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MiRNA-191 functions as an oncogene in primary glioblastoma by directly targeting NDST1

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Abstract. – OBJECTIVE: Glioblastoma is identified as the most aggressive primary brain tumor. Growing evidence has demonstrated that aberrant expression of miR-191 has oncogenic potentiality in many cancers. However, the effects and the underlying mechanisms of miR-191 in the development of glioblastoma remain largely unknown. The aim of this study was to elucidate the pathobiological functions of miR-191 expression by targeting N-deacetylase/N-sulfotransferase 1 (NDST1) in human glioblastoma.

PATIENTS AND METHODS: The miR-191 level in human glioblastoma tissues and four cell lines was examined using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Animals study and MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay were used to examine the effects of miR-191 on human glioblastoma proliferation. Western blot and Luciferase reporter were used to confirm that miR-191 could regulate NDST1 expression.

RESULTS: We found that the miR-191 expression was upregulated in human glioblastoma tissues and cells. MiR-191 over-expression was sufficient to promote human glioblastoma cells growth *in vivo* and *in vitro*. We also found that miR-191 directly targeted NDST1 and negatively regulated the NDST1 expression in human glioblastoma cells.

CONCLUSIONS: In summary, our finding suggested that miR-191 acted as an important regulator in promoting glioblastoma cell proliferation *in vivo* and *in vitro*, and this cellular function may be because of its negative regulation of NDST1.

Key Words:
Glioblastoma, MiR-191, NDST1, Proliferation.

Introduction

Approximately 70% of human malignant primary brain tumors are gliomas[1]. Unfortunately, the most common histological subtype of malignant glioma is the glioblastoma multiforme (GBM), which has the highest morbidity and mortality among humans[2]. GBM is featured with a large degree of tumor heterogeneity and easy invasion into surrounding tissues[3,4]. In spite of many advanced protocols of surgical and radiotherapeutic techniques, the long-term outcomes of clinical therapy remain unsatisfactory, although the results have been remarkably improved[5]. Nevertheless, with the intensive studies correlated to glioblastoma progression, it is urgent to find underlying molecular mechanisms of glioblastoma and identify the promising therapeutic targets to improve treatment strategies.

MicroRNAs (miRNAs) are a subset of a small non-coding RNA molecule, which could interact with target messenger RNAs (mRNAs) in the 3'-untranslated region (3'-UTR) to regulate the transcriptional degradation or repression[6]. MiRNAs seem to play a crucial role in diverse regulatory pathways including developmental timing, growth, cell differentiation, apoptosis and fat storage[7]. Moreover, accumulating evidence showed that the miRNA expression was a significantly different cause of various tumors, and the certain tumors progression lineage and differentiation state can be reflected by miRNA profiles[8]; moreover, miRNAs aberrant expressions have been associated with the development and metastasis of cancer[9], including human primary glioblastoma[10]. A previous research[11] has suggested that the deregulation of miR-191 was common in various types of human cancers, and this deregulation can affect the disease prognosis, clinical stage and patient survival. However, the functional molecular mechanisms of miR-191 in glioblastoma are still

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elusive, and the function of miR-191 acting as a therapeutic target of glioblastoma remains to be detected.

Heparan sulfate (HS) proteoglycans, predominantly in basement membranes, are produced as cell surfaces and extracellular matrix ubiquitous components by most mammalian cells[12]. During the biosynthesis of HS, four known N-deacetylase/N-sulfotransferases (NDSTs) enzymes are demonstrated to play a critical step in constructing the sulfation pattern, and then affecting the HS structure[13]. Among the four vertebrate NDSTs, the expression patterns of NDST1 and NDST2 are much broad and we can see in all embryonic and adult tissues examined, whereas the expression pattern of NDST3 and NDST4 are much more restricted[14]. In the previous work, microRNA-24 targeted NDST1 to reduce HS sulfation and thereby the binding affinity of HS for vascular endothelial growth factor A (VEGFA)[15].

We found that miR-191 was over-expressed in glioblastoma, compared to normal non-cancer tissue and a normal human glioblastoma cell line. MiR-191 can promote glioblastoma cell growth *in vitro* and *in vivo*. The expression level of NDST1 was inversely correlated with miR-191, and NDST1 may be a downstream target gene of miR-191. MiR-191/ NDST1 axis appeared to be the underlying target for human glioblastoma therapy.

Patients and Methods

Tissue Samples

From Jining No. 1 People's Hospital (Jining, Shandong Province, China), a total of 46 cases of tissue specimens including human glioblastoma tissues and paired normal adjacent tissues (NATs) were obtained between February 2013 and September 2015. Liquid nitrogen was used to flash-frozen all the tissue specimens, and the specimens were stored at -80°C. None of the spinal glioblastoma patients had received radiotherapy or chemotherapy before surgery. The use of human tissues was approved by the Ethics Committee of Jining No. 1 People's Hospital.

Cell Lines and Transfection

The four human glioblastoma cell lines (U87, U251, A172, U118) and primary normal human astrocytes (NHAs) were obtained from Cell Bank, Chinese Academy of Sciences (Xuhui District, Shanghai, China). The NHAs cells were cultured under the conditions according to the manufacturer's instruction. Human glioblastoma cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) at 37°C and 5% CO_2. Transfection of cells was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the glioblastoma tissue specimens and cells using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was examined using SYBR Green Premix ExTaq (TaKaRa, Dalian, China) with the Roche Light Cycler 480 system (Roche, Basel, Switzerland). TaqMan gene assays were provided for determination of NDST1 expression from Applied Biosystems (Foster City, CA, USA). U6 snRNA was used as endogenous control for normalization of miR-191 expression, and β-actin was chosen for NDST1 expression as endogenous control. The expression ratios were calculated by the $2^{-\Delta\Delta CT}$ method. Each experiment was repeated at least in triplicates. The following primers were used: miR-191 forward, 5'-CGGAATTCGAGCGTCTTGTTCCCTCTAGACTC-3', and reverse, 5'-GCAGACTCGAGGGAGTCACTACCATTGCAG-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAA-3', and reverse, 5'-AAAATATGGAACGCTTCACGA-3'; NDST1 forward, 5'-CGAGGATCCGAGCAATACTCTGTGGAG-3', and reverse, 5'-CGGAATTCACATATGGCAGCGAATACTGAG-3'; β-actin forward, 5'-CGTGACATTAAGGAGAAGCTG-3'; and reverse, 5'-CTAGAAGCATTTGCGGTGGAC-3'. All primers used were offered by RiboBio (Guangzhou, China).

Western Blot Analysis

Human glioblastoma cells or tissues were lysed with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) by semidry blotting (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk and incubated with a specific primary antibody rabbit polyclonal anti- NDST1 antibody (dilution, 1:1,000; Cat # PA5-48535; Invitrogen, Carlsbad, CA, USA) at 4°C overnight, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary

antibody (dilution, 1:2,000; Cat # 14708, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive bands were visualized by autoradiography using enhanced chemiluminescence (ECL) Western blotting kit (Thermo Fisher Scientific, Waltham, MA, USA). The bands were subjected to quantification using the Image J imaging processing program (National Institutes of Health, Bethesda, MD, USA).

Cell Proliferation Assays

To monitor cell proliferation, 24 h after transfection with miRNA mimics, cells were plated in 96-well plates. Cells were seeded and DMEM medium was subsequently added into 96-well plates and incubated for 24, 48, and 72 h at 37°C with 5% CO_2. MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for incubation for 4 h. After centrifugation, the culture medium was removed and dimethyl sulfoxide (DMSO; 100 μL, Sigma-Aldrich, St. Louis, MO, USA) was added into the plates to dissolve the crystals. The absorbance value of each well was measured at the optical density (OD) 450 nm using enzyme-linked immunoassay.

Dual-Luciferase Assay

To illustrate the mechanisms underlying miR-191 functions as an oncogene of human glioblastoma pathology, bioinformatics software (Target Scan, http://www.targetscan.org/) was employed to identify potential target genes for miR-191. For Dual-Luciferase assays, the 3'-UTR of human NDST1 containing miR-191 binding sites were cloned into a pMir-Report (Ambion, Austin, TX, USA) to generate wild-type (WT) pMir-NDST1 3'-UTR. The NDST1 mutant (MUT) 3'-UTR with miR-191 target sites was produced by replacement of wild-type sequence to prevent the binding of miR-191. We used the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) to detect the Luciferase activities. All the experiments were performed in triplicates.

In Vitro Tumor Growth Analysis

For *in vitro* tumor growth analysis, U251 cells (1 million in 100 μL DMEM) with stably miR-191 over-expression or control transfection were subcutaneously inoculated into the female athymic mice (8-week old). Mice were sacrificed after 25 days after inoculation. Tumors were retrieved and prepared as formalin-fixed. All experimental procedures involving animals were approved by the Animal Care and Use Committee of Jining No. 1 People's Hospital.

Statistical Analysis

All the results analyses were examined by Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS, Chicago, IL, USA) and outcomes are presents as mean ± SD (Standard Deviation). Student's *t*-test and one-way analysis of variance (ANOVA) in SPSS were used to analyze the differences between the groups. The correlation between mRNA and miRNA were estimated using Spearman's correlation method. p-value < 0.05 was considered statistically significant.

Results

MiR-191 Expression Was Higher and NDST1 Was Lower in Human Glioblastoma Tissues and Cells

To investigate the miR-191 expression levels in human glioblastoma, the research first evaluated miR-191 expression level in 46 paired glioblastoma and their NATs specimens using qRT-PCR. The miR-191 expression was markedly higher in glioblastoma tissues compared with NATs specimens (Figure 1A). MiR-191 was also increased in four glioblastoma cell lines (A172, U87, U118 and U251) compared with NHAs (Figures 1B).

Furthermore, the levels of NDST1 in the glioblastoma tissues were also assessed by qRT-PCR. The NDST1 relative expression level was significantly decreased in the glioblastoma tissues and cell lines as shown in Figure 1C-D. Then, we found miR-191 was negatively correlated with the NDST1 level in these glioblastoma clinical specimens (Figure 1E).

Overexpression of MiR-191 Promoted Growth of Glioblastoma Cells

To assess the effect of miR-191 on glioblastoma, the miR-191 mimic or NC were constructed and transfected into U251 and U118 cells. The successful over-expression of miR-191 in glioblastoma cells was detected by qRT-PCR (Figure 2A). The proliferative abilities were increased in miR-191-mimic transfected cell lines (U251, U118) when compared with miR-NC transfected cells, as measured by MTT assays (Figure 2B-2C). The cell growth curves showed ectopic expression of miR-191 promoted the growth of

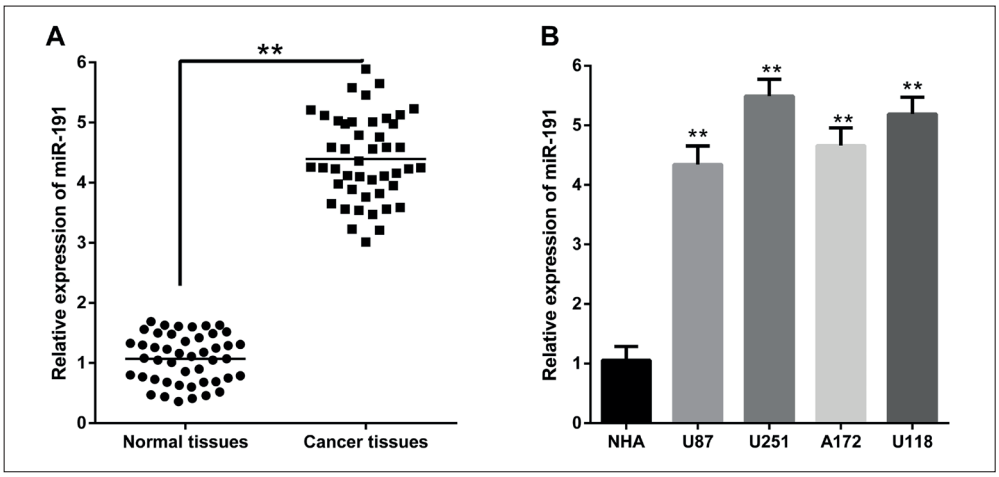

Figure 1. MiR191 expressions in human glioblastoma tissues and cells. ***A,*** MiR191 expression was detected by qRT-PCR in human glioblastoma tissues and their paired tissues (normal). ***B,*** The expression level of miR-191 in four glioblastoma cells (U87, U251, A172, U118) and primary normal human astrocytes (NHA) measured by qRT-PCR. ***C-D,*** The expression level of NDST1 in four glioblastoma cells (U87, U251, A172, U118) and primary normal human astrocytes (NHA) measured by qRT-PCR. ***E,*** Spearman correlation analysis examined the miR-191 and NDST1 expression in glioblastoma tissues. $**p<0.01$, $*p<0.05$.

U251 and U118 cells, indicating that the over-expression of miR-191 promoted cell proliferation of glioblastoma.

MiR-191 Directly Targeted NDST1 and Regulated the NDST1 Expression in Human Glioblastoma Cells

To illustrate the mechanisms of miR-191 which function as an oncogene in human glioblastoma pathology, bioinformatics software (Target Scan) was employed to identify potential target genes for miR-191. The binding site of NDST1 mRNA for miR-191 locates at its 3'-UTR (Figure 3A). We engineered Luciferase reporter constructs, containing either NDST1 WT 3'-UTR or NDST1 3'-UTR to confirm the direct connection between NDST1 and miR-191. Figure 3B showed that the miR-191 expression level was inversely correlated with NDST1 level, miR-191 over-expression decreased the Luciferase activity of the NDST1 WT 3'-UTR, whereas it was not affected the Luciferase activity of the MUT NDST1 3'-UTR, which was consistent with our previous findings as shown in Figure 1E. In addition, the over-expression of miR-191 significantly inhabited NDST1 expression at the protein levels in U251 cells as confirmed by Western blot (Figure

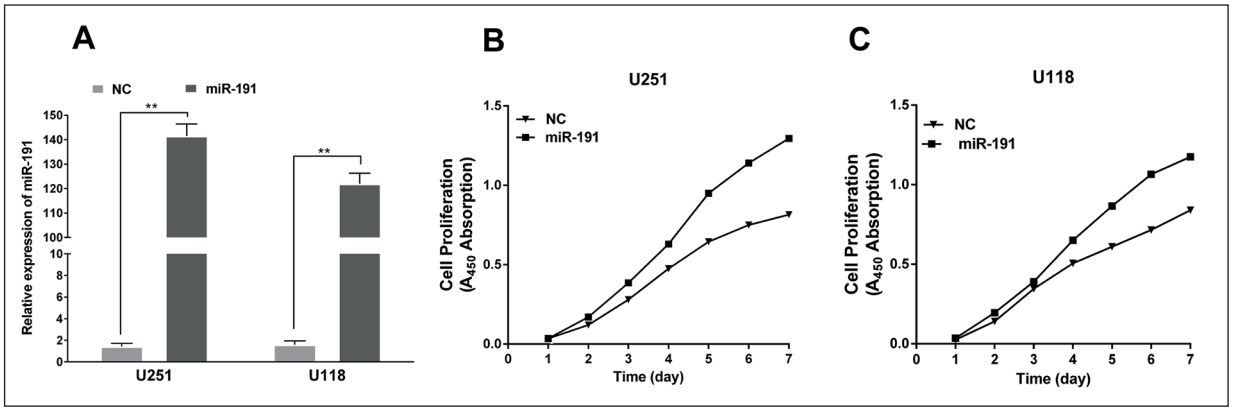

Figure 2. Over-expression of miR191 promotes cell proliferation. ***A,*** MiR-191 is successfully re-expressed in U251 and U118 cells as confirmed by qRT-PCR. ***B-C,*** Growth curves of miR-191 in U251 and U118 cells are measured by MTT assay. $**p<0.01$, $*p<0.05$.

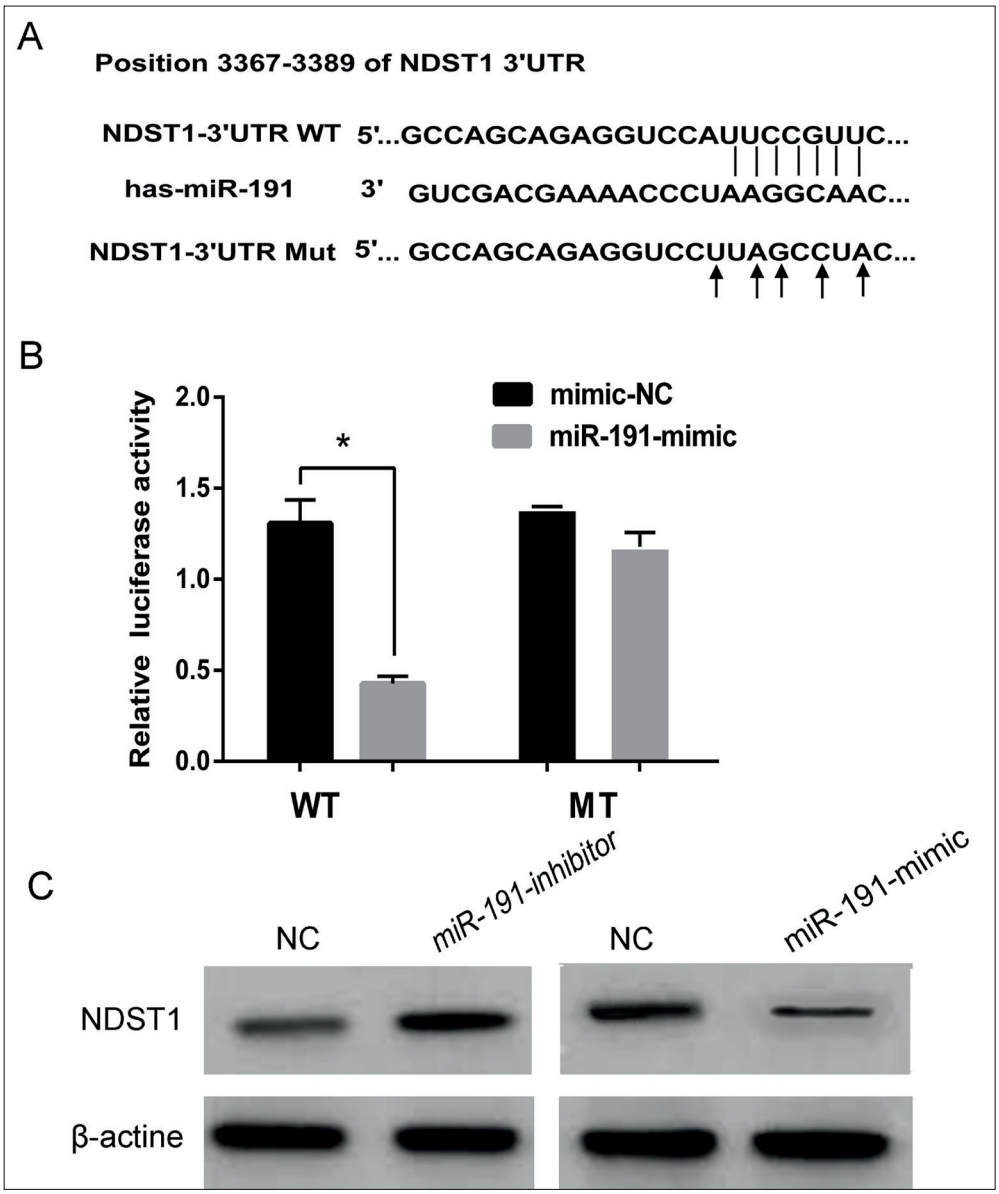

Figure 3. MiR-191 directly targeted NDST1 and negatively regulated the endogenous NDST1 expression in human glioblastoma cells. ***A,*** The NDST1 WT 3'-UTR carries a binding site of miR-191. ***B,*** Luciferase reporter assay showed the effect of miR-191 on NDST1 3'UTR Luciferase activity in U251 cells. ***C,*** The expression levels of NDST1 protein transfected with miR-191 mimics and miR-191 inhibitor by Western blot. *$p<0.05$.

3C). These findings strongly demonstrated that miR-191 could affect the endogenous NDST1 expression negatively and NDST1 was a target gene of miR-191.

Effects of MiR-191 Over-expression on Glioblastoma Xenograft Tumor Growth in Nude Mice

The above findings have indicated that miR-191 can remarkably promote the glioblastoma cells growth *in vitro*. To further investigated miR-191 function in glioblastoma Proliferation, we examined whether the forced expression of miR-191 could increase U87 cells ability to form xenograft tumors in nude mice. After 25 days of inoculation, the results showed that over-expression of miR-191 in U87 cells increased tumor growth compared with control group *in vivo* (Figure 4A-4B), suggesting that miR-191 acts as a promoter among tumor growth *in vivo*.

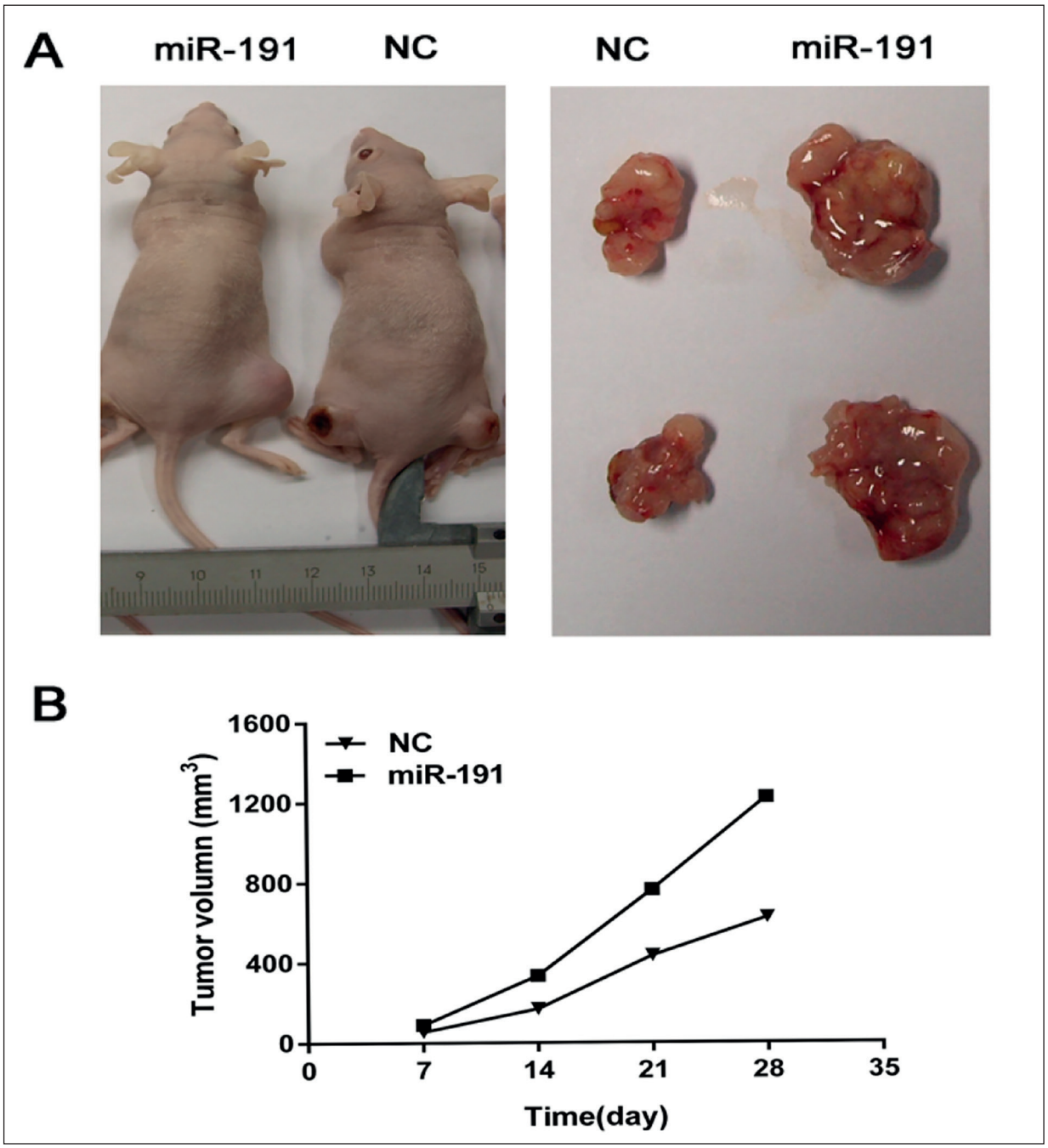

Figure 4. Effects of miR-191 over-expression on glioblastoma xenograft tumor growth *in vitro*. ***A,*** Tumors formed in nude mice. U251 cells stably over-expressing miR-191 or empty vector were injected into the flanks of nude mice (n = 10), and the mice were sacrificed after 25 days. ***B,*** The volume of the tumors injected with miR-191-overexpressing U251 cells was increased than that in those injected with miR-ctrl-expressing cells. The data are shown as the means ± SD.

Discussion

Glioblastoma is identified as the most common and aggressive primary brain tumor and it is characterized by the hallmarks of hypoxia and necrosis, rapid proliferation, extensive invasion and strong resistance to radiation and chemotherapy[16-18]. Due to the highly invasive and diffusely infiltrate of glioblastomas, the radical resection of this type of tumor mass cannot be permanently

curative[10]. MiRNAs indeed play a decisive role in tumor progression because of the miRNAs misregulation results in their target genes expression aberrantly which may function as tumor suppressors or oncogenes. Increasing evidence has shown abnormal expressions of miRNAs have been connected with the development of human glioblastoma[6,19]. Although we have constructed miRNA signatures for glioblastoma, it still in the early stage of elucidation of the deregulated miRNAs role in glioblastoma tumorigenesis and progression.

Previous studies have reported miR-191 functions differentially in various types of tumors, such as gastric cancer[20], pancreatic cancer[21], colorectal cancer[22], breast cancer[23]. Furthermore, Li et al[24] have reported that miRNAs, such as miR-191, can be considered biomarkers for prognosis stratification in each subtype of glioblastoma, increasing evidence indicated that miRNAs play vital roles as diagnostics biomarkers for human cancers[25]. In our work, we used qRT-PCR, MTT assay and animals studies to detect the functional molecular mechanisms of miR-191 in glioblastoma tissues and U251 and U118 cells. The results showed the high-expression levels of miR-191 in human glioblastoma tissues and U251 and U118 cells, suggesting its potential oncogenic activity. The over-expression of miR-191 could promote cell proliferation, which indicated that miR-191 was a tumor promoter in glioblastoma. Furthermore, animal studies showed that high-expression levels of miR-191 could also promote tumor growth *in vivo*, which further supported its oncogenic role in glioblastoma.

NDST1 is an enzyme in the heparan sulfate biosynthetic pathway whose function has been shown to be a key part in embryonic development of the skeletal system, vascular system, craniofacial region and lungs[26-29]. A significant decrease in the NDST1 expression levels in gliomas was demonstrated, and low expression levels of the heparan sulfate biosynthesis-involved genes NDST1 in glioblastoma were changed in gliomas of different grades. In our study, we predicted that NDST1 was acting as one target of miR-191 with microRNA Targetscan software. MiR-191 expression inversely correlated with NDST1 in human glioblastoma tissues and cells, and Western blot and Luciferase reporter assay also showed that NDST1 was regulated by miR-191. These results indicate that miR-191 may function as a tumor promoter partly mediated by repressing NDST1 expression in glioblastoma development.

Conclusions

Our findings identified the specific functional molecular mechanisms of miR-191 in human glioblastoma proliferation, and verified NDST1, a multifaceted oncogene, as the downstream target gene of miR-191, whose expression was suppressed by miR-191. The study indicated that miR-191 acted as an oncogene and was a promising therapeutic target in human glioblastoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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