European Review for Medical and Pharmacological Sciences 2019; 23: 96-104

LncSNHG16 promotes proliferation and migration of osteosarcoma cells by targeting microRNA-146a-5p

S.-N. ZHENG[1], D.-W. GE[1], J. TANG[2], J. YANG[3], J.-W. YAN[1], J.-J. QIU[1], Z.-W. YIN[1], Y. NI[4], L. ZHAO[1], X. CHEN[1], L. YANG[1]

[1]Department of Orthopedic Surgery, Nanjing First Hospital, Nanjing Medical University, Nanjing, China
[2]Department of Orthopedics, The First Affliated Hospital of Nanjing Medical University, Nanjing, China
[3]Department of Pathology, Wuxi Third People's Hospital, Wuxi, China
[4]Department of Nursing, Nanjing First Hospital, Nanjing Medical University, Nanjing, China

Shengnai Zheng and *Dawei Ge* contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to elucidate the regulatory role of lncSNHG16 in the progression of osteosarcoma (OS) and its underlying mechanism.

MATERIALS AND METHODS: Expressions of lncSNHG16, microRNA-146a-5p and NOVA1 in OS tissues and adjacent normal tissues were determined by quantitative Real-time polymerase chain reaction (qRT-PCR). Their expressions in OS cell lines were detected by qRT-PCR as well. We analyzed the relationship between lncSNHG16 expression and tumor stage, diagnosis and survival prognosis of OS patients, respectively. Cell counting kit-8 (CCK-8) and transwell experiments were conducted to explore proliferative and migratory changes of OS cells. Dual-luciferase reporter assay was used to verify the binding relationship of lncSNHG16 to microRNA-146a-5p, and microRNA-146a-5p to NOVA1. Finally, rescue experiments were performed to elucidate the regulatory effect of lncSNHG16 on the cellular behaviors of OS cells.

RESULTS: LncSNHG16 was highly expressed in OS tissues and cell lines. Its expression was positively correlated with the tumor stage of OS patients. Receiver operating characteristic (ROC) curves suggested that lncSNHG16 can be used as a clinical indicator to distinguish OS patients from healthy controls. Survival analysis indicated a negative correlation between lncSNHG16 expression and survival of OS patients. Overexpression of lncSNHG16 enhanced the proliferative and migratory potentials of OS cell lines 143B and MNNG/HOS. MicroRNA-146a-5p was predicted to be the target gene of lncSNHG16, which was lowly expressed in OS tissues and cell lines. Overexpression of lncSNHG16 downregulated the expression of microRNA-146a-5p in 143B and MNNG/HOS cells. Furthermore, we verified that lncSNHG16 could bind to microRNA-146a-5p. The promotive role of lncSNHG16 in proliferative and migratory potentials of OS cells was reversed by microRNA-146a-5p. Subsequently, NOVA1 was predicted to be the target gene of microRNA-146a-5p, and was further verified by dual-luciferase reporter gene assay. Correlation analysis showed that microRNA-146a-5p expression was negatively correlated with NOVA1 expression in OS. More importantly, NOVA1 reversed the inhibitory effect of microRNA-146a-5p on the proliferative and migratory capacities of 143B and MNNG/HOS cells.

CONCLUSIONS: LncSNHG16 is highly expressed in OS tissues and cell lines, participating in the development of OS by downregulating microRNA-146a-5p to upregulate NOVA1 expression.

Key Words:
Osteosarcoma, LncSNHG16, MicroRNA-146a-5p, NOVA1.

Introduction

Osteosarcoma (OS) is a common primary malignancy, showing an increased trend in recent years. Its incidence accounts for 20% of primary malignancies[1,2]. OS is particularly common among adolescents and children, especially in those with 15-19 years old, with a prevalence of 5% in childhood tumors[3,4]. OS originates from

Corresponding Author: Lei Yang, Ph.D; e-mail: leiyang@njmu.edu.cn

immature tissues, such as osteoid tissues and mesenchymal tissues. Due to the rapid differentiation, proliferation, growth and invasion of OS cells, normal skeletal tissues are infiltrated and destroyed by malignant tumor tissues[5]. OS is a bone tumor that occurs in the proximal end of the long bones and growing epiphysis, which seriously affects the life quality of affected patients. At present, surgical resection and adjuvant radiotherapy and chemotherapy for OS have been greatly advanced. However, the tumor recurrence rate and mortality still remain high, leading to an unsatisfied prognosis of OS[6]. Due to the high degree of malignant invasion, early distant metastasis through blood flow and lymphatics, the 5-year survival of OS is only 10%. It is reported[7] that 40% of OS patients die from lung metastasis. About 5-10% of the human genomes are stably transcribed, and the vast majority of these transcribed sequences are non-coding transcripts[8-10]. Non-coding transcripts can be divided into housekeeping non-coding RNA and regulatory non-coding RNA. The most transcribed non-protein coding sequences are lncRNAs[11]. LncRNAs are a class of non-coding RNAs without protein coding ability due to the lack of an open reading frame. They are greater than 200 nucleotide (nt) in length. LncRNA is involved in tumor biological function mainly through epigenetic, transcriptional and post-transcriptional regulations of gene expression and translation. In the past, lncRNA was originally thought to be transcriptional noise. Recent studies have focused on the expression patterns and regulatory roles of lncRNAs in tumors[12]. LncRNA SNHG16, also known as small nucleolar RNA host gene 16, is highly expressed in aggressive neuroblastoma, bladder cancer and colorectal cancer as an oncogene[13-15]. This work mainly elucidated the potential role of lncSNHG16 in the development of OS.

Patients and Methods

Patients Collection

OS tissues and adjacent normal tissues were surgically resected from OS patients who received the surgical treatment in our hospital for the first time. All samples were placed in cryotubes immediately, and preserved in liquid nitrogen. None of OS patients received preoperative chemotherapy, radiotherapy, targeted therapy, biological therapy, etc. Meanwhile, they did not suffer from other diseases. All patients were followed up for recording the survival time. This study was approved by the Ethical Committee of Nanjing Medical University and Wuxi Third People's Hospital.

Cell Culture and Transfection

Osteoblasts hFOB1.19 and OS cell lines MG-63, U2OS, 143B, and MNNG/HOS were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C, 5% CO_2 incubator. One day prior to transfection, cells were seeded in a 6-well plate with 1×10^6 cells/mL. When the cell confluence reached about 80%, transfection plasmid was diluted in serum-free DMEM, and added in each well accompanied by diluted Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced at 6 h. After 48 hours, transfection efficacy was verified by quantitative Real-time polymerase chain reaction (qRT-PCR).

Cell counting kit-8 (CCK-8)

Transfected cells for 48 h were seeded in each well of the 96-well plate with 5×10^3 cells per well, and 5 replicates in each group. 10 μL of CCK-8 reagent (Dojindo, Kumamoto, Japan) was supplied in each well. After cell culture for 2 h, the absorbance value at 450 nm wavelengths was measured by a microplate reader for plotting a growth curve.

Transwell

200 μL of a suspension containing 5×10^4 cells was supplied into the apical chamber and 600 μL of complete medium was added in the basolateral chamber. At the other day, un-penetrating cells above the chamber were wiped off. Subsequently, the chamber was fixed in 4% paraformaldehyde for 30 min and dyed with 1% crystal violet for another 10-15 min. Six randomly selected fields in each sample were captured using an inverted microscope (magnification 20×).

RNA Extraction and qRT-PCR

Total RNA from OS tissues or cells was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) and quantified using a UV spectrophotometer. RNA samples with 1.8-2.1 of A260/280 value were considered as qualified. 1 μg of total RNA sample was used for reverse transcription to obtain complementary deoxyribose nucleic acid (cDNA). QRT-PCR reaction solution was

prepared according to the instructions of the SYBR fluorescence quantitative premixing kit (TaKaRa, Otsu, Shiga, Japan), and the total system was 10 μL. PCR conditions were pre-denaturation at 95°C for 30 s, 95°C for 5 s, and 60°C for 31 s, for a total of 40 cycles. Each experiment was repeated for three times. U6 was utilized as the internal reference to microRNA-146a-5p, whereas glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference to lncSNHG16 and NOVA1. The primer sequences were as follows: LncSNHG16, F: CTTCTGGGCTCAAGTGATCCT, R: TGTCATCATACTTG; MicroRNA-146a-5p, F: GCCGAGAGGTAGTAGGTTGCATAG, R: AGTGCAGGGTCCGAGGTATT; NOVA1, F: AGGCAGAAGACTTGGTGGC, R: GGTCAGGGACTGGGAGATTC; GAPDH, F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC; U6, F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT.

Western Blot

Total protein was extracted using the cell lysate for determining protein expression. The protein sample was quantified by bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhanced chemiluminescence (ECL).

Dual-Luciferase Reporter Gene Assay

143B and MNNG/HOS cells were seeded in 12-well plates. They were co-transfected with lncSNHG16-WT/NOVA1-WT or lncSNHG16-MUT/NOVA1-MUT and microRNA-146a-5p mimics or NC, respectively. At 24 hours, cells were lysed and centrifuged at 10,000 g for 5 min. 100 μL of the supernatant were collected for determining the luciferase activity.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 12.0 software (SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for analyzing the measurement data. Receiver operating characteristic (ROC) curves were plotted for analyzing the specificity and sensitivity of lncSNHG16 in diagnosing OS. Kaplan-Meier was introduced for calculating the overall survival of OS patients based on their lncSNHG16 expressions. $p < 0.05$ was considered statistically significant.

Results

LncSNHG16 Expression was Upregulated in OS

First of all, we determined the expression of lncSNHG16 in OS tissues and adjacent normal tissues by qRT-PCR. LncSNHG16 was highly expressed in OS tissues compared with adjacent normal tissues (Figure 1A). Furthermore, we found that advanced OS patients had a higher expression of lncSNHG16 than those early-stage OS patients, suggesting that lncSNHG16 expression may be related to tumor stage of OS (Figure 1B). ROC curves revealed that lncSNHG16 was capable of distinguishing OS patients from healthy controls, showing a potential in clinical monitoring of OS (Figure 1C). Moreover, survival analysis found a negative correlation of lncSNHG16 expression with the survival of OS patients. The higher the expression level of lncSNHG16, the worse the prognosis of OS (Figure 1D). The above data demonstrated the potential tumor-promoting role of lncSNHG16 in the development of OS.

LncSNHG16 Accelerated the Proliferation and Migration of OS Cells

We further detected lncSNHG16 expression in OS cell lines MG-63, U2OS, 143B, MNNG/HOS and osteoblasts hFOB1.19 by qRT-PCR. LncSNHG16 was highly expressed in OS cells, especially in 143B and MNNG/HOS cells (Figure 2A). We next constructed the overexpression plasmid of lncSNHG16 and verified its transfection efficacy in 143B and MNNG/HOS cells (Figure 2B). CCK-8 data showed that 143B and MNNG/HOS cells overexpressing lncSNHG16 possessed higher viability than controls, suggesting an increased proliferative potential (Figure 2C and 2D). Similarly, the results of transwell assay indicated that lncSNHG16 overexpression markedly enhanced the migratory potential of 143B and MNNG/HOS cells (Figure 2E and 2F).

MicroRNA-146a-5p was the Target Gene of lncSNHG16

Previous studies have shown that lncRNA can exert biological functions by competitively ad-

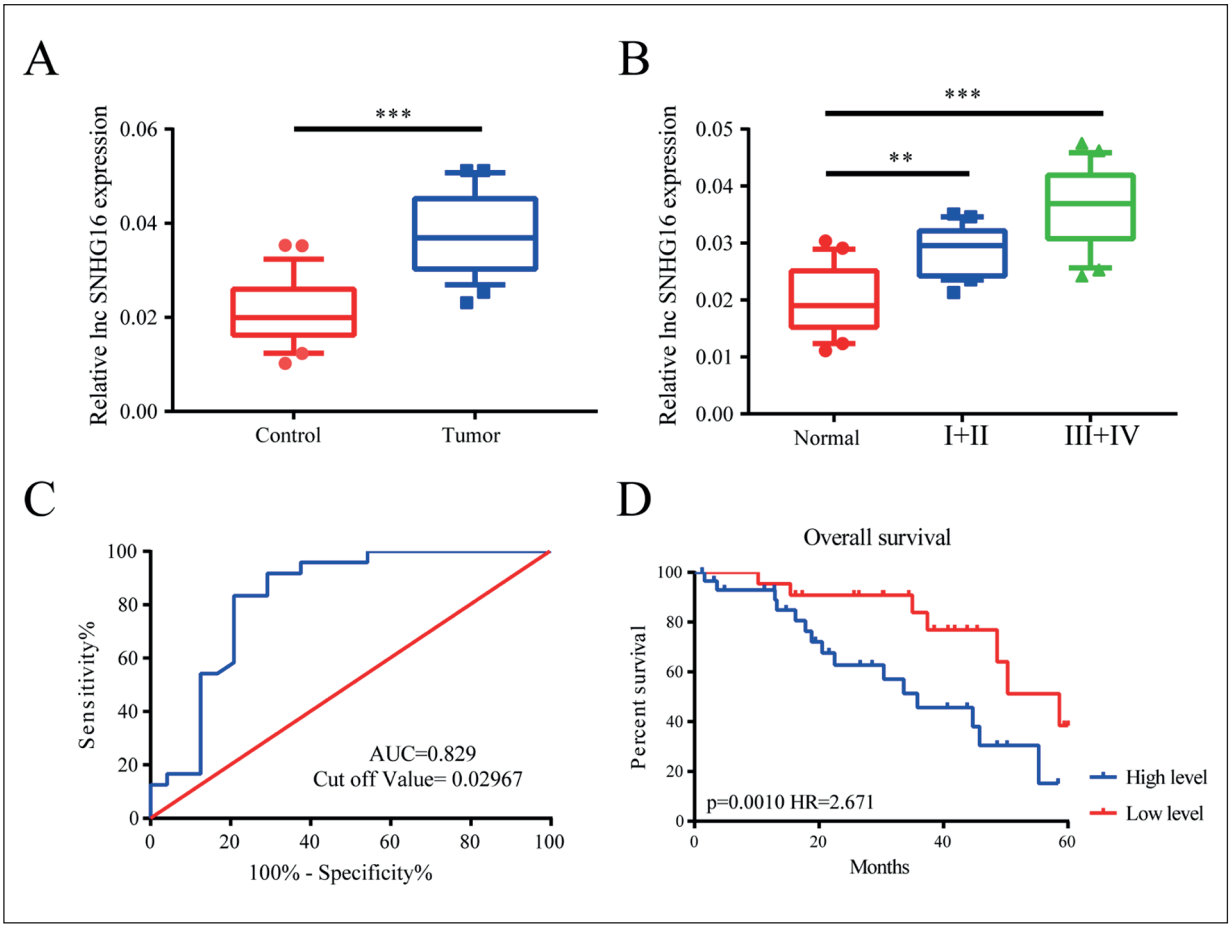

Figure 1. LncSNHG16 expression was upregulated in OS. ***A,*** LncSNHG16 was highly expressed in OS tissues relative to controls detected by qRT-PCR. ***B,*** Advanced OS patients had a higher expression of lncSNHG16 than those early-stage OS patients. ***C,*** ROC curves revealed that lncSNHG16 was capable of distinguishing OS patients from healthy controls (AUC = 0.829, cut-off value = 0.02967). ***D,*** Survival analysis found a negative correlation between lncSNHG16 expression with the survival of OS patients ($p = 0.0171$, HR = 2.871). ***$p < 0.001$.

sorbing miRNAs. Through bioinformatics prediction, we found that microRNA-146a-5p may be the potential target of lncSNHG16. QRT-PCR data showed that microRNA-146a-5p exhibited a low expression in OS tissues compared to adjacent normal tissues (Figure 3A). Identically, the microRNA-146a-5p expression also decreased in OS cell lines (Figure 3B). Furthermore, luciferase activity was markedly reduced in wild-type group, while we did not observe significant changes of luciferase activity in mutant-type group. Those results confirmed that lncSNHG16 could bind to microRNA-146a-5p (Figure 3C). After overexpression of lncSNHG16, the expression level of microRNA-146a-5p was markedly downregulated in 143B and MNNG/HOS cells (Figure 3D). To further verify the potential interaction of lncSNHG16 and microRNA-146a-5p in regulating behaviors of OS cells, microRNA-146a-5p mimics was constructed. Transfection of microRNA-146a-5p mimics efficiently upregulated its expression in OS cells (Figure 3E). Rescue experiments clarified that co-overexpression of lncSNHG16 and microRNA-146a-5p reversed the promotive role of microRNA-146a-5p overexpression in regulating proliferative (Figure 3F and 3G) and migratory potentials of OS cells (Figure 3H and 3I).

NOVA1 was the Target Gene of microRNA-146a-5p

RT-PCR and Western blot were performed to detect mRNA and protein levels of NOVA1 in OS tissues, respectively. The expression of NOVA1 was higher in OS tissues when compared to their adjacent normal tissues (Figure 4A). Correlation

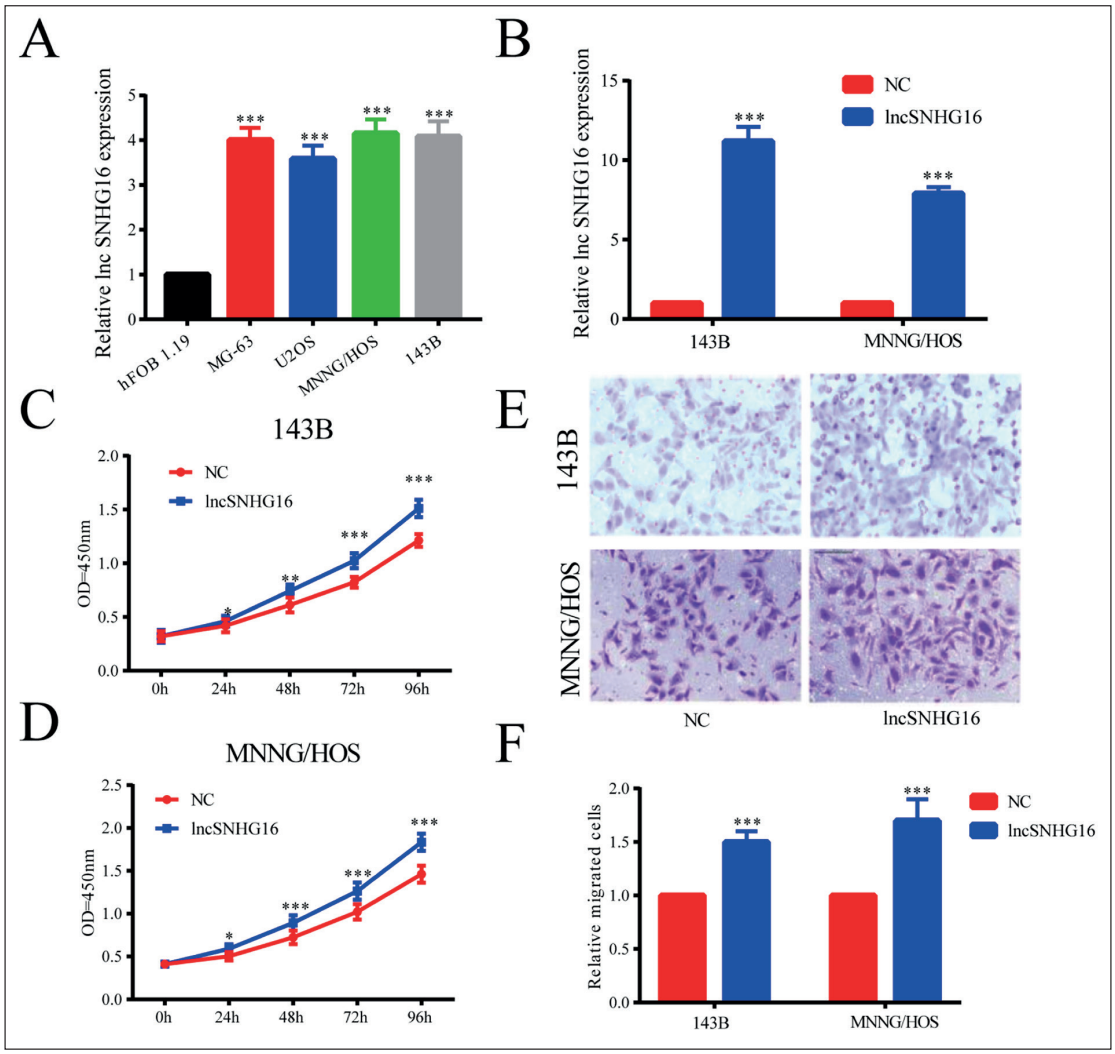

Figure 2. LncSNHG16 accelerated the proliferation and migration of OS cells. ***A,*** LncSNHG16 was highly expressed in OS cell lines MG-63, U2OS, 143B, MNNG/HOS compared with osteoblasts hFOB1.19 detected by qRT-PCR. ***B,*** Transfection efficacy of overexpression plasmid of lncSNHG16 in 143B and MNNG/HOS cells. ***C,*** and ***D,*** CCK-8 data showed that 143B and MNNG/HOS cells overexpressing lncSNHG16 had higher viability than that in controls. ***E,*** and ***F,*** Transwell assay indicated that lncSNHG16 overexpression markedly enhanced migratory potential of 143B and MNNG/HOS cells. $^{**}p < 0.01$, $^{***}p < 0.001$.

analysis further suggested a negative correlation between microRNA-146a-5p expression and NOVA1 expression in OS (Figure 4B). NOVA1 was predicted to be a target gene of microRNA-146a-5p through bioinformatics. We thereafter performed dual-luciferase reporter gene assay to clarify their binding condition. NOVA1 could bind to microRNA-146a-5p, suggesting that microRNA-146a-5p may regulate its biological function through targeting NOVA1 (Figure 4C). After exogenous overexpression of microRNA-146a-5p, the expression of NOVA1 remarkably decreased in 143B and MNNG/HOS cells (Figure 4D). Overexpression plasmid of NOVA1 was then constructed, which could effectively upregulate NOVA1 expression in OS cells (Figure 4E). Of note, rescue experiments revealed that co-overexpression of microRNA-146a-5p and NOVA1 reversed the inhibited proliferative (Figure 4F and 4G) and migratory potentials of OS cells (Figure 4H and 4I) induced by microRNA-146a-5p overexpression.

Discussion

Researches have shown that lncRNA, a key regulator in tumor development and metastasis, serves as an oncogene or tumor-suppressor gene

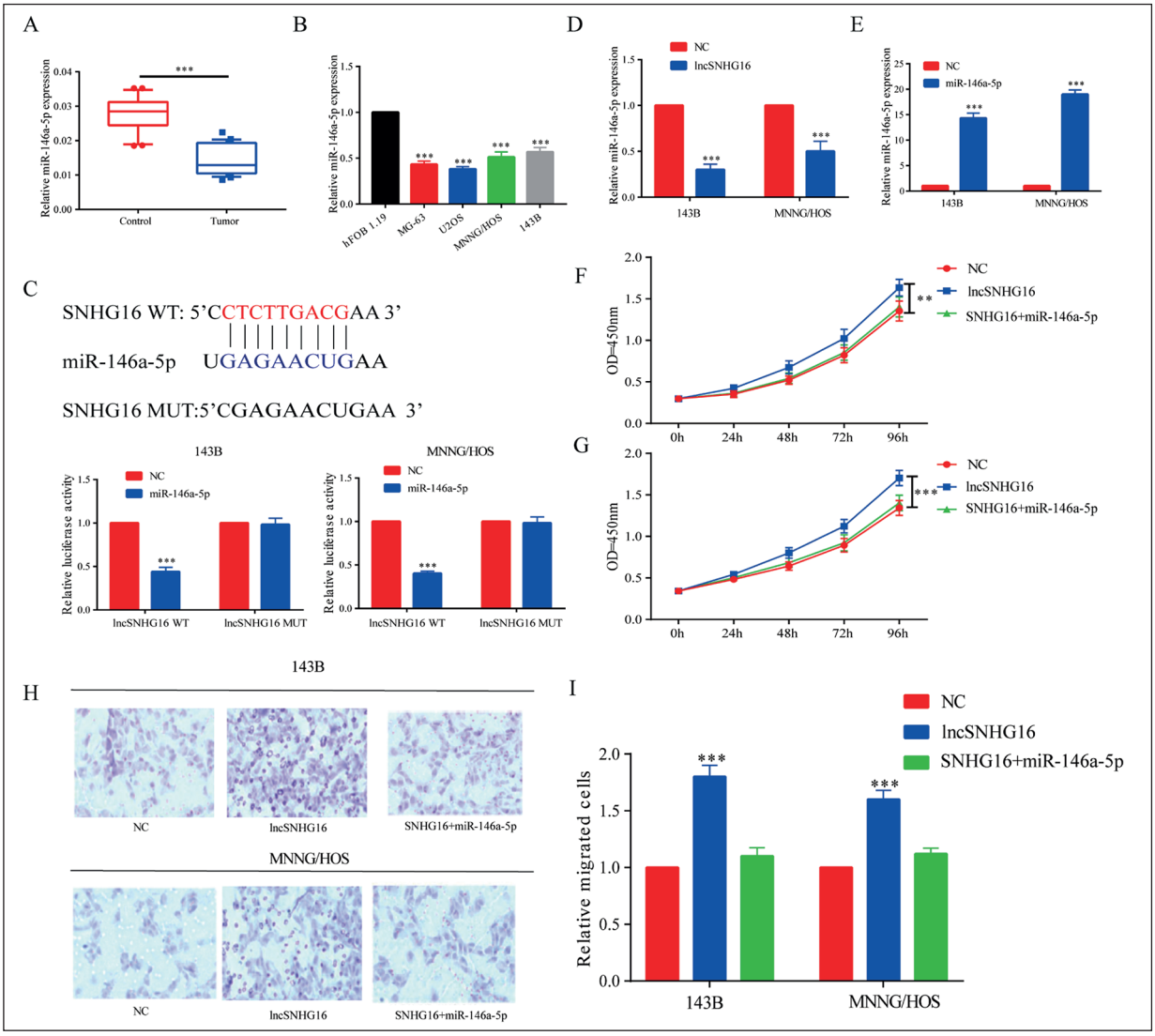

Figure 3. MiR-146a-5p was the target gene of lncSNHG16. ***A,*** QRT-PCR data showed that miR-146a-5p exhibited low expression in OS tissues compared with adjacent normal tissues. ***B,*** QRT-PCR data showed that miR-146a-5p expression decreased in OS cell lines. ***C,*** Dual-luciferase reporter gene assay confirmed that lncSNHG16 could bind to miR-146a-5p. ***D,*** Overexpression of lncSNHG16 downregulated miR-146a-5p expression in 143B and MNNG/HOS cells. ***E,*** Transfection of miR-146a-5p mimics efficiently upregulated miR-146a-5p expression in 143B and MNNG/HOS cells. ***F,*** and ***G,*** Co-overexpression of lncSNHG16 and miR-146a-5p reversed the promotive role of miR-146a-5p overexpression in regulating proliferative potential of 143B and MNNG/HOS cells. ***H,*** and ***I,*** Co-overexpression of lncSNHG16 and miR-146a-5p reversed the promotive role of miR-146a-5p overexpression in regulating migratory potential of 143B and MNNG/HOS cells. $^{**}p < 0.01$, $^{***}p < 0.001$

based on its functions[16]. It is reported that TUG1 may be a therapeutic target for OS, which is closely related to the disease state[17]. Plasma TUG1 could be utilized as a diagnostic and prognostic indicator for dynamic monitoring of OS. Upregulation of UCA1 expression may be associated with tumor enlargement and distant metastasis, serving as an independent prognostic biomarker for adverse outcomes[18]. Upregulation of lncRNA 91H expression is associated with tumor growth and postoperative chemotherapy, which can be used as an independent predictor of survival status in OS patients[19]. MALAT1 is highly expressed in patients with distant metastatic OS[20]. OS patients with high expression of MALAT1 are tended to have a shorter survival, indicating its potential as an independent prognostic factor for predicting patient survival. In this study, we first examined the expression of lncSNHG16 in OS tissues and adjacent normal tissues. QRT-PCR data

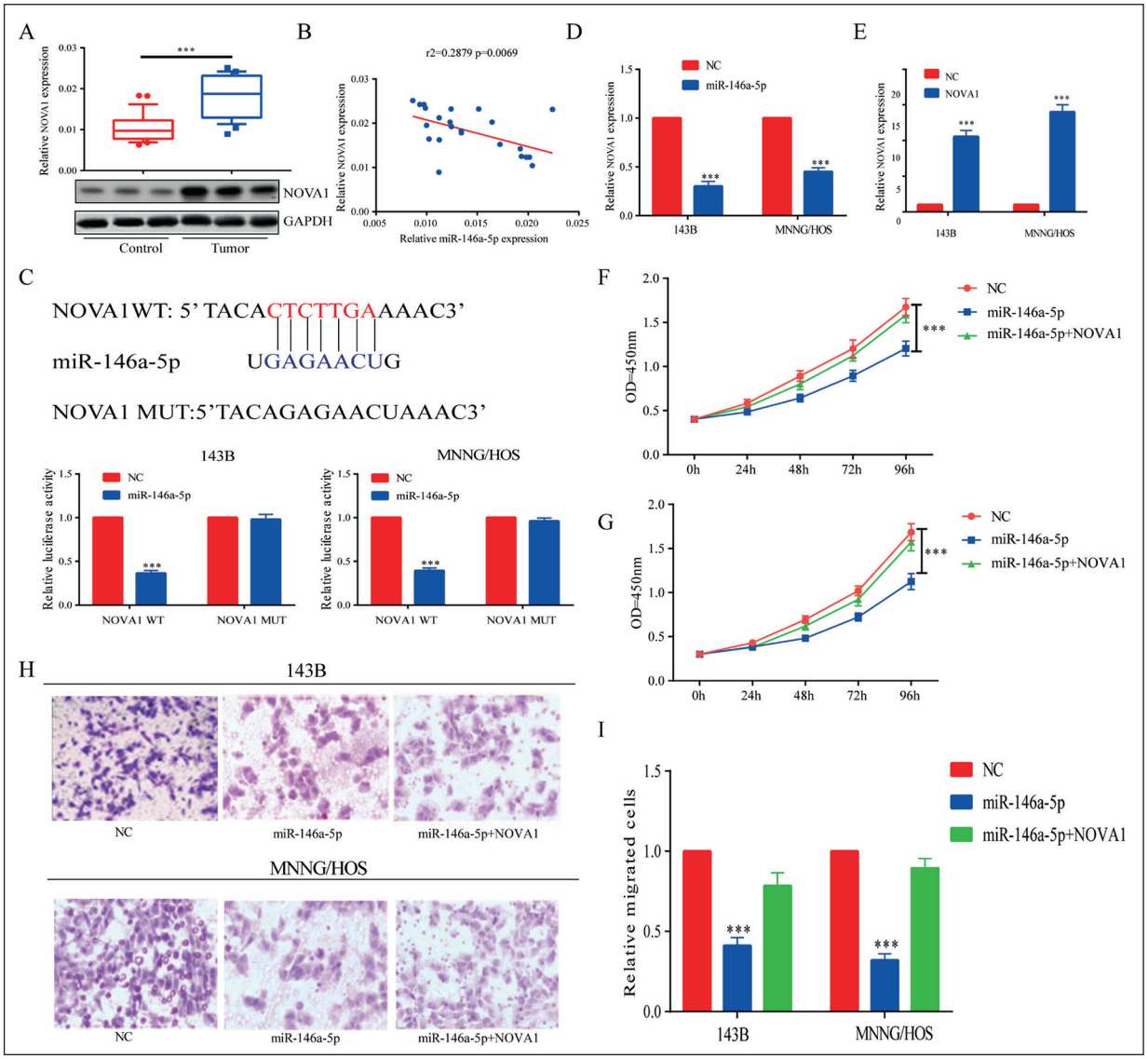

Figure 4. NOVA1 was the target gene of miR-146a-5p. ***A,*** NOVA1 was highly expressed in OS tissues compared with adjacent normal tissues at mRNA and protein levels. ***B,*** Correlation analysis suggested a negative correlation between miR-146a-5p and NOVA1 expression in OS. ***C,*** Dual-luciferase reporter gene assay confirmed that NOVA1 could bind to miR-146a-5p. ***D,*** Overexpression of miR-146a-5p downregulated the expression of NOVA1 in 143B and MNNG/HOS cells. ***E,*** Transfection of overexpression plasmid of NOVA1 efficiently upregulated its expression in 143B and MNNG/HOS cells. ***F,*** and ***G,*** Co-overexpression of miR-146a-5p and NOVA1 reversed the inhibited proliferative potential of 143B and MNNG/HOS cells induced by miR-146a-5p overexpression. ***H,*** and ***I,*** Co-overexpression of miR-146a-5p and NOVA1 reversed the inhibited migratory potential of 143B and MNNG/HOS cells induced by miR-146a-5p overexpression. ***$p < 0.001$.

found that its expression level in tumor tissues was markedly higher than that in adjacent normal tissues. Moreover, expression level of lncSNHG16 was negatively correlated with the tumor stage of OS. ROC curves indicated the potential of lncSNHG16 to distinguish OS patients from healthy controls. Furthermore, survival analysis suggested that OS patients with high expression of lncSNHG16 were expected to have a worse prognosis. We suggested that lncSNHG16 exerted a tumor-promoting role in the development of OS. In the next *in vitro* experiments, we found that lncSNHG16 was also highly expressed in OS cells. LncSNHG16 was capable of accelerating the proliferative and migratory potentials of 143B and MNNG/HOS cells. Through biological prediction, microRNA-146a-5p was screened out to be the target gene of lncSNHG16. Previous

experiments have showed that upregulation of miR-146b-5p could inhibit the proliferative, invasive and adherent capacities of Caski cervical cancer cells. Meanwhile, miR-146b-5p overexpression arrests cell cycle in G0/G1 phase, inhibits secretion of TGF-β, MCP-1 and TNF-α, and suppresses telomerase activity, thereby inhibiting the growth of Caski cells[21]. It is believed that microRNA-146a-5p plays a crucial role in the development and progression of various tumors. Our results showed that microRNA-146a-5p was lowly expressed in OS tissues and cell lines. LncSNHG16 negatively regulated the expression level of microRNA-146a-5p. Of note, microRNA-146a-5p reversed the promotive effects of lncSNHG16 on proliferative and migratory capacities of 143B and MNNG/HOS cells. To explore the mechanism of microRNA-146a-5p in regulating the cellular behaviors of OS cells, we predicted that NOVA1 was a potential target gene of microRNA-146a-5p through bioinformatics. NOVA1 has been identified in the occurrence and development of various tumors[22,23]. The expression of NOVA1 is downregulated in the tumor microenvironment of gastric cancer, and its low expression is associated with the progression and poor prognosis of gastric cancer[24]. MiR-339 can inhibit the proliferative, invasive and migratory potentials of gastric cancer cells by inhibiting the expression of NOVA1[25]. High expression of NOVA1 indicates a high risk of liver cancer recurrence, suggesting a poor prognosis[26]. A relative research[27] proved the tumor-promoting role of NOVA1 in OS, which is capable of enhancing the proliferative and migratory potentials of OS cells. Here, we found that NOVA1 was highly expressed in OS tissues and cell lines, showing a negative correlation with microRNA-146a-5p. Subsequently, dual-luciferase reporter gene assay verified the binding between microRNA-146a-5p and NOVA1. Interestingly, NOVA1 reversed the inhibitory effect of microRNA-146a-5p on the proliferative and migratory capacities of 143B and MNNG/HOS cells, suggesting that microRNA-146a-5p exerted its function in OS cells through targeting NOVA1.

Conclusions

We first found that lncSNHG16 was upregulated in OS tissues and cell lines, which participates in the development of OS by downregulating microRNA-146a-5p to upregulate NOVA1 expression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

This study was supported by the National Natural Science Fund from the National Natural Science Foundation of China (Grant No. 81802149) and Natural Science Foundation of Jiangsu Province (Grant No. BK20171089).

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