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lncRNA MNX1-AS1 Promotes Glioblastoma Progression Through Inhibition of miR-4443

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Long noncoding RNAs (lncRNAs) have been acknowledged as important regulators in various human cancers. lncRNA MNX1-AS1 has been shown to be an oncogene in epithelial ovarian cancer. However, the function of MNX1-AS1 in glioblastoma (GBM) remains largely unknown. Here we found that the expression of MNX1-AS1 was significantly upregulated in GBM tissues and cell lines. Knockdown of MNX1-AS1 significantly inhibited the proliferation, migration, and invasion of GBM cells. In terms of mechanism, we found that MNX1-AS1 could bind to miR-4443 in GBM cells. Overexpression of miR-4443 significantly inhibited the expression of MNX1-AS1 and vice versa. Moreover, there was an inverse correlation between the expression levels of MNX1-AS1 and miR-4443 in GBM tissues. We found that overexpression of miR-4443 inhibited the proliferation, migration, and invasion of GBM cells. We also showed that inhibition of miR-4443 reversed the effects of MNX1-AS1 knockdown on GBM cell proliferation, migration, and invasion. Taken together, we found that MNX1-AS1 promoted the proliferation, migration, and invasion of GBM cells through inhibiting miR-4443.

Key words: MNX1-AS1; Proliferation; Migration; miR-4443; Glioblastoma (GBM)

INTRODUCTION

Glioblastoma (GBM) is the most prevalent and lethal brain tumor around the world¹. Nowadays, the main therapeutic methods for GBM contain surgical resection, radiation, and chemotherapy². However, the outcomes of GBM patients are not satisfactory. The 5-year survival rate of GBM patients is rather low^{3,4}. Therefore, there is a necessary requirement to investigate the underlying mechanism of GBM progression and develop novel effective therapeutic targets.

In the past decades, the functions of long noncoding RNAs (lncRNAs) have attracted much attention. lncRNAs belong to a noncoding RNA family and have a length of over 200 nucleotides⁵. Emerging evidence indicates that lncRNAs have very important functions in the regulation of cellular proliferation, migration, and death⁶⁻⁹. Abnormal expression of lncRNAs is usually correlated with the development or progression of cancers¹⁰. For example, Wei reported that lncRNA MEG3 inhibits proliferation and metastasis of gastric cancer via p53 signaling pathway¹¹. Zhu et al. reported that LINC00052 enhances EPB41L3 expression to inhibit the migration and invasion of hepatocellular carcinoma

by binding miR-452-5p¹². Additionally, Zhang and colleagues reported that the lncRNA CASC2 inhibits tumorigenesis through modulating the expression of PTEN by targeting miR-18a-5p in esophageal carcinoma¹³. A study indicated that lncRNAs may serve as biomarkers for cancer diagnosis and prognosis¹⁴. Therefore, it is important to define the mechanism of lncRNA-mediated tumor progression.

A previous study showed that MNX1-AS1 was involved in ovarian cancer progression¹⁵. Nevertheless, whether MNX1-AS1 regulates other cancers remains largely unknown. In this study, we found that MNX1-AS1 was upregulated in GBM tissues and cell lines. Moreover, we found that knockdown of MNX1-AS1 significantly inhibited GBM cell proliferation, migration, and invasion. Mechanistically, we found that MNX1-AS1 sponged miR-4443 in GBM cells. We showed that there was an inverse correlation between the expression levels of MNX1-AS1 and miR-4443 in GBM tissues. Finally, we demonstrated that inhibition of miR-4443 rescued the proliferation, migration, and invasion of GBM cells transfected with shMNX1-AS1. Taken together, our findings indicated that MNX1-AS1 promoted the

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proliferation, migration, and invasion of GBM cell via sponging miR-4443.

MATERIALS AND METHODS

Patient Samples and Cell Lines

Forty-four pairs of GBM samples and adjacent normal tissues were obtained from the Affiliated Hospital of China Medical University. Sections of all specimens were confirmed according to the WHO criteria. Prior to tumor resection, none of the patients received radiotherapy (RT) or chemotherapy. All tissues were immediately frozen in liquid nitrogen and then stored at -80°C . This study was approved by the ethics committee of the Affiliated Hospital of China Medical University. Written informed consent was obtained from all involved patients.

Human GBM cell lines U138, LN229, T98, and U251 were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, P.R. China) and were cultured in Dulbecco's modified Eagle's medium (DMEM; SH30022.01B; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; 10082147; Gibco, Bethesda, MD, USA), 100 U/ml of penicillin, and 100 ng/ml of streptomycin. Normal human astrocytes (NHAs) were obtained from Lonza (Basel, Switzerland) and cultured in the provided astrocyte growth media supplemented with rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine, and 5% FBS. All the cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 .

Construction and Infection

The oligonucleotides of siMNX1-AS1 were transfected, according to the product specification, into the cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

CCK-8 Assay

We used the CCK-8 assay to determine the viability of T98 and U251 cells. The two cell lines were seeded into 96-well plates 72 h after transfection. A CCK-8 kit (DOJINDO, Kumamoto, Japan) was used to detect cell proliferation at 24, 48, and 72 h, following the manufacturer's instructions.

In Vitro Migration and Invasion Assays

Twenty-four-well Transwell chambers with 8- μm pore size polycarbonate (Corning Incorporated, Corning, NY, USA) were used for cell migration and invasion assays. For invasion assays, the top side of the membrane was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and then 1×10^5 cells (in each well) in serum-free DMEM or RPMI-1640 medium were seeded on the chambers. DMEM or RPMI-1640 containing 10% FBS was added to the wells under the chamber. For migration analysis, 5×10^4 cells (in each well) in serum-free DMEM

or RPMI-1640 medium were seeded on the chambers without Matrigel. After 24 h of incubation, cotton swabs were used to remove the cells inside the upper chamber, while the cells on the other side of the membrane surface were fixed and stained with 0.5% crystal violet solution. Five random fields were counted in each well.

Reverse Transcription and Real-Time PCR

Total RNA of glioma samples, NHA samples, and cultured cells were extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The expression levels of MNX1-AS1 were assessed by SYBR Green real-time quantitative reverse transcription (qRT)-PCR and normalized with GAPDH.

Luciferase Reporter Assay

U251 and T98 cells were seeded into a 24-well plate. Cells were cotransfected with wild-type (WT) or mutated MNX1-AS1 reporter plasmid and miR-4443 mimics or negative controls (NCs). Luciferase assays were conducted 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

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

MNX1-AS1 Was Upregulated in GBM Tissues

To explore the function of MNX1-AS1 in GBM, we analyzed its expression in paired GBM tissues and adjacent normal tissues using qRT-PCR. We found that the expression of MNX1-AS1 was significantly upregulated in GBM tissues compared to adjacent normal tissues (Fig. 1A). Then we analyzed the expression patterns of MNX1-AS1 in GBM tissues. qRT-PCR analysis also indicated that the expression of MNX1-AS1 was markedly upregulated in GBM cell lines compared with NHA cells (Fig. 1B).

MNX1-AS1 Knockdown Inhibited the Proliferation, Migration, and Invasion of GBM Cells

To further explore the physiological function of MNX1-AS1, we knocked down MNX1-AS1 in U251 and T98 cells. qRT-PCR analysis indicated that the expression of MNX1-AS1 was significantly downregulated in U251 and T98 cells transfected with shMNX1-AS1 (Fig. 2A). Then we performed the CCK-8 assay. As shown, knockdown of MNX1-AS1 significantly suppressed the proliferation of U251 and T98 cells (Fig. 2B). Moreover, Transwell assay showed that knockdown of MNX1-AS1 markedly reduced the numbers of migrated and invaded

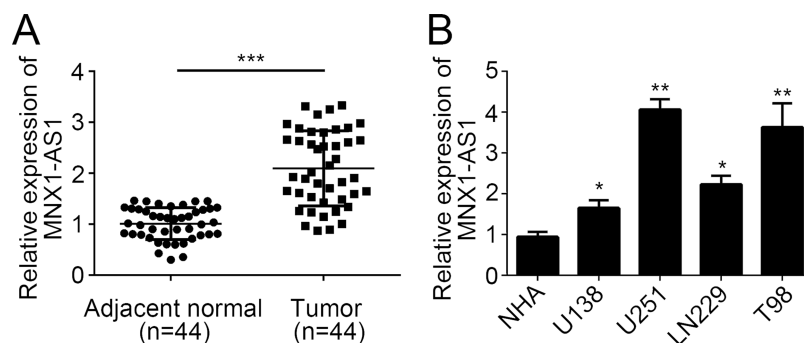


Figure 1. MNX1-AS1 was upregulated in glioblastoma (GBM) tissues. (A) Quantitative reverse transcription and real-time (qRT)-PCR was used to analyze the expression of MNX1-AS1 in paired GBM tissues and adjacent normal tissues. (B) qRT-PCR analysis of MNX1-AS1 expression in GBM cell lines and normal human astrocyte (NHA) cells. All data are representative of three independent experiments and expressed as mean \pm standard deviation (SD). * p <0.05, ** p <0.01, and *** p <0.001.

cells (Fig. 2C and D). Taken together, our results indicated that knockdown of MNX1-AS1 inhibited the proliferation, migration, and invasion of GBM cells.

MNX1-AS1 Acted as a Sponge of miR-4443

To investigate the mechanism of MNX1-AS1, we made a prediction and found that miR-4443 was a potential target of MNX1-AS1. There were two potential binding sites of miR-4443 in MNX1-AS1 (Fig. 3A). Through luciferase reporter assay, we found that overexpression of miR-4443 significantly inhibited the luciferase activity in U251 and T98 cells transfected with WT MNX1-AS1, while mutation of these two binding sites abrogated the

inhibitory effects of miR-4443 (Fig. 3B). Moreover, we found that overexpression of miR-4443 significantly inhibited the expression of MNX1-AS1 in U251 and T98 cells (Fig. 3C). Besides, MNX1-AS1 knockdown promoted the expression of miR-4443 in U251 and T98 cells (Fig. 3D). Finally, through qRT-PCR analysis, we found that there was an inverse correlation between the expression of MNX1-AS1 and miR-4443 in GBM tissues (Fig. 3E).

Overexpression of miR-4443 Suppressed the Proliferation, Migration, and Invasion of GBM Cells

In the next step, we evaluated the function of miR-4443 in GBM cells. We found that the expression of miR-4443

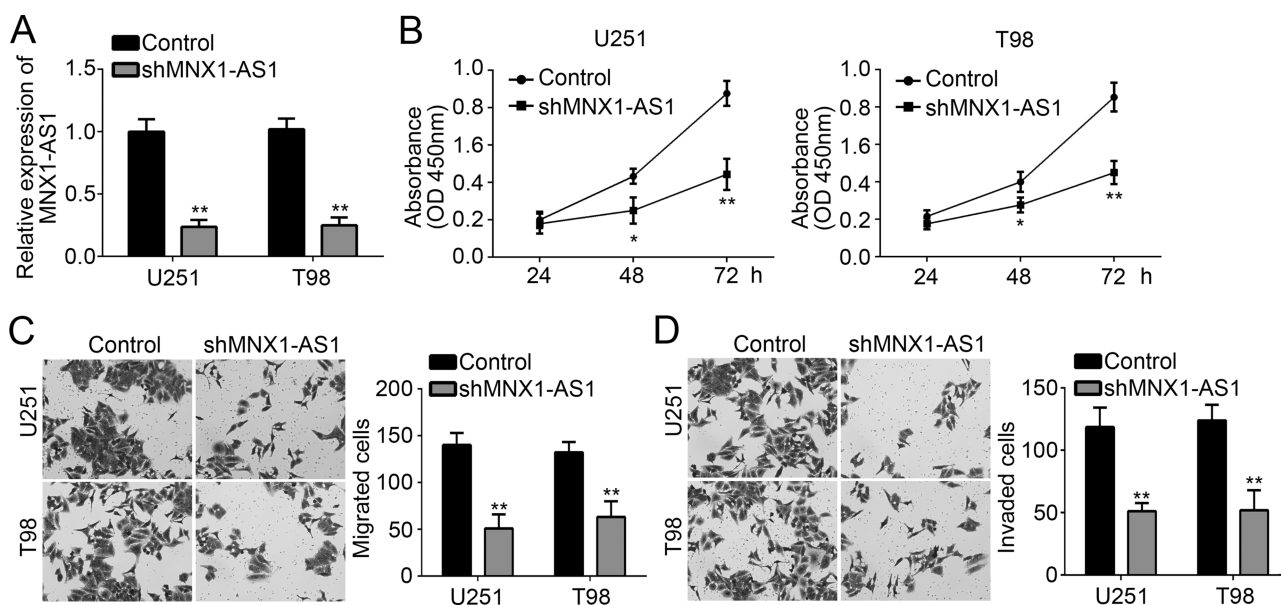


Figure 2. MNX1-AS1 knockdown inhibited the proliferation, migration, and invasion of GBM cells. (A) qRT-PCR analysis of MNX1-AS1 expression in U251 and T98 cells transfected with shMNX1-AS1 or control. (B) CCK-8 assay was used for the analysis of U251 and T98 cell proliferation. (C, D) Transwell assay indicated that knockdown of MNX1-AS1 inhibited the migration and invasion of U251 and T98 cells. All data are representative of three independent experiments and expressed as mean \pm SD. * p <0.05 and ** p <0.01.

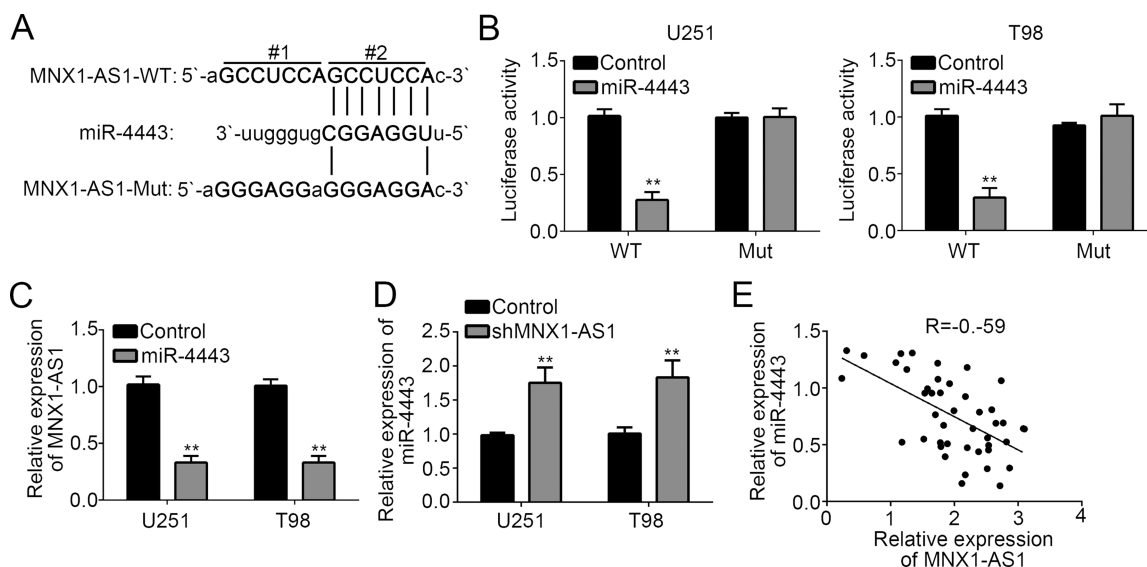


Figure 3. MNX1-AS1 acted as a sponge of miR-4443. (A) A diagram for the binding sites of miR-4443 in MNX1-AS1. (B) Luciferase assay indicated that overexpression of miR-4443 inhibited the luciferase activity in U251 and T98 cells transfected with wild-type (WT) MNX1-AS1. (C) Overexpression of miR-4443 inhibited the expression of MNX1-AS1 in U251 and T98 cells. (D) Knockdown of MNX1-AS1 promoted the expression of miR-4443 in U251 and T98 cells. (E) There was an inverse correlation between the expression of MNX1-AS1 and miR-4443 in GBM tissues. All data are representative of three independent experiments and expressed as mean \pm SD. $**p < 0.01$.

was significantly downregulated in GBM tissues compared to adjacent normal tissues (Fig. 4A). In addition, the expression of miR-4443 was also downregulated in GBM cell lines compared to NHA cells (Fig. 4B). Then we overexpressed miR-4443 in U251 and T98 cells by transfection

with miR-4443 mimics (Fig. 4C). By CCK-8 and Transwell assays, we found that overexpression of miR-4443 significantly inhibited the proliferation, migration, and invasion of GBM cells (Fig. 4D–F), which is consistent with the effects of MNX1-AS1 knockdown on GBM cells.

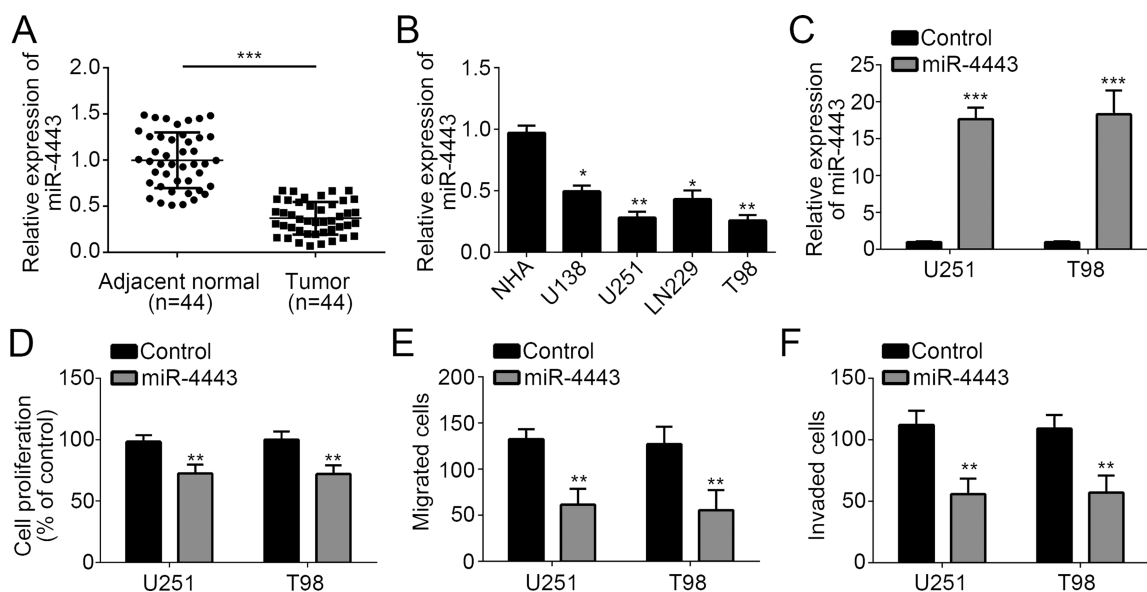


Figure 4. Overexpression of miR-4443 suppressed the proliferation, migration, and invasion of GBM cells. (A) qRT-PCR analysis indicated that miR-4443 was downregulated in GBM tissues compared with normal tissues. (B) Downregulated expression of miR-4443 in GBM cell lines as shown by qRT-PCR. (C) qRT-PCR analysis indicated that miR-4443 was overexpressed in U251 and T98 cells transfected with miR-4443 mimics. (D) CCK-8 assay indicated that overexpression of miR-4443 inhibited the proliferation of U251 and T98 cells. (E, F) Transwell assay indicated that overexpression of miR-4443 inhibited the migration and invasion of U251 and T98 cells. All data are representative of three independent experiments and expressed as mean \pm SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

MNX1-AS1 Promoted the Proliferation, Migration, and Invasion of GBM Cells by Inhibition of miR-4443

To further determine whether MNX1-AS1-induced effects depend on miR-4443, we inhibited miR-4443 in MNX1-AS1-silenced U251 and T98 cells (Fig. 5A). Through CCK-8 and Transwell assay, we found that inhibition of miR-4443 reversed the inhibitory effects of MNX1-AS1 knockdown on the proliferation, migration, and invasion of U251 and T98 cells (Fig. 5B–D). In a word, our results indicated that MNX1-AS1 promoted the proliferation, migration, and invasion of GBM cells by suppressing miR-4443.

DISCUSSION

GBM is the most prevalent and lethal brain tumor around the world¹. However, the pathogenesis of GBM remains largely unknown. In order to screen out novel diagnostic and prognostic biomarkers of GBM and develop effective therapeutic methods, it is urgently required to determine the underlying molecular mechanism of GBM progression. In the present study, we identified lncRNA MNX1-AS1 as an oncogene that could promote the proliferation, migration, and invasion of GBM cells.

During recent decades, lncRNAs have become one of the hottest topics in the field of biology. Because of its

essential function in the regulation of cellular survival, apoptosis, and mobility, lncRNA expression is closely linked to human cancers, including chronic myeloid leukemia¹⁶, breast cancer¹⁷, chondrosarcoma¹⁸, gallbladder carcinoma¹⁹, and GBM²⁰. In GBM, increasing evidence shows that lncRNAs act as vital regulators²¹. For example, Su et al. reported that knockdown of SOX2OT inhibits the malignant biological behaviors of GBM stem cells via upregulating the expression of miR-194-5p and miR-122²¹. Li et al. showed that MALAT1 decreases the sensitivity of resistant GBM cell lines to temozolomide²². Ma et al. reported that long noncoding RNA AC023115.3 suppresses chemoresistance of GBM by reducing autophagy²³. Additionally, Liu et al. showed that lncRNA RP11-838N2.4 enhances the cytotoxic effects of temozolomide by inhibiting the functions of miR-10a in GBM cell lines²⁴. In our study, we demonstrated the oncogenic role of MNX1-AS1, which is consistent with its function in ovarian cancer¹⁵. Through the CCK-8 and Transwell assays, we proved that knockdown of MNX1-AS1 significantly suppressed GBM cell proliferation, migration, and invasion.

miRNAs are another member of noncoding RNAs, which have a length of 18–22 nucleotides²⁵. miRNAs could regulate the expression of target genes by associating with the complementary site in the 3'-UTR of

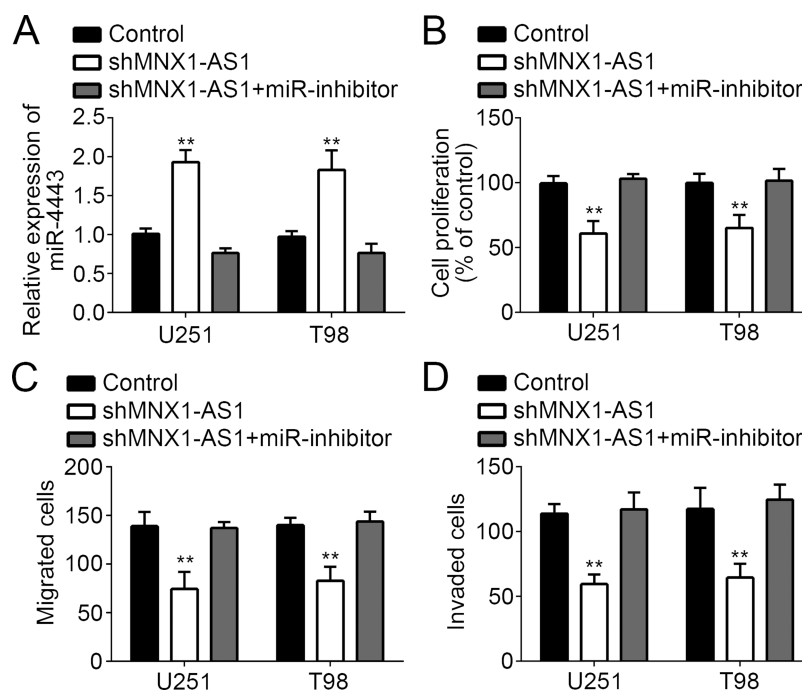


Figure 5. MNX1-AS1 promoted the proliferation, migration, and invasion of GBM cells by inhibition of miR-4443. (A) qRT-PCR analysis for the expression of miR-4443 in indicated cell lines. (B) CCK-8 assay for the detection of the proliferation of U251 and T98 cells transfected with shMNX1-AS1 and miR-4443 inhibitor or control. (C, D) Transwell assay was used for the evaluation of the migration and invasion of U251 and T98 cells transfected with shMNX1-AS1 and miR-4443 inhibitor or control. All data are representative of three independent experiments and expressed as mean \pm SD. ** $p < 0.01$.

specific mRNAs. Similar to lncRNAs, miRNAs are also reported to be widely involved in the regulation of cell development, survival, and death^{26–28}. Abnormal expression of miRNAs is also observed in nearly all kinds of cancers, including GBM²⁹. For instance, Chen et al. reported that downregulation of miR-205 is associated with GBM cell migration, invasion, and the epithelial–mesenchymal transition, by targeting ZEB1 via the Akt/mTOR signaling pathway²⁹. Another study indicated that miR-135a regulates NHE9 to inhibit proliferation and migration of GBM cells³⁰. In addition, Li et al. showed that miR-376a inhibits cell proliferation and invasion in GBM multiforme by directly targeting specificity protein 1³¹. Recently, evidence shows that lncRNAs could serve as miRNA sponges to exert biological functions³². In our study, we found that MNX1-AS1 could directly associate with miR-4443 in GBM cells. Through luciferase activity reporter assay, we proved their direct interaction. Meerson and colleagues showed that overexpression of miR-4443 suppresses NCOA1 and TRAF4 expression and decrease the invasiveness of human colon cancer cells³³. In addition, Chen et al. showed that miR-4443 induced malignancy of breast cancer³⁴. However, the function of miR-4443 in GBM requires to be investigated. By CCK-8 and Transwell assays, we showed that overexpression of miR-4443 inhibited the proliferation, migration, and invasion of GBM cells. Moreover, we demonstrated that knockdown of miR-4443 in MNX1-AS1-silenced GBM cells promoted cellular proliferation, migration, and invasion, which suggested that miR-4443 also acted as a tumor suppressor in GBM.

In conclusion, our findings demonstrated that MNX1-AS1 serves as an oncogene in GBM and promotes cancer cell proliferation, migration, and invasion by sponging miR-4443. Our study suggested that the MNX1-AS1/miR-4443 axis may be a novel therapeutic target for GBM intervention.

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