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NSD2 promotes osteosarcoma cell proliferation and metastasis by inhibiting E-cadherin expression

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Abstract. – OBJECTIVE: Osteosarcoma is one of the most common malignant bone tumors. The mechanisms of osteosarcoma development and invasion have been studied for periods of time, yet targeted therapy for improving survival has not been well established. Histone lysine methyltransferase NSD2 was frequently overexpressed in multiple types of cancer such as multiple myeloma, stomach and colon cancer, and the overexpression of it usually associated with aggressiveness tumor type. However, the expression status and function of NSD2 are still ambiguous in osteosarcoma.

MATERIALS AND METHODS: Here, we evaluate the abnormal expression levels of NSD2 in osteosarcoma samples and cell lines. The higher expression of NSD2 in tumors resulted in a poorer outcome and a worse 5-year overall survival. To investigate the role of NSD2 in osteosarcoma cell proliferation and invasion *in vitro*, MTT assay, cell cycle distribution, wound healing, transwell assay was performed in relative cell lines, using a recombinant lentivirus expressing NSD2 short hairpin RNA or NSD2 construction.

RESULTS: Our results imply that NSD2 promotes osteosarcoma cell proliferation and invasion, and the mechanism was possibly through the suppression of E-cadherin and induction of the epithelial mesenchymal transition, further to proceed invasion of osteosarcoma cells.

CONCLUSIONS: NSD2 may work as a novel repression of E-cadherin; therefore, NSD2 has potential as a target of OS therapy. In the future, the monitoring of NSD2 in the serum/plasma from the RNA level may be used as a non-invasive method for selecting patients for target therapy.

Key Words:
NSD2, Osteosarcoma, Proliferation, Metastasis, E-cadherin.

Introduction

Osteosarcoma is one of the most frequently primary malignant solid bone tumors, with rapid growth and a high tendency for invasion and metastasis[1,2]. Nuclear receptor-binding SET domain protein 2(NSD2), together with NSD1 and NSD3, belongs to the SET histone methyltransferase family[3-5]. By mediation of dimethylation and trimethylation of H3K36, or dimethylation of H4K20, NSD2 usually works as a transcription repressor[6,7]. Although several studies[8] have indicated that NSD2 was the overexpression of some types of human cancers, such as neuroblastoma, stomach cancer, colon cancer, and small-cell lung cancers. However, whether NSD2 is deregulated and whether NSD2 plays a role in the osteosarcoma, it has not been explored.

The concept of epithelial-mesenchymal transition (EMT) has been widely accepted in embryogenesis and cancer metastasis[9,10]. In osteosarcoma, during EMT, the epithelial cells lost cell-to-cell adhesion and converted into motile mesenchymal cells type[11-13]. The lost of epithelial marker E-cadherin is considered as the hallmark of EMT[14,15]. Transcription factor snail is the first identified transcriptional repressor of E-cadherin[16], and now, more and more studies are interested in the new regulator of E-cadherin in cancer progress.

Materials and Methods

Materials

50 osteosarcoma samples and their pairs of adjacent non-tumor tissues were collected at the Department of Orthopedics, Affiliated Hospital of Nantong University (Nantong, China). This study was approved by the review board of the Ethics Committee of Nantong University. Each patient has written the informed consent. All samples were obtained from the legs, and the tissues were obtained and stored at -80°C immediately after surgery until used for mRNA isolation. The survival times were calculated from operation to metastasis-related death.

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Cell Culture and Reagents

Human osteosarcoma cell line Saos-2, MG-63, and human osteoblastic cell line hFOB1.19 were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C with 5% CO_2 humidified atmosphere, in DMEM HyClone (South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) Gibco (Rockville, MD, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Antibodies used are as follows: anti-NSD2 antibody (Abcam, Burlingame, CA, USA); anti-E-cadherin antibody BD Biosciences (Bedford, MA, USA); and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA), and secondary antibody were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RNA Isolation and qRT-PCR

Total RNA of cell lysates was extracted in accordance with TRIzol solution (Invitrogen, Carlsbad, CA, USA). 2 μg of RNA was taken to synthesize cDNA using M-MLV reverse transcription reaction system (Invitrogen, Carlsbad, CA, USA). Oligo $(deoxythymidine)_{20}$ was used as primers.

Quantitative Real-time PCR was conducted using BioRad Real-Time PCR detection system, using SYBR (Hoffmann-La Roche, Basel, Switzerland). Relative expression levels were determined using $2^{-\Delta\Delta Ct}$ methods. GAPDH was applied as the internal control and amplified. The amplification protocol included denaturation at 95°C for 1 min, annealing at 60°C for 10 sec, extension at 72°C for 30 sec for 35 cycles. All the experiments were performed for three times.

MTT Analysis

MTT assay was performed to measure proliferation of Saos-2 cells. Saos-2 cells (5000 per well) were placed in 96-well plates, after transfected with relative siRNAs, each well was treated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) at 24 h, 48 h, 72 h or 96 h, the treated cells were further incubated for 4 h at a final concentration of 1 mg/ml. The culture medium was removed and each well had 200 μl of acidic isopropanol to terminate the reaction. The absorbance was red with an ELISA plate reader at 570 nm. Each assay was done in triplicate and the result was taken as the average of three.

Cell Cycle Analysis

The effect of NSD2 on cell cycle distribution was measured by flow cytometry. Briefly, $2x10^5$ cells were seeded into the 12-plates and allowed to attach overnight. Cells were transfected with control siRNAs or siNSD2#1, siNSD2#2, 48 h after transfection, cells were harvested and centrifuged for 5 min at 2000 rpm and then washed with PBS and fixed using 70% ethanol at 4°C overnight. The cells were incubated with 100 mg/ml RNase for 30 min and collected with FACS can flow cytometer. The data were analyzed with FlowJo7.6.

Western Blot

Cells were collected and lysed in SDS lysis buffer. Followed by centrifugation and heated at 95°C for 10 min, the protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), following transferred onto nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in tris-buffered saline (PBS) containing tween 20 (0.05%). The membrane was incubated with the primary antibody at 4°C overnight, following incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody for 1 h. Finally, the immune-reactive bands were visualized with the enhanced chemiluminescence system.

Wound Healing

2×10^6 cells per well were seeded in 6-well plates and cultured for approximately 24 h to be nearly confluent. A p200 pipette tip was used to make the scratch "wound", the FBS-free medium was changed to remove detached cells, and phase contrast micrographs were taken immediately. 24 h later, the wound was taken photos again, and the number of migrated cells was compared. For each sample, three representative fields were counted.

Transwell Assay

5×10^4 transfection cells of each group were washed with PBS and seeded on the top of BD Matrigel™ invasion chambers without serum, according to the manufacturer's introductions. 10% fetal bovine serum (FBS) media was placed in the lower chamber. After allowed to invade for 10 h, the cell invasion was assessed by counting the cells that migrated to the bottom of the chamber using crystal violet staining. 5 random fields per well for each replicate.

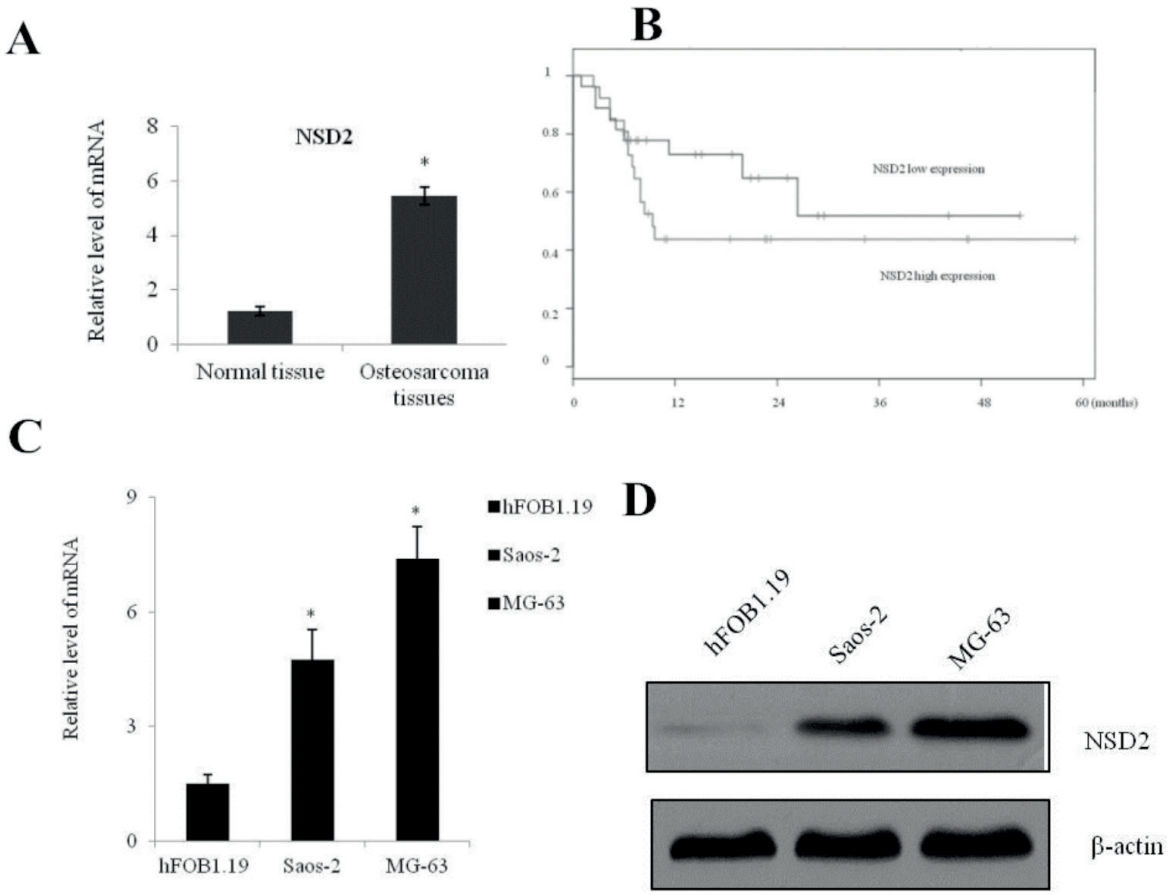

Figure 1. High-expression of NSD2 was observed in human osteosarcoma tissue and osteosarcoma cells. ***A,*** qRT-PCR was used to measure NSD2 expression in paired human osteosarcoma tissues and adjacent normal tissues, all experiments were repeated three times. Each bar indicates as the mean ± SD. $^{*}p<0.05$. ***B,*** The relationship between NSD2 expression and survival times in osteosarcoma patients was measured by Kaplan-Meier curve. ***C,*** qRT-PCR analysis of NSD2 expression in human osteoblastic cell line hFOB1.19 and osteosarcoma cell lines, including Saos-2 and MG-63. GAPDH was used as an internal control. $^{*}p<0.05$. ***D,*** Western blotting analysis of NSD2 expression in human osteoblastic cell line hFOB1.19 and osteosarcoma cell lines, including Saos-2 and MG-63. β-actin was used as an internal control.

Statistical Analysis

Data were presented as the means±SD, of three independent experiments. All experiments were performed at least three times. Statistical analysis was evaluated by Student's *t*-test. $p<0.05$ was considered as significant.

Results

High Expression of NSD2 was Observed in Human Osteosarcoma Tissue and Osteosarcoma Cells

To learn the role of NSD2 in osteosarcoma, the NSD2 mRNA level was detected in the 50-osteosarcoma samples, compared with the adjacent normal tissues. The expression of NSD2 was higher in the tumor samples (Figure 1A), and the higher expression of NSD2 was correlated with poorer survival of the patients, as shown by the survival curve (Figure 1B). In the osteosarcoma cell lines, we detected that the expression level of NSD2 was higher in the Saos-2, MG-63 compared with the human osteoblastic cell line hFOB1.19, qRT-PCR was used to detect the mRNA level (Figure 1C), Western blot was used to detect the protein level (Figure 1D). Together, the above results showed that NSD2 is upregulated in osteosarcoma cell lines and tumor tissues.

ShRNA Targeting NSD2 Inhibited the Expression of NSD2 in Osteosarcoma Cells

As the expression of NSD2 was markedly higher in the osteosarcoma tissue and cell lines compared with the adjacent non-tumor tissues,

which indicated that overexpression of NSD2, this is important in osteosarcoma tumorigenesis. So, two different shRNA targeting NSD2 were developed and transfected into the relative cell lines to explore its function. For the mRNA level, shNSD2#1 was about 20% of the control group, shNSD2#2 was about 15% of the control group (Figure 2A). Whereas regarding the protein level, this inhibitory ratio was 18.2% or 13.5% according to the Western blot gray analysis in the Saos-2 cell line (Figure 2B). Furthermore, another osteosarcoma cell line (MG-63 cells) was used to confirm the efficacy of shNSD2 by qPCR (Figure 2C). The Western blot analysis (Figure 2D) was performed and the similar tendency was observed in the MG-63 cells. The above data indicated that the shRNAs were efficient to knockdown the NSD2.

Inhibition of NSD2 Represses the Proliferation ability of Osteosarcoma Cell Line

We investigated whether inhibiting NSD2 expression could influence osteosarcoma cell survival. Firstly, MTT assay was used to investigate the role of NSD2 on cell pro liferation. As shown in Figure 3A, Saos-2 cell proliferation was markedly attenuated in the cells transfected with shNSD2 compared with the control group. Furthermore, assay was performed in Saos-2 cells, knockdown of NSD2 caused G0/G1 arrest compared with the control group (Figure 3B). Furthermore, MG-63 cells were used to investigate the function of NSD2 in osteosarcoma progress, similarly, as shown in Figure 3C, the growth of the MG-63 cells was inhibited significantly when NSD2 was inhibited, cell

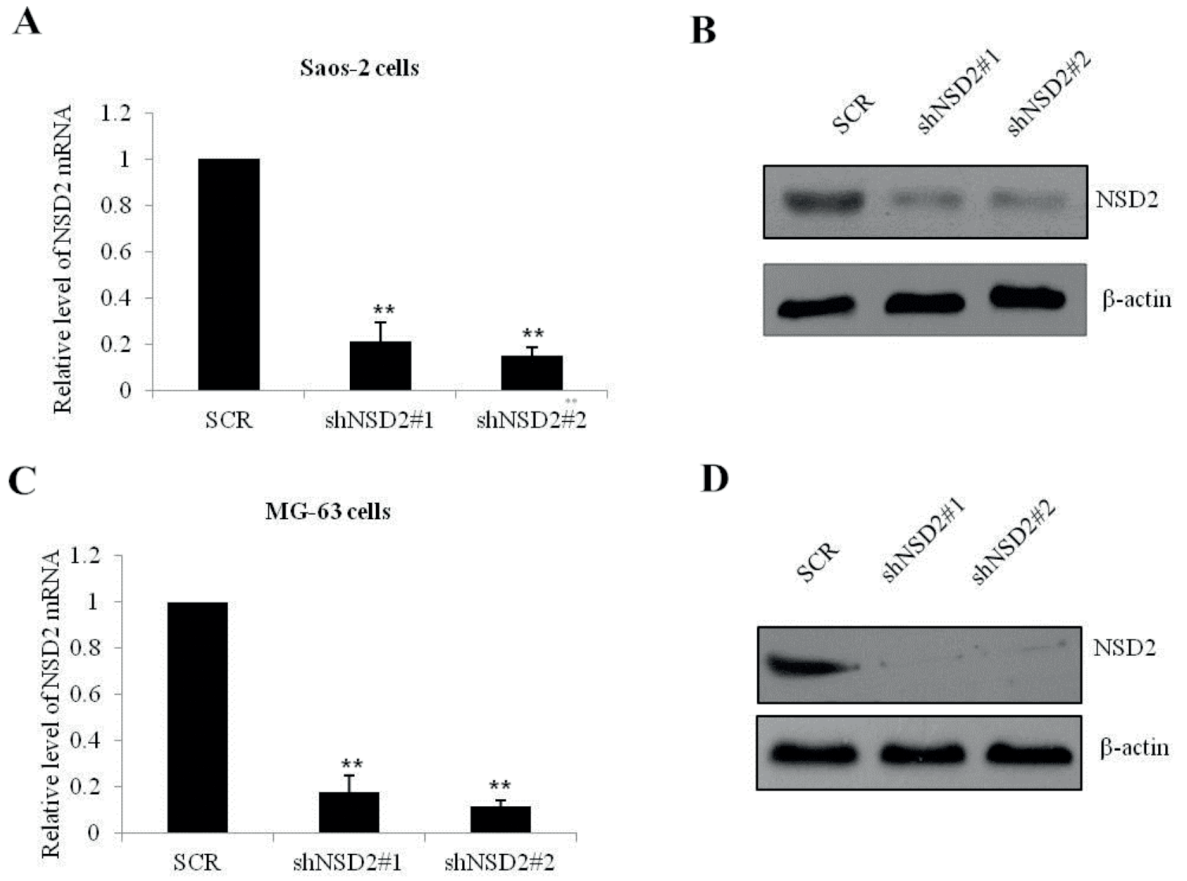

Figure 2. shRNA targeting NSD2 inhibited the expression of NSD2 in osteosarcoma cells. ***A,*** RT-PCR analysis was used to validate NSD2 mRNA level in osteosarcoma cells SaOS-2; the results are presented as the mean ± SD. GAPDH was used as an internal control. $^{*}p<0.05$. ***B,*** Western blot analysis was used to measure the NSD2 protein expression in osteosarcoma cells, β-actin was used as an internal control. ***C,*** NSD2 expression levels following inhibition of NSD2 in MG-63 cells analyzed by qRT-PCR. GAPDH was used as a loading control. The experiments were performed in triplicate. ***D,*** The relative protein expression levels of NSD2 were measured following the above treatment in MG-63 cells.

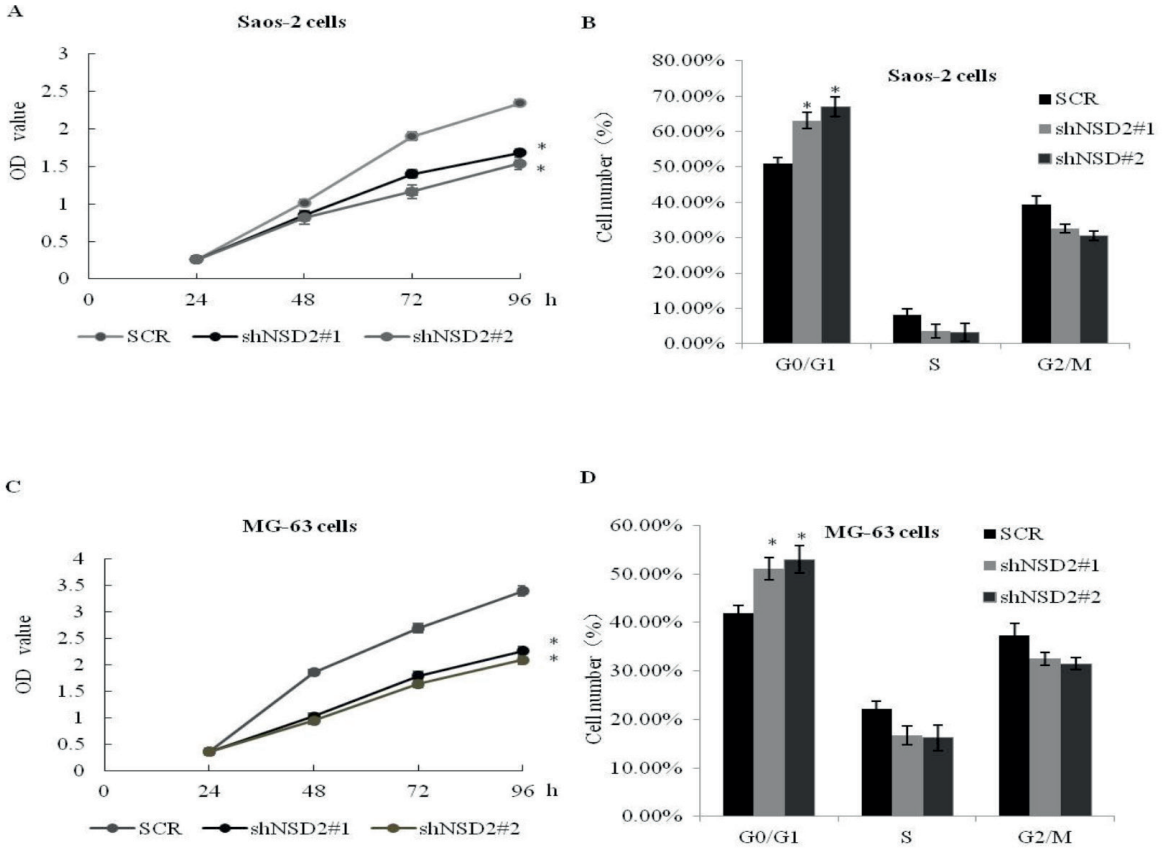

Figure 3. Inhibition of NSD2 represses the proliferation ability of osteosarcoma cell line. ***A,*** MTT assay was performed in Saos-2 cells, cells were transfected with control siRNA, siNSD2#1, or siNSD2#2 comparison of the growth inhibition in different time periods. In triplicate for each group, *p<0.05. ***B,*** Cell cycle distribution was performed using flow cytometry (FCM) in siNSD2 transfected Saos-2 cells. The percentage of cells were shown as indicated. *p<0.05. **p<0.01. ***C,*** MTT assay was performed in MG-63 cells following the above treatment. ***D,*** Cell cycle distribution was performed in MG-63 cells.

cycle distribution (Figure 3D) revealed that knockdown of NSD2 also caused G0/G1 arrest in MG-63 cells. The above results illustrated that the inhibition of NSD2 resulted in the repression of osteosarcoma cells proliferation.

NSD2 Increases the Migration and Invasive Activity of osteosarcoma cells in vitro

Based on the Kaplan-Meier analyses results, the expression of NSD2 might be a stimulatory factor in the regulation of osteosarcoma metastatic activity. The SaOS-2 cells and MG-63 cells were used to measure the migration and invasion ability. Since the invasive ability was weak, wound healing was performed in SaOS-2 cells stably overexpressing FLAG-NSD2 or stably transfected with empty vector. The expression levels of NSD2 in SaOS-2/NSD2 cells were 10 times higher than the endogenous group (Figure 4A, left panel), as to MG-63 cells, the NSD2 expression was 8.8 times higher (Figure 4A, right panel). The migration rate was measured, as shown in Figure 4B, SaOS-2/NSD2 cells were obviously higher than that of control SaOS-2/vector cells (p<0.05). The invasion of SaOS-2 was further measured by Transwell assay, there were more cells crossed the membranes in the NSD2 overexpression group than the control groups (Figure 4C). In the MG-63 cells, compared with the vector groups, cells transfected with NSD2 also showed enhancive migration and invasive activity in wound-healing assay (Figure 5A) and Transwell assay (Figure 5B).

Inhibition of NSD2 Increases the Expression Levels of E-cadherin in Osteosarcoma Cells

To determine the mechanism in NSD2 promoting osteosarcoma cell proliferation and

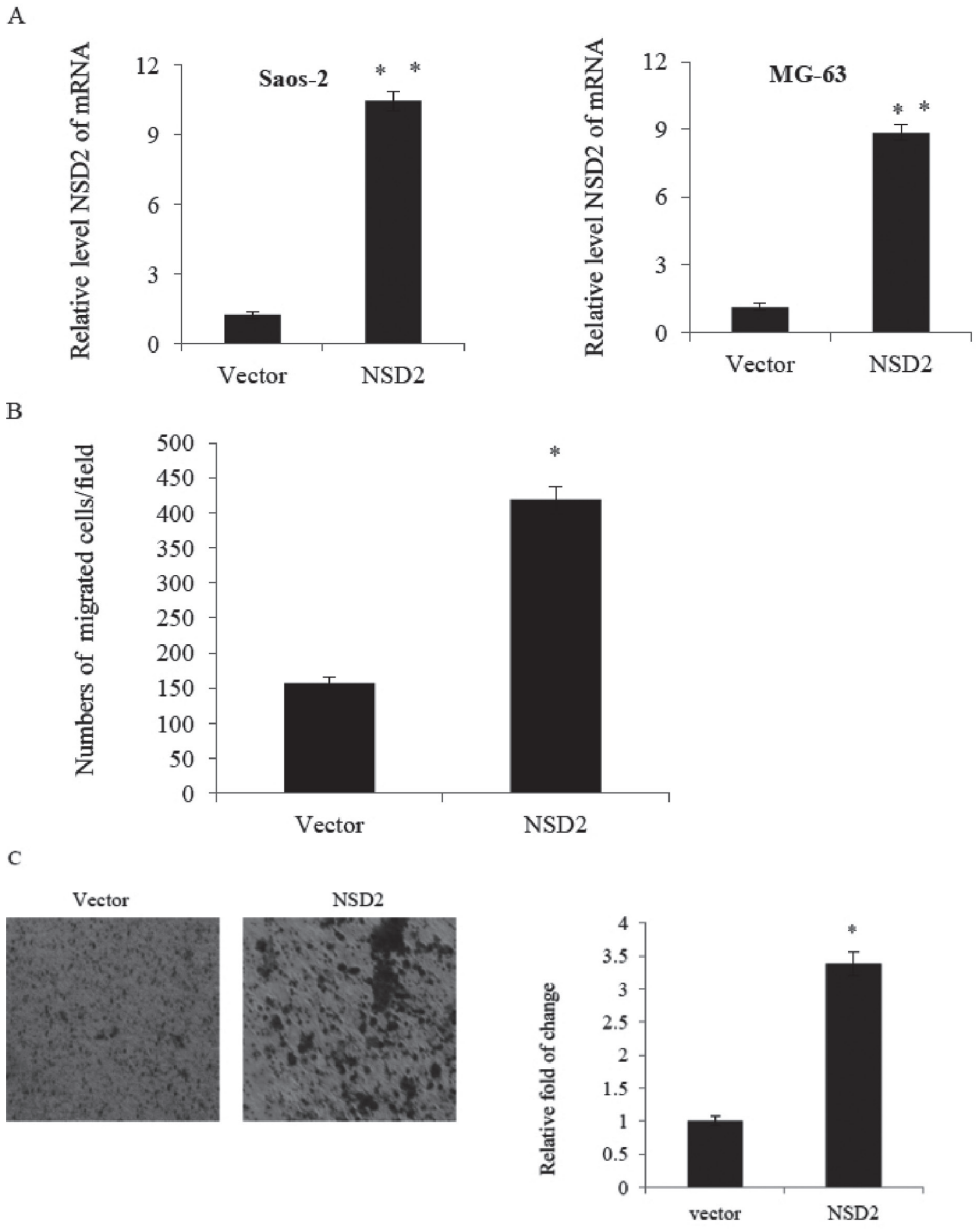

Figure 4. NSD2 increases the migration and invasive activity of osteosarcoma cells *in vitro*. ***A,*** Real-time PCR showed an increase of NSD2 mRNA expression in Saos-2 cells (*left panel)* and MG-63 cells (*right panel*) stably expressing control or FLAG-NSD2 construction. ***B,*** Saos-2 cells migration analysis was performed using the wound-healing assay. A scratch "wound" was made and numbers of migrated cells were counted using phase contrast micrographs, 0 h and 24 h after making the wound. The three experiments are shown. $^{*}p<0.05$. ***C,*** Transwell assay was performed inSaos-2 cells. The assay was done in modified Boyden chambers consisting of transwell with matrigel coated 8-μm filter insert in 24-well plates for 24 h. Average numbers from three experiments are shown. $^{**}p<0.001$, Student's *t*-test.

metastasis, shRNA targeting NSD2 was successfully transfected into SaOS2 cells, the expression levels of E-cadherin were detected by RT-qPCR and Western blot analysis. As shown in Figure 6A and Figure 6B, the expression levels of E-cadherin in the NSD2 shRNA group were significantly higher than those infected with the negative control shRNA, in the NSD2 overexpression group, E-cadherin reduced. In MG-63 cells, qRT-PCR analysis revealed that NSD2 knockdown induced E-cadherin mRNA upregulation, while the overexpression of NSD2 caused the opposite tendency (Figure 6C). Similar results were found by Western

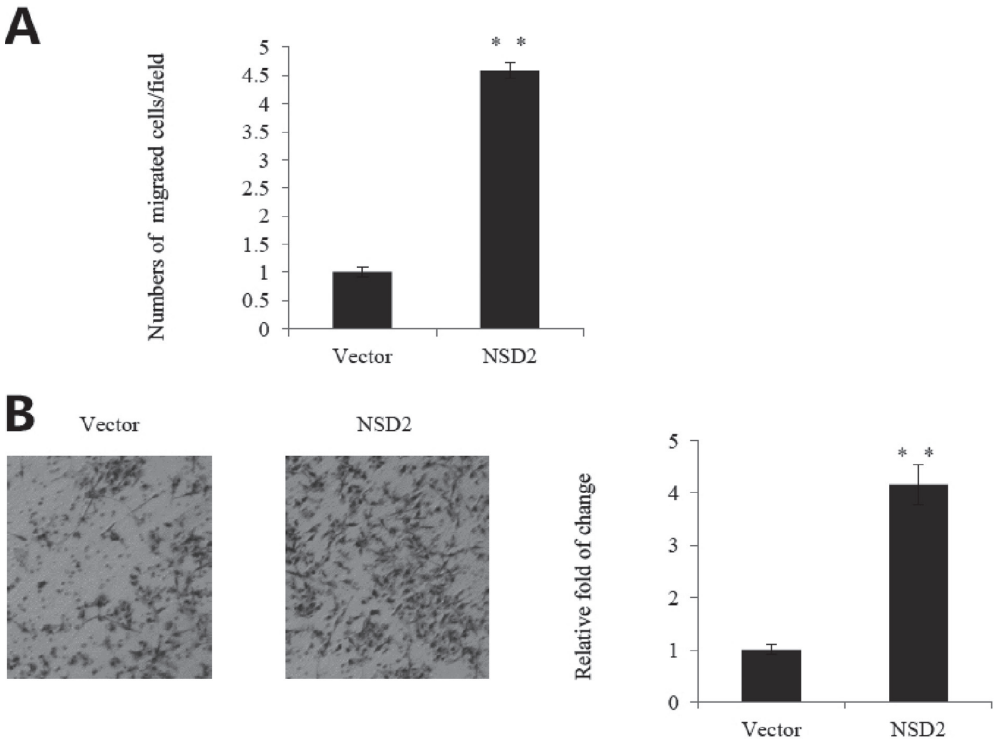

Figure 5. ***A,*** MG-63 cells migration analysis was performed using the wound-healing assay, cells were transfected with FLAG-NSD2 or stably transfected with empty vector. ***B,*** Relative transwell assay was performed in MG-63 cells.

blot assay (Figure 6D). Therefore, inhibition of NSD2 expression resulted in higher levels of E-cadherin expression.

Discussion

The molecular mechanism of osteosarcoma is still unclear, to more effectively control OS and improve the survival rate, new molecular targeted therapy novel treatment options are in emergency needed, since the loss of E-cadherin was usually considered as an indicator of poor clinical prognosis in Osteosarcoma as well as other solid tumors[17,18]. To understand the molecular mechanism that regulates E-cadherin is of great importance.

In the present study, we identify that high expression of NSD2 was observed in human osteosarcoma tissue and osteosarcoma cells, and it was correlated with poor survival of the OS patients. To undercover the mechanism, SaOS-2 cells were treated with shRNA-NSD2, following the inhibition of NSD2, the proliferation ability of osteosarcoma cell line was remarkably repressed. Furthermore, in SaOS-2 cells stably overexpressing FLAG-NSD2 or stably transfected with empty vector, wound-healing assay and transwell assay were performed. The result indicated that NSD2 could increase the migration and invasive activity of osteosarcoma cells *in vitro*. Since as reported by different groups, the loss of E-cadherin was associated with the acquisition of migration capacity[19,20]. Interestingly, following inhibition of NSD2, the expression levels of E-cadherin increased, which indicated that the repression of E-cadherin may be the mechanism for NSD2 in promoting osteosarcoma cell proliferation and metastasis.

Conclusions

Our study indicated that NSD2 may work as a novel repression of E-cadherin. Therefore, NSD2 has potential as a target of OS therapy. In the future, the monitoring of NSD2 in the serum/plasma from the RNA level may be used as a non-invasive method for selecting patients for target therapy.

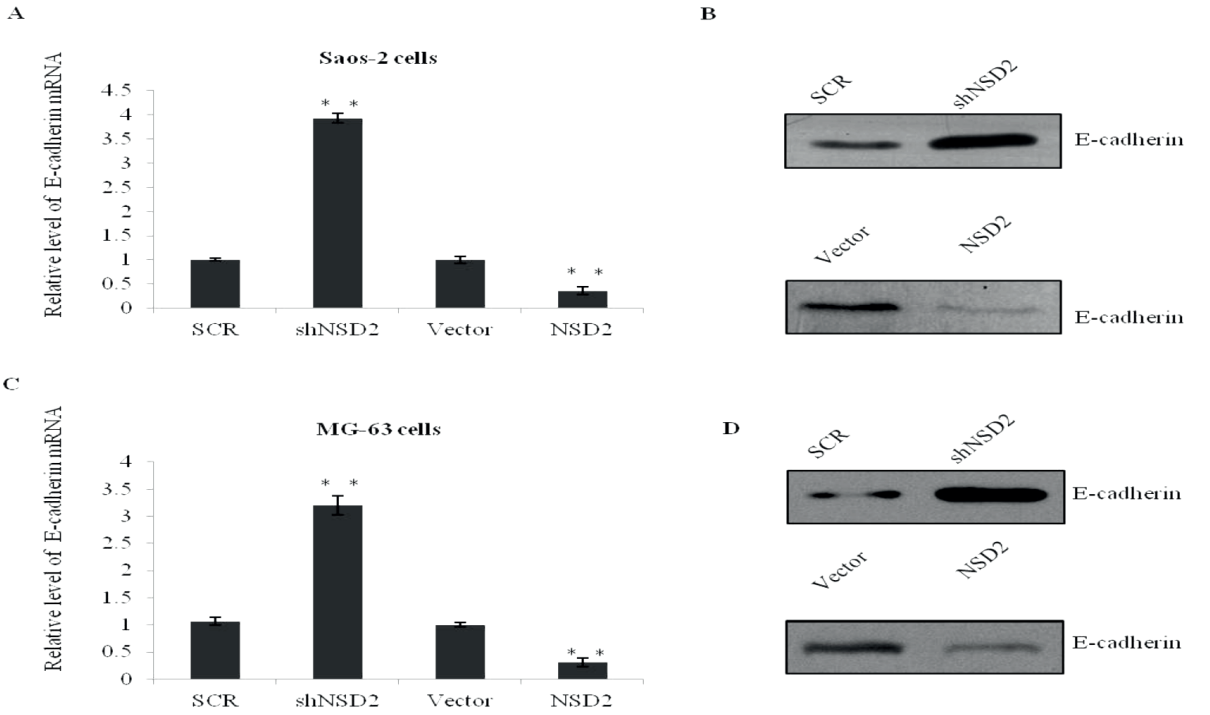

Figure 6. Inhibition of NSD2 increases the expression levels of E-cadherin in osteosarcoma cells. ***A*,** Real-time PCR showed a significant effect on E-cadherin expression in Saos-2 cells stably expressing control or NSD2-specific shRNA. ***B*,** Western blot showed increased E-cadherin protein expression in Saos-2 cells stably expressing control or NSD2-specific shRNA. β-Actin was as a loading control. ***C*,** Real-time PCR and (D) Western blot showed the increased expression level of E-cadherin in MG-63 cells, of three independent experiments, $^{*}p<0.05$.

Ethics Approval

The study was approved by the Ethics Committee of Linyi People's Hospital and informed consent was obtained from each participant prior to the trial.

Availability of data and material

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contributions

ML guarantees the integrity of the entire study, carried out the experiment and drafted the manuscript. QL carried out statistical analysis, searched and arranged references, and modified the manuscript further. XY conceived of the study, participated in its design and coordination, and carried out final proofreading of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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