

Targeting effect of microRNA on *CD133* and its impact analysis on proliferation and invasion of glioma cells

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ABSTRACT. MiR-200b, a member of the microRNA-200 family, has been identified to be capable of suppressing glioma cell growth through targeting *CREB1* or *CD133*. However, whether miR-200b affects the biological behavior (proliferation, invasion, and migration) of glioma cells is poorly understood. The aim of this study was to evaluate the effect of miR-200b on the biological behavior of glioma cells *in vitro*. MiRNA-200b mimics, miRNA-200b inhibitor, and mimic control were transfected into conventionally cultured glioma U251 cells, followed by measuring the expression of miR-200b and *CD133* in transfected cells by RT-PCR; effect of miR-200b on *CD133* mRNA 3'-UTR luciferase activity by luciferase reporter assay; proliferation activity of transfected

U251 cells by MTT method; and changes in U251 cell invasion and migration by Transwell method after transfection. Compared to that in the miRNA-200b inhibitor, mimic control, and blank control groups, miRNA-200b expression was significantly increased and *CD133* mRNA expression was significantly decreased in the mimic miRNA-200b group in a time-dependent manner ($P < 0.05$). Meanwhile, dual luciferase reporter assay showed that miR-200b could inhibit *CD133* activity through binding to the 3'-UTR of *CD133* mRNA ($P < 0.05$). Furthermore, the proliferation activity and invasion and migration abilities of U251 cells transfected with miRNA-200b mimic were significantly decreased ($P < 0.05$). In conclusion, overexpression of miR-200b inhibited the proliferation, invasion, and migration of glioma cells possibly through targeting *CD133*.

Key words: miR-200b; CD133; Glioblastoma U251 cells; Proliferation; Invasion; Migration

INTRODUCTION

Glioma is the most common type of malignant tumor of the nervous system with a WHO classification of grade I-IV. The postoperative recurrence rate is still very high along with a poor prognosis and decreased short- and long-term survival times (Wu et al., 2011). The mean survival time of patients with malignant gliomas was only 14 months with the 5-year survival rate being less than 5% (Tabatabai and Weller, 2011). Previous studies have demonstrated that activation of proto-oncogenes and inactivation of tumor suppressor genes may induce malignant tumor cells to escape regulation, leading to uncontrolled proliferation and ultimately resulting in the development of glioma (Lei and Canoll, 2011).

MicroRNAs (miRNAs) are small non-coding RNA molecules containing a hairpin loop structure with a length of about 16-24 nucleotides, which has a strong regulatory effect on gene expression (Yamamoto et al., 2008). MiRNAs are involved in the occurrence and development of cancers through regulation of the growth and development of cells (Brandsma et al., 2008; Li et al., 2016; Wang et al., 2016). The expression of miR-200b, a member of the microRNA-200 family, has been found to be decreased in various tumor tissues and cells and capable of inhibiting the proliferation and growth of glioma cells through targeting *CREB1* or *CD133* (Mongroo and Rustgi, 2010; Zhao et al., 2014). However, whether miR-200b affects the biological behavior (proliferation, invasion, and migration) of glioma cells is poorly understood. The aim of this study was to evaluate the role of miR-200b in the biological behavior of glioma cells *in vitro* after transfection with miR-200b mimic or inhibitor.

MATERIAL AND METHODS

Test cells, reagents, and instruments

miRNA-200b, *CD133*, *β -actin*, and RT-PCR reagent kit were purchased from Takara Shuzo (Kyoto, Japan); PCR amplification instrument PTC-100, from PerkinElmer, Inc. (Waltham, MA, USA); TRIzol reagent, from Gibco (Thermo Fisher Scientific, Waltham, MA,

USA); RPMI-1640 culture medium and MTT, from Sigma-Aldrich (St. Louis, MO, USA); and carbon dioxide incubator and inverted microscope, from Sanyo (Osaka, Japan).

Experimental methods

Cell transfection

Human malignant glioma cell line U251 (Qingdao University, Shandong, China) was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 U/mL penicillin and streptomycin (Gibco) at 37°C with 5% CO₂. Tumor cells were seeded in the cell culture plate, and when the cell growth rate reached about 60% confluence, miRNA-200b mimics, miRNA-200b inhibitor, and mimic control (GenePharma, Shanghai, China) were transfected into U251 cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific). After 4-6 h, the medium was replaced. The cells were cultured for 48 h. Non-transfected U251 cells constituted the blank control group.

Detection of miRNA-200b and CD133 expression by RT-PCR in the transfected U251 cell strain

U251 cells were transfected with miRNA-200b mimics, miRNA-200b inhibitor, and mimic control for 72 h. Total RNA extraction was performed according to the manufacturer's instructions of TRIzol reagent. The concentration of total RNA was determined to calculate the OD260/280 ratio. Total RNA (200 ng) was extracted and the polyA tail of miRNA was synthesized, followed by cDNA synthesis according to the kit manufacturer's instructions (Takara Shuzo). PCR amplification was performed. Table 1 lists the primer sequences designed for the amplification process. PCR conditions were as follows: first step: 95°C, 30 s, and second step (for 40 amplification cycles): 95°C, 5 s and 60°C, 30 s. *U6* was used as the internal reference gene because of its stable expression. The relative mRNA expression of target genes (normalized as a ratio to *U6*) was calculated by the comparative Ct method using the following formula: relative expression = $2^{-\Delta\Delta C_t}$.

Table 1. Primer design.

Gene		Sequences (5'-3')
miRNA-200b	Upstream	5'-AGCCGTCAAGAGCAATAACGAA-3'
	Downstream	5'-GTGCAGGGTCCGAGGT-3'
CD133	Upstream	5'-TGGTGGGCAGTATGTTGT-3'
	Downstream	5'-GCTATTGGCATTGGTGAA-3'
U6	Upstream	5'-CTCGCTTCGGCAGCACA-3'
	Downstream	5'-AACGCTTCACGAATTTGCGT-3'

Luciferase reporter assay

Genomic DNA containing miRNA-509-5p binding sites in the 3'-UTR of *CD133* was amplified. The control vector was inserted, and the control vector carrying mutated *CD133* 3'-UTR was regarded as the control. MiRNA-200b was co-transfected using Lipofectamine 2000 as per the manufacturer's instructions. After 24 h of U251 cell transfection, results were detected by the dual luciferase reporter gene assay.

Detection of the proliferation activity of transfected HGC-27 cells

The cells transfected with miRNA-200b mimics, miRNA-200b inhibitor, and mimic control and the blank control group in the logarithmic phase of growth were collected in suspension. The suspension concentration was adjusted to 8×10^4 cells/well and incubated at 37°C with 5% CO₂. After 24, 48, and 72 h, proliferation activity of the cells was measured. MTT solution (20 µL 5 mg/mL concentration) was added to the cells, which were incubated for 4 h, followed by addition of 150 µL dimethyl sulfoxide and shaking of the mixture for 10 min. Absorbance was measured at 570 nm. The growth curve was prepared by plotting the absorbance values (Y-axis) against time (X-axis).

Detection of invasion and migration of U251 cells by the Transwell method after transfection

Invasion experiment: Matrigel was added into the Transwell chamber, and the chamber was kept overnight at 4°C. Cells were added to serum-free water and incubated at 37°C for 1 h. After transfection, the cells were seeded into the upper compartment of the Transwell chamber, and RPMI-1640 culture medium was added to the lower compartment. Giemsa staining was then performed to observe and enumerate the cells using an inverted microscope.

Migration experiment: This experiment was similar to the invasion experiment. Cells were seeded into the chamber; however, this method needed an artificial basement membrane.

Data processing

The SPSS 17.0 statistics software was used for data processing. Data are reported as means ± standard deviation. The two-way ANOVA was used for comparison of the expression levels of miRNA-200b and *CD133* and reporting the proliferation activity of the U251 cell strain after transfection with the different groups over time. Unpaired Student *t*-test was performed to compare the luciferase activity of U251 cells between control and transfected cells. $P < 0.05$ was considered as statistically significant.

RESULTS

Expression of miRNA-200b and *CD133* in U251 cell strain after transfection

The concentration of extracted RNA was 500 mg/mL (in a total volume of 50 µL) with an OD_{260/280} ratio of 1.9. The expression of miRNA-200b and *CD133* in U251 cells after transfection were detected by RT-PCR. Compared to that in the miRNA-200b inhibitor, mimic control, and control groups, we observed that miRNA-200b expression was significantly increased in the miRNA-200b mimic group, while *CD133* mRNA expression was decreased in a time-dependent manner ($P < 0.05$) (Table 2). However, in the miRNA-200b inhibitor group, miRNA-200b expression was significantly decreased and *CD133* mRNA expression was increased in a time-dependent manner ($P < 0.05$) (Table 2).

Table 2. Expression of miRNA-200b and *CD133* in the U251 cells after transfection.

Incubation period	miRNA-200b mimic	miRNA-200b inhibitor	Mimic control	Blank control
miRNA-200b				
24 h	1.102 ± 0.014 ^{abc}	0.232 ± 0.017 ^{bc}	0.703 ± 0.010	0.765 ± 0.011
48 h	1.547 ± 0.045 ^{abcd}	0.058 ± 0.009 ^{bcd}	0.697 ± 0.011	0.744 ± 0.012
72 h	1.972 ± 0.063 ^{abcde}	0.031 ± 0.001 ^{bcd}	0.656 ± 0.012	0.758 ± 0.009
<i>CD133</i> mRNA				
24 h	1.124 ± 0.023 ^{abc}	1.174 ± 0.036 ^{bc}	1.133 ± 0.025	1.151 ± 0.031
48 h	0.732 ± 0.013 ^{abcd}	1.561 ± 0.065 ^{bed}	1.141 ± 0.017	1.154 ± 0.028
72 h	0.304 ± 0.005 ^{abcde}	1.932 ± 0.087 ^{bede}	1.138 ± 0.022	1.157 ± 0.027

^aCompared with the miRNA-200b inhibitor group, the difference was statistically significant, $P < 0.05$; ^bcompared with the mimic control, the difference was statistically significant, $P < 0.05$; ^ccompared with the blank control, the difference was statistically significant, $P < 0.05$; ^dcompared with the 24 h incubation period, the difference was statistically significant, $P < 0.05$; ^ecompared with the 48 h incubation period, the difference was statistically significant, $P < 0.05$.

Targeting effect of miRNA-200b on *CD133* 3'-UTR

The luciferase reporter assay results showed that the luciferase activity of U251 cells was decreased with a statistically significant difference after co-transfection of the 3'-UTR of *CD133* and miRNA-200b mimic ($P < 0.05$) (Figure 1). Then, the binding site of 3'-UTR of *CD133* and miRNA-200b was mutated and transfected into U251 cells. As shown in Figure 1, luciferase activity increased after mutation since the mutation inhibited the binding of miRNA-200b and the 3'-UTR of *CD133*.

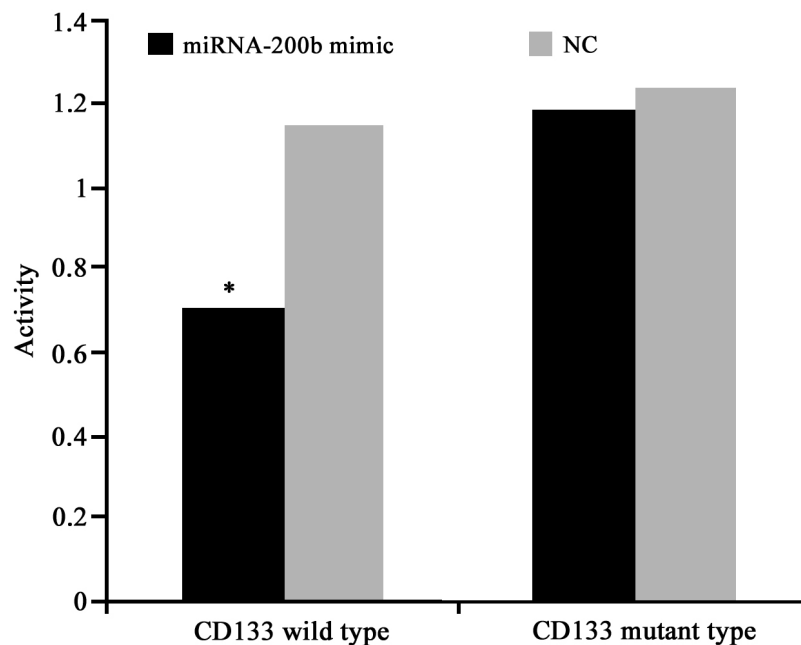


Figure 1. Targeting effect of miRNA-200b on the 3'-UTR of *CD133*. *Compared with the control group, the difference was statistically significant ($P < 0.05$).

Detection of the proliferation activity of U251 cells by MTT after transfection

The proliferation activity of U251 cells was detected by MTT after transfection. Compared to those of the miRNA-200b inhibitor, mimic control, and blank control groups, the OD value and proliferation ratio of the miRNA-200b mimic group were significantly decreased. However, the OD values and proliferation ratios of the miRNA-509-5p inhibitor, mimic control, and blank control groups increased gradually and had statistically significant values ($P < 0.05$) (Table 3).

Table 3. Proliferation activities of U251 cells after transfection.

Incubation period	miRNA-200b mimic	miRNA-200b inhibitor	Mimic control	Blank control
OD value				
24 h	0.781 ± 0.043 ^{abc}	1.029 ± 0.018 ^{bc}	1.010 ± 0.012	1.009 ± 0.016
48 h	0.421 ± 0.021 ^{abcd}	1.273 ± 0.024 ^{bcd}	1.125 ± 0.027 ^d	1.135 ± 0.024 ^d
72 h	0.231 ± 0.011 ^{abcde}	1.424 ± 0.055 ^{bcd}	1.174 ± 0.038 ^{de}	1.181 ± 0.031 ^{de}
Proliferation ratio (%)				
24 h	109 ^{abc}	128 ^{bc}	123	120
48 h	82 ^{abcd}	149 ^{bcd}	137 ^d	138 ^d
72 h	68 ^{abcde}	167 ^{bcd}	153 ^{de}	156 ^{de}

^aCompared with the miRNA-200b inhibitor group, the difference was statistically significant, $P < 0.05$; ^bcompared with the mimic control, the difference was statistically significant, $P < 0.05$; ^ccompared with the blank control, the difference was statistically significant, $P < 0.05$; ^dcompared with the 24 h incubation period, the difference was statistically significant, $P < 0.05$; ^ecompared with the 48 h incubation period, the difference was statistically significant, $P < 0.05$.

Detection of invasion and migration of U251 cells by the Transwell method after transfection

The invasion and migration abilities of U251 cells of each group were detected by the Transwell method. Results showed that the invasion and migration of U251 cells transfected with the miRNA-200b inhibitor were significantly enhanced compared to those of the control (Table 4), while the invasion and migration of U251 cells transfected with the miRNA-200b mimic was significantly decreased ($P < 0.05$) (Table 4). Results are shown in Figure 2.

Table 4. Invasion and migration of U251 cells after transfection.

	miRNA-200b mimic	miRNA-200b inhibitor	Mimic control	Blank control
Invasion	10.8 ± 4.7 ^{abc}	37.4 ± 8.6 ^{bc}	20.4 ± 5.8	21.5 ± 6.5
Migration	8.2 ± 2.1 ^{abc}	31.3 ± 7.4 ^{bc}	18.6 ± 4.1	19.2 ± 4.3

^aCompared with the miRNA-200b inhibitor group, $P < 0.05$; ^bcompared with the mimic control group, $P < 0.05$; ^cCompared with the blank control group, $P < 0.05$.

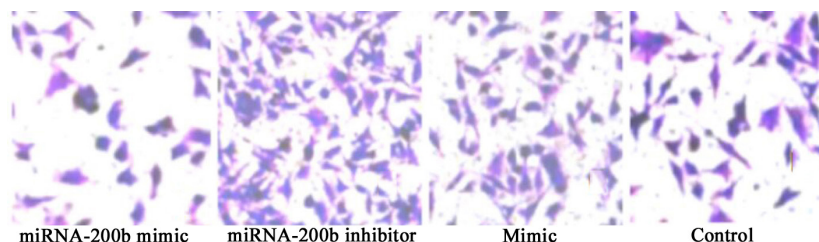


Figure 2. Detection of invasion and migration of U251 cells by the Transwell method after transfection.

DISCUSSION

Glioma is a common malignant tumor of the nervous system with high incidence and relapse rates and metastasis potentials (Sun et al., 2013; Ahmed et al., 2014). Surgery, radiotherapy, and chemotherapy are common glioma treatment strategies used in clinics. However, the survival of patients is short with a median survival period of 9-12 months (Stupp et al., 2005). MiRNA is an endogenous non-coding single stranded type of RNA that can partially or completely bind to the nucleotide sequence of the 3'-UTR of target mRNA, leading to the degradation of target mRNA, blockage of the translation process, as well as inhibition of gene expression (Kurashige et al., 2012). Several miRNAs have been found to be involved in the development of gliomas, such as miR-29a, miR-29c, miR-195, miR-200a, miR-181a, miR-181b, miR-26a, and miR-218 (Xia et al., 2010; Feng et al., 2012). MiR-200 family members such as miR-200b/c/429 and miR-200a/141 can regulate cell cycle, affect the differentiation and apoptosis of tumor cells, and participate in the occurrence and development of malignant tumors (Korpala and Kang, 2008). Previous studies have shown that *miR-200* is a potential tumor suppressor gene that may have inhibitory effects on the growth of gliomas (Mongroo and Rustgi, 2010).

In the present study, the human malignant glioma U251 cell line was cultured conventionally to be used for the study. MiRNA-200b mimics, miRNA-200b inhibitor, and mimic control were transfected into U251 cells, and the expression of miRNA-200b and *CD133* was detected by RT-PCR in the transfected cells. Compared to those in the miRNA-200b inhibitor, mimic control, and blank control groups, the miRNA-200b mimic group displayed increased expression of miRNA-200b and decreased expression of *CD133* in a time-dependent manner. However, the miRNA-200b inhibitor group presented significantly reduced miRNA-200b expression and increased *CD133* mRNA expression. The results demonstrated that there was imbalanced expression of miRNA-200b and *CD133* in the glioma cell line U251. In addition, the expression levels of *CD133* were decreased in the U251 cell line after miRNA-200b transfection, suggesting that miRNA-200b has certain inhibitory effects on *CD133* expression. In order to investigate the association between miRNA-200b and *CD133*, the luciferase reporter assay was performed. Results showed that luciferase activity of the U251 cell line was decreased after co-transfection with the 3'-UTR of *CD133* and miRNA-200b mimic, indicating that miRNA-200b negatively regulates the expression of *CD133*. *CD133* is a glycoprotein with 5 transmembrane domains, comprising 865 amino acids (O'Brien et al., 2007). It has been confirmed that tumors in cancers such as gastric cancer, liver cancer, colorectal cancer, etc., have the *CD133* phenotype, suggesting that *CD133* may be enriched on the surface of cancer stem cells, resulting in a broad spectrum of markers (Welte et al., 2013; Wen et al., 2013; Arndt et al., 2013).

In the present study, the proliferation activity of U251 cells was also measured. Compared to those in the miRNA-200b inhibitor, mimic control, and blank control groups, the OD values and proliferation ratios were significantly decreased in the miRNA-200b mimic group and increased gradually in the miRNA-509-5p inhibitor group in a time-dependent manner. Additionally, the Transwell method was used to detect the invasion and migration of U251 cells in each group. Results showed that invasion and migration were markedly enhanced in the miRNA-200b inhibitor transfected U251 cell line and significantly decreased in the miRNA-200b mimic transfected U251 cell line, indicating that the expression levels of miRNA-200b could inhibit the invasion and migration of U251 cells. These results

were consistent with results obtained in previous studies (Shimono et al., 2009; Zhao et al., 2014). However, the exact mechanism behind the function of miRNA-200b in the biological behavior of glioma cells was not investigated in our study and is the main study limitation. The mechanism needs to be explored in further studies.

In conclusion, our study demonstrated that miRNA-200b negatively regulates *CD133* expression by binding to the 3'-UTR of *CD133*. Moreover, miRNA-200b inhibited the proliferation, invasion, and migration activities of human glioma cells, suggesting that it might be involved in the occurrence and development of gliomas. Therapeutically targeting miRNA-200b might be beneficial in the prophylaxis and treatment of glioma.

Conflicts of interest

The authors declare no conflict of interest.

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