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MiR-491 functions as a tumor suppressor through Wnt3a/β-catenin signaling in the development of glioma

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Abstract. – OBJECTIVE: Glioma is the most frequent brain tumor that has high invasion and usually disperses to the whole brain through blood and basement membranes. MicroRNA-491 (miR-491) has been reported to have low expression and act as a tumor suppressor in several cancers. The Wnt/β-catenin signaling is a classic signaling pathway that participated in several biological processes. Our purpose was to detect the molecular mechanism of miR-491 in regulating the growth and metastasis of glioma.

MATERIALS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was applied to calculate the mRNA level of miR-491 and target gene. The protein expression of special genes was assessed by Western blot. The proliferation and invasive abilities were measured by the Cell Counting Kit-8 (CCK-8) and transwell assays. The Kaplan-Meier method was conducted to evaluate the association between the expressions of miR-491 with the overall survival of glioma patients.

RESULTS: We discovered that miR-491 was lowly expressed in glioma and downregulation of miR-491 predicted poor outcome of glioma patients. Similarly, a high expression of miR-491 suppressed the growth and metastasis in glioma cell line LN229. MiR-491 high expression inhibited the growth of glioma in a mouse xenograft model. Moreover, Wnt3a was a target gene of miR-491 and miR-491 mediated the invasion-mediated epithelial-mesenchymal transition (EMT) by regulating the expression of Wnt3a. Additionally, miR-491 regulated the proliferation through the Wnt/β-Catenin pathway by targeting Wnt3a.

CONCLUSIONS: MiR-491 overexpression inhibited the proliferation through the Wnt3a/β-catenin pathway and invasion-mediated EMT in glioma. The newly identified miR-491/Wnt3a/β-catenin axis provides novel insight into the pathogenesis of glioma.

Key Words:
MiR-491, Wnt3a/β-catenin, Glioma, Tumor suppressor, EMT.

Introduction

Glioma originated from precursors and the glial cell is the most frequent brain tumor in adults[1,2]. Due to the high relapse rate and the resistance to radiotherapy and chemotherapy, the mortality is high[3,4]. Therefore, to explore the biomarkers for the treatment is needed. MicroRNAs (miRNAs) has been reported to be non-code RNAs with approximately 19-28 nucleotides, regulating the translation and degradation of mRNA by binding to the mRNA 3'-UTR[5,6]. Increasing evidence revealed that several miRNAs act as an oncogene or tumor suppressor and regulate the tumor process in glioma, including miR-93, miR-215, miR-338 and miR-133b[7-10]. MiR-491 is located at fourth intron of FOCAD and act as tumor suppressor in tumors[11]. In osteosarcoma, Wang et al[12] indicated that miR-491 suppressed the lung metastasis and chemosensitivity. Moreover, miR-491 inhibited cellular proliferation and invasion by targeting JMJD2B in gastric cancer[13]. Therefore, we strongly believe that miR-491 may play a great role in tumorigenesis and metastasis. The Wnt family member 3A (Wnt3a), located at 1q42.13, was a key protein in the Wnt signaling pathway associated with the oncogenesis[14,15]. Pacella et al[16] showed that Wnt3a enhanced T-cell responses through indirect mechanisms and restrains tumor growth. Moreover, Oguma et al[17] revealed that Wnt3a was overexpressed in esophageal squamous cell carcinoma and the overexpression of Wnt3a predicted poor prognosis of ESCC patients. In addition, the Wnt/β-Catenin signaling regulated the proliferation and migration in breast cancer[18]. In this study, we discovered that Wnt3a was a direct target gene of miR-491 and miR-491 mediated the cell epithelial-mesenchymal transition (EMT) by regulating the expression of Wnt3a. Low expression of miR-491 inhibited the

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growth of glioma in a mouse xenograft model and downregulation of miR-491 predicted a poor outcome of glioma patients. MiR-491 regulated cellular proliferation through the Wnt/β-Catenin pathway by targeting Wnt3a.

Patients and Methods

Patients and Tissue Samples

A collection of 50 glioma patients who received treatment at the Xiangyang Central Hospital from January 2016 to June 2018 was obtained; we collected 50 pairs of glioma tissues and the corresponding peritumoral normal tissues. The fresh tissues were frozen in liquid nitrogen and then stored at -80°C in a refrigerator. We obtained the informed consents from the patients and the study was approved by the local Human Research Ethics Committee of the Xiangyang Central Hospital.

Cell Lines and Culture Conditions

Two human glioma cell lines LN18 and LN229 and a normal spongiocyte HEB were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Plasmid Construction and Transfection

The miR-491 mimic and miR-491 inhibitor oligo fragments were used to upregulate or downregulate miR-491, which were purchased from GenePharma (Shanghai, China). LN229 cells were seeded in 6-well plate to perform the transfection. The Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) reagent and the oligo fragments were diluted by the Opti-MEM/Reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA) and the two solutions were mixed; then, the mixture was added in the cells. The cells were harvested after transient transfection for 48 h; on the contrary, the Geneticin (G418; Thermo Fisher Scientific, Waltham, MA, USA) was employed to select the cells with stable transfection.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was employed to extract total RNAs while the mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to total miRNAs. The Prime Script RT Reagent Kit (TaKaRa Biotechnology Co. Ltd, Dalian, China) and the miRNA Reverse Transcription Kit (Life Technologies, Gaithersburg, MD, USA) were applied to synthesize the first cDNA chain of mRNA and miRNA, respectively. The SYBR Premix Ex Taq (TaKaRa Biotechnology Co. Ltd, Dalian, China) and the MystiCq microRNA qPCR Assay Primer (Sigma-Aldrich, St. Louis, MO, USA) performed the quantitative Polymerase Chain Reaction (qPCR) using an Applied Biosystems Step One Plus™ Real Time-Polymerase Chain Reaction (RT-PCR) system (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acted as the endogenous control of miR-491 and Wnt3a, respectively. The primers were as follows: miR-491: Forward 5'-GCCCCTTATGCAAGATTCCC-3', reverse GTGCAGGGTCCGAGGT-3'; U6: Forward 5'-CGCTTCACGAATTTGCGTGTCAT-3', reverse 5'-CTCGCTTCGGCAGCACA-3'; Wnt3a: forward: 5'-CCATCCTCTGCCTCAAATTC-3', reverse: 5'-TGGACAGTGGATATAGCAGCA-3'; GAPDH: Forward 5'-TCCACCACCCTGTTGCTGTA-3', reverse 5'-ACCACAGTCCATGCCATCAC-3'. The QRT-PCR reaction program was as follows: 95°C for 20 s; 95°C for 10 s and 60°C for 20 s for 40 cycles; 70°C for 10 s.

Western Blot Analysis

Cells were lysed using radioimmunoprecipitation assay (RIPA) Lysis Buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 10% phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA) on ice for 30 min. The protein solution was centrifugated at 12,000 ×g speeds for 20 min at 4°C and the supernatants were collected. The total amount of proteins was separated using 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) through electrophoresis and then transferred on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After being blocked by 5% fat-free milk at room temperature for 1 h, the blots were incubated the primary antibodies at 4°C overnight. The primary antibodies were against Wnt3a (1:1000; Abcam, Cambridge, MA, USA), E-cadherin (1:1000; Abcam, Cambridge, MA, USA), N-cadherin (1:1000; Abcam, Cambridge, MA, USA), Vimentin (1:1000; Abcam, Cambridge, MA, USA), p-PI3K (1:1000, Abcam, Cambridge, MA, USA), PI3K (1:1000, Abcam,

Cambridge, MA, USA), p-AKT (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), AKT (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (1:2000, Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the membranes were incubated by anti-rabbit or mouse horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h. Finally, enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) was employed to evaluate the signals.

Cell Counting Kit-8 Assay

Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay was employed to evaluate the proliferative ability. LN229 cells were seeded in 96-well plate and cultured at 37°C 24 h, 48 h, 72 h or 96 h. Subsequently, we added 10 μL of the CCK-8 solutions into each well and cultured 1 h at 37°C. The absorbance was assessed at 450 nm using a microplate reader (BioTeke, Beijing, China).

Transwell Assays

The transwell insert (8 μm membrane; Corning, Corning, NY, USA) covered with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), was conducted to evaluate the cell invasive ability. The inserts were placed in a 24-well plate and formed up. The LN229 cells were suspended by RPMI-1640 medium and we added 200 μL of suspensions in the upper chamber. Meanwhile, the lower chamber was filled with 500 μL of medium containing 15% FBS. After incubating the cells at 37°C for 24 h, the non-invasive cells on the upper surface were removed using cotton swabs. For the invasive cells, we fixed and stained by 4% paraformaldehyde and 10% crystal violet respectively; we then counted the cells under a microscope (Olympus Corporation, Tokyo, Japan).

Dual-Luciferase Reporter Assay

TargetScan predicted Wnt3a was a potential target gene of miR-491, and the binding site was located at 2359-2381 on 3'-UTR of Wnt3a mRNA. To verify whether miR-491 direct binding to Wnt3a mRNA 3'-UTR, the binding sequences were mutated from 5'-CCCUGCCCUCGGGUCUCCCCACC-3' to 5'-CCCUGCCCUCGGGUCUCCGGTCC-3' and inserted in the pmirGlo Luciferase reporter vector. The LN229 cells were co-transfected with the miR-491 mimic and the wild type (WT) 3'-UTR or the mutant 3'-UTR of Wnt3a. The Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA) was conducted to measure the Luciferase activity, and the Renilla Luciferase activity acted as normalization.

Tumor Xenograft Model in Nude Mice

We purchased 4 weeks old nude mice from the Charles River Laboratories (Beijing, China) to perform the tumor xenograft model experiment. To elucidate the role of miR-491 on glioma cell growth *in vivo*, followed by the mice culturing one week to adapt the environment, LN229 cells stably expressing miR-491 mimic or NC were subcutaneously inoculated in nude mice. Tumor volumes were evaluated every 3 days, and the mice were sacrificed and got out of the xenograft after implanted cells 26 days. All animal experiments were performed in the Animal Laboratory Center of Xiangyang Central Hospital and approved by the Xiangyang Central Hospital Animal Care and Use Committee.

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as the mean ± standard deviation. The comparison between groups was made using One-way analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference). p-values < 0.05 were considered to be statistically significant.

Results

MiR-491 Was Lowly Expressed in Glioma and Downregulation of MiR-491 Predicted Poor Prognosis

To verify the important roles of miR-491 in glioma, RT-qPCR was employed to evaluate the miR-491 mRNA levels of all 50 pairs of tissues. The expression of miR-491 was lower in glioma than in matched normal tissues ($p<0.0001$; Figure 1A). Moreover, the overall survival of the 50 cases of glioma patients was assessed and we discovered that the downregulation of miR-491 predicted poor prognosis in glioma ($p=0.0317$; Figure 1B).

MiR-491 Inhibited the Proliferation and Invasion

The miR-491 expressions of human astrocyte HEB and two glioma cell lines LN18 and LN229 were assessed by RT-qPCR. As expected, the expression of miR-491 was lower in

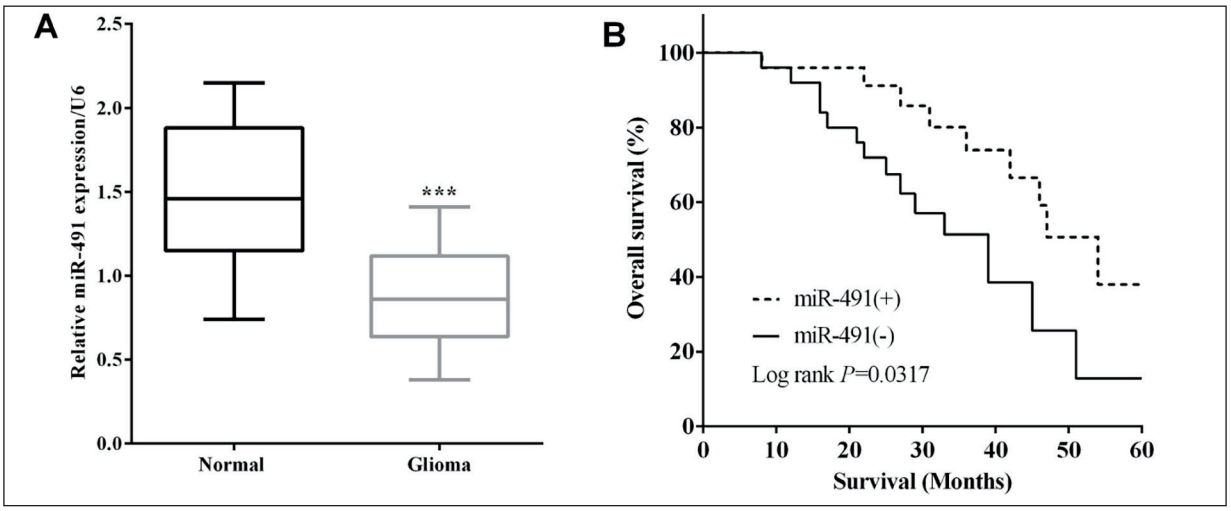

Figure 1. MiR-491 was lowly expressed in glioma and the downregulation of miR-491 predicted poor prognosis. **A,** The expression of miR-491 was lower in glioma than in the matched normal tissues. **B,** Downregulation of miR-491 predicted poor prognosis in glioma.

LN18 (p=0.0022) and LN229 (p=0.0005) than HEB (Figure 2A). To explore the biological mechanism of miR-491 in glioma, the miR-491 mimic or the miR-491 inhibitor were transfected into LN229 cells, to increase (p<0.0001) or decrease (p=0.0031) the miR-491 (Figure 2B). Moreover, CCK-8 data evaluated that the cell viability was reduced (p=0.0084 and 0.0023 of

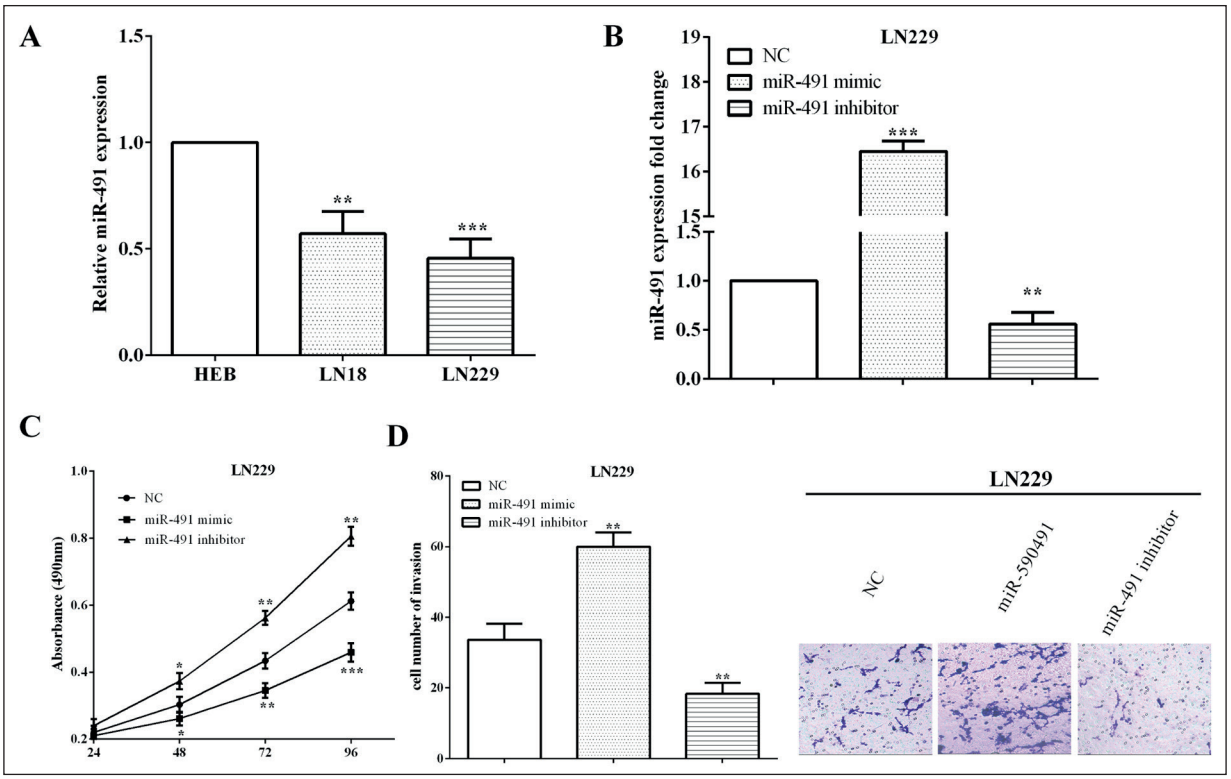

Figure 2. MiR-491 inhibited the proliferation and invasion. **A,** The expression of miR-491 was lower in LN18 and LN229 than HEB. **B,** The miR-491 mimic or the miR-491 inhibitor was transfected into LN229 cells, to increase or decrease the miR-491. **C,** CCK-8 data evaluated that the viability was reduced in miR-491 mimic-transfected LN229 cells, while it exhibited the opposite trend when cells were transfected with miR-491 inhibitor. **D,** The transwell assay revealed that the miR-491 mimic decreased cell invasion whereas the miR-491 inhibitor enhanced the invasive ability in LN229 cells (Magnification × 40).

72 h and 96 h) when transfected with the miR-491 mimic, while it exhibited the opposite trend when transfected with the miR-491 inhibitor in LN229 cells (p=0.0233, 0.0021 and 0.0009 of 48 h, 72 h and 96 h; Figure 2C). In addition, transwell assay revealed similar results with CCK-8, the miR-491 mimic decreased (p=0.0016) cell invasion whereas the miR-491 inhibitor enhanced (p=0.0082) the invasive ability in LN229 cells (Figure 2D).

Wnt3a Was a Direct Target of MiR-491 and the Expression of Wnt3a Was Mediated by MiR-491

Targetscan predicted that Wnt3a was a direct target gene of miR-491 and the binding sequences were located at 2359 to 2381 on mRNA 3'-UTR of Wnt3a. To verify whether miR-491 targeted Wnt3a in glioma, the binding sequences were mutated from 5'-CCCUGCCCUCGGGUCUC-CCCACC-3' to 5'-CCCUGCCCUCGGGUCUC-CGGTCC-3', which were designated as Wnt3a 3'-UTR WT and MUT (Figure 3A). In comparison with the negative control, the Luciferase activity was inhibited by the miR-491 mimic (p=0.0019), while this inhibitory effect disappeared when we mutated the predicted binding site (p=0.8503) (Figure 3B). In addition, the expression of Wnt3a was suppressed by the miR-491 mimic (p=0.0025) whereas enhanced by the miR-491 inhibitor (p=0.0003) in LN229 cells (Figure 3C).

MiR-491 Inhibited Cell Invasion-Mediated EMT and Proliferation Through the PI3K/AKT Signal Pathway

The expression of Wnt3a was calculated in tissues and cell lines by RT-qPCR. The expression of Wnt3a in glioma tissues was higher than that in matched normal tissues (p<0.0001; Figure 4A). Similarly, the Wnt3a was lowly expressed in glioma cells LN18 (p=0.0021) and LN229 (p=0.0005) compared to astrocyte HEB

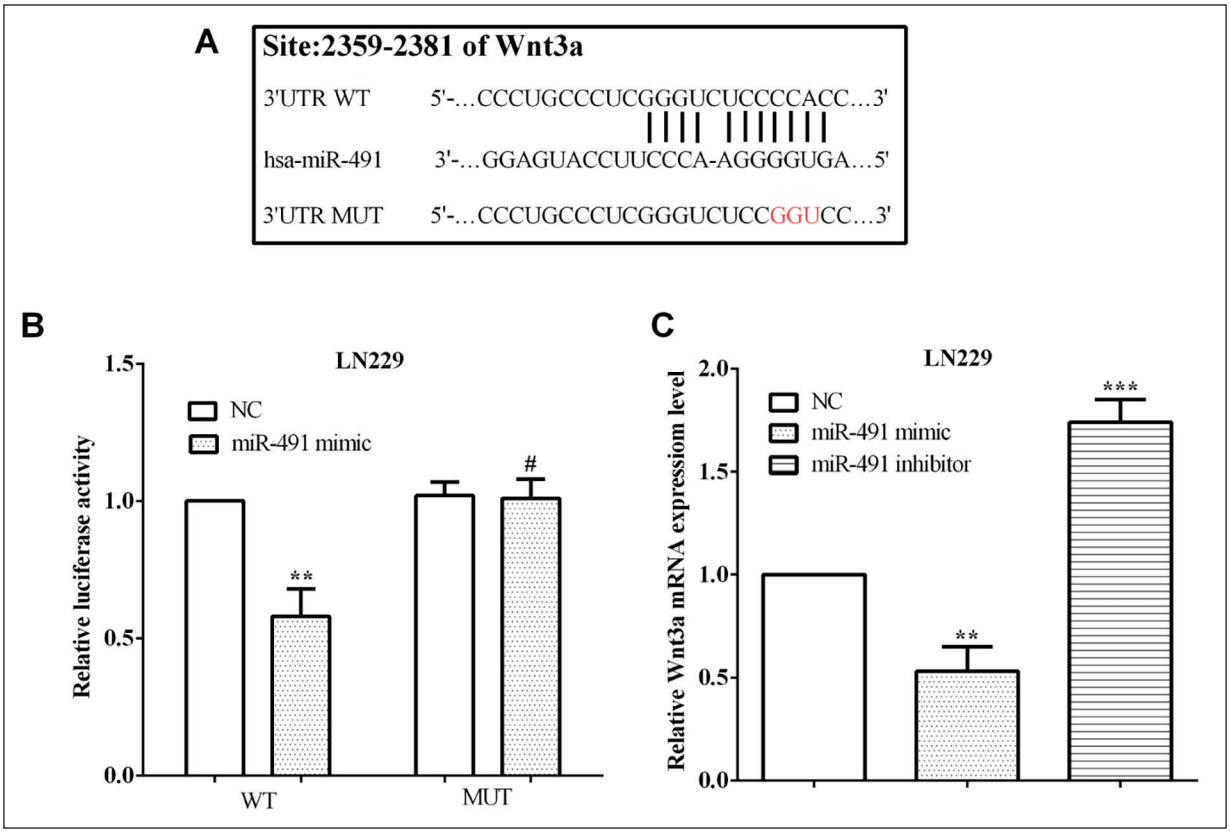

Figure 3. Wnt3a was a direct target of miR-491 and mediated by miR-491. **A,** Targetscan predicted that Wnt3a was a direct target gene of miR-491. **B,** The Luciferase activity was inhibited by miR-491 mimic, while this inhibitory effect disappeared when the predicted binding site was mutated. **C,** The expression of Wnt3a was suppressed by miR-491 mimic whereas it was promoted by miR-491 inhibitor in LN229 cells.

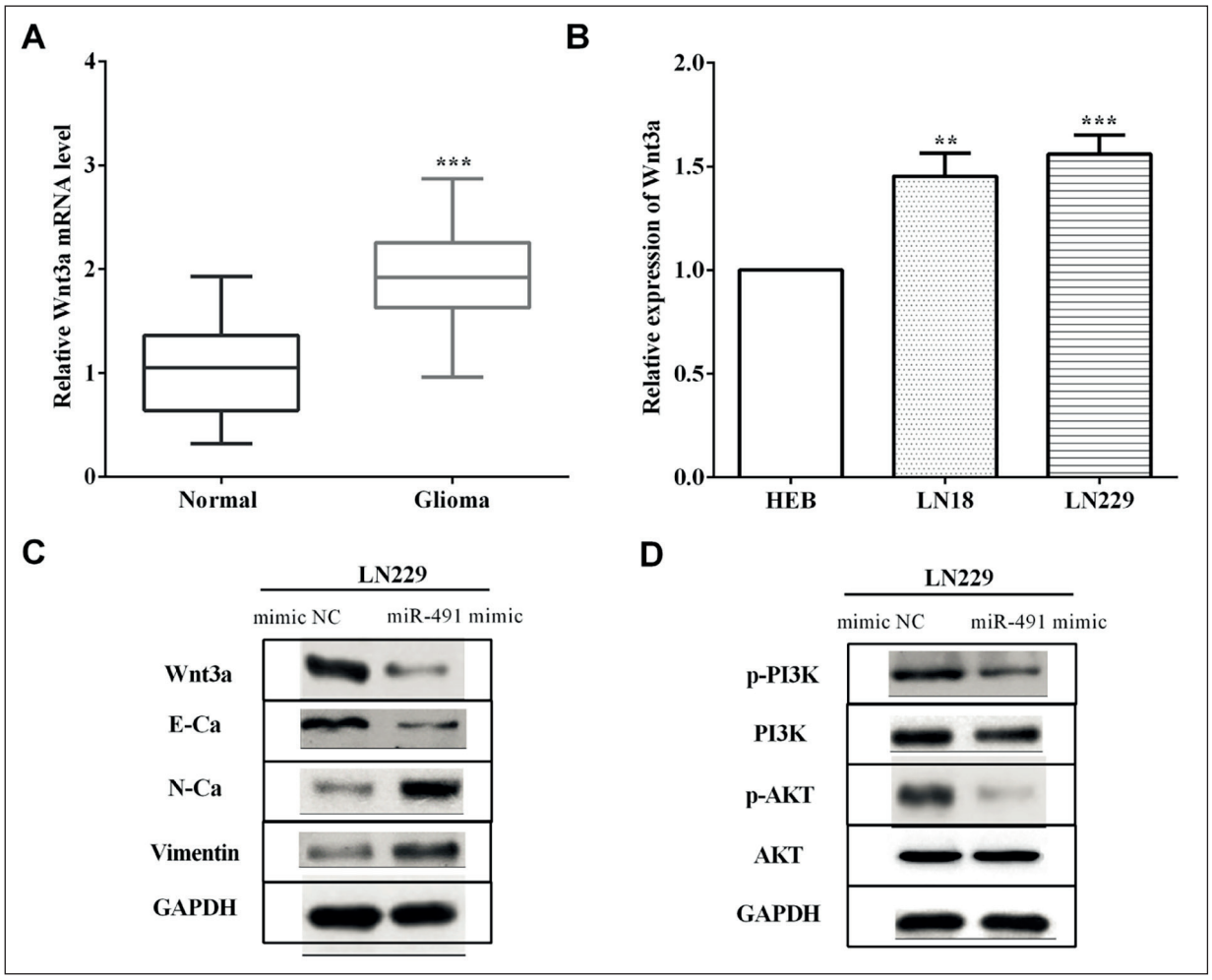

Figure 4. MiR-491 inhibited cell invasion-mediated EMT and proliferation through the PI3K/AKT signal pathway. **A,** The expression of Wnt3a in glioma tissues was higher than that in matched normal tissues. **B,** Wnt3a was lowly expressed in glioma cells than normal cells. **C,** MiR-491 inhibited EMT by regulating the expression of Wnt3a. **D,** MiR-491 suppressed the proliferation *via* the PI3K/AKT pathway through Wnt3a.

(Figure 4B). Moreover, the proteins expression associated with EMT and the PI3K/AKT signaling pathway was calculated by Western blot in LN229 cells. As expected, the miR-491 mimic inhibited the expression of Wnt3a and E-cadherin, while enhanced N-cadherin and Vimentin expression in LN229 cells (Figure 4C), which revealed that miR-491 inhibited the EMT by inhibiting Wnt3a. Meanwhile, up-regulation of miR-491 inhibited the expression of p-PI3K and p-AKT in LN229 cells (Figure 4D), which elucidated that miR-491 suppressed the proliferation *via* the PI3K/AKT pathway through Wnt3a. All the results revealed that miR-491 impaired glioma cell proliferative and invasive abilities by targeting Wnt3a *via* the PI3K/AKT signaling pathway and epithelial-to-mesenchymal transition.

MiR-491 Suppressed the Xenograft Growth In Vivo

LN229 cells that stably transfected the miR-491 mimic or control plasmid, were utilized to subcutaneously injected into one side of the nude mice. The xenograft tumor volumes were calculated every 3 days and the group of transfecting miR-491 mimic had a slower growth rate than the control group (Figure 5A). We also discovered that it had a smaller tumor volume of cells

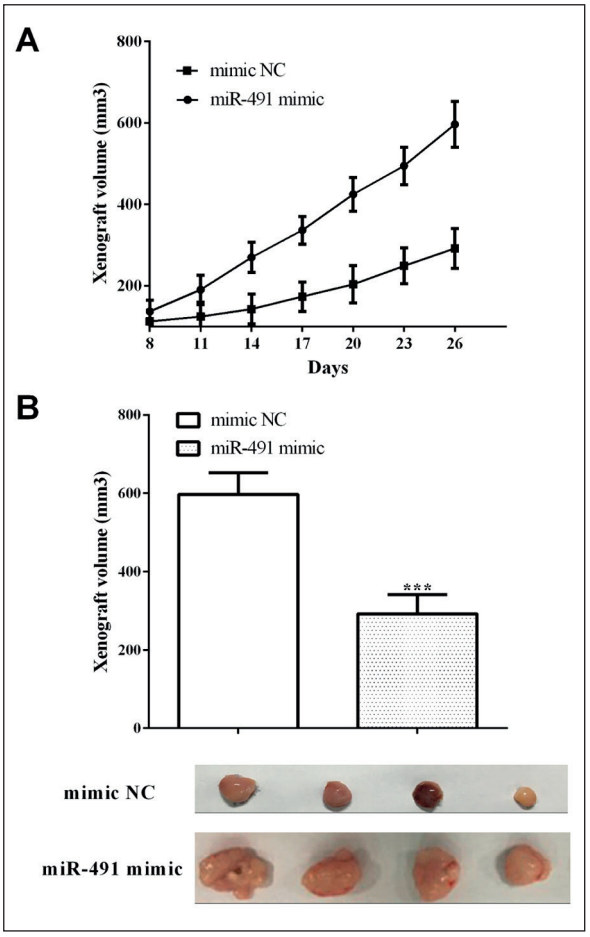

Figure 5. MiR-491 suppressed the xenograft growth *in vivo*. **A,** The tumor volume of cells that overexpressed miR-491 was smaller than that of the control group. **B,** Overexpression of miR-491 inhibited the growth of glioma xenograft.

overexpressed miR-491 than the control group (p=0.0002), which indicated that overexpressed miR-491 inhibited glioma xenograft growth (Figure 5B).

Discussion

Glioma, the most frequent brain tumor in adults, has a high mortality[1,19]. Thus, it is of great importance to explore the biomarkers for its treatment. MiRNAs were important post-transcriptional regulators that regulated mRNA translation and degradation by binding to the mRNA 3'-UTR[20,21]. MiR-491 was downregulated and functioned as a tumor suppressor in breast cancer[22]. MiR-491 inhibited cell proliferation and invasion in osteosarcoma and non-small cell lung cancer[23,24]. Consistent with all the results, we discovered that miR-491 was overexpressed in glioma tissues and cell lines. The overexpression of miR-491 suppressed the growth and metastasis in glioma cell LN229. Moreover, downregulation of miR-491 predicted poor outcome of glioma patients, which agreed with the findings of Wang et al[12] in osteosarcoma. In addition, Xu et al[25] elucidated that miR-491 mediated the cell cycle and metastasis in prostate cancer and inhibited prostate cancer xenograft growth. Consistent with this study, we revealed that the overexpression of miR-491 inhibited glioma growth in a mouse xenograft model. Wnt3a, a member of the WNT gene family, was a key protein in the Wnt signaling pathway[14]. Lu et al[26] showed that Wnt3a promoted cell proliferation, invasion and cell cycle in hepatocellular carcinoma. Moreover, Wnt3a induced the EMT and enhanced cell migratory and invasive capacities in lung adenocarcinoma[27]. In the present research, we found that Wnt3a was overexpressed in glioma tissues and cell lines in comparison with the normal control. Wnt3a was a target gene of multiple miRNAs, including miR-766, mIR-195, miR-15a and miR-214[28-31]. Our results were consistent with the study by Sun et al[32] in gastric cancer; Wnt3a was a direct target gene of miR-491 and miR-491 mediated the cell EMT by regulating the expression of Wnt3a. Wnt3a/β-catenin signaling was a classic signaling pathway which participated in several biological processes, including growth, differentiation and carcinogenesis[33-35]. In summary, we discovered that in glioma miR-491 regulated cell proliferation through the Wnt/β-Catenin pathway by targeting Wnt3a.

Conclusions

The above data indicated that miR-491 overexpression suppressed the growth and metastasis by targeting Wnt3a in glioma cell LN229. Moreover, downregulation of miR-491 predicted poor outcome of glioma patients. The overexpression of miR-491 inhibited glioma growth in a mouse xenograft model. MiR-491 mediated the cell EMT by regulating the expression of Wnt3a. MiR-491 regulated cell proliferation through the Wnt/β-Catenin pathway by targeting Wnt3a.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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