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LncRNA SNHG20 promoted the proliferation of glioma cells via sponging miR-4486 to regulate the MDM2-p53 pathway

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Abstract. – OBJECTIVE: Evidence demonstrated the critical role of long noncoding RNAs (lncRNAs) in the initiation and development of human cancers. LncRNA small nucleolar RNA host gene 20 (SNHG20) was recently reported to promote the progression of several cancers; however, the function of SNHG20 in glioma has not been characterized.

PATIENTS AND METHODS: Differential expression of SNHG20 in glioma tissues and cell lines was analyzed by RT-qPCR. Cell Counting Kit-8 (CCK-8) assay was performed to detect the cell viability. The targets prediction was analyzed with the TargetScan database. Western blot was performed to check the protein expression.

RESULTS: SNHG20 was overexpressed in glioma tissues and cell lines. Down-regulation of SNHG20 suppressed the proliferation, migration and induced apoptosis of glioma cells. Molecular studies uncovered that SNHG20 acted as a competing endogenous RNA (ceRNA) to sponge the expression of miR-4486. MiR-4486 was down-regulated in glioma tissues and significantly inversely correlated with the expression of SNHG20. Restoration of miR-4486 remarkably attenuated the promotion effect of SNHG20 on the growth of glioma cells. Further study revealed that miR-4486 targeted the E3 ubiquitin ligase mouse double minute 2 (MDM2) and negatively regulated the expression of MDM2. Down-regulation of MDM2 by miR-4486 increased the abundance of p53 in glioma cells.

CONCLUSIONS: We identified the functional mechanism by which SNHG20 modulated the malignancy of glioma cells via targeting the miR-4486/MDM2/p53 pathway. Interrupting the expression of SNHG20 might be a novel strategy to suppress the progression of glioma.

Key Words:
Glioma, SNHG20, MiR-4486, MDM2, p53.

Introduction

Glioma is one of the most common lethal and rapidly advanced tumor malignancies[1]. Currently, surgical resection combined with radiotherapy and chemotherapy has been widely applied in the treatment of glioma. However, the mortality rate of the glioma patients is still high and the prognosis of glioma remains poor[2]. Therefore, investigating the molecular mechanisms that are involved in the malignant progression of glioma is urgent. Identification of novel factors that contribute to the initiation and development of glioma would benefit the design of therapeutic strategies. Accounting evidence[3,4] has demonstrated that non-coding RNAs (ncRNAs) play important roles in regulating gene expression and progression of human diseases, especially cancers. Long non-coding RNAs (lncRNAs) are characterized as a class of RNAs longer than 200 nucleotides (nt) without protein-coding ability[5,6]. It has been well documented that lncRNAs play an important role in the establishment and progression of cancers through modulating the expression of genes that are involved in the oncogenesis[7-9]. The small nucleolar RNA host gene 20 (SNHG20, GeneBank Accession No. NR-027058.1) is a newly defined long noncoding transcript localized at 17q25.2, which is originally identified in hepacellular carcinoma (HCC)[10]. Overexpression of SNHG20 was suggested as a potential useful biomarker for the prognosis of HCC patients[10]. Additionally, the oncogenic function of SNHG20 was also found in colorectal cancer (CRC). Highly expressed SNHG20 was remarkably associated with the advanced progression of CRC patients[11]. These studies shed light on the potential involve-

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ment of SNHG20 in the development of human cancers; however, the function of SNHG20 in glioma has not been investigated.

In addition to lncRNAs, microRNAs (miRNAs) are a large part of ncRNA with the length of approximately 22 nucleotides[12,13]. MiRNAs are defined as key post-transcriptional regulators of gene expression through binding to the 3'-untranslated region (3'-UTR) of targeted mRNAs[13-15]. MiRNAs play critical roles in the tumorigenesis[16-21]. Increasing reports[22-26] indicated that lncRNAs act as competitive endogenous RNAs (ceRNAs) to sponge miRNAs and regulate the binding of miRNAs with the targets, which consequently modulate the expression of targeted mRNAs. Guo et al[27] showed that SNHG20 promoted the proliferation and invasion of cervical cancer cells via sponging miR-140-5p. Additionally, SNHG20 modulated the expression of human epidermal growth factor receptor-2 (HER2) via sponging miR-495 and enhanced the malignancy of breast cancer[28]. These findings suggested SNHG20 as a potential oncogene in the progression of cancers by acting as a ceRNA. In this study, we found that SNHG20 was highly expressed in glioma tissues. Down-regulation of SNHG20 suppressed the growth of glioma cells. The underlying molecular mechanism revealed that SNHG20 sponged miR-4486 and modulated miR-4486-targeted expression of MDM2 in glioma cells.

Patients and Method

Patients

Glioma cell lines including U87, U251, SHG-44 and SW-38 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Corning, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were maintained at 37ºC with 5% CO_2. Fifty paired glioma cancer tissues and matched adjacent normal tissues were obtained from glioma patients who were underwent surgical resection between January 2012 and December 2014 at the Lishui People's Hospital. The tissue samples were reviewed independently by two specialists and handled with the approval of the Institutional Review Board of Lishui People's Hospital. All the tissues were stored with liquid nitrogen before RNA extraction. Written informed consents were obtained from all patients.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from tissues or cells using the TRIzol reagent (Invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The concentration of RNA was determined by measuring the absorbance at 260/280 nm using Nano-Drop spectrophotometer (ND-100, Thermo-Fisher, Waltham, MA, USA). cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The expression of SNHG20 was detected by Real-time PCR using the SYBR Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan) on the 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression of U6 RNA was detected as the internal control. The PCR cycling conditions were as follows: 95ºC for 5 min; 40 cycles at 95°C for 10 s and 60°C for 1 min. The relative fold change of gene expression was calculated with the $2^{-\Delta\Delta Cq}$ method. The primers used in this study were summarized as Table I.

Cell Proliferation Assay

U87 cells were seeded in the 96-well plate at the density of 2,000 cells per well and transfected with the corresponding expression vector. 20 μl of CCK-8 solution (Millipore, Billerica, MA, USA) was added into the medium at the indicated time points for 4 h at 37°C. The absorbance of each well at 450 nm was measured with the microplate reader (Bio-Rad, Hercules, CA, USA).

Western Blot

U87 cells transfected with the corresponding expression vector were lysed with the NP-40 lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) on ice of 15 min. Samples were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected and the protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Equal amount of protein was separated with 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membrane was blocked with 5% non-fat milk in Tris-buffered saline and Tween (TBS) for 1 h at room temperature (RT). And then membrane was incubated

Table I. The primers used in this study.

Primers	Sequences (5′--3′)
SNHG20-Forward	ATGGCTATAAATAGATACACGC
SNHG20-Reverse	GGTACAAACAGGGAGGGA
MiR-4486-Forward	ACACTCCAGCTGGGGCTGCGCGA
MiR-4486-Reverse	TGGTGTCGTGGAGTCG
MDM2-Forward	ACCCATCTACCCTGACCACA
MDM2-Reverse	AGAATGCTTTAGTCCACCTAACCT
GAPDH-Forward	GCACCGTCAAGGCTGAGAAC
GAPDH-Reverse	TGGTGAAGACGCCAGTGGA
U6-Forward	CTCGCTTCGGCAGCACA
U6-Reverse	AACGCTTCACGAATTTGCGT
p53-Forward	CCATGAGCGCTGCTCAGATA
p53-Reverse	CAGGCACAAACATGCACCTC

with the primary antibody overnight at 4°C. After washing three times with TBS, membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 1 h at RT. The protein signal was visualized with the enhanced chemiluminescence kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Cell Apoptosis Analysis

Cell apoptosis was determined with the Annexin V-APC/7-AAD kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Briefly, cells were transfected with the indicated expressing vector for 48 h and then cells were resuspended in the binding buffer with the final concentration of 1×10^6 cells/ml. The cells were stained with 5 µl of allophycocyanin (APC) and 5 µl of 7-Amino-Actinomycin D (7-AAD) for 15 min at RT in the darkness. Next, 400 µl of binding buffer was added into the cells and the percentage of cell apoptosis was determined with the flow cytometry (FACScan, BD Biosciences, Franklin Lakes, NJ, USA) according to the guidelines.

Bioinformatics Prediction

The potential binding sites of SNHG20-miR-4486 were predicted using the miRDB database (http://mirdb.org/cgi-bin/custom.cgi). The possible targets of miR-4486 were predicted with the TargetScan database (http://www.targetscan.org).

Dual-Luciferase Reporter Assay

The wild-type or mutant seeding sequences of MDM2 or miR-4486 were synthesized and cloned into the pMIR-REPORT luciferase vector (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). U87 cells were transfected with the corresponding expressing vector using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). After transfection for 48 h, the firefly luciferase activity was measured and normalized to that of Renilla with the Dual Luciferase Reporter Assay Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Statistical Analysis

Results were presented as mean±SD from three independent repeats. Statistical analysis was performed with the SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). Difference between two groups was analyzed with the Student's *t*-test. Comparison between more than two groups was performed with one-way analysis of variance followed by least-significant difference (LSD) post-hoc test. $p<0.05$ was considered as statistical significant.

Results

SNHG20 was Overexpressed in Glioma Tissues and Cell Lines

To investigate the potential involvement of SNHG20 in glioma, the expression of SNHG20 in paired glioma tissues and corresponding normal tissues was analyzed by RT-qPCR. The result showed that the expression of SNHG20 was significantly elevated in glioma tissues compared with that of non-tumor tissues (Figure 1A). Furthermore, the expression of SNHG20 was also significantly increased in glioma cell lines in-

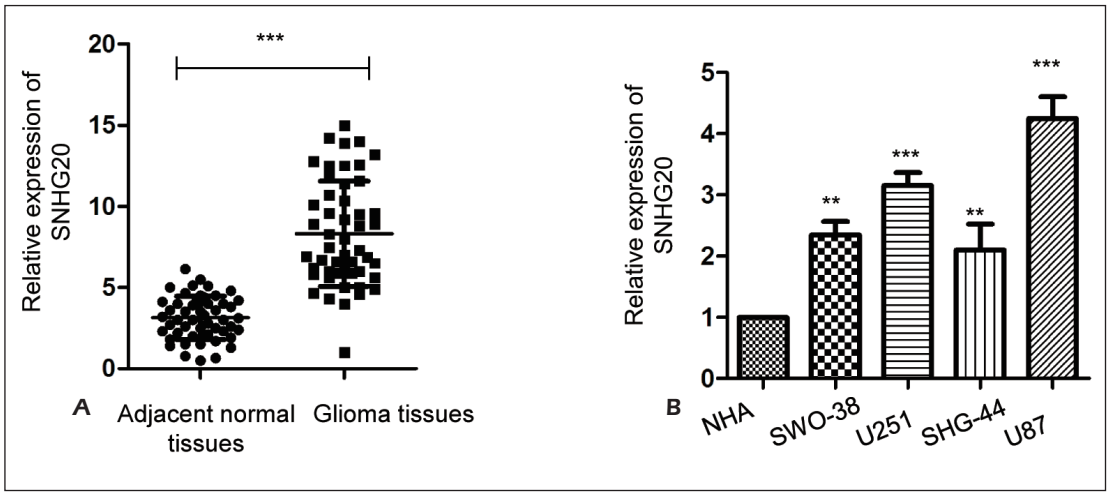

Figure 1. SNHG20 was overexpressed in glioma tissues. **A**, Analysis of the expression of SNHG20 in glioma tissues and adjacent normal tissues by RT-qPCR. **B**, The expression of SNHG20 in glioma cell lines and normal cell NHA.

cluding U87, U251, SWO-38 and SHG-44 cells in comparison with that of the normal control cells (Figure 1B). These results demonstrated the up-regulation of SNHG20 in glioma.

Down-Regulation of SNHG20 Inhibited the Proliferation of Glioma Cells

To access the function of SNHG20 in glioma, the expression of SNHG20 was down-regulated by transfecting shRNA-SNHG20 into U87 cells. The depletion of SNHG20 was confirmed by RT-qPCR and the data showed that the level of SNHG20 was significantly decreased in glioma cells harboring shRNA-SNHG20 (Figure 2A). The CCK-8 assay showed that the proliferation of U87 cells with depleted SNHG20 was decreased compared with that of the cells expressing shRNA-control vector (Figure 2B). The cell migration assay also indicated that down-regulation of SNHG20 inhibited the migration of U87 cells (Figure 2C). Additionally, knockdown of SNHG20 remarkably increased the apoptosis percentage of glioma cells (Figure 2D). These data suggested that down-regulation of SNHG20 suppressed the malignant behaviors of glioma cells.

SNHG20 Bound miR-4486 and Negatively Modulated the Expression of miR-4486

To further understand the role of SNHG20 in regulating the growth of glioma cells, the possible miRNA binding sites of SNHG20 were predicted with the miRDB database. The data indicated miR-4486 containing the putative binding sites of SNHG20 (Figure 3A). To verify this, the wild-type (WT) or mutant sequence of SNHG20 harboring the seeding regions of miR-4486 was constructed into the luciferase reported vector. As shown in Figure 3B, transfection of miR-4486 significantly decreased the luciferase activity of WT but not the mutant SNHG20 in U87 cells. These results suggested the interaction between SNHG20 and miR-4486 in glioma cells. To investigate whether the binding of SNHG20 with miR-4486 affected the stability of miR-4486, the expression of miR-4486 in U87 cells expressing control vector or SNHG20 was detected by RT-qPCR. The data indicated that overexpression of SNHG20 significantly decreased the level of miR-4486 (Figure 3C). To further support the negative modulation of SNHG20 on the expression of miR-4486, the level of miR-4486 in paired glioma tissues and corresponding adjacent normal tissues was detected. As showed in Figure 3D, miR-4486 was significantly down-regulated in glioma tissues in comparison with that of the normal controls. Similarly, decreased expression of miR-4486 was also observed in glioma cell lines (Figure 3E). Additionally, significantly negative correlation between the expression of SNHG20 and miR-4486 was observed in glioma tissues (Figure 3F). Overexpression of miR-4486 reversed the promotion effect of SNHG20 on the proliferation of U87 cells (Figure 3G). These data indicated that SNHG20 sponged miR-4486 and decreased the level of miR-4486 in glioma cells.

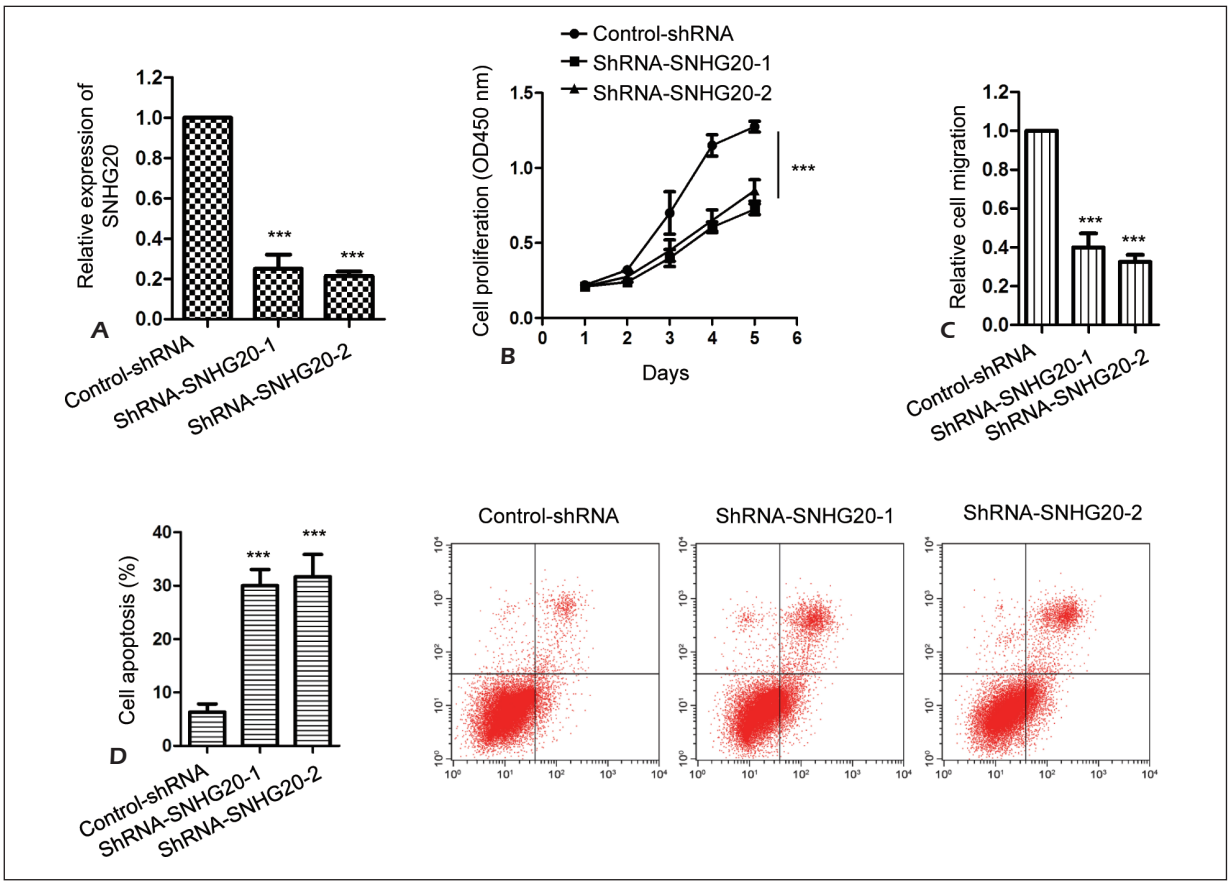

Figure 2. Depletion of SNHG20 inhibited the growth of glioma cells. **A**, RT-qPCR analysis showed SNHG20 was significantly down-regulated with the transfection of SNHG20-shRNA. **B**, CCK-8 assay displayed the decreased proliferation of U87 cells with the depletion of SNHG20. **C**, Quantification of the results of wound healing assay. **D**, Down-regulation of SNHG20 significantly increased the apoptosis of U87 cells.

MiR-4486 Targeted MDM2 and Up-Regulated the Expression of p53 in Glioma Cells

To further understand the molecular mechanism by which SNHG20/miR-4486 regulated the growth of glioma cells, the downstream targets of miR-4486 were predicted with the TargetScan database. The prediction indicated a potential complementary binding site of miR-4486 at the 3'-UTR of MDM2 (Figure 4A). To confirm the regulatory loop between miR-4486 and MDM2, luciferase reporter assay was performed by transfecting the WT or mutant 3'-UTR of MDM2 into U87 cells. The result showed that overexpression of miR-4486 significantly decreased the luciferase activity of WT 3'-UTR of MDM2 (Figure 4B), which suggested the binding of miR-4486 with the 3'-UTR of MDM2. To investigate whether miR-4486 modulated the level of MDM2, U87 cells were transfected with miR-4486 mimics and the mRNA level of MDM2 was examined by RT-qPCR analysis. The data indicated that overexpression of miR-4486 significantly decreased the mRNA level of MDM2 in glioma cells (Figure 4C). Consistently, reduced protein expression of MDM2 was also obtained in U87 cells with the up-regulation of miR-4486 (Figure 4D). MDM2 has been reported as the E3 ubiquitin ligase of p53, which catalyzes the polyubiquitination and degradation of p53. As miR-4486 down-regulated MDM2 in glioma cells, the influence of miR-4486 on the expression of p53 was also investigated. As shown in Figure 4D, highly expressed miR-4486 in U87 cells increased the protein expression of p53 without affecting the mRNA level of p53 (Figure 4E). These findings suggested that miR-4486 negatively regulated MDM2 and enhanced the expression of p53 in glioma cells. Cells were transfected with control vector or SNHG20 and the levels of MDM2 and p53 were detected by

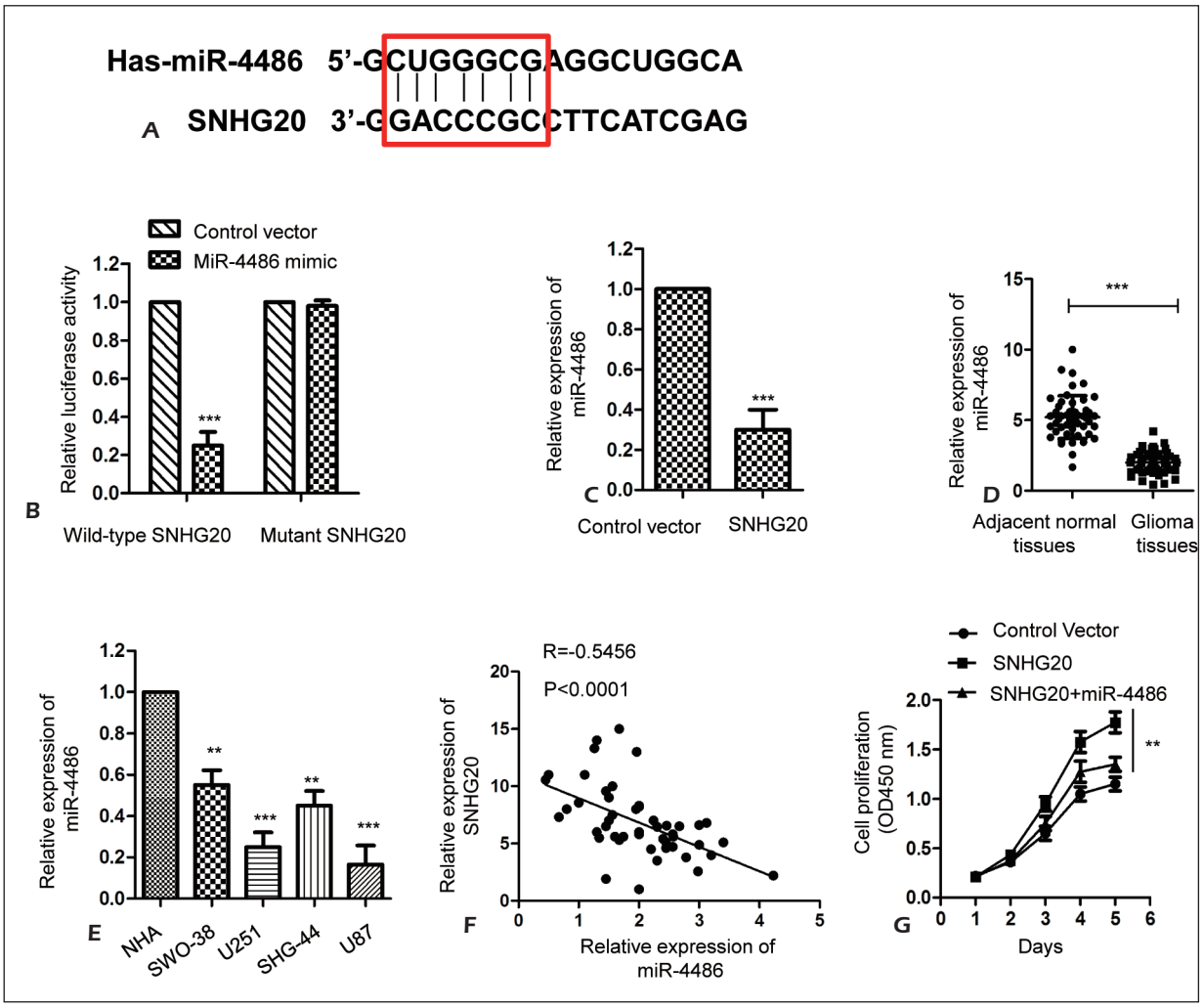

Figure 3. SNHG20 sponged miR-4486 in glioma cells. **A**, Bioinformatic analysis showed the potential binding sites of SNHG20 within miR-4486. **B**, Luciferase reporter assay in U87 cells with wild-type or mutant SNHG20 and miR-4486 mimics or control vector. **C**, RT-qPCR assay was performed to detect the expression of miR-4486 with the transfection of SNHG20. **D**, The expression of SNHG20 in paired glioma tissues and normal tissues. **E**, The level of SNHG20 in control normal cells and glioma cell lines. **F**, The expression of SNHG20 was significantly negatively correlated with that of the miR-4486. **G**, U87 cells were transfected with the indicated expression vector and the proliferation of cells was determined with the CCK-8 assay.

western blot. The results demonstrated that highly expressed SNHG20 up-regulated the level of MDM2 but decreased the expression of p53 in U87 cells (Figure 4F). These data indicated the SNHG20 was an upstream regulator of the miR-4486/MDM2/p53 axis.

Discussion

Evidence suggested the important role of lncRNAs in the progression of human cancers[7]. Understanding the molecular mechanisms by which lncRNAs regulate the growth of glioma cells might provide novel targets for the therapy of glioma. In this study, we found that SNHG20 was highly expressed in glioma tissues and cell lines. Down-regulation of SNHG20 suppressed the proliferation, migration and induced apoptosis of glioma cells. Mechanistically, SNHG20 was found to bind miR-4486, which attenuated miR-4486-mediated decrease of MDM2. The reduced level of MDM2 by miR-4486 up-regulated the expression of p53 in glioma cells (Figure 4G). Our results provided the possible mechanism by which SNHG20 modulated the progression of glioma. The potential function of SNHG20 was initially discovered in hepatocellular carcinoma, which showed that the up-regulated SNHG20 might serve as an independent prognostic pre-

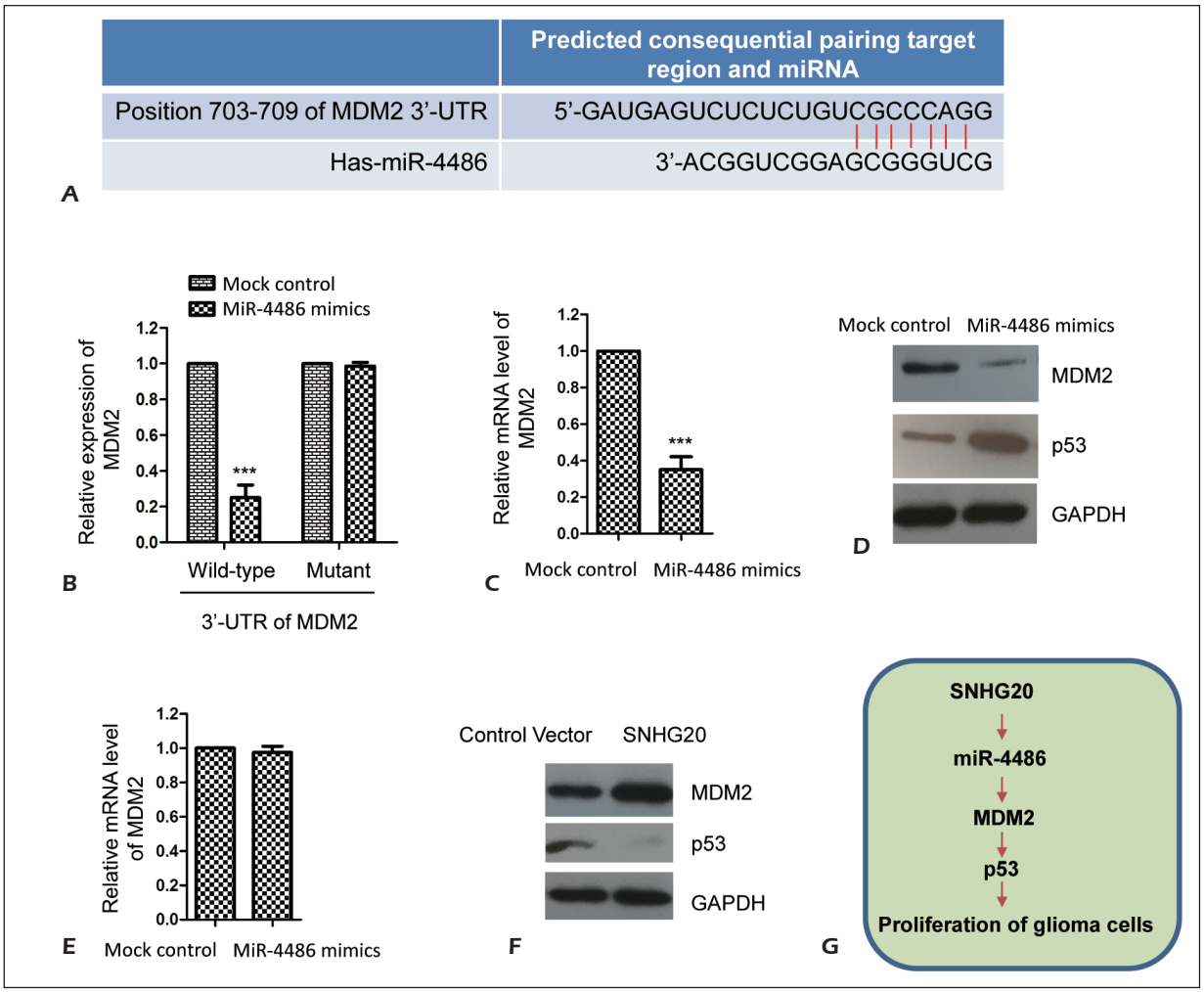

Figure 4. MDM2 was a target of miR-4486. **A**, Predicted binding sites of miR-4486 at the 3'-UTR of MDM2. **B**, Luciferase reporter assay was performed by transfecting wild-type or mutant 3'-UTR of MDM2 with the miR-4486 mimic or control vector. **C-D**, Overexpression of miR-4486 decreased both the mRNA and protein level of MDM2 in U87 cells. **E-F**, Transfection of SNHG20 up-regulated the protein but not the mRNA level of MDM2. **G**, Proposed working model of SNHG20 in regulating the proliferation of glioma cells. SNHG20 was overexpressed in glioma cells. SNHG20 sponged miR-4486 and decreased the expression of miR-4486. MDM2 was identified as the target of miR-4486. Highly expressed SNHG20 increased the expression of MDM2 and inhibited the expression of p53, which finally promoted the growth of glioma cells.

dictor for HCC patients[10]. Aberrant expression of SNHG20 was also found in cervical cancer and regulated the growth of cervical cancer cells through modulating the expression of cell cycle-associated genes[27]. Recent study demonstrated that SNHG20 acted as a ceRNA to sponge the expression of miRNAs that were involved in the progression of cancers[27,28]. For example, SNHG20 modulated the expression of miR-140-5p to promote the proliferation and invasion of cervical cancer cells[27]. In breast cancer cells, SNHG20 sponged miR-495, which affected the expression of HER2 and enhanced the progression of breast cancer[28]. In our study, the potential targets of SNHG20 were predicted with the bioinformatics database. MiR-4486 was uncovered as one of the downstream targets of SNHG20. Further experimental data showed that SNHG20 bound miR-4486 and negatively regulated the abundance of miR-4486 in glioma cells. Consistent with this conclusion, inverse correlation between the expression of SHHG20 and miR-4486 was found in glioma tissues. Our results identified the possible mechanism by which SNHG20 modulated the malignant behaviors of glioma cells via affecting the expression of miR-4486. The function of miR-4486 in cancers has not been explored. In the present study, miR-4486 was down-regulated

in glioma tissues. Overexpression of miR-4486 suppressed the promotion effect of SNHG20 on the growth of glioma cells. To further understand the involvement of miR-4486, the downstream targets of miR-4486 were predicted. Among all the candidates, MDM2, the E3 ubiquitin ligase of p53, was found as the putative target of miR-4486. It has been well documented that MDM2 catalyzed the polyubiquitin of p53 and trigged the 26S proteasome-mediated degradation of p53[29]. Ectopic expression of miR-4486 in glioma cells decreased the expression of MDM2, which consequently up-regulated the level of p53. Our results identified miR-4486 as an upstream regulator of the MDM2-p53 axis. It would be necessary to investigate the expression and function of miR-4486 in other types of cancers.

Conclusions

We showed that SNHG20 was significantly up-regulated in glioma tissues and cell lines. Down-regulation of SNHG20 inhibited the proliferation and migration of glioma cells. Molecular mechanism studies revealed that SNGH20 sponged miR-4486 and modulated the MDM2-p53 pathway. Our findings uncovered the possible mechanism by which SNHG20 regulated the malignancy of glioma cells. These findings suggested SNHG20 as a promising target for the treatment of glioma.

Conflict of Interests

The authors declare no conflict of interest

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