Int J Clin Exp Pathol 2017;10(11):10918-10925
www.ijcep.com /ISSN:1936-2625/IJCEP0064980

Original Article

Long non-coding RNA *lncTCF7 predicts poor prognosis and promotes* tumor metastasis in osteosarcoma

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Received September 5, 2017; Accepted October 18, 2017; Epub November 1, 2017; Published November 15, 2017

Abstract: The 5-year survival rate of patients with metastatic osteosarcoma remains poor. Therefore, the molecular mechanisms underlying metastasis of osteosarcoma need to be investigated. Long non-coding RNA *lncTCF7* promotes tumor metastasis in liver and lung cancers; however, its role in osteosarcoma remains unclear. In this study, we found that *lncTCF7* expression was significantly higher in osteosarcoma tissues than that in adjacent normal osteosarcoma tissues and upregulated *lncTCF7* expression was significantly correlated with tumor metastasis, higher TNM grade and lower survival rate. Additionally, we observed that *lncTCF7* silencing significantly inhibited the migration and invasion of osteosarcoma cells, but showed no effects on the proliferation and apoptosis of these cells. *lncTCF7* silencing markedly increased the expression of E-cadherin and decreased the expressions of N-cadherin, vimentin, matrix metalloproteinase-2 (MMP-2), and MMP-9, which exerted a potentiating effect on EMT. The result was suggested that *lncTCF7* silencing inhibited tumor metastasis in osteosarcoma by possibly inhibiting EMT process. In conclusion, these observations indicated the potential of *lncTCF7* as a biomarker of poor prognosis and promising target for treating osteosarcoma.

Keywords: Osteosarcoma, long non-coding RNA, *lncTCF7*, tumor metastasis, epithelial-mesenchymal transition

Introduction

Osteosarcoma, the most common primary malignant tumor of bone, is a major cause of cancer-related deaths in children and young adults worldwide [1, 2]. To date, standard treatments including surgical resection, neoadjuvant chemotherapy, and radiotherapy have seen great improvement. However, osteosarcoma patients have low overall survival rate owing to tumor recurrence and metastasis [3, 4]. Therefore, it is important to investigate the molecular mechanisms underlying invasion and metastasis of osteosarcoma cells, in order to develop novel therapeutic strategies for treating osteosarcoma patients.

Long non-coding RNAs (lncRNAs), a class of endogenous RNAs (>200 nucleotides in length), are dysregulated in various types of cancers and play critical roles in tumorigenesis and metastasis [5, 6]. Increasing evidence has shown dysregulation of lncRNAs associated with tumor size, clinical stage, post-operative chemotherapy, recurrence, and poor prognosis in osteosarcoma [7-9]. The dysregulation of lncRNAs also affected proliferation, apoptosis, cell cycle, migration, and invasion of osteosarcoma cells [10-12].

The expression of *lncTCF7* was dysregulated in human non-small-cell lung cancer, hepatocellular carcinoma, and liver cancer. The dysregulated *lncTCF7* promoted invasiveness and aggre-ssiveness of these cancer cells [13-15]. However, the role of *lncTCF7* in osteosarcoma remains unclear. In this study, we explored the expression of *lncTCF7* in osteosarcoma tissues and the relationship between *lncTCF7* and clinicopathologic characteristics of osteosarcoma patients. Further, we explored the expression and biological function of *lncTCF7* in osteosarcoma cells and investigated the effect of *lncTCF7* on EMT. Thus, we aimed at investigating a novel therapeutic target for treating osteosarcoma.

Table 1. SiRNA sequences and qRT-PCR primers

Gene	Sequence (5'-3')
si-lncTCF7-1	AGCCAACATTGTTGGTTAT
si-lncTCF7-2	CACCTAGGTGCTCACTGAA
si-NC	UUCUCCGAACGUGUCACGUTT
lncTCF7 forward	AGGAGTCCTTGGACCTGAGC
lncTCF7 reverse	AGTGGCTGGCATATAACCAACA
GAPDH forward	CCCATCACCATCTTCCAGGAG
GAPDH reverse	GTTGTCATGGATGACCTTGGC

Materials and methods

Patients and tissue samples

A total of 104 tissue samples were obtained from osteosarcoma patients who were recruited from January 2013 to December 2016 in the Hunan University of Chinese Medicine, Luoyang Orthopedic Hospital of Henan Province, and No.91 central hospital of liberation army before receiving chemotherapy or radiation therapy. All participants signed informed consent forms. Diagnosis and clinicopathological characteristics were confirmed by two pathologists. Osteosarcoma tissues and adjacent normal osteosarcoma tissues (at least 5 cm away from the primary site) were obtained during radical resection and immediately stored at -80°C until used for total RNA extraction. All experiments were approved by the Ethics Committee of the Luoyang Orthopedic Hospital of Henan Province. After radical resection, all of the patients were followed up with 2-50 months.

Cell culture and siRNA transfection

Human osteoblast (hFOB1.19) and osteosarcoma (MG-63 and Saos-2) cell lines were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences (Shanghai, China). The hFOB1.19 cells were cultured in DMEM/F12: Ham's F12 medium (1:1 v/v; Hyclone Laboratories Inc., Camarillo, CA, USA) supplemented with G418 (0.3 μg/mL) and fetal bovine serum (FBS, 10%). The MG-63 and Saos-2 cells were cultured in RPMI 1640 medium with FBS (10%; GIBCO BRL, Gaithersburg, MD, USA), supplemented with penicillin G (100 U/mL) and streptomycin (100 μg/mL) (Sigma-Aldrich Corp., St. Louis, MO, USA). Cells were maintained in 5% CO_2 at 37°C in a humidified incubator. The siRNAs for *lncTCF7* (si-lncTCF7) and negative control (si-NC) were synthesized by GenePharma Co., Ltd. (Shanghai, China). The cells were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The siRNA sequences have been shown in **Table 1**.

Quantitative real-time polymerase chain reation (qRT-PCR)

The expression level of *lncTCF7* was measured using qRT-PCR. Firstly, total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Next, cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, Liaoning, China). qRT-PCR was performed using the SYBR Premix Ex Taq (Takara) via ABI 7500 Fast Real-Time PCR system (Foster City, CA, USA). The GAPDH was used as an endogenous control. The relative expression was calculated in terms of the fold change using the $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicates. The sequences of all primers are shown in **Table 1**. Each experiment was repeated three times.

Proliferation and apoptosis assays

The CCK-8 assay kit reagent (Beyotime, Shanghai, China) was used for measuring cell proliferation. The transfected cells (5×10^3 per well) were seeded in a 96-well plate in triplicates and incubated at 37°C. After culturing for 24, 48, and 72 hours, the CCK-8 reagent (10 μL) was added to each well and the plates were incubated at 37°C for 4 h. Absorbance at 450 nm was measured using the MK3 microplate reader (Thermo Fisher Scientific, Rockford, IL, USA). For measuring apoptosis, 1×10^5 transfected cells per well were seeded in 6-well plates and the plates were incubated at 37°C for 48 h. Next, the transfected cells were harvested, digested with trypsin, and centrifuged at 2,000 × g for 5 min. The cell pellet (5×10^3 cells) was resuspended in the binding buffer (500 μL) and incubated with Annexin V-FITC (5 μL) and PI (5 μL) for 15 min in dark. Apoptotic cell death was analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA). Each experiment was repeated thrice.

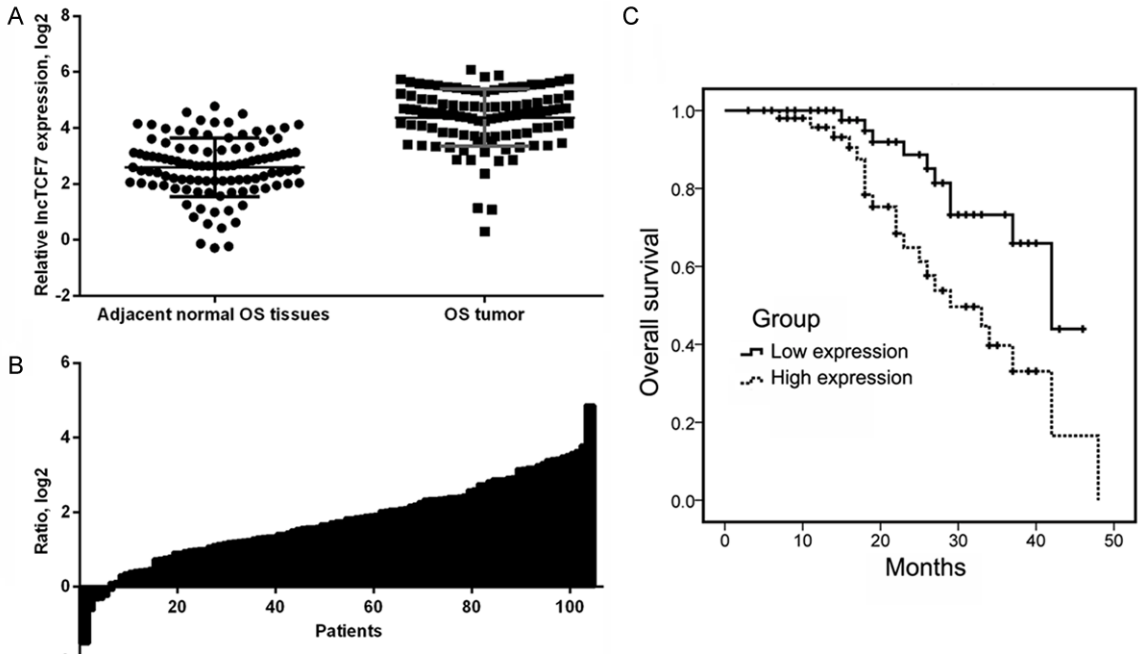

Figure 1. Expression of *lncTCF7* is up-regulated and *lncTCF7* is a prognostic marker in osteosarcoma. A: The Expression of *lncTCF7* was significantly higher in osteosarcoma tissues than that in adjacent normal osteosarcoma tissues. B: The number of osteosarcoma patients *vs.* the ratio (*lncTCF7* expression in osteosarcoma tissues/in adjacent normal osteosarcoma tissues). C: High *lncTCF7* expression group had a significantly lower survival rate than those of the low expression group.

Migration and invasion assays

The migration and invasion assays were performed using the Transwell chambers, as described previously [16]. For the cell migration assay, 5 × 10^4 transfected cells were resuspended in serum-free RPMI 1640 medium and seeded in the upper Transwell chamber (24-well insert, 8 mm pore size; Corning Costar, Cambridge, MA, USA). Next, RPMI 1640 medium (500 mL) containing FBS (10%) was added to the lower Transwell chamber. For the cell invasion assay, the Transwell chamber membrane was precoated with Matrigel (30 mL; BD Biosciences, Franklin Lakes, NJ, USA). Next, 1 × 10^5 transfected cells were seeded in the upper Transwell chamber, and DMEM (500 mL) containing FBS (10%) was added to the lower Transwell chamber. After culturing for 48 h, the cells in the lower chambers were fixed and counted in six independent microscopic fields per well, using an Olympus microscope (magnification, 200 ×; Olympus, Japan).

Western blotting

Western blotting was performed to analyze the expression of MMP-2, MMP-9, E-cadherin, N-cadherin, and vimentin, as described previously [16]. Briefly, total protein was extracted using RIPA buffer (Takara), estimated using the BCA Protein Assay kit (Thermo Fisher Scientific), separated on 10% SDS-PAGE, and transferred onto nitrocellulose membranes. The mebranes were incubated with specific primary antibodies against MMP-2 (1:1000), MMP-9 (1:900), E-cadherin (1:10000), N-cadherin (1:500), and vimentin (1:600) respectively. Next, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG H&L secondary antibody (1:10000; Southern Biotech, Birmingham, AL, USA) for 40 min. Protein bands were visualized using ECL (Thermo Fisher Scientific). Protein expression was normalized relative to GAPDH expression. All primary antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

All data were expressed as mean ± standard deviation (SD) and analyzed using the SPSS 19.0 software (IBM, Chicago, IL). The differences of *lncTCF7* expression between osteosarcoma tissues and adjacent normal osteosarcoma tissues were evaluated using independent t-tests. Survival rates were estimated using the

Table 2. Association of lncTCF7 expression with clinical parameters in osteosarcoma patients (mean ± SD)

Characteristics	Number (104)	Expression (log2)	*t*	*P*
Age			0.996	0.321
<20	70	4.31±1.044		
≥20	34	4.53±1.002		
Gender			0.069	0.945
Male	54	4.34±1.093		
Female	50	4.36±0.970		
Location			1.065	0.289
Femur/Tibia	67	4.25±1.021		
Elsewhere	37	4.48±1.042		
Tumor size			0.900	0.370
<5 cm	63	4.30±1.032		
≥5 cm	41	4.48±1.010		
Metastasis			5.442	<0.001*
Yes	29	5.15±0.586		
No	75	4.07±0.999		
Differentiation			1.317	0.191
Well + moderate	78	4.29±0.993		
Poor	26	4.60±1.095		
TNM stage			2.173	0.032*
I+II	68	4.29±0.993		
III+IV	36	4.60±1.095		

*P<0.05.

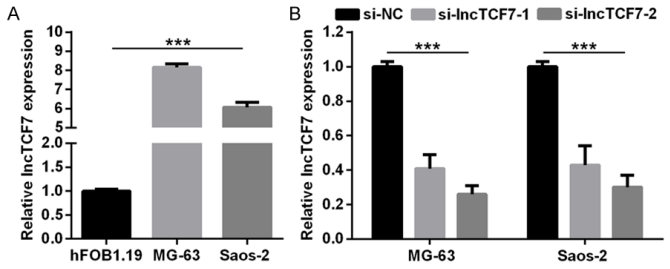

Figure 2. *LncTCF7* expression is upregulated in osteosarcoma cell lines and is downregulated upon si-lncTCF7 transfection. A: qRT-PCR analysis showing the expression of *lncTCF7* in osteoblast cells and osteosarcoma cell lines (MG-63 and Saos-2) after culturing the cells for 24 h. B: qRT-PCR analysis showing the expression of *lncTCF7* in MG-63 and Saos-2 cells transfected with si-lncTCF7-1, si-lncTCF7-2, or si-NC (48-h post-transfection). ***, *P*<0.001.

Kaplan-Meier method. The differences in *lncTCF7* expression between hFOB1.19, MG-63, and Saos-2 cells were evaluated using one-way analysis of variance (ANOVA), followed by the LSD post-hoc test. The differences between si-NC and si-lncTCF7 treatments were evaluated using independent t-tests. P<0.05 was considered to be statistically significant.

Results

Expression of lncTCF7 is up-regulated and lncTCF7 is a prognostic marker in osteosarcoma

qRT-PCR results indicated that *lncTCF7* expression in osteosarcoma tissues was significantly higher than that in adjacent normal osteosacoma tissues (P<0.01, **Figure 1A**). Osteosarcoma patients whose ratio (*lncTCF7* expression in osteosarcoma tissues/that in adjacent normal osteosarcoma tissues) higher than 1 [log2(1)>0] accounted for 94.23% (98/104, **Figure 1B**). Additionally, the expression of *lncTCF7* was significantly correlated with tumor metastasis and TNM grade, in contrast, there was no significant correlation between *lncTCF7* expression and age, gender, tumor location, tumor size, tumor differentiation (**Table 2**). Furthermore, to evaluate the prognostic value of *lncTCF7* in patients with osteosarcoma, we collected survival period data from all 104 patients included in this study. Osteosarcoma patients were divided into the low expression group (*lncTCF7* expression <22.92) and the high expression group (lncTCF7 expression ≥22.92) according to median lncTCF7 expression value (22.92). At the time of the last follow-up (3-48 months after surgery), the numbers of alive patients in the low and the high expression groups were 42 (80.76%) and 33 (63.46%), respectively. The survival rates for each group were estimated using the Kaplan-Meier method and results are shown in **Figure 1C**. Osteosarcoma patients of the high group had a significantly lower survival rate than the patients of the low group (log rank X^2=7.471, P=0.006).

LncTCF7 expression is upregulated in osteosarcoma cell lines and is downregulated upon si-lncTCF7 transfection

After cultured 24 hours, the expression of lncTCF7 in normal osteoblasts cell hFOB1.19 and osteosarcoma cell lines MG-63 and Saos-2 was measured by qRT-PCR. It was observed that *lncTCF7* expression was obviously higher

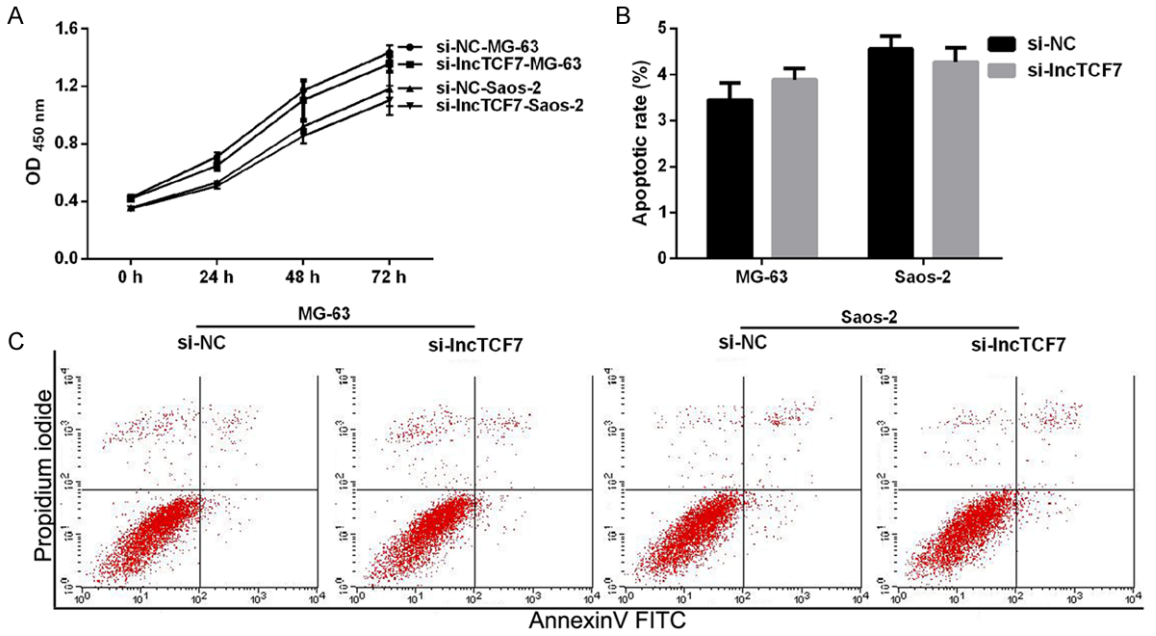

Figure 3. *LncTCF7* silencing shows no significant effect on proliferation and apoptosis in the MG-63 and Saos-2 cells. A: Proliferation of MG-63 and Saos-2 cells in si-NC and si-lncTCF7 groups were determined using the CCK-8 assay post-transfection. B: The apoptosis of MG-63 and Saos-2 cells in the si-NC and si-lncTCF7 groups were determined using flow cytometry, 48-h post-transfection. Data are represented as mean ± SD. C: Representative images showing apoptosis in MG-63 and Saos-2 cells.

in the osteosarcoma cell lines (MG-63 and Saos-2 cells) than that in the hFOB1.1 osteoblast cells (P<0.05, **Figure 2A**). To study the biological function of *lncTCF7*, si-lncTCF7-1 and si-lncTCF7-2 were transfected in the MG-63 and Saos-2 cells to knockdown the expression of *lncTCF7*. At 48-h post-transfection, qRT-PCR results confirmed that si-lncTCF7-1- and si-lncTCF7-2-transfected MG-63 and Saos-2 cells showed lower expression of *lncTCF7* than the si-NC-transfected cells. It was also observed that si-lncTCF7-2 showed higher *lncTCF7* knockdown efficiency than si-lncTCF7-1 (P<0.05, **Figure 2B**). Thus, si-lncTCF7-2 (henceforth referred to as si-lncTCF7) was used for further experiments.

LncTCF7 silencing shows no effect on cell proliferation and apoptosis

After transfection, cell proliferation and apoptosis were analyzed using the CCK-8 assay and flow cytometric analysis, respectively. The CCK-8 assay results showed that the proliferation of MG-63 and Saos-2 cells (24, 48, and 72-h post-transfection) did not decrease significantly in the si-lncTCF7 group compared to the si-NC group at the same time intervals (P>0.05, **Figure 3A**). Similarly, flow cytometric analysis showed that the apoptosis of MG-63 and Saos-2 cells did not differ significantly between the si-lncTCF7 and si-NC groups 48-h post-transfection (P>0.05, **Figure 3B** and **3C**).

LncTCF7 silencing inhibits cell migration and invasion

Cell migration and invasion were assessed using the Transwell chamber, 48-h post-transfection. The results showed that *lncTCF7* silencing drastically decreased migration and invasion of the MG-63 cells (P<0.05, **Figure 4A**). Similar results were observed for the Saos-2 cells (P<0.05, **Figure 4B**).

LncTCF7 silencing inhibits the EMT process

The expressions of E-cadherin, N-cadherin, and vimentin were measured using western blot analysis to evaluate the effect of *lncTCF7* silencing on EMT process. As shown in **Figure 5**, the expression of E-cadherin was obviously higher in the si-lncTCF7 group than those in si-NC group. In contrast, the expressions of N-cadherin and vimentin were obviously lower in the si-lncTCF7 group compared to the si-NC group. As expected, the expressions of MMP-2

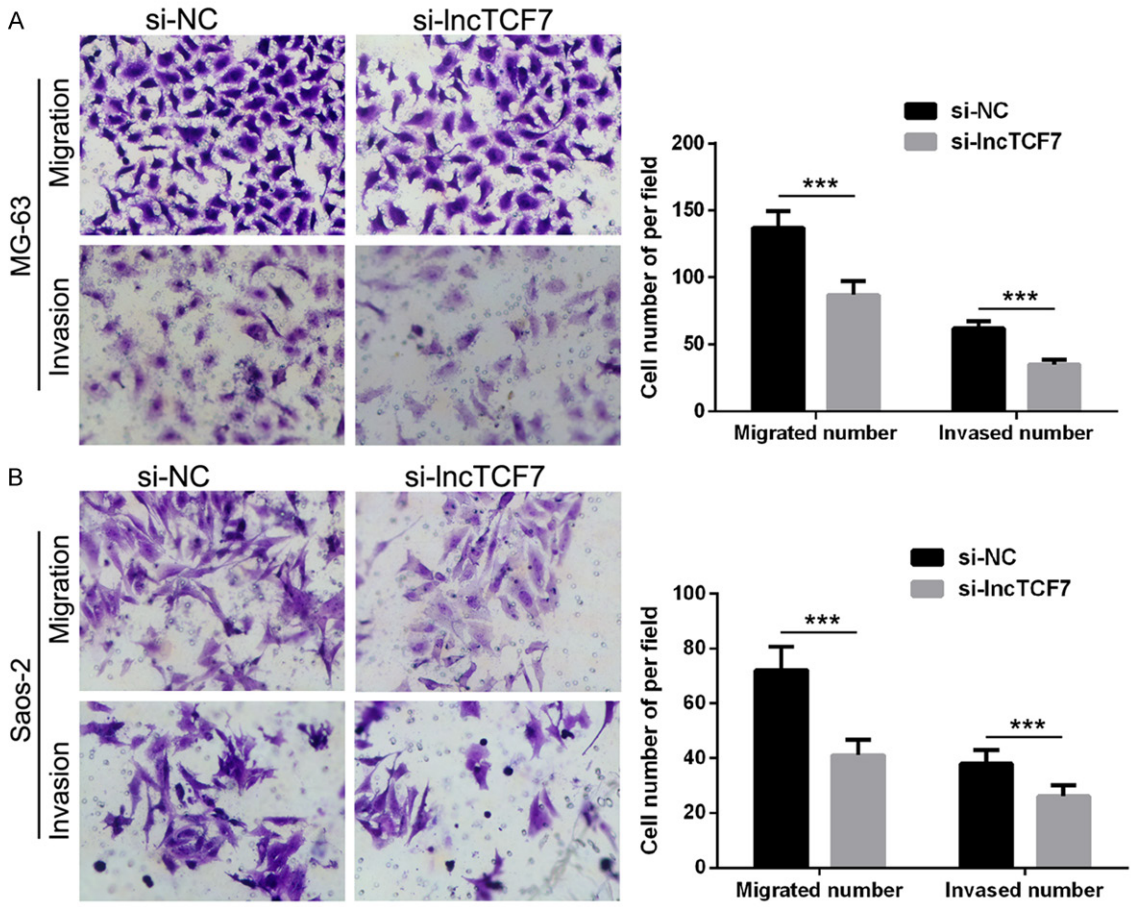

Figure 4. *LncTCF7* silencing inhibits the migration and invasion of (A) MG-63, and (B) Saos-2 cells. Data are represented as mean ± SD. ***, $P<0.001$, compared with si-NC.

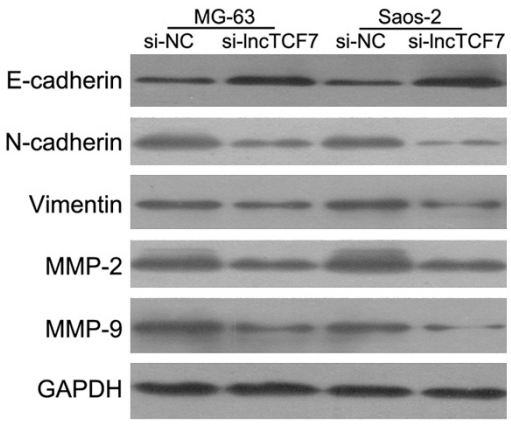

Figure 5. *LncTCF7* silencing increased E-cadherin expression and decreased N-cadherin, vimentin, MMP-2, and MMP-9 expressions.

and MMP-9 were considerably lower in the si-lncTCF7 group compared to the si-NC group.

Discussion

The 5-year survival rate of metastatic osteosarcoma patients remains poor [17]. Therefore, understanding the molecular mechanisms underlying metastasis in osteosarcoma cells will be useful in developing novel therapeutic targets for treating metastatic osteosarcoma. In the present study, we found that *lncTCF7* expression was enhanced in osteosarcoma tissues and upregulated *lncTCF7* expression was significantly correlated with tumor metastasis, higher TNM grade, and lower survival rate. Furthermore, *lncTCF7* silencing obviously inhibited the migration, invasion, and EMT in osteosarcoma cells but showed no effect on the proliferation and apoptosis. These results suggested *lncTCF7* to be a biomarker of poor prognosis and a novel therapeutic target for treating metastatic osteosarcoma patients.

Previous studies have indicated that *lncTCF7* expression was significantly increased in tumor tissues and *lncTCF7* promotes migration and invasion of the liver and lung cancer cells [13, 15, 18]. In this study, we found that *lncTCF7* expression was significantly higher in osteosarcoma tissues than that in adjacent normal osteosarcoma tissues. Its expression was significantly correlated with tumor metastasis and TNM grade but had no significant correlation with age, gender, tumor location, tumor size, tumor differentiation. High *lncTCF7* expression group had a significantly lower survival rate than those of the low expression group. Our results indicated that *lncTCF7* acts as a biomarker of poor prognosis.

We also found that *lncTCF7* expression were significantly higher in osteosarcoma cells than that in human osteoblast (hFOB1.19), similar with the result of *lncTCF7* expression in osteosarcoma tissues and adjacent normal osteosarcoma tissues. *lncTCF7* silencing obviously inhibited the migration and invasion of MG-63 and Saos-2 osteosarcoma cells, decreased the expressions of MMP-2 and MMP-9; however, it showed no effect on the proliferation and apoptosis in these cells. The results consistent with the clinical results which showed *lncTCF7* expression was significantly correlated with tumor metastasis and TNM grade but had no significant correlation with tumor size. Our present study demonstrated that *lncTCF7* promote osteosarcoma metastasis, which was similar with the results of previously studies in liver and lung cancer cells [13, 15, 18].

EMT plays a key role in regulating the invasion and metastasis of cancer cells. During EMT process, the expression of epithelial markers (such as, E-cadherin and other cell junction proteins) were inhibited and the expression of mesenchymal markers (such as, N-cadherin and vimentin) were enhanced [19]. A previous study demonstrated that *lncTCF7* activated EMT in hepatocellular carcinoma cells [12]. In this study, we found that *lncTCF7* silencing markedly increased E-cadherin expression, and decreased N-ca-dherin and vimentin expressions in osteosarcoma cells. The result was suggested that *lncTCF7* silencing inhibited tumor metastasis in osteosarcoma by possibly inhibiting EMT process.

Overall, our results indicated that *lncTCF7* acts as a biomarker of poor prognosis and a novel therapeutic target for treating metastatic osteosarcoma patients. However, the molecular mechanisms underlying the regulati on of EMT by *lncTCF7* in metastatic osteosarcoma need to be investigated further.

Acknowledgements

The authors thank the sponsor: The Cultivation Project of Chinese Medicine Clinical Leading Talents of Henan Province (HNZYLJ201301-009). We thank the members of our laboratories for their insight and technical support.

Disclosure of conflict of interest

None.

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