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LncRNA SNHG7 participates in osteosarcoma progression by down-regulating p53 via binding to DNMT1

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Abstract. – OBJECTIVE: This study aims to explore the role of lncRNA SNHG7 in the development of osteosarcoma, and its underlying mechanism.

PATIENTS AND METHODS: The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to detect SNHG7 expression in tumor tissues and paracancerous tissues harvested from osteosarcoma patients. Meanwhile, the relationship between SNHG7 expression and tumorigenesis was analyzed. The effects of SNHG7 and p53 on cell proliferation, cell cycle and apoptosis were detected by plate cloning and flow cytometry, respectively. The binding relationship between SNHG7 and DNMT1, as well as the regulatory mechanism of DNMT1 on p53, were detected by RIP and ChIP. Western blot was conducted to detect the expression of p53 after the knockdown of SNHG7 in osteosarcoma cells. Rescue experiments were finally conducted to verify whether SNHG7 exerted its biological function by targeting p53.

RESULTS: QRT-PCR results demonstrated that the expression of SNHG7 in osteosarcoma tissues was remarkably higher than that in paracancerous tissues. Moreover, SNHG7 expression in osteosarcoma with stage III and IV was higher than those in stage I and II. The inhibition of SNHG7 in osteosarcoma cells U2OS and HOS promoted cell proliferation, arrested cell cycle in the G0/G1 phase and induced apoptosis. RIP and ChIP experiments illustrated that SNHG7 inhibited the expression of p53 by binding to DNMT1. The overexpression of p53 in U2OS cells partially reversed the promoted cell proliferation and apoptosis caused by SNHG7.

CONCLUSIONS: Highly expressed SNHG7 can promote the proliferation and inhibit apoptosis of osteosarcoma cells by regulating p53 expression by binding to DNMT1.

Key Words:
SNHG7, Osteosarcoma, Cell proliferation, Apoptosis, P53.

Introduction

Osteosarcoma is a primary malignant bone tumor originating from the mesenchymal tissue. The frequent onset age is 13-25 years, and the incidence rate of males is higher than that of females. Osteosarcoma is more common in the metaphyseal end of the long tubular bone, distal femur and proximal tibia. The histological feature is that the hyperplastic tumor cells can directly produce bone-like tissue or tumor-like bone[1]. Lungs are the most common metastatic organ of osteosarcoma, with other organs such as the kidney, heart, and brain[2]. Osteosarcoma is characterized by a high degree of malignancy and poor prognosis. Current therapies of osteosarcoma include the combination of surgery and neoadjuvant chemotherapy or biological therapy. However, the 5-year survival rate of osteosarcoma patients is only 60% to 70%[3]. It has been confirmed that cancers are essentially a genetic disease. The occurrence, development and metastasis of tumors involve genetic changes *in vivo*, such as mutations and deletions of normal genes, abnormal amplification and expression of oncogenes, and the synergy of multiple regulatory genes. The pleiotropic effects of the gene and the immune factors ultimately determine the expression of the tumor phenotype. In view of the etiology of tumorigenesis, research on genetic changes in tumors has always been the

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focus of life sciences. Therefore, in-depth exploration of genes related to the pathogenesis of osteosarcoma has significant clinical value.

Long non-coding RNA (lncRNA) is a kind of non-coding RNA with greater than 200 nucleotides in length[4]. Liz and Esteller[5] have demonstrated that lncRNAs can act as a crucial role in many life activities, such as dose compensation effects, epigenetic regulation, cell cycle and cell differentiation[5]. Currently, it has been found that lncRNAs participate in the normal physiological activities of cells through chromosome modification, shear splicing, transcriptional activation, mRNA degradation and translational regulation. Besides, they are closely related to the pathological mechanism, progression and prognosis of many diseases such as pancreatic cancer, bladder and gastric cancer, etc[6-8]. SNHG7 has been confirmed to exert a regulatory role in many tumors, but its mechanism in osteosarcoma remains to be studied.

P53 is a tumor-suppressor gene. More than 50% of malignancies have p53 mutations. P53 is also a transcription factor involved in the regulation of cell cycle[9]. When cells are undergoing damage and cannot be repaired, p53 induces the regulation of cell apoptosis[10]. P53-deficiency cells continue to divide under injury, finally leading to cell death. P53 can also act as a monitoring role in cell division under normal conditions. It can determine the degree of DNA variation in cells. If the variation is small, this gene will prompt the cell to repair itself; otherwise, p53 will induce cell apoptosis[11]. DNA methyltransferases (DNMTs) are a significant family of enzymes that catalyze and maintain DNA methylation in epigenetics. In mammals, it is divided into three families: DNMT1, DNMT2, and DNMT3[12], of which DNMT1 is the key enzyme for DNA replication repair and maintenance of its normal methylation[13]. Numerous authors[14,15] have demonstrated that DNMT1 is associated with aberrant DNA methylation. A large number of research has illustrated that both p53 and DNMT1 are closely related to the occurrence and development of tumors. The purpose of this work was to elucidate the effect of SNHG7 on the proliferation and apoptosis of osteosarcoma cancer cells.

Patients and Methods

Sample Collection

Fresh tumor and adjacent tissues were collected from 48 patients diagnosed with osteosarcoma by pathological diagnosis and received surgery from July 2012 to August 2017. None of the patients received any treatment before surgery, and they had no family history. All patients voluntarily participated in this study and signed informed consent. Our study has been approved by the Ethics Committee of Yantaishan Hospital. After radical surgery, the collected samples were collected in liquid nitrogen.

Cell Culture and Transfection

Osteosarcoma cells U2OS, HOS, MG-63 and Saos-2 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured with Modified Eagle's Medium (MEM; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% (penicillin + streptomycin), incubated in a 37°C, 5% CO_2 incubator. The medium was changed every other day. When cells were grown to 70%-80% confluency, 0.25% trypsin was used to digest cells and passaged at a 1:2 or 1:3. Cells with better vitality in log phase were selected and transfected according to Lipofectamine 3000 instructions. Cells were transfected with SNHG7 siRNA, pcDNA-SNHG7, pcDNA-p53 or the corresponding negative control. SNHG7 siRNA, pcDNA-SNHG7 and pcDNA-p53 were all designed and synthesized by GenePharma (Shanghai, China). After 48 hours of transfection, cells were used for other experiments.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The tissue and cellular RNA were extracted according to the instructions for rapid extraction of total RNA by TRIzol (Invitrogen, Carlsbad, CA, USA). After centrifugation at 4 °C, the supernatant was taken and precipitated with isopropyl alcohol, washed and dried at room temperature. Finally, 20-30 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China) was added and the RNA concentration was measured. All RNAs were stored in a -80 °C freezer. Reverse transcription experiments were performed following the TaKaRa OneStep PrimeScript® miRNA complementary Deoxyribose Nucleic Acid (cDNA) Synthesis Kit instructions (TaKaRa, Otsu, Shiga, Japan). SYBR Green I was used for PCR detection. The PCR amplification conditions were: pre-denaturation at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and 30 s. The primer sequences were: SNHG7

(F: 5'-AGGCTGGCTGGAATAAAGGT-3', R: 5'-TATGAAAAGGGAGGCGTGGT-3'), p15 (F: 5'-CGGCGGTCAACCTGGAGGACTCC-3', R: 5'-CCAGTGCAGGGTCCGAGGTAT-3'), P21 (F: 5'-CGACGCGTCGTTGTAATAAAGCCTCCAG-3',R: 5'-GACTAGTCGTTTTCATTTCAATCGTAG-3'), p27 (F: 5'-TGGAAGACTAGTGATTTTGTTGT-3', R: 5'-TACTGGCACCACTGGAAACC-3'), p53 (F: 5'-GGACTCTGCCCTGCCACCATTTA-3', R: 5'-CTTGTGCCCTGTGAGGTCGTTGA-3'), DNMT1 (F: 5'-AGGCGGCTCAAAGATTTG-GAA-3', R: 5'-GCAGAAATTCGTGCAAGAGAT-TC-3'), GAPDH (F: 5'-AGCCACATCGCTCAGA-CAC-3', R: 5'-GCCCAATACGACCAAATCC-3'), U6 (F: 5'-CTCGCTTCGGCAGCAGCACATA-TA-3', R: 5'-AAATATGGAACGCTTCACGA-3').

Plate Colony Experiment

HOS and MG-63 cells in logarithmic growth phase were collected, and the cell suspension concentration was adjusted to 1×10^4 cells/mL. 2×10^3 cells were cultured in six-well plates per well, and incubated 3-4 days in 37 °C incubator. After fixation with 4% paraformaldehyde for 30 min, the cells were stained with 0.1% crystal violet for another 30 min. The number of colonies per well were counted and photographed.

Cell Apoptosis

Cells were digested with pancreatin without Ethylene Diamine Tetraacetic Acid (EDTA) (Beyotime, Shanghai, China), and the suspended cells were adjusted to 1×10^5 cells/mL. 100 μL of 1×Annexin buffer was added to suspend the cells. Annexin V-fluorescein isothiocyanate (FITC) was used for labeling. The cells were then added with 5 μL of Annexin V and 1 μL of Propidium Iodide (PI) staining (Solarbio, Beijing, China) in the dark. After gentle mixture, the cells were incubated at room temperature for about 15 min. Before testing, 400 μL of 1× buffer was added to flow cytometer. Each group had 3 replicates and the experiment was repeated 3 times.

Cell Cycle

The cells were harvested for adjusting the cell concentration to 1×10^5/mL. After fixation with 1 mL of 75% ice-cold at -20 °C, cells were then placed in a refrigerator at 4 °C overnight. Before testing, the cells were washed with Phosphate-Buffered Saline (PBS) twice. Then, the supernatant was discarded and 100 μL of RNaseA was added. The solution was incubated in a water bath at 37°C for 30 min. 400 μL Propidium Iodide (PI) staining was added and mixed, incubated in the dark at 4°C for 30 min. The samples were tested at 488 nm red fluorescence to record the cell cycle using flow cytometry. The experiment was repeated three times.

Western Blot

Collected cells were lysed on the ice and centrifuged for collecting the supernatant. The protein concentration was measured according to the bicinchoninic acid (BCA) protein quantification Kit instructions (Pierce, Waltham, MA, USA). After the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein loading buffer was added, the protein was denatured by heating at 100°C. After gel electrophoresis, the membrane was transferred and the corresponding size of polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) was cut according to the molecular weight. The membrane was then blocked in 5% skim milk. Primary antibody was added overnight. At the other day, the secondary antibody was incubated and exposed.

Nucleoplasm Separation

After the cells were grown to 1×10^6 cells/mL, 200 μL of Lysis Buffer J was added to the flask to lyse the cells. After centrifugation, the supernatant containing the cytoplasmic RNA was collected, and the remaining liquid contained nuclear RNA. Buffer SK and absolute ethanol were added to the liquid containing cytoplasmic RNA and nuclear RNA, respectively. Then, the cytoplasmic RNA and nuclear RNA were eluted by column centrifugation.

RNA Binding Protein Immunoprecipitation (RIP) and Chromatin Immunoprecipitation (ChIP)

The RIP experiment was performed strictly in accordance with the Millipore Kit instructions (Billerica, MA, USA). After cell lysis, the detection antibody was added and the working concentration of the antibody was 8 μg per reaction system. After overnight incubation on a shaker at 4°C, cells were warmed at room temperature for 1 h. Protein G beads were added to capture the complex. After washing with the wash buffer, cellular RNA was extracted. Cellular RNA levels were detected using fluorescence quantitative PCR.

ChIP experiment was performed strictly following the Millipore kit instructions. Cells were

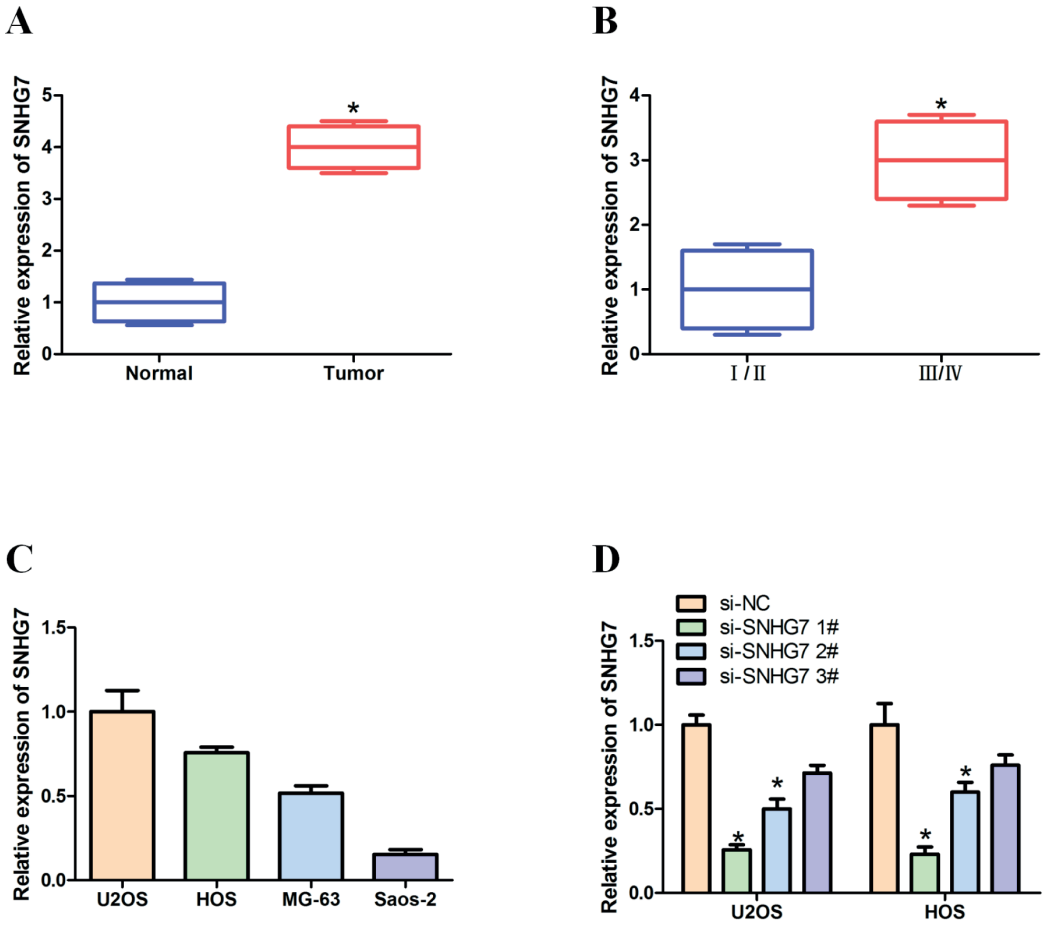

Figure 1. SNHG7 was highly expressed in osteosarcoma tissue. ***A,*** SNHG7 was highly expressed in osteosarcoma tissues. ***B,*** SNHG7 expression was significantly higher in early osteosarcoma tissues than early. ***C,*** Expression of SNHG7 in osteosarcoma cells, with higher expression in U2OS and HOS. ***D,*** After SNHG7 interference, the expression of SNHG7 was significantly downregulated, with si-SNHG7-1and si-SNHG7-2 being the most efficient.

sonicated, cross-linked, and incubated with DNMT1 antibody overnight at 4°C on a shaker. On the second day, magnetic beads were added again to capture and extract DNA. The PCR was used to detect the binding level of DNMT1 to the p53 promoter region.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA) was used for analysis. Chi-square test was used for classification data, and *t*-test was used for measurement data. Data were expressed as mean ± standard deviation. The difference was statistically significant at $p<0.05$.

Results

SNHG7 was Highly Expressed in Osteosarcoma

The expression of SNHG7 was detected in 48 osteosarcoma tissues and corresponding para-cancerous tissues. The results demonstrated that SNHG7 was highly expressed in osteosarcoma. Besides, it was remarkably higher in advanced osteosarcoma than those in early stage (Figure 1A, 1B). We then examined the expression of SNHG7 in osteosarcoma cell lines and found that the expression of SNHG7 was higher in U2OS and HOS (Figure 1C). Three SNHG7 siRNAs (si-SNHG7-1, si-SNHG7-2, si-SNHG7-3)

were transfected into U2OS and HOS cell lines, respectively. Both si-SNHG7-1 and si-SNHG7-2 can significantly inhibit SNHG7 expression (Figure 1D), and their co-transfection was selected as a follow-up study. These results suggested that SNHG7 was highly expressed in osteosarcoma and the relative cell lines.

Interference with SNHG7 Can Inhibit Osteosarcoma Cell Proliferation, Induce Apoptosis and Cell Cycle Arrest

We detected cell proliferation after SNHG7 knockdown in U2OS and HOS cell lines. Plate cloning experiments demonstrated that SNHG7 knockdown significantly inhibited cell proliferation (Figure 2A). Subsequently, we examined the effect of SNHG7 on cell apoptosis and cell cycle. The results showed that SNHG7 knockdown remarkably induced osteosarcoma cell apoptosis (Figure 2B), and arrested cell cycle in the G0/G1 phase (Figure 2C).

SNHG7 Can Bind to DNMT1 to Inhibit p53 Expression

After SNHG7 knockdown in U2OS and HOS, we detected the expression of tumor-suppressor genes p15, p21, p27 and p53. The results demonstrated that SNHG7 interference strikingly upregulated p53 expression in both cell lines (Figure 3A, 3B). Western blot demonstrated that p53 expression decreased after SNHG7 knockdown=in U2OS and HOS cell lines (Figure 3C). To further explore the regulatory mechanism of SNHG7 on p53, nuclear separation assay was performed to detect the subcellular localization of SNHG7. The results illustrated that SNHG7 mainly distributed in the nucleus (Figure 3D), suggesting that SNHG7 may exert its regulation at the transcriptional level. Many previous researchers[16,17] have illustrated that DNMT1 can inhibit the expression of p53 by binding to p53 promoter DNA. We speculated that SNHG7 can be stably expressed by binding to DNMT1 to downregulate p53. We then

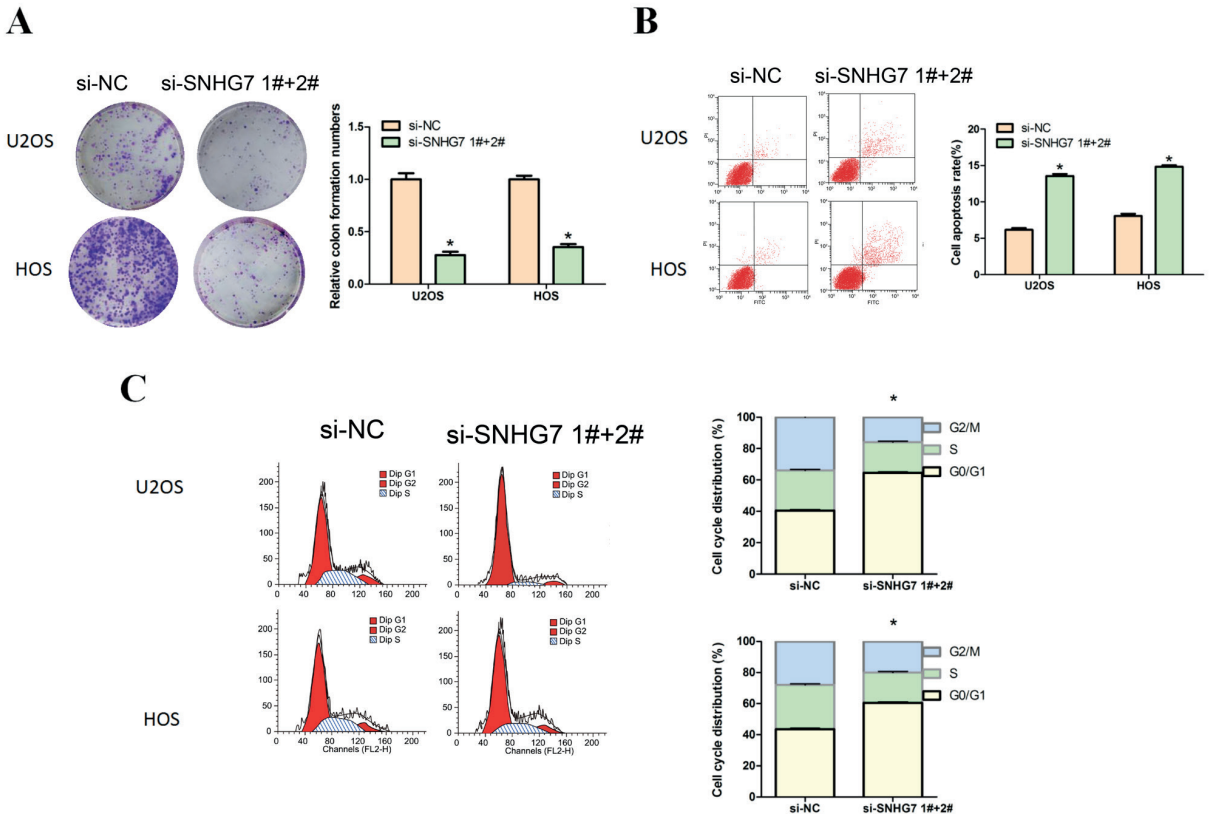

Figure 2. Interfering with SNHG7 inhibited osteosarcoma cell proliferation and induced apoptosis and cell cycle arrest. ***A,*** After interference with SNHG7, osteosarcoma cell proliferation was significantly downregulated. ***B,*** After interference with SNHG7, osteosarcoma cell apoptosis increased significantly. ***C,*** After interference with SNHG7, the cell cycle arrested at G0/G1 phase.

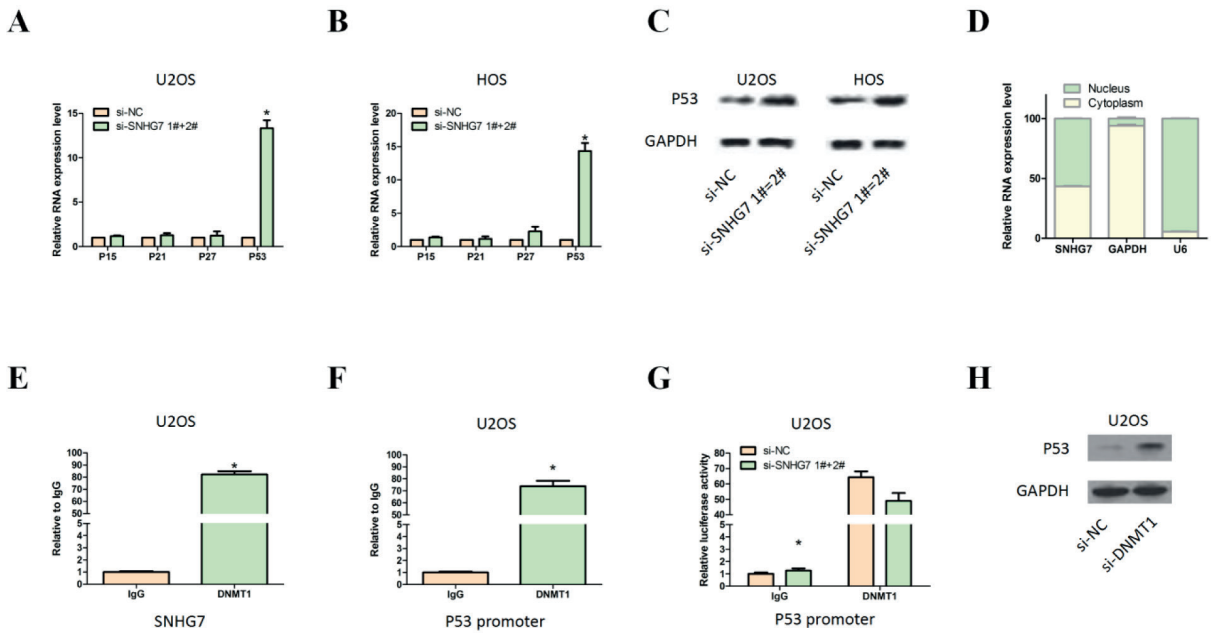

Figure 3. Binding to DNMT1 by SNHG7 inhibited p53 expression. ***A-B,*** After interference with SNHG7, the expression of p53 was up-regulated in both U2OS and HOS. ***C,*** After interference with SNHG7, the expression of p53 protein was significantly up-regulated in both cell lines. ***D,*** Nucleoplasmic separation assay showed that SNHG7 was mainly distributed in the nucleus. ***E,*** RIP experiment proved that SNHG7 can bind to DNMT1. ***F,*** CHIP assay demonstrated that DNMT1 can bind to p53 promoter region. ***G,*** After interference with SNHG7, the binding of DNMT1 to p53 promoter was downregulated. ***H,*** After interference with DNMT1, p53 expression was up-regulated.

performed RIP and ChIP studies in U2OS cell lines. RIP results demonstrated that SNHG7 can bind to DNMT1 (Figure 3E). ChIP results demonstrated that DNMT1 can bind to the p53 promoter region (Figure 3F). After SNHG7 knockdown in the U2OS cell line, we performed a ChIP work to detect the binding of DNMT1 on p53 promoters (Figure 3G). Besides, DNMT1 knockdown in U2OS cell lines strikingly upregulated the expression of p53 (Figure 3H). These findings indicated that SNHG7 can inhibit p53 expression by binding to DNMT1.

P53 Can Reverse the Carcinogenesis of SNHG7

After transfection of pcDNA-p53 in U2OS cell lines, mRNA and protein expression of p53 were remarkably up-regulated (Figure 4A, 4B). We explored the effect of p53 on cell cycle by flow cytometry. The results illustrated that the overexpression of p53 arrested cell cycle in the G0/G1 phase (Figure 4C). After SNHG7 was over-expressed, CCK8 assay was conducted to detect the cell proliferation ability. We found that the over-expression of SNHG7 enhanced the cell proliferation ability. Moreover, p53 overexpression weakened the cell proliferation ability, but still higher than that in the control group (Figure 4D). The results showed that SNHG7 can promote tumor cell proliferation by inhibiting the expression of p53.

Discussion

In recent years, long non-coding RNA has become a hot topic in the research field as a new tumor-related factor. Previous studies have demonstrated that lncRNA can act as a crucial role in the occurrence and development of osteosarcoma. As pointed out by Sun et al[18], up-regulated lncRNA HULC indicated a poor prognosis of osteosarcoma and significantly promoted cell metastasis in osteosarcoma. Dong et al[19] found that MALAT1 promoted the proliferation and metastasis of osteosarcoma cells by activating the PI3K/Akt pathway. Recently, Tian et al[20] pointed out that the down-regulated lncRNA MEG3 may serve as a predictive biomarker for osteosarcoma progression and prognosis. Some works showed that SNHG7 was involved in the lung cancer progression. She et al[21] found that lncRNA-SNHG7 can promote cell proliferation, invasion and migration

but inhibit apoptosis of lung cancer cells by inhibiting the expression of FAIM2. LncRNA-SNHG7 can participate in the development of osteosarcoma. However, its regulatory role and its specific mechanisms have not been reported yet.

In this paper, we detected the expression of SNHG7 in osteosarcoma tissues, corresponding precancerous tissues and osteosarcoma cells. The results illustrated that SNHG7 expression was remarkably higher in osteosarcoma tissues and osteosarcoma cells. Subsequently, we researched the function of SNHG7 in osteosarcoma cells. SNHG7 promoted proliferation and inhibited cell apoptosis osteosarcoma cells. Studies have shown that lncRNA can be controlled by inhibiting the expression of target genes. Based on this data, we found that SNHG7 exerted its biological functions by inhibiting the expression of p53. QRT-PCR and Western blot results demonstrated that SNHG7 could affect the expression of p53. P53 may be a target gene of SNHG7. It has been reported that P53 is a tumor-suppressor gene, and its low expression is associated with the occurrence of numerous tumors. Evidence has shown that the abnormally low expression of p53 is involved in the occurrence, progression and prognosis of osteosarcoma[22].

LncRNAs can form regulatory networks with transcription factors. To explore the mechanism of SNHG7 function, we considered whether SNHG7 could be combined with a certain transcription factor to alter p53 expression to ultimately lead to the biological changes of osteosarcoma. RIP experiments verified that DNMT1 could bind to SNHG7, and SNHG7 could stabilize the expression of DNMT1. DNMT1 (Persistent DNA Methyltransferase) can act on DNA duplexes with only one strand of methylation, making it fully methylated and participates in the methylation of newly synthesized strands in the DNA duplex. DNMT1 may directly block transcription in concert with HDAC (histone deacetylase)[23]. In tumor cells, hypermethylation of tumor-suppressor genes and abnormal proliferation and differentiation of cells are all associated with increased DNMT1 activity. Cacan et al[24] have demonstrated that the ab-

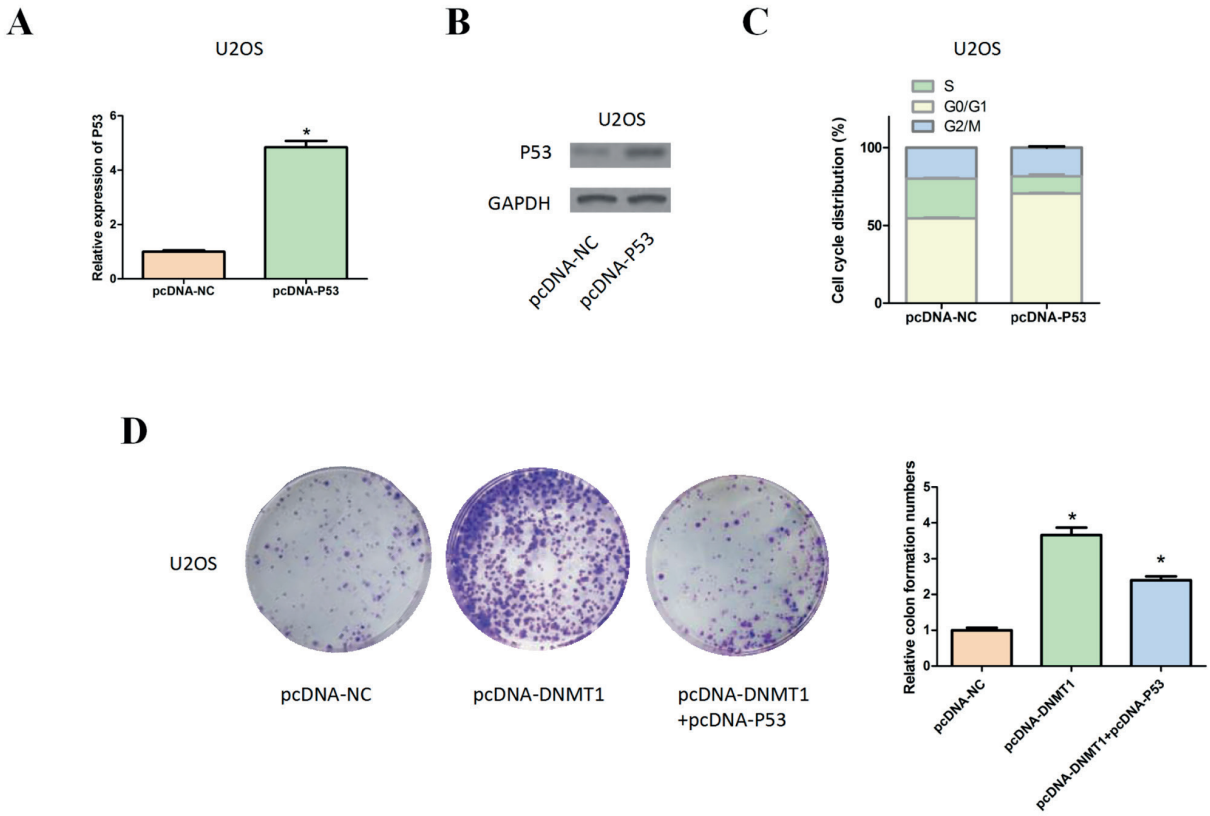

Figure 4. P53 can reverse the cancerous effect of SNHG7. ***A-B,*** After transfection of pcDNA-p53, the expression of p53 RNA ***(A)*** and protein (***B***) were significantly up-regulated. ***C,*** After overexpression of p53, the cell cycle arrested at G0/G1 phase. ***D,*** The overexpression of p53 can partially reverse the role of SNHG7 in promoting osteosarcoma cell proliferation.

normal expression of DNMT1 is related to the occurrence and prognosis of ovarian cancer[24], breast cancer[25] and colorectal cancer[26]. We then verified that DNMT1 could bind to the p53 promoter region and inhibit p53 expression. SNHG7 downregulated expression of p53 by binding to DNMT1. These results explained why SNHG7 could cause a biological change in osteosarcoma.

In this work, we described the function of SNHG7, DNMT1 and p53 in the development of osteosarcoma. However, there was only a small part of the lncRNA study. We believe that we will be able to introduce new targets for the early diagnosis and even treatment of patients with osteosarcoma from the perspective of epigenetics in the future.

Conclusions

We demonstrated that SNHG7 was highly expressed in osteosarcoma, and high expression of SNHG7 can promote the proliferation of osteosarcoma cells and inhibit apoptosis, which may be related to the inhibition of p53 expression by binding to DNMT1.

Conflict of interest

The authors declare no conflicts of interest.

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