

Original Article

Overexpression of miR-221 inhibits proliferation and promotes apoptosis of human astrocytoma cells

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Abstract: microRNAs (miRNAs) play tumor-promoting roles in a variety of tumors. This study investigated the expression of miRNA-221 (miR-221) in human astrocytoma, and its effect on proliferation and apoptosis of human astrocytoma cells in vitro. miR-221 expression was detected in 10 astrocytoma tissues and 4 adjacent tissues by real-time quantitative PCR (qRT-PCR). miR-221 expression in situ was significantly higher in astrocytoma tissues than in adjacent tissues ($P<0.05$). To determine whether the upregulation of miR-221 could be associated with tumor development or progression, a synthetic miR-221 mimic was transiently transfected into U251 astrocytoma cells in vitro. qRT-PCR confirmed that the mimic significantly increased the expression of miR-221 in these cells. An MTT colorimetric assay indicated that proliferation was significantly higher in U251 cells transfected with miR-221 mimic than in scramble-transfected control cells ($P<0.05$). Further analysis of miR-221 transfected cells by flow cytometry revealed an altered cell cycle progression, with more cells in S and G1 phase, as well as an inhibition of apoptosis ($P<0.05$). These findings indicate that the upregulation of miR-221 in astrocytoma tissues may be associated with development or progression of these tumors. Thus, miR-221 should be explored as a potential molecular marker for the diagnosis and treatment of astrocytoma.

Keywords: miR-221, astrocytoma, cell proliferation, apoptosis

Introduction

Astrocytoma is a common neuroepithelial tumor originating in astrocytes of the white matter or grey matter of the central nervous system. These tumors account for 13.0 to 26.0% of intracranial tumors and 21.2 to 51.6% of neuroepithelial tumors, and are the most common type of glioma. Interestingly, astrocytoma occurs more frequently in males than in females (approximately 3:2 males : females). Additionally, although these tumors develop in people of all ages, it is more common in those ages 31 to 40. Finally, this cancer can occur in any region of the central nervous system [1]. It is important to note that, according to the 2000 World Health Organization Classification of Tumors, astrocytoma is divided into four grades (Grade I-IV) and Grades II-IV are malignant and have a poor prognosis [2]. Thus, improving the prognosis for patients with these tumors requires a good understanding of their etiology.

The roles of microRNAs (miRNAs) in cancer etiology are increasingly evident. These endogenous, non-coding, single-stranded, short RNAs, which are highly conserved in evolution, regulate gene expression [3]. In inhibiting gene expression, miRNAs participate in the regulation of complex life processes like growth and development [4], particularly in the early embryo, as well as in cell proliferation, differentiation, apoptosis, and metabolism [5]. The abnormal expression of miRNAs is correlated with the occurrence and development of a variety of tumors [6], therefore, miRNAs can serve as diagnostic and prognostic markers. Some work has demonstrated roles for at least one miRNA, miR-181b-5p, in promoting the features of astrocytoma [7].

Another miRNA, miR-221, has been linked to features of glioblastoma [8, 9], though it remains unknown whether it contributes to the development of astrocytoma. miR-221 plays an important role in regulating gene expression in cells involved in angiogenesis and erythroid dif-

ferentiation [10, 11] as well as cell proliferation and tumor formation [12, 13]. To determine whether miR-221 plays a role in the development of astrocytoma, this study investigated expression levels of miR-221 in astrocytoma samples. Astrocytoma U251 cells in culture were transiently transfected with an exogenous miR-221 mimic to determine the effect of highly-expressed miR-221 upon the proliferation, cell cycle progression, and apoptosis of U251 cells, thereby exploring the possibility that miR-221 served as a potential target for gene therapy of glioma.

Materials and methods

Materials

Samples of astrocytoma tissues from 10 individuals and 4 tumor-adjacent brain tissues were obtained from the Department of Neurosurgery of Shanghai Punan Hospital. All these samples were taken from the patients having surgery for tumor resection, confirmed by pathological examination. The study was approved by the Ethics Committee for Clinical Investigation of Shanghai Punan Hospital and written informed consent was obtained from all the patients after complete explanation. Human astrocytoma U251 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science.

Synthesis of miR-221 mimic

The sequence of mature miR-221 was identified from miRBase (www.mirbase.org). The miR-221 mimic was then synthesized by GenePharma (Shanghai, China). The mimic sequence was 5'-AGCUACAUUGUCUG CUGG-UUUUC-3', and the random sequence (scramble control) was 5'-UUCUCCGAACGUGUCA-CGUTT-3'.

Cell culture and transfection

Astrocytoma U251 cells were suspended in DMEM high glucose (Gibco) plus 100 mL/L FBS (HyClone), then placed in a 5% CO₂ incubator (Thermo, 310 Direct-Heat CO₂ Incubator) at 37°C for culture. Logarithmic growth phase cells were taken and seeded on a 6-well plate with the density of 1.0×10⁶/mL. When the cells were 50-70% confluent, they were transfected

with 100 nmol/well miR-221 mimic according to the instructions in Lipofectaminetm™ 2000 (Invitrogen); 48 h later, the cells were collected for follow-up detection.

Real-time PCR (qRT-PCR) detection of miR-221 expression

Total RNA was extracted from astrocytoma tissues and cells using TRIzol (Invitrogen)-chloroform-isopropanol extraction method. NanoDrop 2000 (Thermo) was used for quantification, and then reverse transcription (RT) solution was used according to the instructions in MicroRNA Reverse Transcription Kit (TaKaRa). Quantitative analysis was performed for miR-221 by qRT-PCR using the upstream primer, 5'-AGCUACAUUGUCUGCUGGGUUUC-3', and the universal downstream primer provided in the kit. U6 was used as an internal reference; the primer sequences were: upstream, 5'-CTCGCTT-CGGCAGCACA-3', and downstream, 5'-AACGC-TTCACGAATTTGCGT-3'. The primer sequences were synthesized by Sangon Biotech (Shanghai). The reaction was performed over 40 cycles under following conditions: denaturation at 95°C for 3 min, 95°C for 10 s, and 55°C for 30 s. Each group involved 3 wells adjacent to each other, and the test was repeated 3 times. Relative expression of miR-221 was calculated using the comparative $\Delta\Delta CT$ method.

Tetrazolium-based colorimetric (MTT) assay for the detection of U251 cell proliferation

U251 cells during logarithmic growth phase were collected to be prepared into a unicellular suspension, and then seeded in a 96-well plate (4×10³ cells/well); each group involved 6 wells, and a blank control was designed (culture medium only). When the cell density reached approximately 50%, the cells were transfected with the control scramble sequence or miR-221 mimic. At 0, 24, 48, 72 and 96 h, 20 μ L (5 mg/mL) of MTT solution (Promega) were added to each well; cells were cultured for another 4 h. Then 150 μ L of DMSO were added to each well, and the mixture was vibrated for 10 min to make purple crystals dissolve completely. A microplate reader (Thermo) was used to detect the absorbance (A570) of each well, then the maximum and minimum were removed, and other data were measured using GraphPad Prism 5 to draw cell growth curves. The test was repeated 3 times.

miR-221 in astrocytoma cells

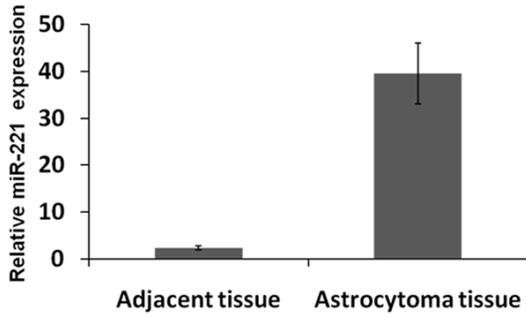


Figure 1. Expression of miR-221 in astrocytoma tissues (n=10) and tumor-adjacent brain tissues (n=4). Expression was detected using quantitative PCR. Expression depicted as mean \pm S.D. of experiments performed in triplicate.

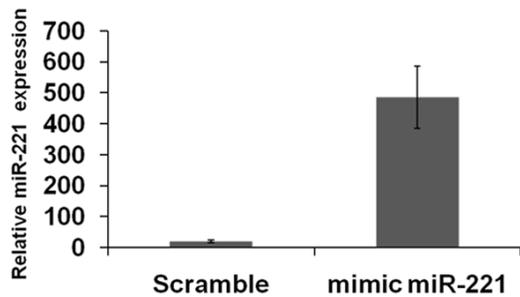


Figure 2. Transfection efficiency of miR-221 mimic in U251 cells. U251 astrocytoma cells in culture were transfected with miR-221 mimic or scramble RNA, and miR-221 expression was measured by quantitative PCR.

Flow cytometry (FCM) detection of cell cycle

Forty-eight hours after transfection, U251 cells were digested into a unicellular suspension using 2.5 g/L pancreatin, and then washed 3 times using 1 \times PBS. The supernatant was discarded and 1 mL of 1 \times PBS was added. After even mixture, 2 mL of absolute alcohol were added, then immediately the mix was vibrated to an even state. Next, it was sealed with sealing membranes, and fastened overnight at 4°C. Before detection, the mix was centrifuged at 800 rpm for 5 min and washed 3 times with PBS, and 100 μ L of 1 \times PBS were added to suspend the cells; 0.1 g/L RNase and 5 g/L propidium iodide (BD) were used to treat the cells at 4°C for 20 min. The cells were then filtered with a 300-mesh nylon net. The DNA content was measured using an excitation wavelength of 488 nm, and the proliferation index (PI) was calculated, which was expressed using (S+G2)/(G1+S+G2). The test was repeated 3 times.

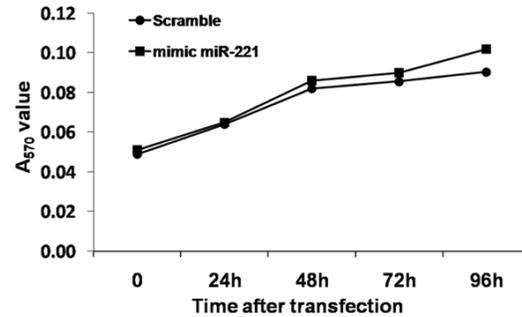


Figure 3. U251 cell proliferation promoted by miR-221 expression. U251 astrocytoma cells were transfected with scramble RNA or miR-221 mimic and proliferation was measured by flow cytometry at the indicated time points after transfection.

FCM detection of the apoptosis of U251 cells

Forty-eight hours after transfection, U251 cells were digested into a unicellular suspension using 2.5 g/L pancreatin, then resuspended with 100 mL/L FBS; the culture solution was centrifuged at 1000 rpm for 5 min, washed once using an incubation buffer, and centrifuged again at 1000 rpm for 5 min. Cells were resuspended in 100 μ L of Annexin V (Beijing 4A Biotech Co., Ltd, Beijing, China), and incubated at room temperature in the dark for 15 min. The suspension was centrifuged at 1000 rpm for 5 min to pellet the cells, washed once using an incubation solution. Cells were treated with propidium iodide for detection and incubated with vibration at 4°C away from light for 20 min. The excitation wavelength of the flow cytometer was 488 nm, a 515 nm band-pass filter was used to detect the FITC fluorescence, and propidium iodide was detected using a filter with an excitation wavelength greater than 560 nm. In the bivariate flow cytometric dot plots, the upper left quadrant showed cell debris, the left lower quadrant showed living cells, the upper right quadrant showed late apoptotic cells, and the right lower quadrant showed early apoptotic cells. The test was repeated 3 times.

Statistical analysis

SPSS17.0 was used for statistical treatment of data. Measurement data are expressed using mean \pm standard deviation ($\bar{X} \pm s$). An independent samples t-test was used to analyze the differences between various groups. The test was two-tailed, $\alpha=0.05$ was set as the level of sig-

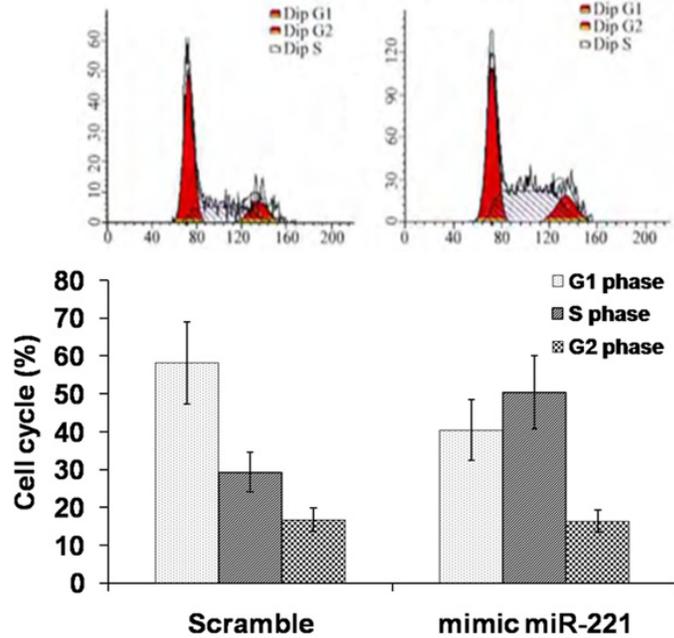


Figure 4. U251 cell cycle regulated by miR-221. Astrocytoma cells were transfected with scramble RNA or miR-221 mimic and cell cycle progression was assessed by flow cytometry.

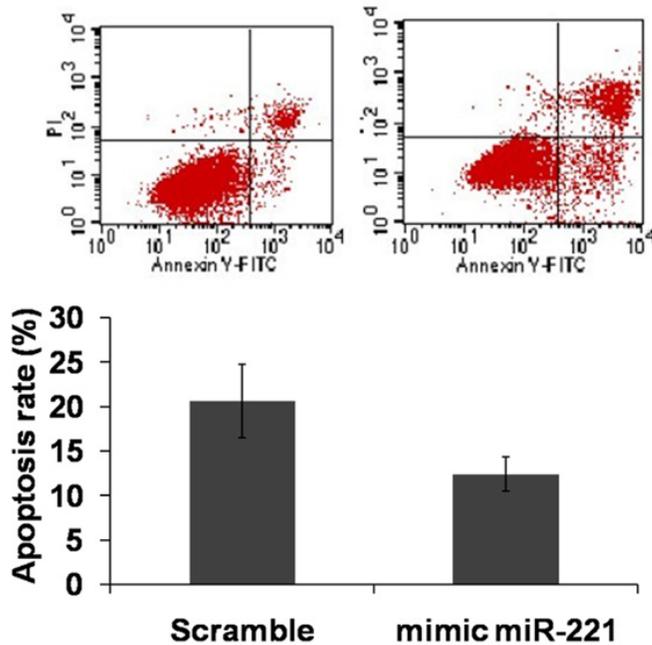


Figure 5. U251 cell apoptosis regulated by miR-221. Astrocytoma cells transfected with scramble RNA or miR-221 mimic were assessed for apoptosis by flow cytometry.

nificance and $P < 0.05$ was considered to indicate the difference was statistically significant.

Results

High expression of miR-221 in astrocytoma tissues

The expression of miR-221 was detected and compared in 10 astrocytoma tissues and 4 tumor-adjacent brain tissues (**Figure 1**). The expression level of miR-221 was significantly higher in the astrocytoma tissues ($P < 0.05$).

Expression of miR-221 in U251 cells is up-regulated by miR-221 mimic

Real-time PCR was used to detect the expression level of miR-221 in U251 astrocytoma cells in culture 48 h after transfection with miR-221 mimic. As expected the expression level of miR-221 was significantly higher in the mimic group than in the control scramble group ($P < 0.05$; **Figure 2**).

U251 cell proliferation promoted by miR-221 mimic

The increased miR-221 expression produced by transfection of the mimic miR-221 enabled further investigation of the role of this miRNA in astrocytoma progression. Cell proliferation indexes were measured by flow cytometry at various times in the scramble group and mimic group to determine whether the miRNA influences proliferative capacity of the tumor cells. The proliferative capacity of astrocytoma cells was markedly enhanced in the mimic group 48 h after transfection compared with that in the scramble group ($P < 0.05$; **Figure 3**).

U251 cell cycle progression promoted by miR-221 mimic

To determine whether cell cycle progression is also altered when miR-221 is highly expressed, flow cytometry was used to detect stages of the cell cycle.

Compared with the scramble group, U251 cells in the mimic group were significantly enriched in S phase and were decreased in G0/G1 phase ($P < 0.05$; **Figure 4**).

U251 cell apoptosis inhibited by miR-221 mimic

Finally, to determine whether astrocytoma cells undergo normal apoptotic entry in the presence of high miR-221 expression, flow cytometry was used to detect cell apoptosis. The proportion of apoptotic cells in the mimic group was significantly lower than that in the scramble group ($P < 0.05$; **Figure 5**).

Discussion

miRNAs are correlated with tumor formation, where they can play a role as tumor suppressor genes; they can also act as oncogenes. Therefore, cancer-related miRNAs are named oncomirs [14]. Of known miRNAs, 75% are located in chromosomal regions that are already known to be changed in tumors, and the genetic variation, amplification, deletion, or gene silencing of miRNA will contribute to specific oncogenesis or improve susceptibility to tumors in individuals [15]. In tumor cells, the expression level of mature miRNAs or their precursors is dysregulated, leading to downstream effects on their target RNAs.

miR-221 is mapped within a proto-oncogene cluster on chromosome Xp11.3 [16]. It displays markedly abnormal expression in multiple tumors such as primary hepatocellular carcinoma, breast cancer, thyroid cancer, and pancreatic cancer [17-20]. The role of miR-221 overexpression in these tumors appears to be to downregulate the expression of tumor suppressor proteins and cell-cycle regulators (p27^{Kip1}, p57^{Kip2}), thereby promoting the proliferation of tumor cells [21].

In this study, miR-221 was found to show high expression in situ in astrocytoma tissue compared to adjacent normal tissue, suggesting the dysregulation of the miRNA in the tumor cells. Further investigation into the contribution of miR-221 overexpression in astrocytoma by transient transfection of an astrocytoma cell line with miR-221 mimic, which significantly increased the expression of miR-221, revealed that miR-221 upregulation was associated with significantly higher proliferative capacity, cell

cycle progression, and inhibition of apoptosis. Future work to identify the targets of miR-221 will reveal the molecular pathways by which these changes occur. However, these results provide a basis for further exploration of miR-221 as a marker for the diagnosis and treatment of astrocytoma.

Disclosure of conflict of interest

None.

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