Original Article LncRNA small nucleolar RNA host gene 8 promotes cell growth and migration of osteosarcoma *in vitro and in vivo* by functioning as a ceRNA of microRNA-876-5p

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Abstract: Osteosarcoma (OS) is the most leading primary malignant tumor of the bone in adolescents and young adults worldwide. Increasing data have suggested that long non-coding RNA (IncRNA) small nucleolar RNA host gene 8 (SNHG8) plays a key role in the progression of various types of human malignancy. However, the roles and potential mechanisms of SNHG8 in OS remain unclear. In this study, we found that SNHG8 levels were obviously upregulated in OS tissues and cell lines. High expression of SNHG8 was significantly correlated with increased tumor size and advanced Enneking stage, and predicted a poor prognosis of OS patients. Functional assays revealed that SNHG8 knockdown inhibited OS cell growth and migration *in vitro*, and restrained tumor growth of OS in nude mice *in vivo*. Mechanistically, SNHG8 functioned as a competing endogenous RNA (ceRNA) of miR-876-5p in OS cells. Notably, knockdown of miR-876-5p reversed the inhibitory effects of SNHG8 inhibition on OS cell proliferation and migration. In conclusion, our study suggested that SNHG8 stimulates cell growth and migration of OS cells by functioning as a ceRNA of miR-876-5p, indicating SNHG8 may be served as a novel prognostic biomarker and therapeutic target for the treatment of OS.

Keywords: Osteosarcoma, SNHG8, prognosis, miR-876-5p, ceRNA

Introduction

Osteosarcoma (OS) is the most leading primary malignancy of bone and the most cause of cancer-associated death in adolescents and young adults worldwide [1, 2]. OS is characterized by malignant osteoid production and resistance to conventional treatments. Despite continuous progresses in OS therapy, such as surgical resection, radiotherapy, and chemotherapy, the overall survival of OS patients, especially those cases with recurrent and metastatic sarcomas. still remains unsatisfactory [3, 4]. Although numerous efforts have been taken to explore the pathogenesis of OS, the molecular mechanisms involved in the tumorigenesis of OS are limitedly clarified. Thus, it is essential to identify new biomarkers related to the carcinogenesis of OS and to provide reliable therapeutic strategies for the treatment of this cancer.

Long non-coding RNAs (IncRNAs) are a family of longer than 200 nt in length with non-protein coding capacity [5, 6]. Growing data have suggested that IncRNAs are emerging as a new regulator within a large range of biological processes, such as cell growth, differentiation, migration, and metastasis [7]. Recent studies have found that aberrantly expressed IncRNAs plays a crucial role in initiation and tumorigenesis of OS [8, 9]. For instance, downregulation of IncRNA DBH antisense RNA 1 inhibits OS progression by targeting PI3K-AKT signaling pathways and indicates a good prognosis [10]. LncRNA small nucleolar RNA host gene 16 increases OS cell migration and invasion via sponging miR-340 [11]. The epigeneticallyinduced IncRNA1 suppresses the growth and invasion of OS cells by upregulating myocyte enhancer factor 2D ubiquitylation [12]. LncRNA X inactive specific transcript indicates a poor prognosis and promotes aggressive phenotypes in OS [13]. Hence, detection of key IncRNAs involved in OS development is needed.

Interesting, IncRNA small nucleolar RNA host genes (SNHGs) have been reported to involve in

tumor cell growth and metastasis [14]. Deng et al [15] showed that IncRNA SNHG1 promotes cell growth, migration and invasion of OS cells by negatively regulating miR-101-3p to upregulate the Rho associated coiled-coil containing protein kinase 1 (ROCK1) expression. Xu et al [16] demonstrated that IncRNA SNHG4 facilitates tumor growth by sponging miR-224-3p and predicts a poor overall survival and recurrence in OS. Wang et al [17] suggested that IncRNA SNHG5 sponges miR-26a to increase the development of OS by targeting ROCK1. SNHG8 is a new small nucleolar guide RNA located on 4q26 position of human chromosome. More evidences have confirmed that SNHG8 serves as an oncogenic IncRNA that elevates tumorigenesis and tumor progression in various types of human cancer [18-20]. Previous sequencing for IncRNAs expression has shown that SNHG8 is upregulated in OS [21, 22]. However, its biological roles and potential mechanisms in OS development have not yet been clarified. In our study, we detected the effects of SNHG8 on the growth and migration of OS cells in vitro and in vivo. We further identified that SNHG8 stimulates tumor cell growth and migration of OS cells by functioning as a ceRNA of miR-876-5p. These findings suggested that SNHG8 may be a new biomarker for the prognosis and therapy of OS.

Materials and methods

Cell lines and culture conditions

The human OS cell lines, including SaOS2, U2OS, MG63, HOS, and a normal human osteoblast (hFOB 1.19) were purchased and authenticated from Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (Shanghai, China). Cells were seeded and cultured in DMEM medium (Gibco, CA, USA) with 10% FBS (Gibco, CA, USA) containing 100 units/ml of penicillin + streptomycin (Sigma, MO, USA), and maintained in an incubator at 37°C with 5% CO_2 .

Transfection

Three special sequences of short-hairpin RNA (shRNA) target SNHG8 were synthesized and obtained from RiboBio Co., Ltd (Guangzhou, China) referred as to shSNHG8-1# and scrambled shRNA-1#, shSNHG8-2# and scrambled shRNA-2#, shSNHG8-3# and scrambled sh-

RNA-3#. For knockdown the expression of miR-876-5p, antagomir and antagomir control were purchased from GenePharma Co., Ltd (Shanghai, China). U2OS and HOS (4 × 10⁵) were seeded in six-well plates overnight. When cells reached 80% confluence, RNA oligonucleotides were diluted in a Opti-MEM I Reduced Serum Media (Thermo Fisher Scientific, MA, USA) to obtain a concentration of 200 pmol and incubated for 20 min at 37°C. Then, cells were treated with the RNA oligonucleotides using Lipofetamine 2000 reagent (Invitrogen, CA, USA), following the manufacturer's protocols and cultured at 37°C in 5% CO₂ for 6 h. Then, the supernatants were discarded, and 10% serum-containing DMEM medium was added. The transfected cells were collected at 48 h of transfection for RNA extraction.

OS patients

The human OS tissues and matched normal bone tissues (located > 3 cm away from the tumor) were collected from 60 OS patients who received surgical treatment at Department of Orthopedics, Shanxi Academy of Medical Sciences & Shanxi Bethune Hospital (Taiyuan, China) between June 2010 and October 2013. The OS patients included 37 males and 23 females, with average age of 24.16 ± 4.92 years. None of patients received radiotherapy and/or chemotherapy prior to operation. All samples were snap frozen in liquid nitrogen immediately after operation and stored at -80°C for further use. All tissue were reviewed and confirmed by two independent pathologists, and the clinicopathologic data were recorded. The research was approved by the Ethics Committee of Shanxi Academy of Medical Sciences & Shanxi Bethune Hospital (No. 2016-1433-02) and complied with the Declaration of Helsinki (version 2002). All patients had received a follow-up every month, which ranged from one to sixty months since the day of operation. The overall survival times were defined as the time interval between the operation and the death.

RNA extraction and quantitative real-time-polymerase chain reaction (qRT-PCR) assay

Total RNA from OS tissues and cells were extracted with a TRIzol® reagent (Invitrogen, CA, USA) according to the manufacturer's protocols. For SNHG8 analysis, the complementa-

Table 1. The philler sequences used for given		e primer se	quences	useu i	лик	-PUR
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Gene	Sequences
SNHG8	Forward: 5'-AAGTTTACAAGCATGCGCGG-3'
	Reverse: 5'-TCAAACTGACGGTTCTCGGG-3'
GAPDH	Forward: 5'-AGCCACATCGCTCAGACAC-3'
	Reverse: 5'-GCCCAATACGACCAAATCC-3'
miR-876-5p	Forward: 5'-TGAAGTGCTGTGGATTTCTTTGTGA-3'
	Reverse: 5'-TGAAGCACTATGAATTACTTTGTAA-3'
U6	Forward: 5'-ATTGGAACGATACAGAGAAGATT-3'
	Reverse: 5'-GGAACGCTTCACGAATTTG-3'

qRT-PCR, quantitative real-time-polymerase chain reaction; SNHG8, small nucleolar RNA host gene 8; GAPDH, glyceraldehyde-3-phos-phate dehydrogenase; U6, U6 small nuclear RNA.

ry DNA (cDNA) was synthesized by using the Reverse Transcriptase M-MLV (Takara, Otsu, Japan). For miR-876-5p analysis, the cDNA was obtained by using the Mir-X[™] miRNA First Strand Synthesis Kit (Takara, Otsu, Japan). qRT-PCR assay was conducted by using SYBR® Premix Ex Taq[™] (Takara, Otsu, Japan) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA). The amplification conditions of gRT-PCR were as follows: 10 min predenaturation at 98°C followed by 40 cycles of (30 s at 95°C, 30 s at 55°C, and 60 s at 72°C). The special primer sequences of SNHG8, glyceraldehyde-3-phosphate dehydrogenase (GA-PDH), miR-876-5p, and U6 small nuclear RNA (U6) were shown in Table 1. GAPDH was used as the internal control for SNHG8 expression, and U6 was used as the internal control for miR-876-5p expression. The experiments were performed at least three times and the relative levels of SNHG8 and miR-876-5p were quantified using the $2^{-\Delta\Delta CT}$ method [23].

Cell migration assay

Cell migration was detected by using transwell migration assay. Briefly, the transfected U2OS and HOS cells were seeded in transwell inserts with a 8.0 μ m poly-carbonate membrane (Corning, NY, USA). A total of 200 μ l serum-free DMEM medium was added in the upper chamber, and 500 μ l 10% serum-containing DMEM medium was added to the lower chamber. Then cells were incubated in 5% CO₂ at 37°C for 48 h. After that, the migratory cells were fixed with 100% methanol for 30 min at 37°C, and washed twice and stained with 0.5% crystal violet for 30 min at 37°C. The excess crystal violet stain was removed by dipping the upper cham-

ber inserts in double distilled water and cleaned with cotton swabs from the upper chamber. A Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) at $200 \times$ magnification was used to observe the stained cells. Five different fields were chose and the average number of cells per field were calculated.

Cell proliferation assay

The ability of OS cell growth was evaluated through cell counting kit-8 (CCK-8) assay. In brief, U2OS and HOS cells were diluted to 8 × 10³ cells/well and seeded in 96-well plates during logarithmic phase. After 12, 24, 48, and 72 h of post-transfection, 20 µl of CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well. After 4 h of incubation, cell proliferative activity was detected by measuring the optical density (OD) values at 450 nm using a Multiskan Spectrum plate reader (ThermoElectron Corporation, Vantaa, Finland).

Tumour xenograft assay

Male athymic BALB/c nude mice (4-6 weeks old, weighing 20-26 g) were purchased from Experimental Animal Center of Shanxi Medical University (Taiyuan, China). Animals were housed and kept in a specific pathogen-free (SPF) suite with ad libitum access to food and water under a 12-h light/dark cycle, relative humidity (40-70%), temperature (18-22°C), and noise (< 50 dB) conditions. The animal experiment was approved by the Institutional Animal Care and Use Committee of Shanxi Academy of Medical Sciences & Shanxi Bethune Hospital and was in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guideline. 24 mice were randomly assigned to the control and experimental group (12 mice per group). RNA oligonucleotides transfected HOS cells were diluted and suspended in PBS reagent and the cell concentration was adjusted to 1×10^7 /ml. 100 µl cell suspension was subcutaneously injected into the left side of posterior flank of each mouse. Tumor volume of each mice was observed every 7 days, and calculated as $L \times W^2 \times 0.5$, where L is the length of tumor and W is the width of tumor. When mice presenting with multiple tumors, the number of tumors and their total volumes were calculated.

28 days after the injection, all mice were anesthetized with intraperitoneal injection of sodium pentobarbital (35 mg/kg) and then sacrificed by cervical dislocation, and tumors were separated and weighed. The tumor tissues were immediately snap frozen in liquid nitrogen, and stored at -80°C for RNA extraction. Each experiment was conducted at least three times from individual experiments.

Bioinformatics and luciferase reporter assay

A target gene prediction tool starBase v2.0 (http://starbase.sysu.edu.cn/starbase2/) was performed to predict potential SNHG8 targets, and we found that SNHG8 could potentially bind miR-876-5p. The luciferase assay was conducted to confirm the prediction of binding sites between SNHG8 and miR-876-5p. Briefly. the SNHG8 transcript 1 containing the potential binding sites for miR-876-5p was artificial synthesized and inserted into the psiCHECK2 reporter vector (Promega, WI, USA) and named as SNHG8-wild-type (Wt) reporter vector. The mutant (Mut) version of the SNHG8 transcript 1 was obtained by using the Mutagenesis kit (Stratagene, CA, USA) and named as SNHG8-Mut reporter vector. The U2OS and HOS cells were seeded into 24-well plates and treated with the different reporter vectors including SNHG8-Wt vector, SNHG8-Mut vector, together with antagomir or antagomir control using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols. Forty-eight hours of post-transfection, luciferase activities were determined by using the dual-luciferase reporter assay (Promega, WI, USA). The renilla luciferase activity was normalized against that of firefly luciferase activity. Each experiment was conducted at least three times from individual experiments.

Statistical analysis

All data was displayed as the mean \pm standard deviation (SD). Statistical analyses were conducted by using Statistical Product and Service Solutions 17.0 software (SPSS Inc., IL, USA) and GraphPad Prism 6.0 (GraphPad, CA, USA). To compare SNHG8 expression levels in OS tissues vs. normal bone tissues, a paired Student's t-test was used. Differences between the remaining data containing two groups were assessed using an unpaired Student's t-test. One-way ANOVA followed by the Bonferroni post

hoc test was used for comparisons among more than two groups. Relationship between SNHG8 expression and the clinicopathologic characteristics of OS patients was analyzed using chi-square test. Correlation between SNHG8 and miR-876-5p expression was assessed by Spearman's correlation analysis. Survival curves were evaluated using Kaplan-Meier method and log-rank test, and two-stage test was used when survival plots cross. Difference was defined to be significant at P < 0.05.

Results

The SNHG8 expression was elevated in OS tissues and cell lines

The expression levels of SNHG8 in 60 paired OS tissues and adjacent normal bone tissues were examined by using gRT-PCR. The results indicated that SNHG8 expression was obviously higher in OS tissues than in matched normal bone tissues (Figure 1A, P < 0.01). Besides, SNHG8 levels were significantly upregulated in SaOS2, U2OS, MG63 and HOS cell lines compared with the hFOB1.19 cell line (Figure 1B, P < 0.05). Among these OS cell lines, U2OS and HOS cells had higher SNHG8 expression than SaOS2 and MG63 cells, and were chosen for further experiments. In addition, the 60 OS patients were classified into two groups (low SNHG8 levels group and high SNHG8 levels group) based on the median expression level of SNHG8 in OS tissues as the cutoff value. Results revealed that high expression of SN-HG8 was significantly correlated with increased tumor size (P = 0.018) and advanced Enneking stage (P < 0.001), but not correlated with sex (P = 0.426), age (P = 0.301), location (P = 0.195), histological grade (P = 0.194), and distant metastasis (P = 0.181) of the patients with OS (Table 2). As shown in Figure 2, the OS patients with high SNHG8 levels had a distinctly shorter overall survival compared to those patients with low SNHG8 levels (P < 0.05). Thus, upregulation of SNHG8 may be a novel prognostic biomarker for OS.

The knockdown of SNHG8 suppressed OS cell proliferation and migration in vitro

To investigate the effects of SNHG8 on the proliferation and migration of OS cells, U2OS and HOS cells were transfected with shSNHG8-1# or scrambled shRNA-1#, shSNHG8-2# or



Figure 1. The expression levels of IncRNA SNHG8 from OS tissues and cell lines. A. The relative levels of SNHG8 in 60 pairs of OS tissues and matched normal bone tissues were quantified by qRT-PCR. GAPDH was used as the internal control for SNHG8 expression. B. The relative SNHG8 levels were analyzed in four OS cell lines (SaOS2, U2OS, MG63 and HOS) and a normal human osteoblasts hFOB 1.19. LncRNA, long non-coding RNA; SNHG8, small nucleolar RNA host gene 8; OS, osteosarcoma; qRT-PCR, quantitative real-time-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data were shown as the mean \pm standard deviation (SD), n = three independent repeats. **P* < 0.05 and ***P* < 0.01 vs. normal bone tissues or hFOB 1.19 cell line.

in OS patients	inneepa			
Characteristics	Cases	Low	High	Р
Age (y)				
< 20	28	12	16	0.301
≥20	32	18	14	
Gender				
Male	37	20	17	0.426
Female	23	10	13	
Location				
Femur/tibia	54	29	25	0.195
other	6	1	5	
Tumor size (cm)				
< 8	35	22	13	0.018
≥8	25	8	17	
Enneking stage				
I-IIA	22	18	4	< 0.001
IIA-III	38	12	26	
Histological grade				
G1-G2	27	11	16	0.194
G3-G4	33	19	14	
Distant metastasis				
Absent	49	27	22	0.181
Present	11	3	8	
IncDNA long non-oodi		o		

Table 2. LncRNA SNHG8 expression and its association with clinicopathological features in OS patients

LncRNA, long non-coding RNA; OS, osteosarcoma.

scrambled shRNA-2#, and shSNHG8-3# or scrambled shRNA-3#. Images of U2OS and HOS cells successfully transfected with shSN- HG8-1-3# and scrambled shRNA-1-3# were shown in **Figure 3A** and **3B**. The results revealed that SNHG8 expression was remarkably reduced in the shSNHG8-3# group compared with the scrambled shRNA-3# group in U2OS and HOS cells (**Figure 3C** and **3D**, P < 0.05), which indicated that SNHG8 was successfully silenced by shSNHG8-3#. Therefore, shSN-HG8-3# and scrambled shRNA-3# was chosen for further experiments.

Then, we used CCK-8 assay to detect cell proliferative activity. The results revealed that SNHG8 knockdown markedly inhibited the growth of U2OS and HOS cells (**Figure 4A** and **4B**, P < 0.01). After that, the effects of SNHG8 knockdown on cell migration in OS cells were detected by using transwell migration assay. The results showed that SNHG8 knockdown in U2OS and HOS cells suppressed the cell migration abilities compared with the scrambled shRNA-3# groups (**Figure 4C**, P < 0.01). These data suggested that knockdown of SNHG8 inhibits OS cell proliferation and migration *in vitro*.

The knockdown of SNHG8 inhibited OS tumor growth in vivo

To further confirm whether SNHG8 played an inhibitory role in OS cell proliferation *in vivo*, the subcutaneous xenograft model of HOS cells in BALB/c nude mice was established. The HOS



Figure 2. High expression of SNHG8 predicted a poor prognosis of OS patients. OS patients were classified into two subgroups (low and high SNHG8 levels group) using the median level of SNHG8 as a cut-off value. Survival curves were evaluated using Kaplan-Meier method and log-rank test, and high SNHG8 levels in OS patients had a obviously shorter overall survival compared to those patients with low SNHG8 levels.

cells treated with shSNHG8-3# and scrambled shRNA-3# were subcutaneously injected into the left side of posterior flank of each mouse. The maximum diameter and largest volume exhibited by a single subcutaneous tumor in the rats were 23 mm and 781 mm³, respectively. 11 rats (36.67%, 11/30) with 2-3 tumors were observed. As shown in Figure 5A, knockdown of SNHG8 significantly decreased tumor growth compared with the scrambled shRNA-3# group (P < 0.05). Furthermore, the tumor weight of mice inoculated with HOS cells transfected with shSNHG8-3# was significantly lower than that of the scrambled shRNA-3# group (Figure 5B, P < 0.05). Moreover, we analyzed the expression levels of SNHG8 in tumor tissues from the xenograft model. The results indicated SNHG8 possessed lower expression in the shSNHG8-3# group compared with the scrambled shRNA-3# group (Figure 5C, P <

0.01). Interestingly, the expression of miR-876-5p was upregulated when SNHG8 was inhibited (**Figure 5D**, P < 0.01). These *in vivo* results indicated that SNHG8 knockdown represses OS cell growth *in vivo*.

The SNHG8 act as a ceRNA of miR-876-5p in OS

Bioinformatics analysis revealed that SNHG8 transcript 1 contains a conserved target site of miR-876-5p (Figure 6A). Spearman's correlation analysis showed that a significant inverse correlation between SNHG8 levels and miR-876-5p expression in OS (Figure 6B, P < 0.01). We then determined the effects of SNHG8 knockdown on the expression of miR-876-5p in OS cells. After transfection with shSNHG8-3#, the expression levels of miR-876-5p in U2OS and HOS cells were significantly increased compared with the scrambled shRNA-3# groups (Figure 6C, P < 0.01). Subsequently, dual-luciferase reporter assay was conduct to confirm whether SNHG8 directly interacts with miR-876-5p. We transfected the antagomir and antagomir control into U20S and HOS cells, and the results showed that miR-876-5p expression was markedly decreased in the antagomir group compared to the antagomir control group (Figure 6D, P < 0.01). As shown in Figure 6E, the luciferase activity was obviously increased in antagomir and SNHG8-Wt vector co-transfected U2OS and HOS cells, which had no effects on the SNHG8-Mut vector (P < 0.01). Combined with the findings above, we concluded that SNHG8 acts as a ceRNA of miR-876-5p in OS.

The SNHG8 promoted OS cell proliferation and migration by sponging miR-876-5p

To study whether miR-876-5p was a functional target of SNHG8 in OS development, we down-regulated SNHG8 expression and inhibited miR-876-5p at the same time in U2OS and HOS cells. As expected, knockdown of miR-876-5p reversed the inhibitory effects of SNHG8 inhibition on OS cell proliferation (**Figure 7A** and **7B**, P < 0.05). In addition, cotransfection with shSNHG8-3# + antagomir significantly increased OS cells migration when compared with cells transfected with shSNHG8-3# + antagomir control (**Figure 7C**, P < 0.01). These results strong demonstrated that miR-876-5p is a



Figure 3. Knockdown of SNHG8 in OS cell lines by using special shRNA against SNHG8. (A) U2OS cells were successfully transfected with shSNHG8-1-3# and scrambled shRNA-1-3#, and the representative images were shown. (B) Images of HOS cells transfected with shSNHG8-1-3# and scrambled shRNA-1-3#. Bar = 10 μ m. The expression levels of SNHG8 in U2OS (C) and HOS (D) cells were determined using qRT-PCR after being transfected with shSNHG8-1#, shSNHG8-2# or scrambled shRNA-2#, and shSNHG8-3# or scrambled shRNA-3#. ShRNA, short-hairpin RNA; shSNHG8, shRNA against SNHG8. **P* < 0.05 and ***P* < 0.01 vs. scrambled shRNA.

functional target of SNHG8, indicating SNHG8 promotes OS cell proliferation and migration by sponging miR-876-5p.

Discussion

Accumulating data have demonstrated that IncRNAs can regulate various oncogenes and tumor suppressors in tumor cells, and lead to initiation and tumorigenesis of human cancers [5, 6]. Nowadays, several IncRNAs have been found as critical regulators of OS development and promising predictors for prognosis in OS patients [8, 9]. SNHGs have been widely found to be upregulated in OS tissues and to promote tumor initiation and development [16]. SNHG8, as a member of SNHGs, has been suggested to play an important role in several types of tumor [18-20]. However, no literature has reported the roles of SNHG8 in OS. To our knowledge, this is the first research that indicated that SNHG8 is involved in the OS development. In our study, we studied the functional effects of SNHG8 knockdown in OS cells *in vitro and in vivo*, and identify the potential mechanisms of SNHG8 by sponging miR-876-5p, which might give a better understanding of SNHG8 in the regulation of OS development.



Figure 4. SNHG8 knockdown suppressed OS cell proliferation and migration *in vitro*. A. A CCK-8 assay was applied to detect cell proliferation, and SNHG8 knockdown inhibited the proliferation of U2OS cells. B. SNHG8 knockdown significantly suppressed the growth of HOS cells. C. Transwell migration assay was used to investigate the effects of SNHG8 knockdown on OS cell migratory capacity, and the representative images were shown. Results indicated that SNHG8 knockdown in U2OS and HOS cells suppressed the cell migration abilities. CCK-8, Cell Counting Kit-8. Experiments were replicated for three times, bar = 100 μ m. ***P* < 0.01 vs. scrambled shRNA-3#.

In fact, SNHG8 was reported to be upregulated in some solid tumors, such as hepatocellular carcinoma [18], pancreatic adenocarcinoma [19], and non-small-cell lung cancer [20]. In addition, the expression of SNHG8 was upregulated in OS by microarray expression profiles [21, 22]. Consistent with previous sequencing [21, 22], our study showed that SNHG8 expression was obviously increased in OS tissues and cells compared to match normal bone tissues and hFOB1.19 cell line, respectively. High expression of SNHG8 was significantly correlated with increased tumor size and advanced Enneking stage, and predicted a poor prognosis of OS patients. These data indicated that SNHG8 may be used as a promising biomarker for the prognosis of OS. Because U2OS and HOS cells had higher SNHG8 expression than SaOS2 and MG63 cells, we selected the two cells for further research. Subsequently, we used a specially shRNA to target SNHG8 expression in OS cells, and found that SNHG8 knockdown significantly inhibited OS cell growth and migration *in vitro*, and restrained tumor growth of OS of nude mice *in vivo*. These results demonstrated that SNHG8 stimulates tumor cell growth and migration in OS cells, which were consistent with previous researches of SNHG8



Figure 5. Effects of SNHG8 knockdown on OS growth *in vivo*. A. The tumor volumes were measured every seven days in BALB/c nude mice after inoculation. Knockdown of SNHG8 markedly repressed tumor growth compared with the scrambled shRNA-3# group. B. The tumor weight on day 28 in mice inoculated with HOS cells transfected with shSN-HG8-3# was distinctly lower than that of the scrambled shRNA-3# group. C. qRT-PCR analysis of SNHG8 expression levels in tumor tissues from the xenograft model. D. The expression of miR-876-5p was upregulated when SNHG8 was inhibited. W: week. Data were shown as mean \pm SD, n = 3. **P* < 0.05 and ***P* < 0.01 vs. scrambled shRNA-3#.

in other tumors [18-20]. For example, Yang et al [24] demonstrated SNHG8 regulates the development of endometrial carcinoma through regulating MET proto-oncogene (c-Met) expression by miR-152. Liu et al [25] concluded that knockdown of SNHG8 represses cell growth in Epstein-Barr virus-associated gastric carcinoma. Song et al [19] suggested that SNHG8 promotes the development and chemo-resistance of pancreatic adenocarcinoma. These data implicated that SNHG8 acts as an oncogenic IncRNA in the development of OS. LncRNAs were reported to carry out their biological effects by functioning as a ceRNA to negatively regulate miRNAs expression [26]. For example, long intergenic non-protein coding RNA 336 inhibits ferroptosis by functioning as a ceRNA of miR-6852 in lung cancer [27]. Upregulated colon cancer associated transcript 1 accelerates hepatocellular carcinoma progression by functioning as a ceRNA of miR-30c-2-3p [28]. Another study showed ZNFX1 antisense RNA 1 increases the tumor progression and metastasis of colorectal cancer by acting as a



Figure 6. SNHG8 act as a ceRNA for miR-876-5p in OS cells. A. Sequences alignment of miR-876-5p with the putative binding sites of the sequences of SNHG8 transcript 1. B. Spearman's correlation analysis of correlation between SNHG8 and miR-876-5p expression in 60 cases of OS samples. C. qRT-PCR analysis of the expression levels of miR-876-5p in U2OS and HOS cells after transfected with shSNHG8-3# and scrambled shRNA-3#. D. The expression levels of miR-876-5p were markedly decreased in the antagomir group compared to the antagomir control group in U2OS and HOS cells. E. The OS cells were treated with the different reporter vectors including SNHG8-wild-type (Wt) vector, SNHG8-mutant (Mut) vector, together with antagomir or antagomir control, and the luciferase activity was obviously increased in antagomir and SNHG8-Wt vector co-transfected U2OS and HOS cells, which had no effects on the SNHG8-Mut vector. ceRNA, competing endogenous RNA. Experiments were replicated for three times. **P < 0.01 vs. scrambled shRNA-3# or antagomir control.

ceRNA of miR-144 [29]. Although a great number of IncRNAs act as ceRNAs to control the progression of OS [11], it is not clear about the potential molecular mechanisms of SNHG8 in OS. Thus, the mechanisms involved in the functional roles of SNHG8 in OS was further investigated. We used a bioinformatics tool (starBase v2.0) to predict the potential targets of SNHG8. Among these target miRNAs, miR-876-5p was chosen as a candidate miRNA for further investigation based on its biological roles in OS. Xie et al [30] showed that miR-876-5p suppresses OS cell growth, migration and invasion by regulating c-Met. miR-876-5p has been shown to function as a tumor suppressor miRNA in other cancers, such as lung cancer [31], hepatocel-



Figure 7. SNHG8 promoted OS cells proliferation and migration by sponging miR-876-5p. A CCK-8 assay was applied to detect cell proliferation when downregulated SNHG8 expression and inhibited miR-876-5p at the same time in U2OS and HOS cells. Knockdown of miR-876-5p reversed the inhibitory effects of SNHG8 inhibition on U2OS (A) and HOS (B) cell proliferation. (C) Transwell migration assay was used to detect cell migration, and the representative images were shown. Results showed that cotransfection with shSNHG8-3# + antagomir significantly increased cell migration when compared with cells transfected with shSNHG8-3# + antagomir control. Experiments were replicated for three times, bar = $50 \ \mu$ m. **P* < 0.05 and ***P* < 0.01 vs. shSNHG8-3# + antagomir control.

lular carcinoma [32], and head and neck squamous cell carcinoma [33]. In this present study, miR-876-5p was identified as a downstream target of SNHG8 by luciferase reporter assay. SNHG8 silencing resulted in an increased expression of miR-876-5p in OS cells and tumor tissues from the xenograft model.

More importantly, we conducted rescue experiments to confirm whether miR-876-5p was a functional target of SNHG8 in OS development. We found that inhibition of OS cell proliferation *in vitro* induced by SNHG8 knockdown was reversed by knockdown of miR-876-5p in OS cells. In addition, transwell migration assay further confirmed inhibitory effects of SNHG8 on OS cell migration *in vitro* were attenuated when miR-876-5p was downregulated. These results strong demonstrated that miR-876-5p was a functional target of SNHG8, indicating SNHG8 regulated OS cell proliferation and migration by sponging miR-876-5p. Several shortcoming of the present study should be noted. The OS samples are relatively small in our study, and a larger sample should be used to further confirm the clinical significances of SNHG8 in OS. Another concern is that the target gene of miR-876-5p was not identified in the current study. Although previous literatures have reported that c-Met [24], DNMT3A [32], and vimentin [33] are target genes of miR-876-5p, whether these genes regulated by SNHG8 in OS remains to be further studied.

In conclusion, SNHG8 is found to be significantly elevated in OS tissues and cell lines. High expression of SNHG8 predicts a poor prognosis of OS patients. SNHG8 acts as an oncogenic IncRNA in OS by facilitating cell proliferation and migration via sponging miR-876-5p. These findings suggested that SNHG8 may be a new clinical therapeutic target and a promising biomarker for the prognosis and treatment of OS.

Disclosure of conflict of interest

None.

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References

- Siegel RL, Miller KD and Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
- [2] Moore DD and Luu HH. Osteosarcoma. Cancer Treat Res 2014; 162: 65-92.
- [3] Zhang Y, Yang J, Zhao N, Wang C, Kamar S, Zhou Y, He Z, Sun B, Shi X, Han L and Yang Z. Progress in the chemotherapeutic treatment of osteosarcoma. Oncol Lett 2018; 16: 6228-6237.
- [4] Otoukesh B, Boddouhi B, Moghtadaei M, Kaghazian P and Kaghazian M. Novel molecular insights and new therapeutic strategies in osteosarcoma. Cancer Cell Int 2018; 18: 158.
- [5] Mercer TR, Dinger ME and Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.
- [6] Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q, Guigo R and Shiekhattar R. Long noncoding RNAs with enhancer-like function in human cells. Cell 2010; 143: 46-58.
- [7] Fatica A and Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. Nat Rev Genet 2014; 15: 7-21.
- [8] Smolle MA and Pichler M. The role of long noncoding RNAs in osteosarcoma. Noncoding RNA 2018; 4: 7.
- [9] Yang Z, Li X, Yang Y, He Z, Qu X and Zhang Y. Long noncoding RNAs in the progression, metastasis, and prognosis of osteosarcoma. Cell Death Dis 2016; 7: e2389.

- [10] Liu ZB, Wang JA and Lv RQ. Downregulation of long non-coding RNA DBH-AS1 inhibits osteosarcoma progression by PI3K-AKT signaling pathways and indicates good prognosis. Eur Rev Med Pharmacol Sci 2019; 23: 1418-1427.
- [11] Su P, Mu S and Wang Z. Long noncoding RNA SNHG16 promotes osteosarcoma cells migration and invasion via sponging miRNA-340. DNA Cell Biol 2019; 38: 170-175.
- [12] Zhao W, Zhang D, Qin P, Zhang J, Cui X, Gao J, Wang J and Li J. Long non-coding RNA EPIC1 inhibits viability and invasion of osteosarcoma cells by promoting MEF2D ubiquitylation. Int J Biol Macromol 2019; 128: 566-573.
- [13] Wang W, Shen H, Cao G and Huang J. Long non-coding RNA XIST predicts poor prognosis and promotes malignant phenotypes in osteosarcoma. Oncol Lett 2019; 17: 256-262.
- [14] Dupuis-Sandoval F, Poirier M and Scott MS. The emerging landscape of small nucleolar RNAs in cell biology. Wiley Interdiscip Rev RNA 2015; 6: 381-397.
- [15] Deng R, Zhang J and Chen J. IncRNA SNHG1 negatively regulates miRNA1013p to enhance the expression of ROCK1 and promote cell proliferation, migration and invasion in osteosarcoma. Int J Mol Med 2019; 43: 1157-1166.
- [16] Xu R, Feng F, Yu X, Liu Z and Lao L. LncRNA SNHG4 promotes tumour growth by sponging miR-224-3p and predicts poor survival and recurrence in human osteosarcoma. Cell Prolif 2018; 51: e12515.
- [17] Wang Z, Liu J and Yang H. Long non-coding RNA SNHG5 sponges miR-26a to promote the tumorigenesis of osteosarcoma by targeting ROCK1. Biomed Pharmacother 2018; 107: 598-605.
- [18] Dong J, Teng F, Guo W, Yang J, Ding G and Fu Z. LncRNA SNHG8 promotes the tumorigenesis and metastasis by sponging miR-149-5p and predicts tumor recurrence in hepatocellular carcinoma. Cell Physiol Biochem 2018; 51: 2262-2274.
- [19] Song Y, Zou L, Li J, Shen ZP, Cai YL and Wu XD. LncRNA SNHG8 promotes the development and chemo-resistance of pancreatic adenocarcinoma. Eur Rev Med Pharmacol Sci 2018; 22: 8161-8168.
- [20] Chen C, Zhang Z, Li J and Sun Y. SNHG8 is identified as a key regulator in non-small-cell lung cancer progression sponging to miR-542-3p by targeting CCND1/CDK6. Onco Targets Ther 2018; 11: 6081-6090.
- [21] Liu SH, Zhu JW, Xu HH, Zhang GQ, Wang Y, Liu YM, Liang JB, Wang YX, Wu Y and Guo QF. A novel antisense long non-coding RNA SATB2-AS1 overexpresses in osteosarcoma and increases cell proliferation and growth. Mol Cell Biochem 2017; 430: 47-56.

- [22] Li JP, Liu LH, Li J, Chen Y, Jiang XW, Ouyang YR, Liu YQ, Zhong H, Li H and Xiao T. Microarray expression profile of long noncoding RNAs in human osteosarcoma. Biochem Biophys Res Commun 2013; 433: 200-206.
- [23] Schmittgen TD and Livak KJ. Analyzing realtime PCR data by the comparative C(T) method. Nat Protoc 2008; 3: 1101-1108.
- [24] Yang CH, Zhang XY, Zhou LN, Wan Y, Song LL, Gu WL, Liu R, Ma YN, Meng HR, Tian YL and Zhang Y. LncRNA SNHG8 participates in the development of endometrial carcinoma through regulating c-MET expression by miR-152. Eur Rev Med Pharmacol Sci 2018; 22: 1629-1637.
- [25] Liu J, Yang C, Gu Y, Li C, Zhang H, Zhang W, Wang X, Wu N and Zheng C. Knockdown of the IncRNA SNHG8 inhibits cell growth in Epstein-Barr virus-associated gastric carcinoma. Cell Mol Biol Lett 2018; 23: 17.
- [26] Cheng Y, Geng L, Wang K, Sun J, Xu W, Gong S and Zhu Y. Long noncoding RNA expression signatures of colon cancer based on the ceR-NA network and their prognostic value. Dis Markers 2019; 2019: 7636757.
- [27] Wang M, Mao C, Ouyang L, Liu Y, Lai W, Liu N, Shi Y, Chen L, Xiao D, Yu F, Wang X, Zhou H, Cao Y, Liu S, Yan Q, Tao Y and Zhang B. Long noncoding RNA LINC00336 inhibits ferroptosis in lung cancer by functioning as a competing endogenous RNA. Cell Death Differ 2019; 26: 2329-2343.

- [28] Zhang J, Cai M, Jiang D and Xu L. Upregulated LncRNA-CCAT1 promotes hepatocellular carcinoma progression by functioning as miR-30c-2-3p sponge. Cell Biochem Funct 2019; 37: 84-92.
- [29] Shi L, Hong X, Ba L, He X, Xiong Y, Ding Q, Yang S and Peng G. Long non-coding RNA ZNFX1-AS1 promotes the tumor progression and metastasis of colorectal cancer by acting as a competing endogenous RNA of miR-144 to regulate EZH2 expression. Cell Death Dis 2019; 10: 150.
- [30] Xie W, Xiao J, Wang T, Zhang D and Li Z. MicroRNA-876-5p inhibits cell proliferation, migration and invasion by targeting c-Met in osteosarcoma. J Cell Mol Med 2019; 23: 3293-3301.
- [31] Bao L, Lv L, Feng J, Chen Y, Wang X, Han S and Zhao H. MiR-876-5p suppresses epithelialmesenchymal transition of lung cancer by directly down-regulating bone morphogenetic protein 4. J Biosci 2017; 42: 671-681.
- [32] Wang Y, Xie Y, Li X, Lin J, Zhang S, Li Z, Huo L and Gong R. MiR-876-5p acts as an inhibitor in hepatocellular carcinoma progression by targeting DNMT3A. Pathol Res Pract 2018; 214: 1024-1030.
- [33] Dong Y, Zheng Y, Wang C, Ding X, Du Y, Liu L, Zhang W, Zhong Y, Wu Y and Song X. MiR-876-5p modulates head and neck squamous cell carcinoma metastasis and invasion by targeting vimentin. Cancer Cell Int 2018; 18: 121.