

miR-30c Impedes Glioblastoma Cell Proliferation and Migration by Targeting SOX9

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miR-30c has been acknowledged as a tumor suppressor in various human cancers, such as ovarian cancer, gastric cancer, and prostate cancer. However, the role of miR-30c in glioblastoma (GBM) needs to be investigated. In our study, we found that the expression of miR-30c was significantly downregulated in GBM tissues and cell lines. We found that overexpression of miR-30c inhibited cellular proliferation of GBM cells *in vitro* and *in vivo*. More GBM cells were arrested in the G₀ phase after miR-30c overexpression. Moreover, we showed that miR-30c overexpression suppressed the migration and invasion of GBM cells. Mechanistically, we found that SOX9 was a direct target of miR-30c in GBM cells. Overexpression of miR-30c inhibited the mRNA and protein levels of SOX9 in GBM cells. Moreover, there was a negative correlation between the expression of miR-30c and SOX9 in GBM tissues. Finally, we showed that restoration of SOX9 in GBM cells reversed the proliferation, migration, and invasion of GBM cells transfected with miR-30c mimic. Collectively, our results demonstrated that miR-30c suppressed the proliferation, migration, and invasion of GBM cells via targeting SOX9.

Key words: miR-30c; Proliferation; Migration; SOX9; Glioblastoma

INTRODUCTION

Glioblastoma (GBM) is the most common and malignant brain tumor to occur in the central nervous system of adults^{1,2}. GBM is characterized with an increased malignant degree of invasion and growth^{3,4}. Combined therapy of surgery, radiotherapy, and chemotherapy is generally used for GBM treatment. However, because of extreme aggression, the outcomes of GBM patients are quite poor⁵. The 5-year survival rate of GBM patients is very low⁶. Therefore, it will be of great significance to explore the underlying molecular mechanism of GBM development and progression.

MicroRNAs (miRNAs) are a class of small noncoding RNAs (ncRNAs). Previous studies have demonstrated that miRNAs could regulate gene expression through associating with the complementary site of the 3'-UTR of target mRNAs^{7,8}. More and more studies show that miRNAs are widely involved in the regulation of a diversity of biological processes including cell proliferation, apoptosis, migration, and invasion^{9,10}. Abnormal expression of miRNAs often leads to occurrence of cancers^{11–14}. Liu et al. reported that miR-200c inhibits epithelial–mesenchymal transition, invasion, and migration of lung

cancer by targeting HMGB1¹². Yang et al. reported that miR-483-5p promotes prostate cancer cell proliferation and invasion by targeting RBM5¹³. Zeng and colleagues showed that miR-378 suppresses the proliferation, migration, and invasion of colon cancer cells by inhibiting SDAD1¹⁴. miR-30c was also reported to inhibit tumor development. For example, Wu et al. showed that miR-30c negatively regulates the migration and invasion by targeting the immediate early response protein 2 in SMMC-7721 and HepG2 cells¹⁵. However, the function of miR-30c remains largely unknown in GBM. miRNAs have been demonstrated to be promising biomarkers and therapeutic targets for cancer intervention. Therefore, it is important to determine the mechanism of miRNAs in cancers.

In this study, we found that miR-30c was underexpressed in GBM tissues and cell lines. Moreover, overexpression of miR-30c significantly inhibited the proliferation, migration, and invasion of GBM cells. Mechanistically, we found that SOX9 is a direct target of miR-30c in GBM cells. We showed that overexpression of SOX9 could rescue the proliferation, migration, and invasion of GBM cells transfected with a miR-30c

mimic. Taken together, our findings demonstrated the key role of miR-30c in GBM cells and explored the functional mechanism.

MATERIALS AND METHODS

Patient Samples

The present study was approved by the Ethics Committee of Linyi Central Hospital (Shandong Province, P.R. China), and written informed consent was obtained from patients with GBM for use of their tissues. A total of 53 paired GBM tissues and adjacent normal brain tissues were obtained from the patients with GBM who underwent surgical resection at Linyi Central Hospital. None of the patients were treated with radiotherapy or chemotherapy prior to surgery. Collected specimens were immediately frozen following surgical resection and stored in liquid nitrogen.

Cell Culture

Human GBM cell lines U87 and U251 obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; SH30022.01B; Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS; Hyclone). Normal human astrocytes (NHAs) obtained from Lonza were cultured in the provided astrocyte growth media and 5% FBS.

Construction and Infection

miR-30c mimic and negative control (NC) were chemically synthesized by Ribobio (Guangzhou, P.R. China). The oligonucleotides of miRNA mimics and plasmids were transfected, according to the product specification, into the cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Cell Proliferation

For the cell counting kit-8 (CCK-8) assay, stable transfected U87 and U251 cells were seeded in 96-well plates, and cells were cultured for 24, 48, 72, and 96 h before performing the CCK-8 assay (DOJINDO, Kumamoto, Japan). After incubation with CCK-8 at 37°C, absorbance (OD value) at a wavelength of 450 nm was detected and used for calculating cell viability.

For the colony formation assay, cells were harvested 24 h after transfection and then seeded in a new six-well plate (300 cells/well) and cultured for approximately 2 weeks until colony formation was observed. Colonies were fixed with methanol and stained with 1% crystal violet. A colony was considered to be 450 cells. Colony formation rate was used to calculate posttransfection cell survival rate.

In Vitro Invasion Assays

Cell invasion assay was performed using 24-well Transwell chambers with polycarbonate membranes containing 8- μ m-diameter pores (Corning Incorporated, Corning, NY, USA). Cells were seeded on the top side of the membrane precoated with Matrigel in DMEM without serum (Becton Dickinson Company, Franklin Lakes, NJ, USA). The lower chambers were filled with DMEM and 10% FBS. After incubation at 37°C for 24 h, the noninvasive cells on the top side of the membrane were removed by scraping. Invasive cells on the lower membrane were fixed with 20% methanol for 30 min and stained with 0.1% crystal violet for 15 min. Invasion was quantified by counting cells in six randomized fields of view in each well under light microscope (Olympus, Tokyo, Japan) at the level of 100 \times magnification.

Reverse Transcription and Real-Time PCR

Total RNA of glioma samples, NHA samples, and cultured cells was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The expression levels of SOX9 were assessed by SYBR green real-time quantitative reverse transcription PCR (RT-qPCR) and normalized with GAPDH.

Dual-Luciferase Reporter Assay

U87 cells were seeded into a 24-well plate. Cells were cotransfected with wild-type (WT), mutated SOX9 reporter plasmid or pMIR vector (Promega, Madison, WI, USA), and miR-30c mimic. Luciferase assays were conducted 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

Statistical Analysis

All data are shown as mean \pm standard deviation (SD). Statistical significance was determined using Student's *t*-test by SPSS 13.0 and GraphPad Prism 6. A value of $p < 0.05$ was considered statistically significant.

RESULTS

miR-30c Was Downregulated in GBM Cells

To investigate miRNA expression in human GBM tissues, we used RT-qPCR to evaluate the expression levels of miR-30c in 53 pairs of GBM tissues and adjacent normal tissues. We found that the expression of miR-30c was significantly downregulated in GBM tissues compared to normal tissues (Fig. 1A). Moreover, the decrease was more pronounced in high-grade GBM tissues compared to low-grade tissues (Fig. 1B). In addition, we chose GBM cell lines to analyze the expression of miR-30c by RT-qPCR. The results indicated that miR-30c was also downregulated in GBM cell lines compared with NHAs (Fig. 1C). These data indicated that miR-30c was

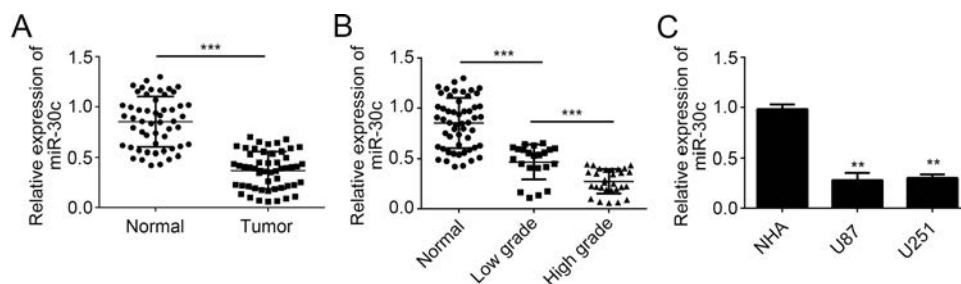


Figure 1. MicroRNA-30c (miR-30c) was downregulated in glioblastoma (GBM) cells. (A) Relative expression of miR-30c in GBM tissues and paired normal tissues. (B) The expression of miR-30c in 53 noncancerous brain tissues, 22 low-grade glioma tissues, and 29 high-grade GBM tissues was measured by real-time quantitative reverse transcription PCR (RT-qPCR). (C) RT-qPCR analysis of miR-30c expression in GBM cell lines (U87 and U251 cells) and normal human astrocytes (NHAs). All data are representative of three independent experiments and expressed as mean \pm standard deviation (SD). ** $p < 0.01$ and *** $p < 0.001$.

downregulated in GBM tissues and negatively correlated with GBM malignance.

Overexpression of miR-30c Suppressed the Proliferation of GBM Cells In Vitro and In Vivo

To explore the function of miR-30c in GBM, we over-expressed miR-30c in U251 and U87 cells by transfection

with a miR-30c mimic. RT-qPCR analysis showed that the expression of miR-30c was significantly higher in U251 and U87 cells transfected with miR-30c mimic than miR-NC (Fig. 2A). Then we performed CCK-8 and colony formation assay to evaluate the effect of miR-30c on GBM cell proliferation. We found that over-expression of miR-30c significantly inhibited the cellular

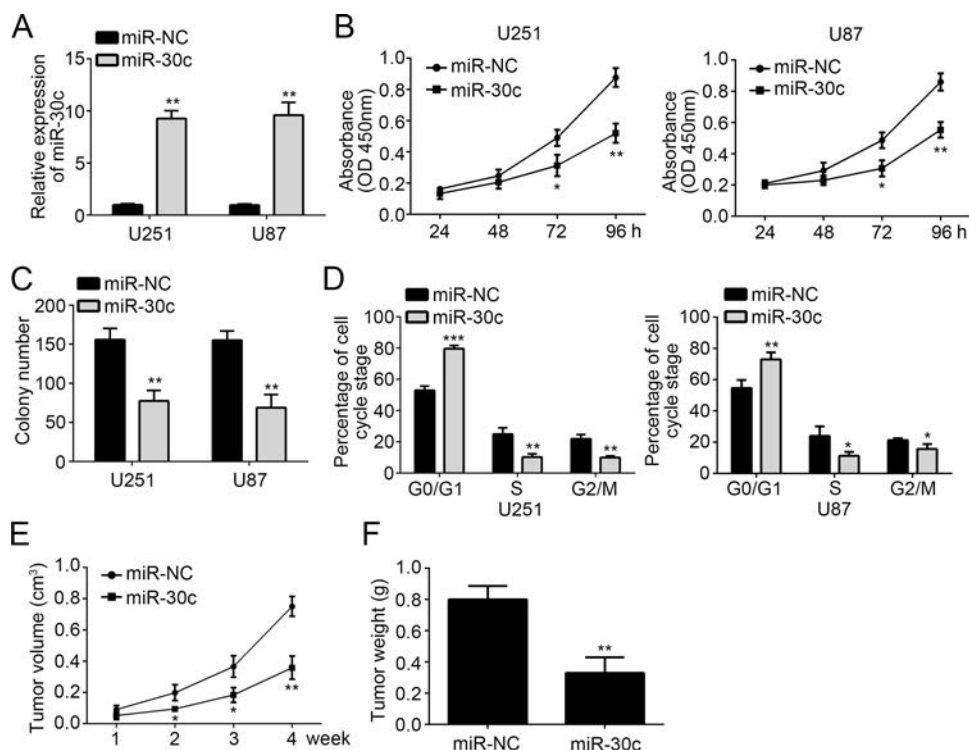


Figure 2. Overexpression of miR-30c suppressed the proliferation of GBM cells in vitro and in vivo. (A) The relative expression level of miR-30c in U251 and U87 cells was analyzed by RT-qPCR after transfection. (B) Overexpression of miR-30c inhibited cell proliferation detected by cell counting kit-8 (CCK-8) assay. (C) Long-term cell viability was evaluated using the colony formation assay. (D) The cell cycle phase of U87 and U251 cells transfected with miR-30c or negative control (miR-NC) was analyzed by flow cytometry. (E) Tumor volumes were measured at the indicated time points. (F) Tumor weight was determined at the end of experiments. All data are representative of three independent experiments and expressed as mean \pm SD. * $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$.

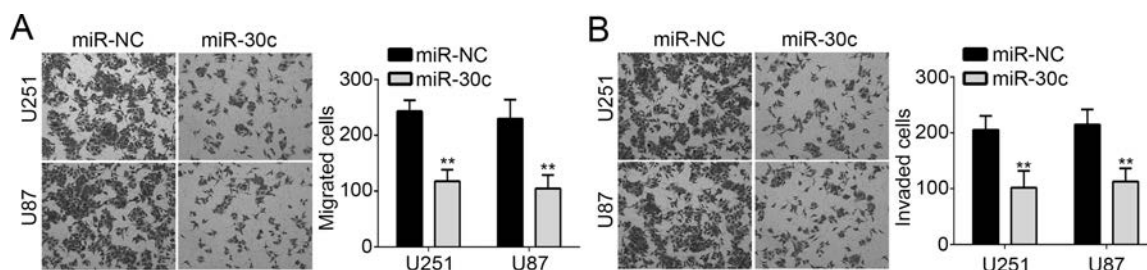


Figure 3. Overexpression of miR-30c inhibited the migration and invasion of GBM cells. (A, B) Cellular migration and invasion of U251 and U87 cells were determined by Transwell assay. All data are representative of three independent experiments and expressed as mean \pm SD. ** $p < 0.01$.

proliferation and colony numbers (Fig. 2B and C). Cell cycle distribution is directly linked to cell proliferation. We then explored the cell cycle distribution of U251 and U87 cells transfected with miR-30c mimic or miR-NC. Flow cytometry analysis (FACS) indicated that overexpression of miR-30c increased the percentage of cells in the G_0/G_1 phase and reduced the percentage in the S and G_2/M phases (Fig. 2D). In order to determine whether miR-30c regulates tumor growth in vivo, we performed a xenograft experiment. At the indicated time points, we measured the tumor volumes and found that overexpression of miR-30c significantly inhibited tumor growth (Fig. 2E). Moreover, we checked the tumor weight at the end of the xenograft experiment and found that the tumor tissues derived from miR-30c-overexpressing cells were smaller (Fig. 2F). Therefore, our data demonstrated that overexpression of miR-30c suppressed cell proliferation in vitro and tumor growth in vivo.

Overexpression of miR-30c Inhibited the Migration and Invasion of GBM Cells

To assess the effect of miR-30c on cell migration and invasion, we performed the Transwell assay. The results demonstrated that overexpression of miR-30c significantly suppressed the migration and invasion of U251 and U87 cells (Fig. 3A and B). These results indicated that miR-30c may serve as a tumor suppressor in GBM progression.

SOX9 Was a Target of miR-30c

To explore the mechanism of miR-30c in GBM, we performed bioinformatics analysis to predict potential targets of miR-30c. Among all targets, SOX9 ranked top and serves as an oncogene in various tumors. There was a potential binding site of miR-30c in the 3'-UTR of SOX9 mRNA (Fig. 4A). Dual-luciferase reporter assays were conducted to verify their interaction. We found

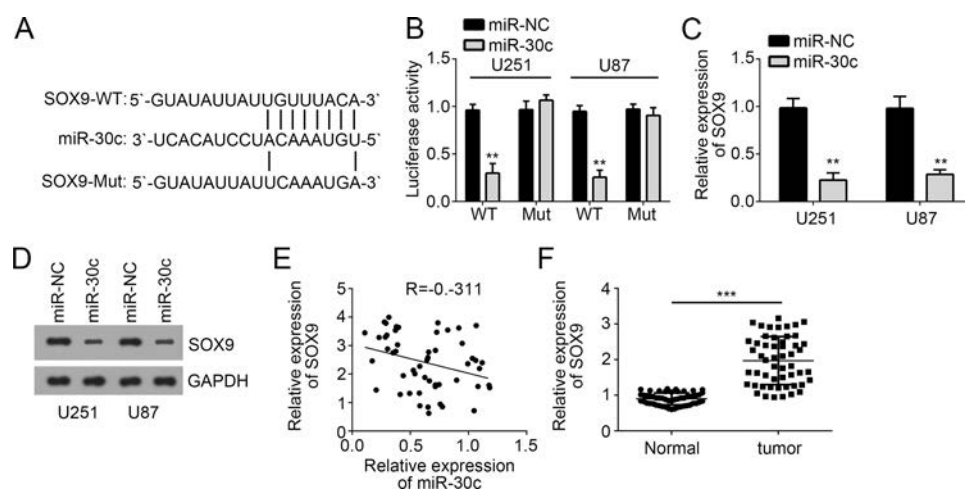


Figure 4. SOX9 was a target of miR-30c. (A) Diagram for the miR-30c binding sites in the 3'-UTR of SOX9 with the wild-type (WT) and mutated (Mut) sequences. (B) U87 and U251 cells were cotransfected with miR-30c and luciferase reporter constructs containing either pGL3-SOX9-3'-UTR-WT or pGL3-SOX9-3'-UTR-Mut. (C) The mRNA and (D) protein levels of SOX9 in U87 and U251 cells transfected with miR-30c mimics were measured by RT-qPCR and Western blot. (E) Pearson's correlation analysis of the relative expression levels of miR-30c and the relative mRNA levels of SOX9. (F) RT-qPCR analysis for SOX9 expression in GBM tissues and adjacent normal tissues. All data are representative of three independent experiments and expressed as mean \pm SD. ** $p < 0.01$ and *** $p < 0.001$.

that overexpression of miR-30c significantly inhibited the luciferase activity in U251 and U87 cells transfected with SOX9-3'-UTR-WT, whereas mutation of the binding site abolished this effect (Fig. 4B). Moreover, through RT-qPCR and Western blot, we found that overexpression of miR-30c inhibited the mRNA (Fig. 4C) and protein levels (Fig. 4D) of SOX9 in U251 and U87 cells. Additionally, we also found that there was an inverse correlation between the expression of miR-30c and SOX9 in GBM tissues (Fig. 4E). Furthermore, through RT-qPCR analysis, we found that SOX9 expression was significantly upregulated in GBM tissues compared with adjacent normal tissues (Fig. 4F). Taken together, our results demonstrated that SOX9 was a direct target of miR-30c in GBM cells.

SOX9 Reintroduction Reverses the Inhibitory Effect of miR-30c

To further determine whether miR-30c inhibited the proliferation, migration, and invasion of GBM cells through targeting SOX9, we restored the protein level of SOX9 in U251 and U87 cells transfected with miR-30c mimic. By Western blot, we found that the expression of SOX9 was upregulated to the level of the control group (Fig. 5A). Then we performed CCK-8 and colony formation assays and found that overexpression of SOX9 really restored the proliferation ability of U251 and U87 cells and promoted colony formation (Fig. 5B and C). Moreover, the Transwell assay demonstrated that restoration of SOX9 in U251 and U87 cells transfected with miR-30c rescued the migration and invasion of GBM

cells (Fig. 5D and E). Therefore, our findings indicated that miR-30c suppressed the proliferation, migration, and invasion of GBM cells through inhibiting SOX9 expression.

DISCUSSION

GBM is the most prevalent and aggressive brain cancer in adults, contributing to a large percentage of cancer-related deaths worldwide¹⁶. Nowadays, how GBM develops and progresses remains largely unknown. Therefore, there is a very urgent requirement to investigate the underlying mechanism of GBM progression. miRNAs have been widely acknowledged as pivot regulators in all kinds of biological processes and are closely linked with the development and progression of cancers¹⁷. miRNAs have been shown to be promising biomarkers for tumor diagnosis or prognosis¹⁸. Thus, understanding the molecular mechanism of miRNAs in the process of cancer development will be of great importance. In this study, we observed that miR-30c was downregulated in GBM tissues and cell lines and acted as a tumor suppressor.

miRNAs are thought to contribute to cancer occurrence due to their key function in the regulation of cell proliferation, apoptosis, and mobility. Dysregulation of miRNAs is often observed in various cancers. For example, miR-154 was downregulated in gastric cancer and inhibits the growth and metastasis of cancer cells by directly targeting MTDH¹⁹. Yang et al. reported that miR-494 is a potential prognostic marker and inhibits cellular proliferation, migration, and invasion by targeting SIRT1 in epithelial ovarian cancer²⁰. In addition, Fan et al. showed that

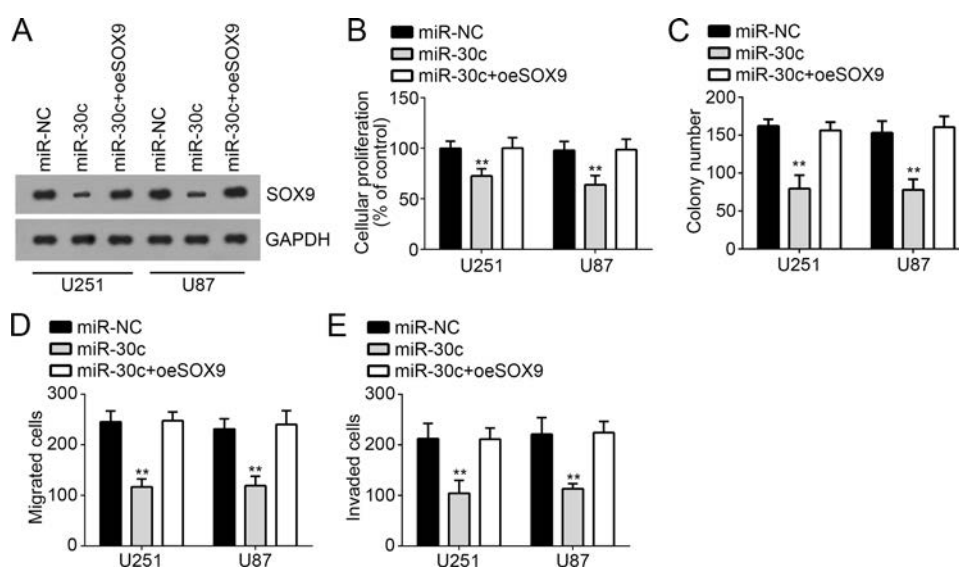


Figure 5. SOX9 reintroduction reversed the inhibitory effect of miR-30c. (A) Protein levels of SOX9 in U251 and U87 cells were measured by Western blot. (B, C) Cell proliferation was measured by CCK-8 and colony formation assays. (D, E) Cell migration and invasion were determined by Transwell assay in U251 and U87 cells. All data are representative of three independent experiments and expressed as mean \pm SD. ** $p < 0.01$.

miR-122 was upregulated in clear-cell renal cell carcinoma and promoted metastasis of cancer cells by downregulating Dicer²¹. Previous studies indicated that miR-30c serves as a tumor suppressor in some tumors. For instance, Ni et al. showed that miR-30c suppressed giant-cell tumor of bone cell metastasis and growth via targeting HOXA1²². Wang et al. reported that miR-30c inhibits metastasis of ovarian cancer by targeting metastasis-associated gene 1²³. Zhang and colleagues also indicated that low expression of miR-30c promotes prostate cancer cell invasion involved in the downregulation of KRAS protein²⁴. Other reports also demonstrated the suppressive role of miR-30c in gastric cancer²⁵, prostate cancer²⁶, liver cancer¹⁵, and non-small cell lung cancer²⁷. However, the role of miR-30c in GBM remains largely unknown. In our study, we demonstrated its low expression in GBM tissues and cell lines. Moreover, through CCK-8, colony formation, and Transwell assays, we demonstrated that overexpression of miR-30c significantly inhibited the proliferation, migration, and invasion of GBM cells, which suggested miR-30c also served as a tumor suppressor in GBM.

Increasing evidence indicates that SOX9 contributes to the progression of some cancers, including renal cell carcinoma²⁸, non-small cell lung cancer²⁹, hepatocellular carcinoma³⁰, and GBM³¹. For example, a report indicated that SOX9-mediated upregulation of LGR5 is important for GBM tumorigenicity³². Another study showed that miR-145 functions as a tumor-suppressive RNA by targeting SOX9 and adducin 3 in human glioma cells³³. However, the relationship between miR-30c and SOX9 remains elusive. In our study, we found that SOX9 was a direct target of miR-30c in GBM cells. Overexpression of miR-30c significantly inhibited the mRNA and protein levels of SOX9 in U251 and U87 cells. Moreover, we showed that there was a negative correlation between the expression of miR-30c and SOX9 in GBM tissues. Through functional experiments, we found that overexpression of SOX9 significantly reversed the effects of miR-30c on the proliferation, migration, and invasion of GBM cells, which suggested SOX9 was indispensable in miR-30c-mediated effects on GBM cells.

In summary, our findings demonstrated the key role of miR-30c on GBM cells. Our results indicated that miR-30c suppressed the development and progression of GBM through targeting SOX9, which provided a novel insight on the pathogenesis of GBM.

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