAs. All 4 tested GBM cell lines showed significantly lower miR-506 levels than those in the NHAs (**Figure 1A**). As shown in **Figure 1B**, miR-506 was also downregulated in glioblastoma tissues compared with that in normal tissues.

Overexpression of miR-506 repressed the glioblastoma cell proliferation

miR-506 was significantly elevated in the U87 cells after mir-506 mimic transfection (**Figure 2A**). CCK-8 assay showed that miR-506-transduced U87 cells exhibited significantly lower growth rates than scramble cells (**Figure 2B**). Cell cycle assays also showed that U87 transfected with miR-506 mimics had an obvious cell cycle arrest at the G0/G1 phase (**Figure 2C**).

Overexpression of miR-506 inhibited the glioblastoma cell invasion

Transwell invasion assays were utilized to examine the effect of miR-506 on cell invasion. As shown in **Figure 3**, the invasive potential of the U87 cells was decreased after transfection with miR-506 mimics when compared with cells transfection with scramble mimics.

IGF2BP1 was a potential target of miR-506 in glioblastoma cells

We found 3'UTR of IGF2BP1 containing the highly conserved putative miR-506 binding

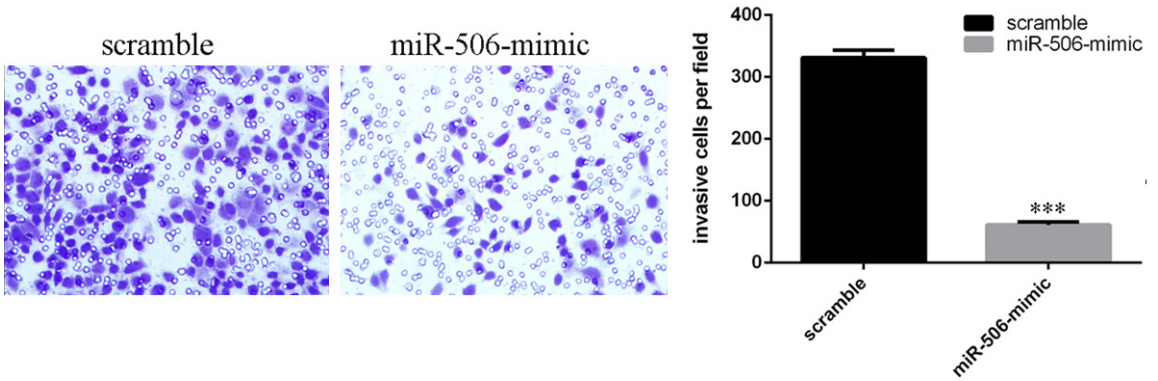

Figure 3. Overexpression of miR-506 inhibited the glioblastoma cell invasion. Cell invasion ability was assessed by an invasion assay after 48 h transfected with miR-506 mimic or scramble.

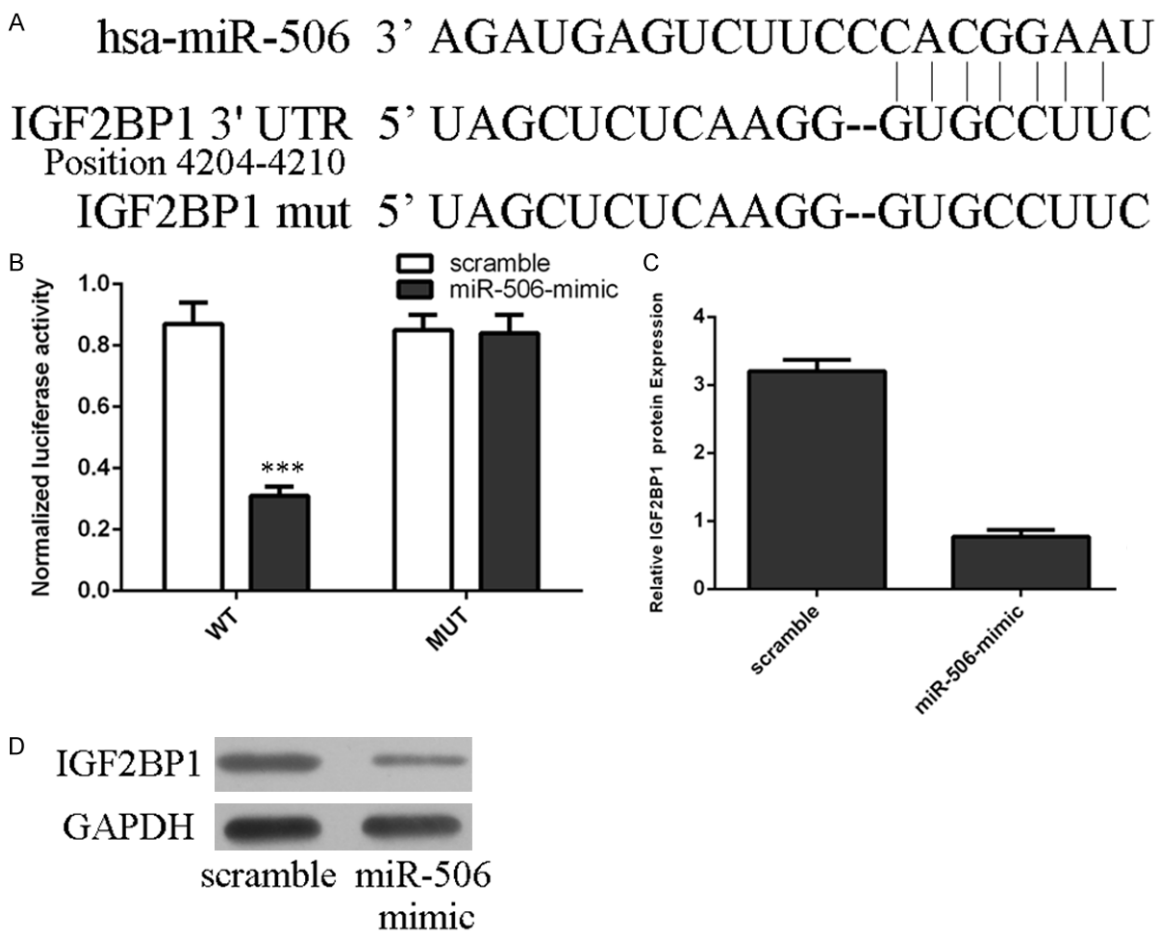

Figure 4. IGF2BP1 is a potential target of miR-506 in glioblastoma cells. A. Schematic representation of the putative binding sites in IGF2BP1 mRNA 3'UTR for miR-506. B. miR-506 mimic repressed luciferase activities controlled by wild-type IGF2BP1-3'-UTR but did not affect luciferase activity controlled by mutant IGF2BP1-3'-UTR. C. The relative mRNA expression level of IGF2BP1 was detected by real-time PCR. D. Western blot analysis was performed to evaluate the expression level of IGF2BP1 in the U87 cell, which was transfected with miR-506 mimic or scramble, respectively. GAPDH was used as a loading control.

sites using the commonly cited programs (TargetScan) (**Figure 4A**). Overexpression of miR-506 repressed the activity of pGL3-WT-IGF2BP1-3'UTR plasmid in U87 cells, without

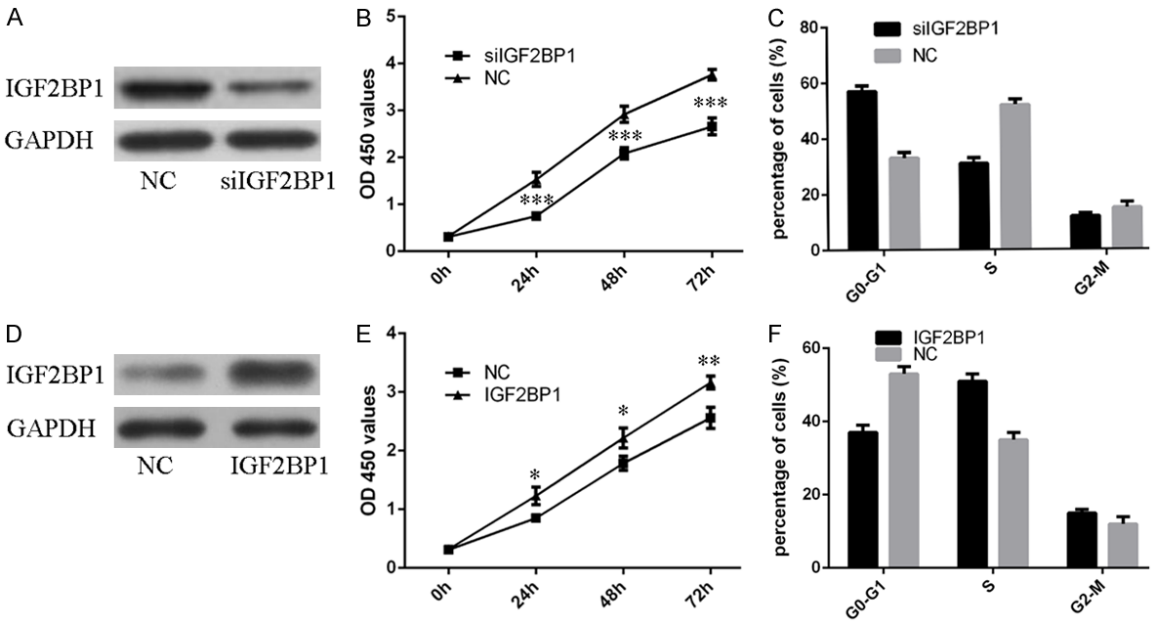

Figure 5. miR-506 suppresses glioblastoma cell growth independent of IGF2BP1 status. A. Western blot analysis was performed to evaluate the expression level of IGF2BP1 in the U87 cell, which was transfected with siIGF2BP1 or NC, respectively. GAPDH was used as a loading control. B. Cell proliferation was measured by CCK8 assay. U87 cells were transfected with siIGF2BP1 or NC. C. U87 cells were treated with the siIGF2BP1 or NC and cell cycle distributions were detected by flow cytometry 48 h later. Percentages of cells in different phases of the cell cycle are shown in the histogram. D. Western blot analysis was performed to evaluate the expression level of IGF2BP1 in the U87 cell, which was transfected with IGF2BP1 vector or NC, respectively. GAPDH was used as a loading control. E. Proliferation abilities of miR-506 overexpressing U87 cells were partially rescued after IGF2BP1 vector transfection. F. miR-506 overexpressing U87 cells were treated with the IGF2BP1 vector or NC and cell cycle distributions were measured by flow cytometry 48 h later. Percentages of cells in different phases of the cell cycle are shown.

changes in luciferase activity of pGL3-MUT-IGF2BP1-3' UTR plasmid (**Figure 4B**). Moreover, miR-506 inhibited the mRNA expression of IGF2BP1 in the U87 cells (**Figure 4C**). Ectopic expression of miR-506 repressed the protein level of IGF2BP1 in the U87 cells (**Figure 4D**).

miR-506 suppressed glioblastoma cell growth independent of IGF2BP1 status

As shown in **Figure 5A**, siIGF2BP1 repressed IGF2BP1 expression. Knockdown of IGF2BP1 inhibited U87 cells proliferation and enhanced cell cycle arrest at the G0/G1 phase (**Figure 5B** and **5C**). This result was consistent with the effect of miR-506 overexpression. Overexpression of IGF2BP1 by IGF2BP1 plasmids enhanced IGF2BP1 protein expression (**Figure 5D**). We restored the expression of IGF2BP1 in miR-506 overexpressing U87 cells by transfecting IGF2BP1 plasmids. Overexpression of IGF2BP1 promoted U87 cells proliferation (**Figure 5E**). Furthermore, the cell cycle arrest at the G0/G1 phase of U87 cells overexpressing miR-506 was partially increased after IGF2BP1 transfection (**Figure 5F**).

Discussion

Accumulated evidence has shown that miRNAs is important molecular markers in almost all types of cancers including glioblastoma [30-34]. This study demonstrated that miR-506 was downregulated in glioblastoma tissues compared with matched normal tissues. Our data also showed that the level of miR-506 was lower in 4 tested GBM cell lines than in the NHAs. Furthermore, ectopic expression of miR-506 inhibited glioblastoma cell proliferation and invasion. IGF2BP1 was found to be the target gene of miR-506 and knockdown of IGF2BP1 had the similar effects with the functions of miR-506 overexpression on the phenotypes in U87 cells. To the best of our knowledge, this is the first study to explore the miR-506 targeting IGF2BP1 expression in glioblastoma.

Previous study has demonstrated that miR-506 as a component of an X chromosome-linked miRNA cluster in the primate testis [35]. However, the expression pattern of miR-506 is

contradictory in different types of malignancies, suggesting the complex role of miR-506 in different cancers [36-38]. Previous studies have shown that miR-506 functions as a tumor suppressor in ovarian, breast and lung cancers [38-40]. However, miR-506 is demonstrated to act as an oncogene in melanomas and colon cancer [41, 42]. In our study, we demonstrated that miR-506 acted as a tumor suppressor in glioblastoma, inhibiting cell proliferation and invasion.

Using miRNA target prediction software, we found the 3'UTR of human IGF2BP1 mRNA had a complementary site for the seed region of miR-506. Luciferase activity assay was performed to validate that the IGF2BP1 was a target of miR-506. In addition, we used qRT-PCR and western blot analysis to confirm whether IGF2BP1 was a target of miR-506 in glioblastoma cell line. We found that overexpression of miR-506 in glioblastoma cells significantly reduced both the mRNA and protein levels of IGF2BP1 expression. These results highlight that IGF2BP1 is a direct target of miR-506 in glioblastoma. Furthermore, our data demonstrated that knockdown of IGF2BP1 inhibited the U87 cells proliferation and invasion, which was consistent with the functions of miR-506 overexpression in the U87 cells. Moreover, we also showed that IGF2BP1 was involved in miR-506-induced glioblastoma cell proliferation.

In conclusion, our results revealed that miR-506 was downregulated in glioblastoma tissues and cell lines. Overexpression of miR-506 dramatically suppressed cell proliferation and invasion by regulating IGF2BP1 expression. Our data suggest that miR-506 may be a promising therapeutic target for glioblastoma treatment in the future.

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