

miR-218-5p inhibits the stem cell properties and invasive ability of the A2B5⁺CD133⁻ subgroup of human glioma stem cells

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Abstract. MicroRNAs (miRs) act as oncogenes or tumor-suppressor genes, and regulate the proliferation, apoptosis, invasion, differentiation, angiogenesis and behavior of glioma stem cells, which are important in glioma development and recurrence. The present study was performed to investigate the impact of miR-218-5p on stem cell properties and invasive ability of the A2B5⁺CD133⁻ human glioma stem cell subgroup. qRT-PCR was used to detect miR-218-5p expression in non-cancerous brain and human glioma tissues, human glioma cell lines and human glioma stem cell lines. Lentivirus vectors encoding miR-218-5p and anti-miR-218-5p were constructed and stably transfected into A2B5⁺CD133⁻ SHG-139s cells. Neurosphere formation Cell Counting Kit-8 (CCK-8) and Transwell assays, immunofluorescence and qRT-PCR analyses were used to explore the role of miR-218-5p in SHG-139s cells. qRT-PCR analysis showed that miR-218-5p expression was lower in human glioma tissues and cells than in non-cancerous brain tissues and normal human astrocyte cells, and lower in A2B5⁺CD133⁻ (SHG-139s) cells than in CD133⁺ (SU2 and U87s) cells. The CCK-8 assay demonstrated that the growth curve was significantly inhibited in the miR-218-5p-SHG-139s cells compared to the miR-control, blank and anti-miR-218-5p groups. The neurosphere formation assay indicated that upregulation of miR-218-5p expression inhibited SHG-139s neurosphere formation. Immunofluorescence staining and qRT-PCR showed that miR-218-5p reduced stem cell marker (A2B5, nestin, PLAGL2, ALDH1 and Sox2)

expression compared with the controls; however, immunofluorescence staining analysis showed that upregulation of miR-218-5p expression led to no difference in CD133 expression. miR-218-5p reduced SHG-139s cell invasiveness in the Transwell assay and reduced MMP9 expression as detected in qRT-PCR and immunofluorescence analyses. All differences were statistically significant. miR-218-5p expression was lower in human glioma tissues, cells and the A2B5⁺CD133⁻ human glioma stem cell subgroup. miR-218-5p may be a tumor-suppressor gene in glioma that functions by upregulating miR-218-5p expression, which inhibits the stem cell properties and invasive properties of SHG-139s cells.

Introduction

Malignant gliomas, which are the most common primary tumors of the central nervous system, are characterized by high capacity for invasion, proliferation and recurrence. Glioblastoma (GBM), which is the highest level of glioma, is the most frequently diagnosed form and one of the least successfully treated human tumors (1); patients with GBM have a median survival of only 15 months (2). Although surgery, chemotherapy and radiation are the most effective therapies available for glioma patients, the outcomes associated with these interventions are far from satisfactory. Over the past 10 years, numerous studies of the molecular mechanisms of malignant gliomas have been performed. A recent theory has been proposed, in which cancer stem cells are the progenitors of tumor occurrence, development and recurrence (3,4). Thus, we hypothesized that the eradication of cancer stem cells may be the most effective approach to the treatment of glioma.

Stem cells have self-renewal, pluripotent and tumorigenic capacity. A number of glioma stem cell markers have been identified, such as nestin, CD133, A2B5, Sox2, NG2 and OCT3/4. Although CD133 expression was thought to be a specific stem cell marker, the existence of CD133-negative stem cells has been confirmed (5,6). Therefore, stem cells are characterized by a variety of markers and can be divided into a plurality of different subpopulations according to the expression of different markers.

Elucidation of the molecular mechanisms of glioma has progressed with the discovery of microRNAs (miRs), which

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act as oncogenes or tumor-suppressor genes in different tumors (7,8). miRs regulate the proliferation, apoptosis, invasion, differentiation, angiogenesis and stem cell behavior of cancer cells by regulating multiple signaling pathways (9-12). Numerous abnormally expressed miRs have been found in gliomas, such as miR-16, miR-650, miR-137, miR-26a, miR-29c and miR-218 (13-18). Although miR-218-5p has not been reported in gliomas, its precursor has been identified in these tumors. The expression of miR-218 was shown to be significantly lower in glioma cell lines than that in normal human astrocytes (NHA) and inhibited the invasive ability of glioma cells by regulating IKK- β and LEF1, involved in the NF- κ B and Wnt signaling pathways (19,20). Among the NF- κ B-regulated genes, matrix metalloproteinases (MMPs) are closely associated with tumor invasion (21,22). In particular, levels of matrix metalloproteinase 9 (MMP9) and 2 (MMP2) increase with tumor progression in gliomas, and are thus implicated as key enzymes required for invasion (23). Furthermore, miR-218 inhibits glioma proliferation and glioma stem cell self-renewal by targeting the polycomb group gene Bmi-1 (24). These studies demonstrated that miR-218 functions as a tumor-suppressor gene. In the present study, we aimed to investigate the role of miR-218-5p, the mature form of miR-218, in human glioma, with particular reference to the stem cell properties and invasive ability of glioma stem cells (GSCs).

The first human glioma cell line (SHG-44) identified in China was established in our laboratory in 1984 and represented a positive contribution to glioma research. In recent years, we obtained a new glioma cell line, SHG-139, from clinical specimens. Subsequently, SHG-139 glioma stem cell spheres (SHG-139s) were successfully collected after culture in neural stem cell medium (NSCM) and propagated for 10 passages. The molecular biological characteristics of SHG-139s, in addition to the pathological and histological characteristics of intracranial xenografted tumors were confirmed in our laboratory. Surprisingly, we identified SHG-139s as an A2B5⁺CD133⁺ glioma stem cell line, which is in contrast to the majority of GSCs, which express the CD133 marker (25). Using GeneChip analysis, we subsequently showed that miR-218-5p was expressed at significantly lower levels in SHG-139s compared with SHG-139 cells. Therefore, the present study was conducted to investigate the hypothesis that miR-218-5p plays an important role in maintaining the stemness and invasive ability of SHG-139s cells.

Materials and methods

Patient samples. The present study was approved by the Ethics Committee of Soochow University (China). A total of 53 glioma samples were collected from patients following the provision of written informed consent between May 2008 and January 2013. The patients underwent surgical resection of tumors at the Department of Neurosurgery of The First Affiliated Hospital of Soochow University (grade I-IV, according to the 2007 WHO classification system: grade I, 6 cases; grade II, 16 cases; grade III, 17 cases; and grade IV, 14 cases). Samples of cancer tissue were confirmed by pathology. No patients received chemotherapy or radiotherapy before surgery. The patients comprised 35 men and 18 women (mean ages at the time of surgery were 44.6 and 46.6 years, respectively). Six

adult non-cancerous brain tissue samples were obtained (with the permission of the family of each patient) from surgical resections carried out on six brain trauma patients to reduce the increased intracranial pressure. All tissue samples were collected and stored in liquid nitrogen prior to analysis.

Cells and cell culture. The human glioma cell lines SHG-44, A172, U373 and U87 and NHA normal human astrocytes were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (CBTCCAS, Shanghai, China). The human glioma cell line SHG-139 was cultured by the Brain and Nerve Research Laboratory of The First Affiliated Hospital of Soochow University. SHG-139s and U87s neurospheres were successfully collected after culture in NSCM. The A172, U373, U87, SHG-44, SHG-139 and NHA cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA). SHG-139s, U87s and SU2 were cultured in DMEM/F12 containing 20 ng/ml basic fibroblast growth factor (bFGF), 1/100 ml N2 supplement, 20 ng/ml epidermal growth factor (EGF) (all from Sigma), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen), and cultured at 37°C under a humidified atmosphere containing 5% CO₂.

Lentiviral vector construction and transfection. The RNA interference (RNAi) candidate sequences to target human miR-218-5p were designed by GeneChem Co. Ltd. (Shanghai, China) and cloned into a GV159-GFP vector. One targeting sequence (5'-ACATGGTTAGATCAAGCACAA-3') showed the best interference efficiency in 293T cells and was selected to knock down endogenous miR-218-5p in SHG-139s cells. The miR-218-2 sequences and nonsense sequence cloned into a GV259-GFP vector (GeneChem Co. Ltd.) were used as controls.

For lentivirus infection, SHG-139s cells were grown to 50-70% confluence and infected with the lentivirus at a multiplicity of infection (MOI) of 1 using an enhanced infection solution. After 8 h, the medium was replaced and the cells were incubated for a further 4-5 days at 37°C under a humidified atmosphere containing 5% CO₂. Infection efficiency was then determined based on the observation of GFP protein expression under an inverted fluorescence microscope (CKX41; Olympus, Shinjuku, Tokyo, Japan). Four groups of cells were cultured for use in subsequent studies: blank (mock infected), lentivirus-miR-218-5p, lentivirus-anti-miR-218-5p and lentivirus-miR-control group (non-sense sequence vector).

Real-time quantitative reverse transcription-polymerase chain reaction. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to analyze the expression of miR-218-5p in human glioma tissue samples and cell lines. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the instructions provided by the manufacturer. miR-218-5p expression was analyzed using the All-in-One™ miRNA qRT-PCR detection kit (GeneCopoeia, Rockville, MD, USA) according to the manufacturer's protocol. The reverse transcription and qRT-PCR kits for analysis of stem cell markers and MMP9 were purchased from Thermo Inc.,

USA. The sequences of the specific primer pairs (designed and synthesized by Sangon, Shanghai, China) used in the present study are shown in Table I. Real-time PCR conditions were as follows: preheating at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 10 sec with the LightCycler 480 (Roche Applied Science). Expression was normalized to that of U6. All qRT-PCR results were presented as Ct values, which were defined as the threshold PCR cycle number at which an amplified product was first detected. The relative quantitative value was expressed as $2^{-\Delta\Delta C_t}$, representing the expression of miR-218-5p, stem cell markers and MMP9. All specimens were analyzed in triplicate.

Cell proliferation. Cell proliferation was measured by a Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China). Cells were disaggregated by treatment with 0.05% pancreatic enzymes + 0.02% EDTA. Cells (2×10^3 /well) were seeded into a 96-well plate. Then, the cells were transfected with miR-218-5p, anti-miR-218-5p or the miR-control group at a final concentration of 50 nmol/l. CCK-8 (10 μ l) was added to each well at 24, 48, 72 and 96 h after transfection, and the plates were incubated for 4 h at 37°C. Absorbance was measured at a wavelength of 450 nm. All specimens were analyzed in triplicate.

Neurosphere formation assay. Neurosphere formation assays were performed according to previously described methods (26). Cells were disaggregated by treatment with 0.05% pancreatic enzymes + 0.02% EDTA. The cells were then added to 24-well plates (100 cells/well) through limiting dilution. Each group was evaluated in 8 different wells and the number of neurospheres was counted after 14 days in culture. Spheres containing >20 cells were scored, and the results were presented as the percentages of the number of neurospheres formed in the control groups.

Immunofluorescence staining. The four groups of SHG-139s cells (blank, lentivirus-miR-218-5p, lentivirus-anti-miR-218-5p and lentivirus-miR-control group) were mounted on glass coverslips precoated with polyornithine and laminin (Sigma, USA). The cells were fixed with 4% paraformaldehyde for 15 min and blocked with bovine serum albumin (5%; Amresco, Solon, OH, USA) for 30 min. The cells were then incubated with primary antibodies for the detection of CD133, PLAGL2, ALDH1, SOX2, A2B5, nestin and MMP9 (Abcam, Tokyo, Japan) (diluted 1:100) at 4°C overnight and then incubated with the corresponding PE-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (diluted 1:1,000) at room temperature for 1 h. Cells were then stained with DAPI to identify the nuclei. Immunofluorescence and DAPI staining were detected by laser scanning confocal microscopy (Leica Inc., Germany). The fluorescence intensity representing the target protein expression was analyzed by ImageJ image analysis software.

Transwell matrix penetration assays. Polycarbonic membrane (diameter 6.5 mm, pore size 8- μ m) Transwell filters (Costar, USA) were coated on the upper side with Matrigel (diluted 1:5, 50 μ l/well) and incubated at 37°C for 30 min to allow solidification. Harvested SHG-139s cells (1×10^5 cells/well) in

Table I. Primer sequences for qRT-PCR analysis of glioma stem cell biomarkers.

| Gene | | Primer sequence (5'→3') |
|--------|---|--------------------------|
| Sox2 | U | TGTCAAGGCAGAGAAGAGAGTG |
| | D | CCCGCCGATGATTGTTATTAT |
| PLAGL2 | U | GGGCTTGCTGACTTCTCTTCAT |
| | D | AATCCTCTTGCCTGTCTTCTGT |
| ALDH1 | U | CGCCAGACTTACCTGTCCTACT |
| | D | TCAACATCCTCCTTATCTCCTTCT |
| Nestin | U | CTTGCCTGCTACCCTTGAGAC |
| | D | GTTTCCTCCACCCTGTGT |
| GAPDH | U | ACCACAGTCCATGCCATCAC |
| | D | TCCACCACCCTGTTGCTGTA |

U, upstream; D, downstream.

200 μ l of serum-free stem cell medium (DMEM/F12, bFGF, EGF and N2) were added into the upper compartment of the chamber, and 500 μ l DMEM containing 10% FBS was added to the lower chamber. After incubation for 48 h at 37°C under a humidified atmosphere containing 5% CO₂, cells were fixed with 4% formaldehyde for 30 min and stained with 0.1% crystal violet staining for 5 min. The upper chamber was gently rinsed and wiped with a cotton swab. Random counts of six fields of vision were made by observation under an inverted microscope (Olympus Inc., Japan), with the average number of cells representing the invasive ability of the cells.

Statistical analysis. Statistical analyses were performed using SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA). Comparisons between two groups were analyzed using t-tests, and between multiple sets of data using ANOVA. P<0.05 was considered to indicate a statistically significant result.

Results

Relative expression levels of miR-218-5p in human glioma samples. qRT-PCR analysis of the relative expression levels of miR-218-5p among glioma tissue and non-cancerous brain tissue samples and glioma cell lines showed that miR-218-5p expression was lower in glioma tissue samples compared with that in non-cancerous brain tissue samples (Fig. 1A). Compared to the NHA cells, miR-218-5p expression was also lower in the glioma cell lines (Fig. 1B). In addition, miR-218-5p expression was lower in the SHG-139s (A2B5⁺CD133⁻) cells than that in the U87s and SU2 (CD133⁺) cells (Fig. 1C). All differences were statistically significant (P<0.05).

Lentivirus infection efficiency. The function of miR-218-5p in A2B5⁺CD133⁻ GSCs (SHG-139s) was investigated by increased or decreased expression mediated by infection with lentivirus-miR-218-5p or lentivirus-anti-miR-218-5p, respectively. Based on the expression of the green fluorescent protein, the transfection efficiency in the lentivirus-miR-218-5p,

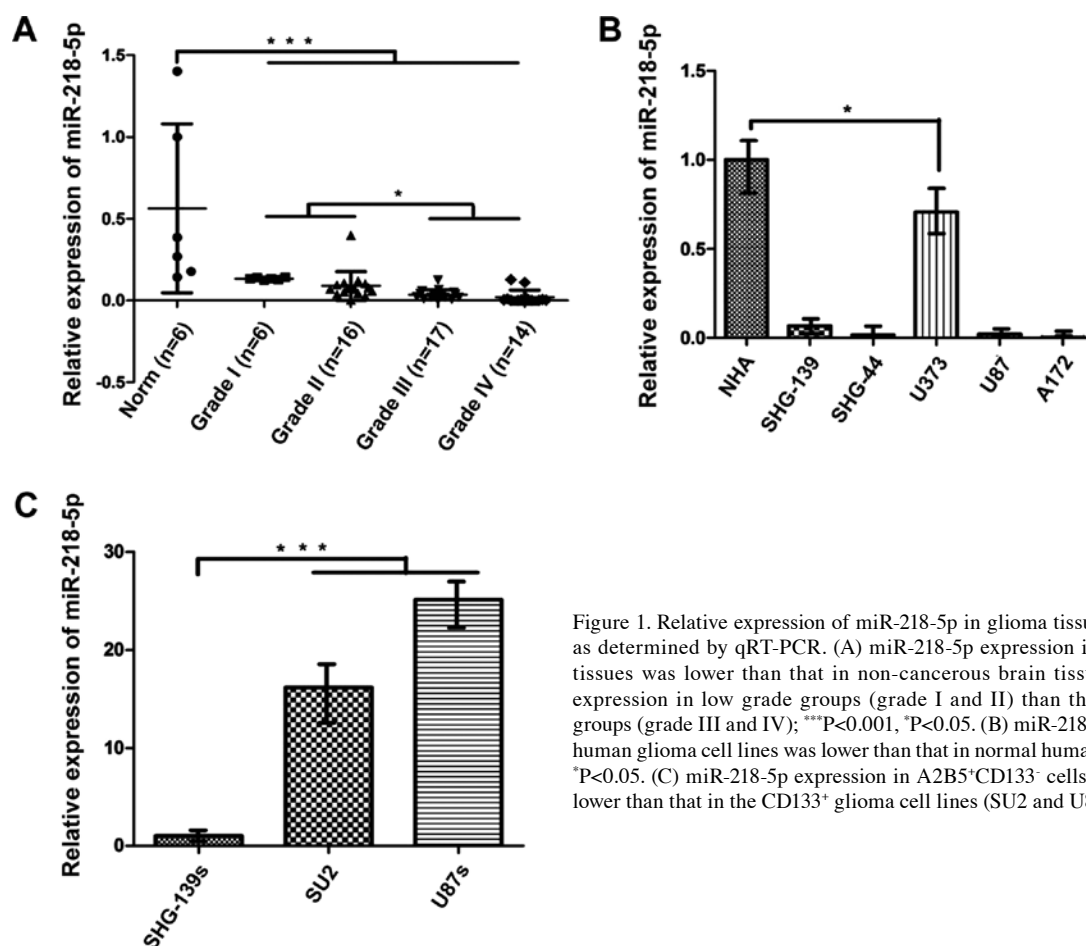


Figure 1. Relative expression of miR-218-5p in glioma tissues and cell lines as determined by qRT-PCR. (A) miR-218-5p expression in human glioma tissues was lower than that in non-cancerous brain tissues, with higher expression in low grade groups (grade I and II) than that in high grade groups (grade III and IV); *** $P < 0.001$, * $P < 0.05$. (B) miR-218-5p expression in human glioma cell lines was lower than that in normal human astrocyte cells; * $P < 0.05$. (C) miR-218-5p expression in A2B5⁺CD133⁺ cells (SHG-139s) was lower than that in the CD133⁺ glioma cell lines (SU2 and U87s); *** $P < 0.001$.

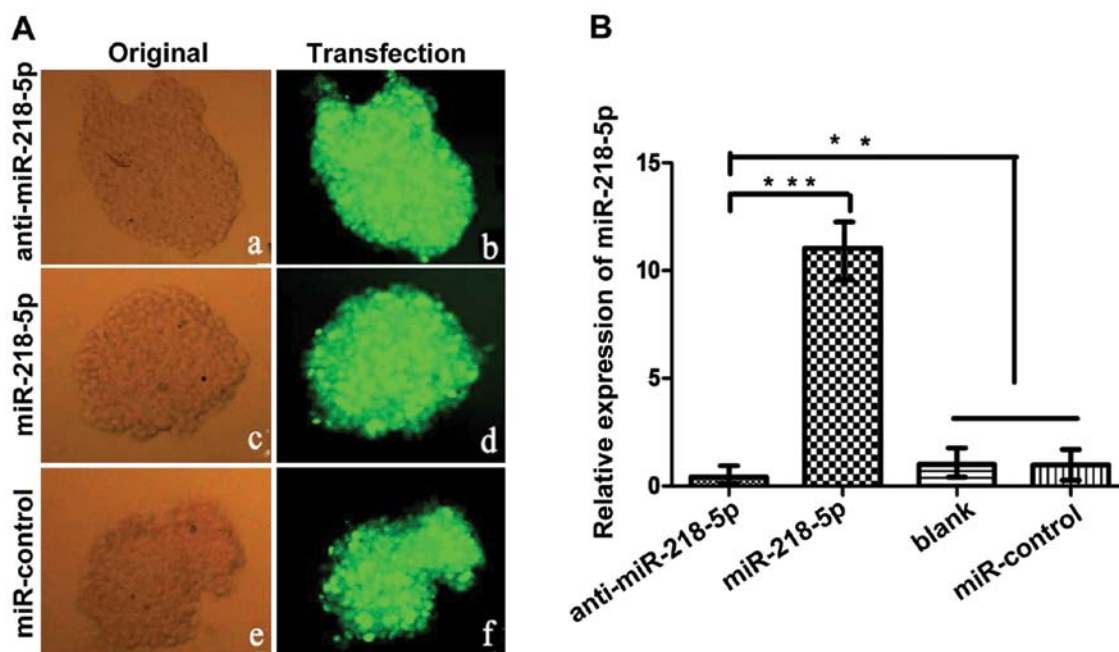


Figure 2. Successful stable transfection of obtained SHG-139s cells with recombinant lentiviruses expressing green fluorescent protein. (A) Strong fluorescence was detected in SHG-139s cells stably transfected with the lentivirus GV159-GFP vector expressing anti-miR-218-5p, miR-218-5p and an miR-control (magnification, $\times 200$). (B) miR-218-5p expression was lower in the anti-miR-218-5p group than that in the miR-218-5p group; *** $P < 0.001$, * $P < 0.05$.

lentivirus-anti-miR-218-5p and lentivirus-miR-control groups was similar (Fig. 2A). qRT-PCR analysis was used

to confirm that the expression of miR-218-5p was higher in lentiviral-miR-218-5p-SHG-139s cells than that in the

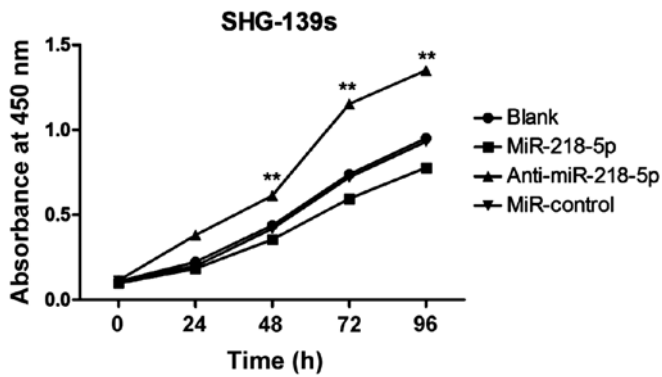


Figure 3. Upregulation of miR-218-5 reduces the growth of SHG-139s glioma stem cells. Cell survival was determined by the CCK-8 assay. Cell proliferation was markedly decreased in the SHG-139s cells after transfection with miR-218-5p. Data are shown as the mean \pm SD (n=3). Differences in the growth of the anti-miR-218-5p and miR-218-5p groups compared to the blank and miR-control groups were statistically significant, **P<0.01.

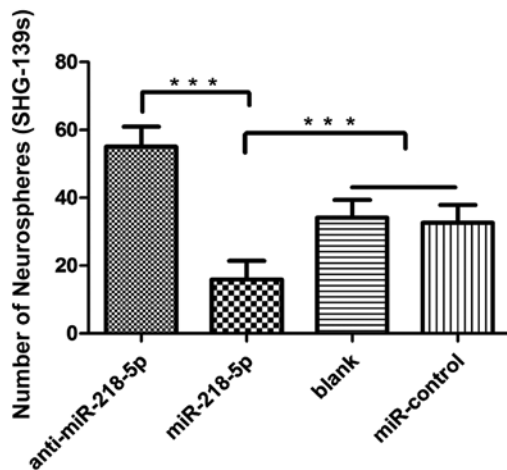


Figure 4. SHG-139s sphere formation assays after upregulation and downregulation of miR-218-5p. The number of neurospheres (SHG-139s) that formed in the anti-miR-218-5p group was significantly higher than those in the blank and miR-control groups, while the number formed in the miR-218-5p group was significantly lower; ***P<0.001.

non-infected SHG-139s cells, with negligible expression in the lentiviral-anti-miR-218-5p-SHG-139s cells (Fig. 2B).

Upregulation of miR-218-5p suppresses glioma cell proliferation *in vitro*. As shown in Fig. 3, the CCK-8 assay was performed to detect the effects of miR-218-5p on SHG-139s cell growth *in vitro*. The data demonstrated that the growth curve was significantly inhibited in the miR-218-5p-SHG-139s cells compared to the miR-control, blank and anti-miR-218-5p groups.

miR-218-5p inhibits stemness maintenance in SHG-139s cells. In the present study, the neurosphere formation assay indicated that upregulated miR-218-5p expression inhibited SHG-139s neurosphere formation (Fig. 4). qRT-PCR analysis of lentivirus infected SHG-139s cells showed lower expression of stem cell markers in the lentivirus-miR-218-5p group than that in the blank and lentivirus-miR-control groups, while the highest expression was detected in the lentivirus-anti-miR-218-5p

group (Fig. 5A). With the exception of CD133, these results were confirmed by immunofluorescence staining (Fig. 5C-G, P<0.05); no significant difference in CD133 expression between the groups was detected by immunofluorescence staining (Fig. 5B, P>0.05).

miR-218-5p inhibits the invasive ability of SHG-139s cells. Transwell matrix penetration assays showed that upregulation of miR-218-5p markedly reduced the invasive ability of SHG-139s cells compared with this ability in the control groups (Fig. 6A). qRT-PCR analysis and immunofluorescence staining results showed that the expression of MMP9 was higher in the lentivirus-anti-miR-218-5p group than that in the blank and lentivirus-miR-control groups, with the greatest effect observed in relation to the lentivirus-miR-218-5p group (Fig. 6B and C). All data differences were statistically significant (P<0.05).

Discussion

Cell culture is one of the most important cancer research methods, with a history of more than 60 years to date (27). After culturing the first human glioma cell line SHG-44 generated in China in our laboratory, more recently, we successfully used NSCM to culture the A2B5⁺CD133⁻ glioma cell line SHG-139s. In the present study, SHG-139s cells were used to investigate the therapeutic effect of miR-218-5p on A2B5⁺CD133⁻ human GSC subsets. Although there are numerous studies of GSCs, investigations of human GSC subpopulations, including the A2B5⁺CD133⁻ subset, are rare. Ogden *et al* (28) and Tchoghandjian *et al* (29) used flow cytometry to identify three groups of stem cells (A2B5⁺CD133⁺, A2B5⁺CD133⁻, A2B5⁻CD133⁻) obtained from fresh glioma tissues according to their expression of the stem cell markers CD133 and A2B5. The A2B5⁺CD133⁺ and A2B5⁺CD133⁻ subsets exhibited tumorigenic ability, while the A2B5⁻CD133⁻ subset did not, implying that the occurrence, development and recurrence of gliomas is related to the existence of A2B5⁺CD133⁺ and A2B5⁺CD133⁻ glioma cells. According to Beier *et al*, the molecular profile of CD133-positive and CD133-negative tumor cells were different; CD133⁻ tumor cells grew adherently *in vitro* and were driven by CD133⁺ tumor cells that fulfilled stem cell criteria. Both subtypes were similarly tumorigenic in nude mice *in vivo*. Clinically, CD133⁻ GBMs were characterized by a lower proliferation index (Ki-67), whereas glialfibrillary acidic protein staining (GFAP) was similar (6). However, SHG-139s cells grew neurosphere-like and SHG-139s cells were positive for A2B5, nestin and neuron-glial antigen 2 (NG2), and negative for vimentin and IDHR132H (isocitrate dehydrogenase), and cells rarely stained for CD133. In SHG-139s xenografts, GFAP and S-100 were expressed, while CD133 was not detected and a few A2B5⁺ cells were found at tumor edges; typical oligodendrogliomas were obtained. A2B5 is a surface glycoside that marks O-2A neural progenitor cells (30), and has been implicated as a marker of poor prognosis, although the statistical evaluation was not rigorous (31,32). Thus, the function of A2B5⁺CD133⁻ GSCs remains to be clarified.

We confirmed that the expression of miR-218-5p in SHG-139s cells was very low (data not shown). Other researchers reported low expression of miR-218, the precursor

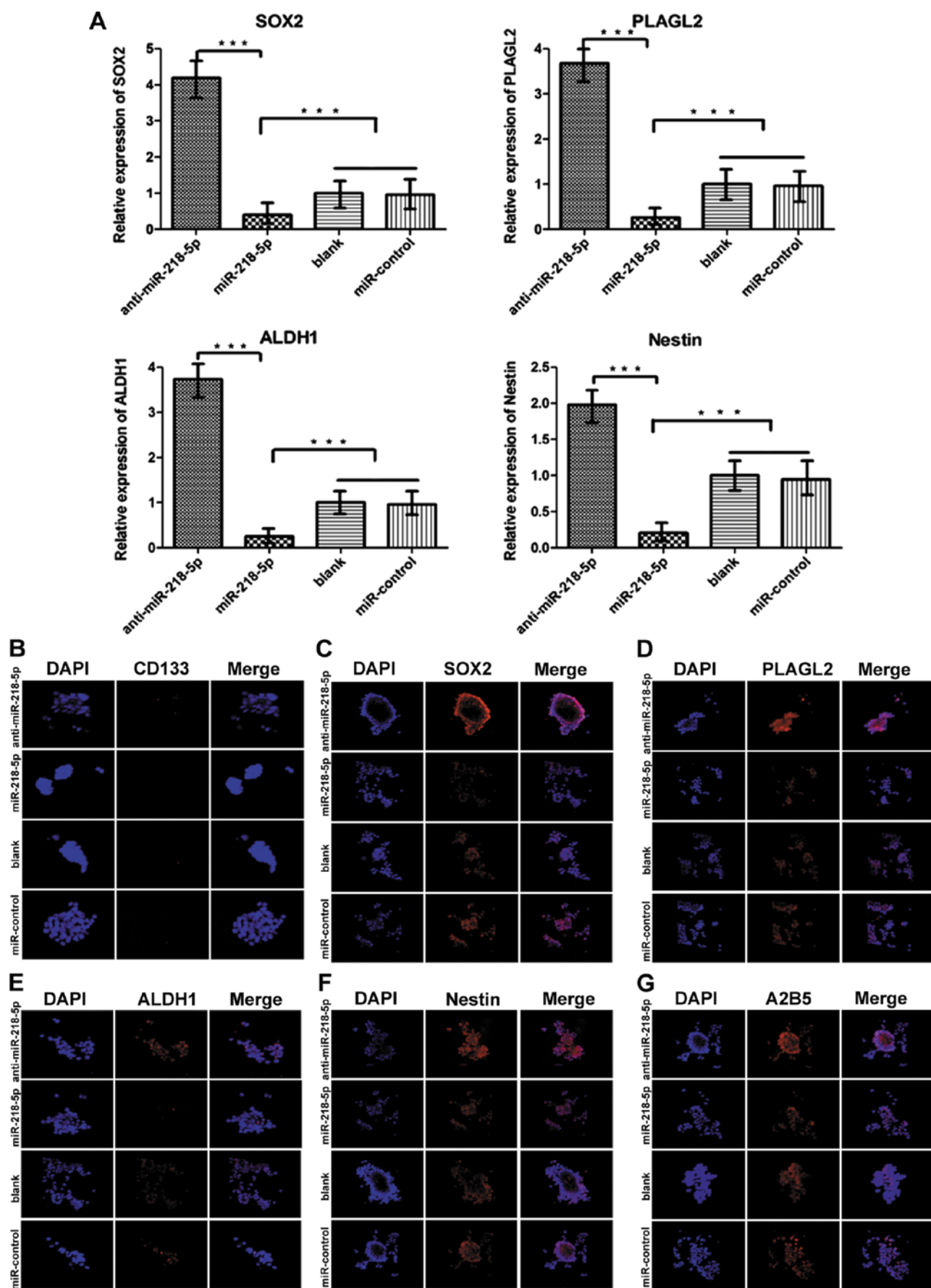


Figure 5. Expression of stem cell markers in SHG-139s cells stably transfected with recombinant lentiviruses. (A) qRT-PCR results show higher expression of glioma stem cell markers (Sox2, PLAGL2, ALDH1 and nestin) in the anti-miR-218-5p group when compared with levels in the blank and miR-control groups, and a much higher expression than that in the miR-218-5p group; **** $P < 0.001$. (B-G) Confocal laser microscopy images of immunofluorescence staining confirming the results of the qRT-PCR analysis, with the exception of CD133, for which no significant difference was detected between the groups. DAPI staining of nuclei observed as blue fluorescence (magnification, x60).

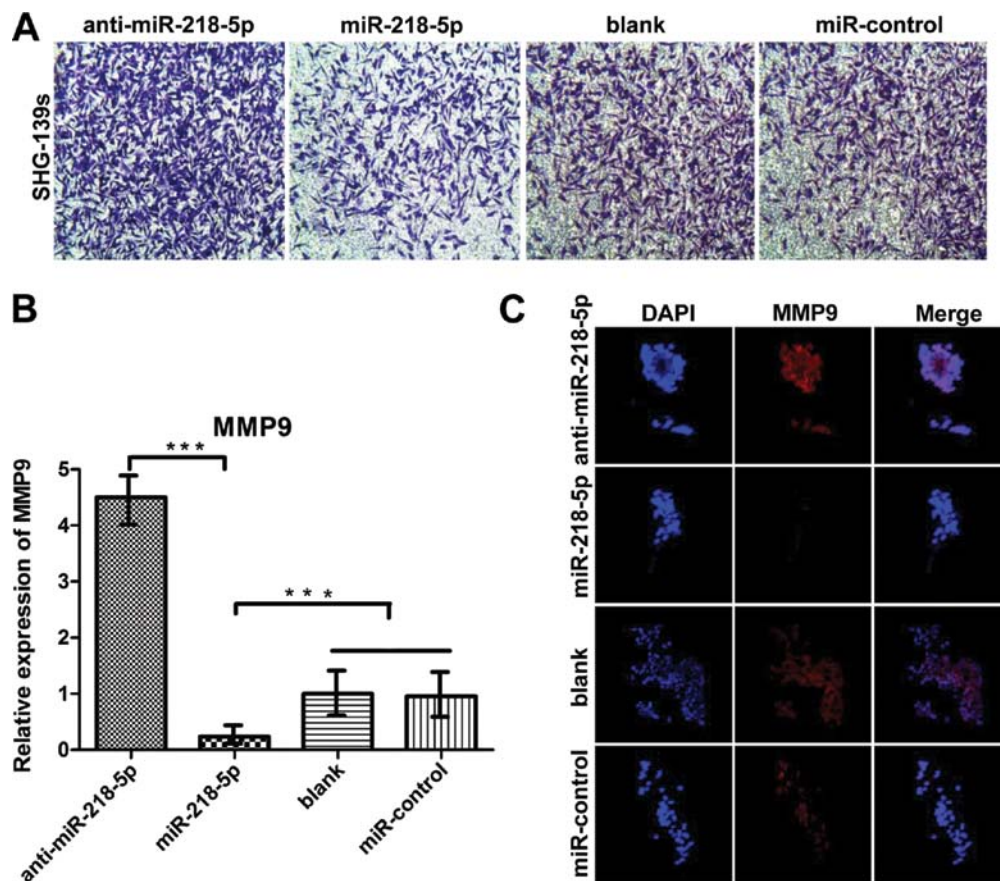


Figure 6. Analysis of the influence of upregulated and downregulated miR-218-5p expression on the invasive ability and MMP9 expression of SHG-139s cells. (A) Transwell experiment shows suppressed invasive ability of SHG-139s cells in the miR-218-5p group when compared with the other groups (magnification, x400). (B) qRT-PCR results show lower MMP9 expression in the miR-218-5p group when compared with the other groups; *** $P < 0.001$. (C) Confocal laser microscopy images of immunofluorescence staining indicating lower MMP9 expression in the miR-218-5p group compared with the other groups. DAPI staining of nuclei observed as blue fluorescence (magnification, x60).

of miR-218-5p, in GSCs and suggested its function as an important tumor-suppressor gene in gliomas. Therefore, we hypothesized that miR-218-5p plays a similar role in glioma.

We detected the expression of miR-218-5p in six non-cancerous human brain tissues and 53 glioma samples using qRT-PCR. Our results showed that the expression of miR-218-5p was lower in glioma samples than that in the non-cancerous brain tissues. Moreover, the expression of miR-218-5p in glioma cell lines was lower than that in the NHA cells. These results suggest that miR-218-5p acts as a tumor-suppressor gene in glioma, which is consistent with previous studies describing the function of miR-218 in glioma. Furthermore, the expression of miR-218-5p in SHG-139s (A2B5⁺CD133⁻) cells was lower than that in U87s and SU2 (CD133⁺) cells (33,34); thus we speculated that miR-218-5p plays a more important role in A2B5⁺CD133⁻ GSCs. To explore this hypothesis, we then investigated the impact of miR-218-5p on the stem cell properties and invasive ability of SHG-139s. Of course, some issues still require further study. The expression levels of miR-218-5p between A2B5⁺CD133⁻ and CD133⁺ cells were examined in two separate groups of cultured cells. It may be important to compare the difference between these two subpopulation of tumor cells derived from the same GBMs, preferably from surgical specimens. Subsequent study will be performed to sort various subsets of cells from fresh glioma tissue samples

by MACS in our laboratory, according to stem cell markers CD133 and A2B5.

In the present study, the neurosphere formation assay was used to demonstrate that upregulation of miR-218-5p expression resulted in a reduced number of neurospheres formed by the SHG-139s cells. Furthermore, qRT-PCR and immunofluorescence analyses showed that the expression of glioma stem cell markers (CD133, PLAGL2, ALDH1, Sox2, A2B5 and nestin) was lowest in the lentivirus-miR-218-5p group, and highest in the lentivirus-anti-miR-218-5p group. It should be noted that the qRT-PCR results for CD133 expression were not confirmed by immunofluorescence staining, with no significant differences in the expression levels of this stem cell marker detected using the latter technique. Nevertheless, these data indicate that miR-218-5p inhibited the stem cell properties of SHG139s cells, and that high expression of miR-218-5p was not conducive to the maintenance of stem cell characteristics of the SHG-139s cells. These findings are consistent with the conclusion reported by Tu *et al* that miR-218 targets the Bmi-1 gene to promote GSC differentiation and maturation of U251s (24). Therefore, these observations indicate that both miR-218-5p and miR-218 regulate stem cell properties in GSCs. Simultaneously, we found that miR-218-5p was expressed both in the CD133-positive (U87s and SU2) and CD133-negative glioma stem cells, while lower miR-218-5p

expression was noted in the A2B5⁺CD133⁻ glioma stem cell subset phenotype. As miR-218 could promote GSC differentiation and maturation of U251s, miR-218-5p may have the same function. Since lower miR-218-5p expression was found in the A2B5⁺CD133⁻ glioma stem cell subset phenotype, we speculated that miR-218-5p may play a more important role in A2B5⁺CD133⁻ glioma stem cell subsets. However, further experiments will need to be carried out. In addition, our results showed that upregulation of miR-218-5p expression inhibited the invasive ability of SHG-139s cells, and that there was a negative correlation between the expression of MMP9 and miR-218-5p. According to previous studies, miR-218 acts on the IKK-B/NF- κ B/MMP9 and LEF1/MMP9 signaling pathways to regulate the invasive ability of glioma cells (19,20). It can be speculated that miR-218-5p has the same mechanism of action but this remains to be confirmed.

In the present study, we found that upregulation of the expression of miR-218-5p inhibited the stem cell properties and invasive ability of SHG-139s cells. Furthermore, miR-218-5p expression in SHG-139s (A2B5⁺CD133⁻) cells was much lower than that in U87s and SU2 (CD133⁺) cells. However, it remains to be confirmed that low miR-218-5p expression is consistently associated with the A2B5⁺CD133⁻ glioma stem cell subset phenotype and that miR-218-5p plays an important role in A2B5⁺CD133⁻ glioma stem cell subsets. Tumorigenicity is the gold standard of cancer stem cells, thus tumorigenic experiments *in vivo* need be carried out in the future. Nevertheless, the important function of miR-218-5p in regulating the stem cell properties and invasive ability of A2B5⁺CD133⁻ glioma stem cell subsets *in vitro* indicates the therapeutic potential of elimination of this subset for improving outcomes in glioma patients.

Using GeneChip analysis, we subsequently showed that various microRNAs are expressed at a significantly lower expression in SHG-139s compared with SHG-139 cells, particularly miR-218-5p. Based on relevant information, it was found that miR-218, the precursor form of miR-218-5p, had specific high expression in normal brain tissues. Thus, miR-218-5p became the object of the present study. Yet, using a single miRNA has its limitation, and more than one miRNA will be used in the same experiment in future research.

In conclusion, the present study provides evidence of an important role for miR-218-5p in the occurrence and development of human gliomas. Furthermore, we provide further clarification of the molecular mechanism underlying the influence of miR-218-5p on glioma cells. Our observations demonstrate that miR-218-5p plays a key role in the maintenance of stem cell properties, invasive ability and self-renewal of A2B5⁺CD133⁻ glioma stem cell subsets. In addition, miR-218-5p is implicated as a tumor-suppressor gene in gliomas. We confirmed the potential of miR-218-5p overexpression for glioma therapy and provided the basis for further research on miR-218-5p and glioma stem cell subsets that will contribute to the advancement of cancer research and treatment.

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