Nasopharyngeal carcinoma-associated gene 6 inhibits cell viability, migration, invasion and induces apoptosis in osteosarcoma cells by inactivating the Wnt/β-catenin signaling pathway

LUN LIU[1], RUIJING WANG[2], ZIAN ZHANG[1] and XIANGDA WANG[1]

Departments of [1]Joint Surgery and [2]Surgery, Qingdao West Coast Hospital, Affiliated Hospital of Qingdao University, Qingdao, Shandong 266555, P.R. China

Received February 6, 2020; Accepted July 16, 2020

DOI: 10.3892/mmr.2020.11732

Abstract. Nasopharyngeal carcinoma-associated gene 6 (NGX6) is associated with the Wnt/β-catenin signaling pathway in a number of different types of cancer, including colorectal cancer (CRC). The present study is aimed to determine the functional role of NGX6 in osteosarcoma (OS) and to investigate the underlying mechanism associated with the Wnt/β-catenin signaling pathway. NGX6 expression levels in tissues derived from patients with OS and cell lines (MG-63, Saos-2, U2OS and HOS) was analyzed using reverse transcription-quantitative PCR. NGX6 expression levels were subsequently overexpressed through transfection of the pcDNA3.1 (pcDNA)-NGX6 overexpression vector into U2OS and HOS cells. BML284 was utilized to activate the Wnt/β-catenin signaling pathway. MTT, wound healing, Transwell and flow cytometry assays were performed to analyze cell viability, migration, invasion and apoptosis, respectively. Western blotting was also used to analyze the protein expression levels of β-catenin, c-Jun and c-Myc. A xenograft model was constructed by injecting pcDNA-NGX6-transfected U2OS cells into nude mice (BALB/c). The results of the present study revealed that the expression levels of NGX6 were downregulated in OS tissues and cell lines. The transfection of pcDNA-NGX6 into OS cells significantly inhibited the cell viability, and migratory and invasive abilities, and induced the apoptosis of U2OS and HOS cells. The expression levels of β-catenin, c-Jun and c-Myc were also significantly downregulated in pcDNA-NGX6-transfected U2OS and HOS cells. Notably, the treatment of transfected cells with BML284 significantly reversed the inhibitory effect of pcDNA-NGX6 on the viability, migration and invasion, and the promoting effect on apoptosis in U2OS cells. Furthermore, NGX6 overexpression also discovered to inhibit the growth of xenograft tumors *in vivo* by inhibiting the Wnt/β-catenin signaling pathway. In conclusion, the findings of the present study suggested that NGX6 may suppress the viability, migration and invasion, while facilitating the apoptosis of OS cells via blocking the Wnt/β-catenin signaling pathway.

Correspondence to: Dr Xiangda Wang, Department of Joint Surgery, Qingdao West Coast Hospital, Affiliated Hospital of Qingdao University, 1677 Wutaishan Road, Huangdao, Qingdao, Shandong 266555, P.R. China
E-mail: wangxiangd225@163.com

Key words: nasopharyngeal carcinoma-associated gene 6, osteosarcoma, Wnt/β-catenin, viability, migration, invasion, apoptosis

Introduction

Osteosarcoma (OS) is an aggressive malignancy that primarily develops in the bones of adults and children (1). Worldwide, an estimated 4 million people die of OS annually, with the disease peaking between the ages of 15-19 years old (2). In Europe and America, the 5-year survival rate for localized OS is ~65-70%, whereas the 5-year survival rate is <20% for metastatic OS (3). Surgical resection is the predominant method used for the treatment of OS (4). However, the incidence (50%) and metastatic rates (70%) following surgery remain high, which seriously affects the health and life of patients with OS (5). Consequently, research into the potential mechanisms of OS and the identification of novel therapeutic targets for OS has become a high priority.

Nasopharyngeal carcinoma-associated gene 6 (NGX6) has been identified as a tumor metastasis suppressor gene, encoding a class of membrane proteins (e.g. catenin-β1, transcription factor 4 and transmembrane protein 8B) primarily located in the nuclear and cell membrane (6). Previous studies have reported a variety of biological functions for NGX6, including an ability to suppress angiogenesis, modulate the protein expression levels of related genes, regulate cell cycle progression, participate in cell signaling transduction pathways (e.g., the epidermal growth factor receptor and Wnt/β-catenin signaling pathways) and enhance the sensitivity of patients to antitumor drugs (7-10). NGX6 was also identified to be aberrantly expressed in numerous different types of human malignancy, such as nasopharyngeal carcinoma (NPC) (11), and gastric (12), colon (12) and lung cancers (13). Wang *et al* (11) reported that NGX6 expression levels were downregulated in NPC, and the overexpression of NGX6 suppressed NPC cell proliferation and invasion. Guo *et al* (10)

revealed that NGX6 expression levels were downregulated in colorectal cancer cells, which suppressed cell invasion and adhesion in colorectal cancer. However, the biological role and regulatory mechanism of NGX6 in OS remains poorly understood.

The Wnt/β-catenin signaling pathway is considered as a β-catenin-dependent extracellular signaling pathway, which exerts a crucial role in regulating numerous biological processes, such as cell proliferation, apoptosis, migration, survival and differentiation (14-17). The aberrant activation of the Wnt/β-catenin signaling pathway has been closely associated with the development of various types of human malignancy, such as hepatocellular carcinoma (HCC), colorectal cancer (CRC) and OS (15-17). For instance, silencing of the sclerostin gene facilitated the proliferation, while repressing the apoptosis, of OS cells by stimulating the Wnt/β-catenin signaling pathway (18). In addition, the knockdown of Sox2 inhibited the migration and invasion of OS cells by blocking the Wnt/β-catenin signaling pathway (19). Moreover, a previous study demonstrated that NGX6 suppresses CRC cell invasion and adhesion by inhibiting the Wnt/β-catenin signaling pathway (10). Nevertheless, to the best of our knowledge, there are few studies on the underlying regulatory mechanism between NGX6 and the Wnt/β-catenin signaling pathway in OS.

The present study aimed to analyze the NGX6 expression levels in OS tissues and cell lines. In addition, *in vitro* experiments were performed to determine the effect of NGX6 on the viability, apoptosis, migration and invasion of OS cells. The association between NGX6 and the Wnt/β-catenin signaling pathway in OS progression was also identified. The findings illustrated the functional role of NGX6 in OS, providing a potential novel therapeutic target for the treatment of OS.

Materials and methods

Clinical specimens. The use of patient tissues was approved by the ethics committee of Qingdao West Coast Hospital, Affiliated Hospital of Qingdao University (approval no. QYFYWZLL25779A) and written informed consent was obtained from all patients. In total, 60 patients (27 males and 33 females; age range, 35-63 years old) histologically diagnosed with OS were recruited in our hospital between February 2017 and April 2019. The inclusion criteria were: i) First-time diagnosis; and ii) no prior history of radiotherapy, chemotherapy and other adjuvant therapy. The exclusion criteria were: i) Presence of other malignant tumors; and ii) treatment for OS before admission. OS tissues and adjacent normal tissues (distance from tumor margin, 2 cm) were collected from these patients prior to the administration of treatment.

Cell culture. The human OS cell lines (MG-63, Saos-2, U2OS and HOS) and the human osteoblast cell line hFOB1.19 were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. All cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva) and 1% penicillin-streptomycin, and maintained at 37˚C with 5% CO_2.

Cell transfection. U2OS and HOS cells ($6x10^5$ cells/well) in the logarithmic growth phase were transfected with 20 nM pcDNA3.1 (pcDNA)-NGX6 or pcDNA-negative control (NC; both Sangon Biotech Co., Ltd.) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were randomly divided into the BLANK (untransfected cells), pcDNA-NC and pcDNA3.1-NGX6 groups. Following transfection for 48 h at 37˚C, the cells were used for follow-up experiments.

To determine whether the regulatory effects of NGX6 on OS cells were associated with the Wnt/β-catenin signaling pathway, U2OS and HOS cells transfected with pcDNA-NGX6 were treated with BML284 (20 μM; Abcam), an activator of the Wnt/β-catenin signaling pathway, for 0, 24, 48, 72 or 96 h at room temperature. Cells were divided into the following groups: i) pcDNA-NC; ii) pcDNA3.1-NGX6; and iii) pcDNA3.1-NGX6 + BML284.

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'-3')
Nasopharyngeal carcinoma-associated gene 6	F: CAACAGCCTCAAGATCATCAGCA R: GAGGAGGGGAGATTCAGTGTGGT
β-catenin	F: TGAGGACAAGCCACAAGATTAC R: TCCACCAGAGTGAAAAGAACG
GAPDH	F: GAGTCAACGGATTTGGTCGT R: TTGATTTTGGAGGGATCTC

F, forward; R, reverse.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tissues or cells using TRIzol® Plus RNA Isolation reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration of total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA (500 ng) was reverse transcribed into cDNA at 42˚C for 45 min using Moloney Murine Leukaemia Virus Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) on a StepOnePlus™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 3 min; followed by 40 cycles at 95˚C for 15 sec, annellation at 60˚C for 30 sec, elongation at 72˚C for 1 min, and a final extension at 72˚C for 5 min. The primer sequences used for the qPCR are listed in Table I. The mRNA expression levels of target genes were quantified using the $2^{-\Delta\Delta Cq}$ method (21). GAPDH or U6 was used as the internal loading control.

Western blotting. Total protein was extracted from tissues or OS cell lines (U2OS and HOS) using RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a BCA Protein assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) and 50 μg protein/lane was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto a polyvinylidene fluoride membrane

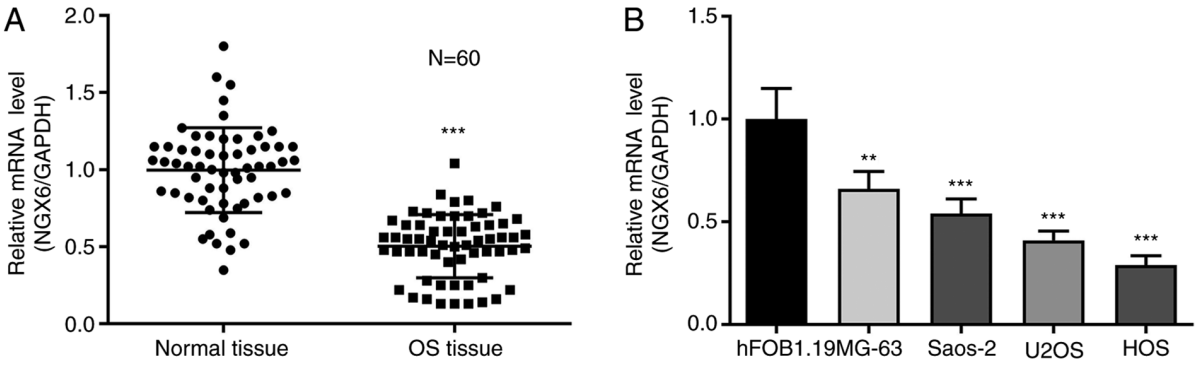

Figure 1. NGX6 expression levels are downregulated in OS tissues and cell lines. (A) mRNA expression levels of NGX6 in OS tissues (n=60) and normal tissues (n=60) were analyzed by RT-qPCR. ***P<0.001 vs. normal tissue. (B) mRNA expression levels of NGX6 in OS cell lines (MG-63, Saos-2, U2OS and HOS) and the human osteoblast cell line hFOB1.19 were analyzed using RT-qPCR. **P<0.01, ***P<0.001 vs. hFOB1.19 cells. OS, osteosarcoma; NGX6, nasopharyngeal carcinoma-associated gene 6; RT-qPCR, reverse transcription-quantitative PCR.

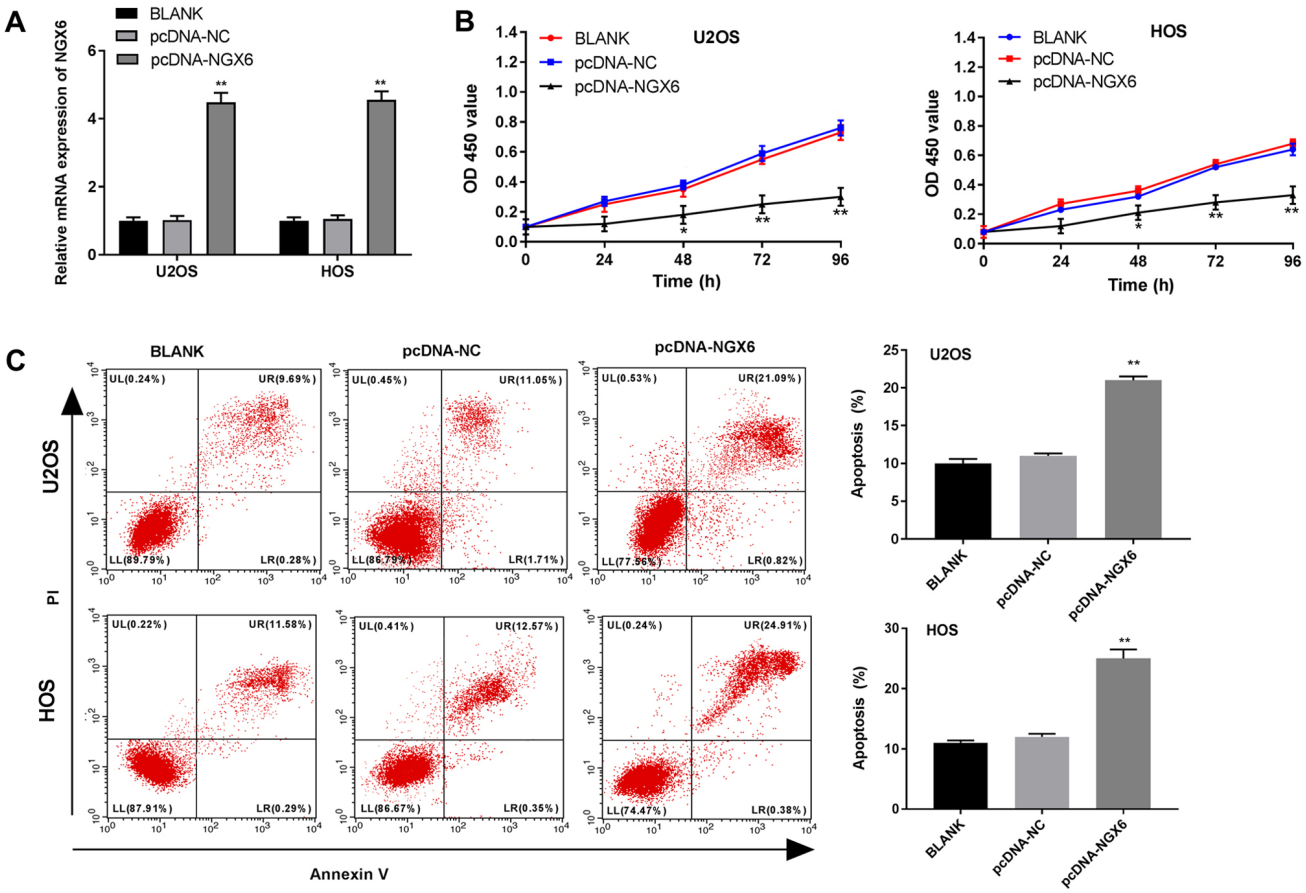

Figure 2. Overexpression of NGX6 inhibits the viability and induces the apoptosis of OS cells. (A) pcDNA-NGX6 overexpression vector was transfected into U2OS and HOS cells, and reverse transcription-quantitative PCR was performed to analyze the transfection efficiency compared with the pcDNA-NC and BLANK groups. (B) Cell viability of transfected U2OS and HOS cells was determined using an MTT assay. (C) Levels of apoptosis of transfected U2OS and HOS cells were analyzed using flow cytometry. *P<0.05, **P<0.01 vs. BLANK. BLANK, untransfected U2OS or HOS cells; NGX6, nasopharyngeal carcinoma-associated gene 6; NC, negative control; OD, optical density; PI, propidium iodide.

and blocked with 5% skimmed milk for 2 h at 25˚C. The membranes were then incubated with the following primary antibodies (Abcam) overnight at 4˚C: Anti-GAPDH (1:1,000; cat. no. ab9485), anti-β-catenin (1:1,000; cat. no. ab32572), anti-c-Jun (1:1,000; cat. no. ab40766) and anti-c-Myc (1:1,000; cat. no. ab32072). After washing three times with TBST (Tween-20; 0.05%) for 5 min, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2,000; cat. no. ab6721; Abcam) for 2 h at 25˚C. Protein bands were visualized using an chemiluminescent substrate kit (Invitrogen; Thermo Fisher Scientific, Inc.) and analyzed using Gel-Pro Analyzer software (version 4.0; Media Cybernetics, Inc.). GAPDH was used as the internal loading control.

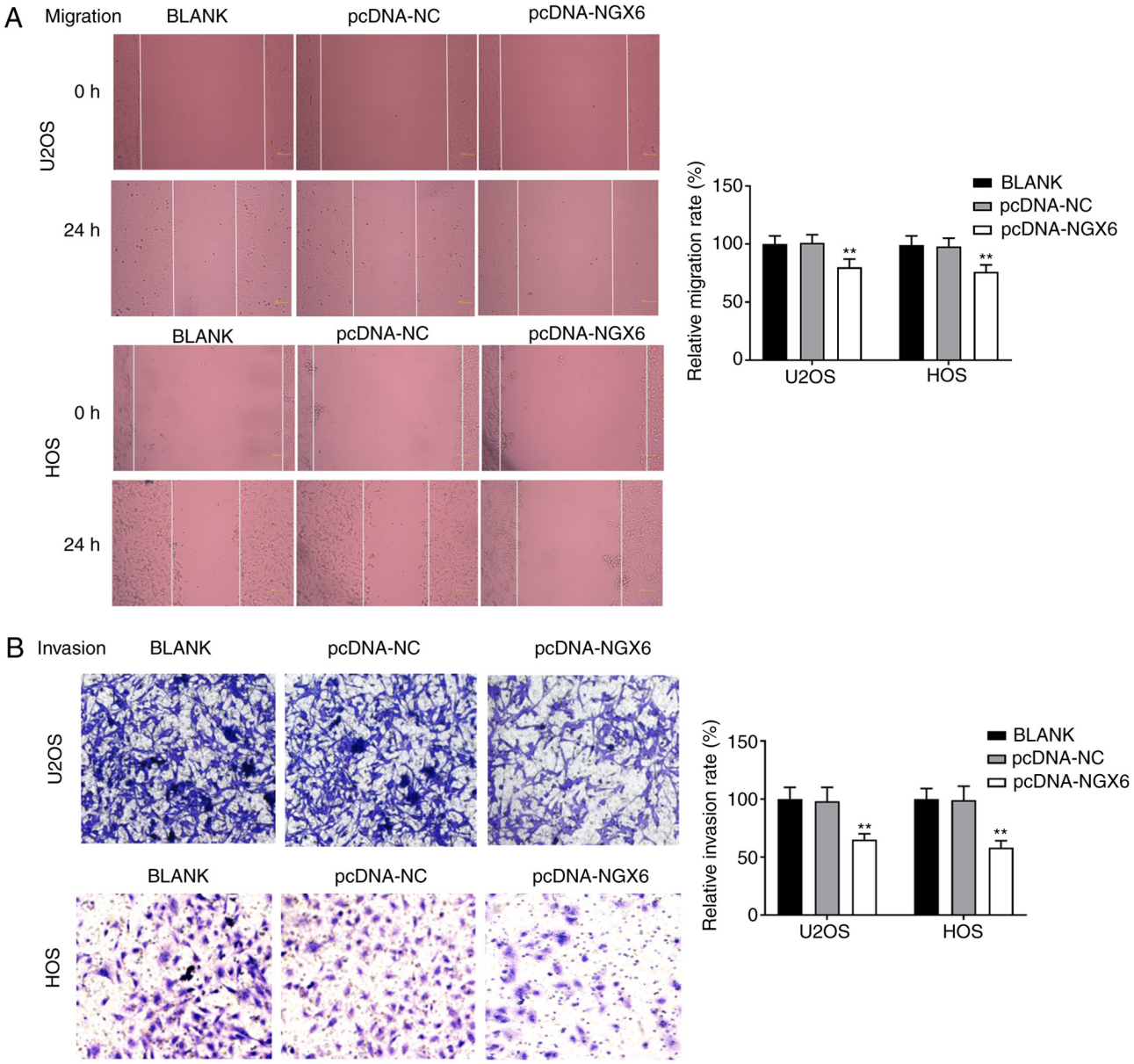

Figure 3. NGX6 overexpression suppresses the migration and invasion of OS cells. (A) Relative migration rate of U2OS and HOS cells transfected with pcDNA-NGX6 was analyzed using a wound healing assay (magnification, x200). (B) Relative invasion rate of U2OS and HOS cells transfected with pcDNA-NGX6 was analyzed using a Transwell Matrigel assay (magnification, x200). **P<0.01 vs. BLANK. BLANK, untransfected U2OS or HOS cells; NGX6, nasopharyngeal carcinoma-associated gene 6; NC, negative control.

MTT assay. The transfected U2OS and HOS cells ($2x10^4$ cells/well) were seeded into 96-well plates. Following incubation for 24, 48, 72 or 96 h at 37˚C, 20 μl MTT reagent (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added into each well. After 4 h of incubation at 37˚C, 150 μl DMSO was added into each well to terminate the reaction. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Flow cytometric analysis of apoptosis. Early apoptosis in transfected U2OS and HOS cells were analyzed using an Annexin V-FITC apoptosis detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, cells were centrifuged at 200 x g for 5 min at 4˚C and resuspended in binding buffer. Cells were subsequently stained with 5 μl Annexin V-FITC and 5 ml propidium iodide for 15 min at 4˚C in a dark room. Apoptosis was detected using a FACScan flow cytometer (version 2.0; BD Biosciences) and the data were analyzed using CellQuest software (version 5.1; BD Biosciences).

Wound healing assay. U2OS and HOS cells were plated into six-well plates containing DMEM at a density of $5x10^5$ cells/well. Upon reaching 90% confluence, the cell monolayer was scratched using a sterile 100-μl pipette tip to form wounds. The cells were then incubated in serum-free DMEM for 24 h at 37˚C. Cells were observed and photographed at 0 and 24 h using an inverted light microscope (Olympus Corporation; magnification, x200) and measured using ImageJ software (version 1.46; National Institutes of Health). The relative migration rate was calculated as follows: (original gap distance-gap distance at 24 h)/original

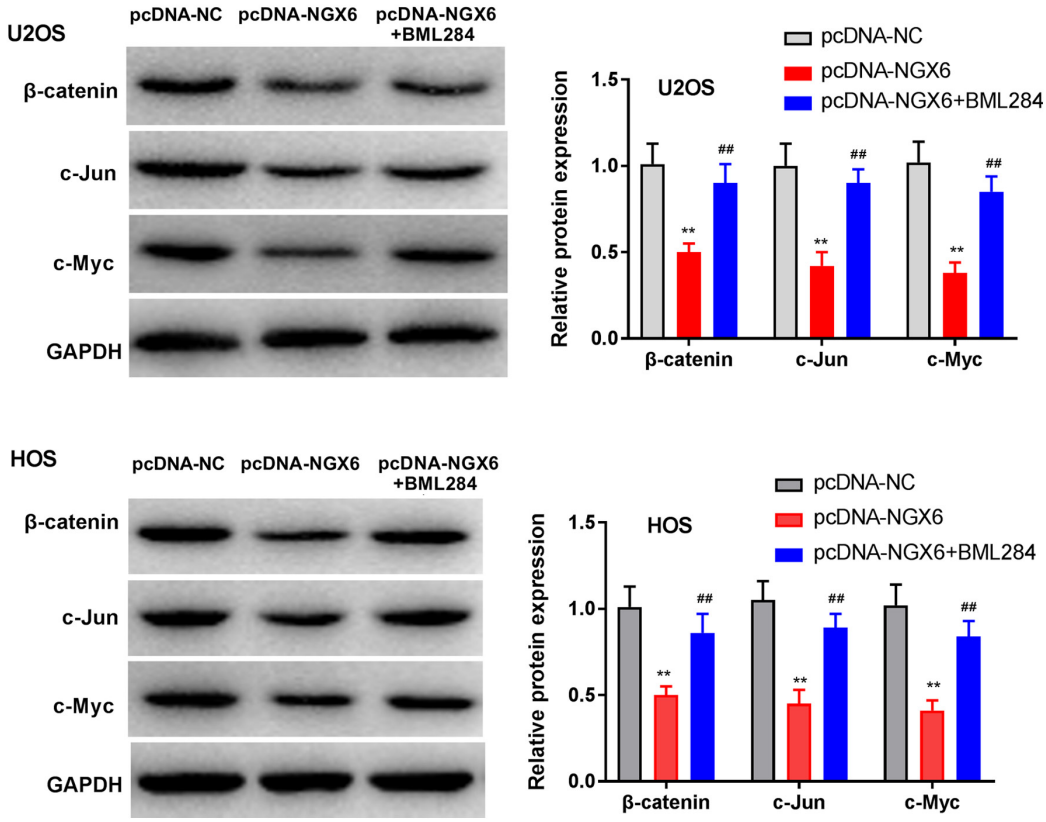

Figure 4. NGX6 overexpression blocks the Wnt/β-catenin signaling pathway in OS cells. The expression levels of β-catenin, c-Jun and c-Myc in U2OS and HOS cells transfected with pcDNA-NGX6 in the presence or absence of BML284 treatment (20 μM) were analyzed using western blotting. **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. pcDNA-NGX6. NGX6, nasopharyngeal carcinoma-associated gene 6; NC, negative control.

gap distance x100. Relative migration was normalized to the BLANK or pcDNA-NC group.

Transwell Matrigel assay. Cell invasion was assessed using Transwell chambers (Corning, Inc.) pre-coated (at 37˚C for 30 min) with Matrigel® (BD Biosciences). Briefly, transfected U2OS and HOS cells were resuspended in serum-free medium and 200 μl cell suspension (1x10^5 cells) was placed in the upper chamber. Subsequently, 600 μl medium containing 10% FBS was plated into the lower chambers. Following 24 h of incubation at 37˚C with 5% CO_2, the invasive cells in the lower chamber were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with 0.5% crystal violet at 37˚C for 30 min. Cells in six independent fields per well were imaged using a light microscope (Olympus Corporation; magnification, x200), and the number of invading cells were counted.

Establishment of xenograft tumor model mice. Animal experiments were approved by the ethics committee of Qingdao West Coast Hospital (approval no. QYFYWZLL25779) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (version 1996) (20). Male specific pathogen free mice (BALB/c; age, 4 weeks; weight, 20-25 g; n=30) were purchased from the Medical College of Shanghai Jiaotong University (Shanghai, China). The animals were housed in a sterile environment at a controlled temperature of 20˚C with 40% relative humidity, 12-h light/dark cycles, and free access to food and water. A volume of 100 μl U2OS cells (0.1x10^8 cells/ml) at the logarithmic growth phase from the different groups (pcDNA-NGX6, pcDNA-NC, pcDNA-NGX6 + BML284) were resuspended in PBS and subcutaneously injected into the posterior limb of mice (n=10/group) to create a subcutaneous tumor-bearing model. The tumor volume was measured with a Vernier caliper every week following injection. At the end of the 4th week, mice were anesthetized with 50 mg/kg pentobarbital sodium and sacrificed by cervical dislocation. The tumors were removed and weighed. The experiment lasted for 4 weeks and no mice died during this period.

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.). All experiments were performed in triplicate and data are presented as the mean ± SD. A paired Student's t-test was used to determine the significant differences between two groups (Fig. 1A), while a one-way ANOVA test followed by Tukey's post hoc test was used to determine the statistical differences between >2 groups. P<0.05 was considered to indicate a statistically significant difference.

Results

NGX6 expression levels are downregulated in OS tissues and cell lines. RT-qPCR analysis revealed that NGX6 expression levels were significantly downregulated in the OS tissues compared with the normal tissues from patients (P<0.001; Fig. 1A). Consistently, NGX6 expression levels were also significantly

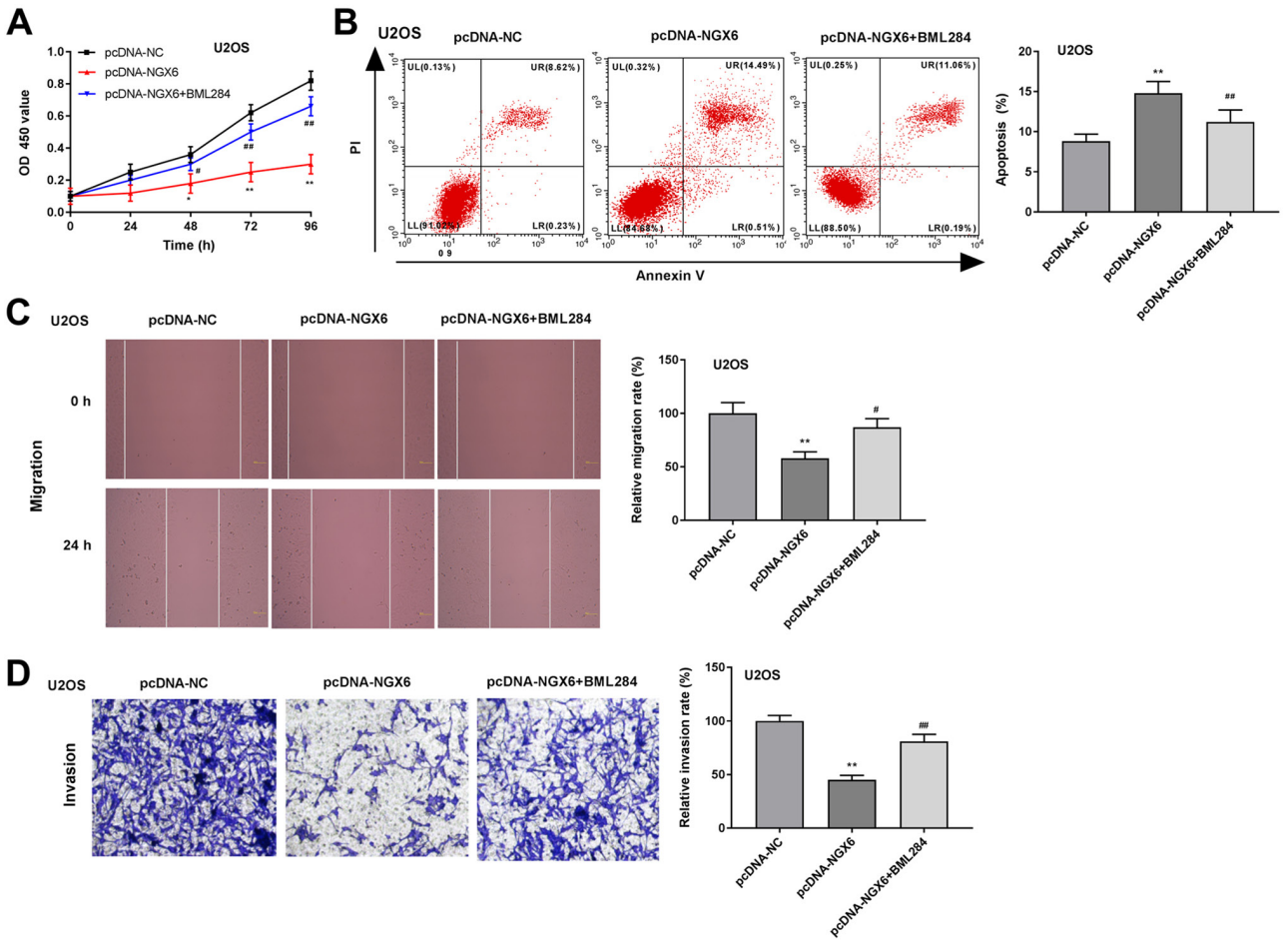

Figure 5. NGX6 overexpression inhibits the viability, migration and invasion, while inducing the apoptosis of OS cells by blocking the Wnt/β-catenin signaling pathway. (A) MTT assay was used to analyze the viability of U2OS cells transfected with pcDNA-NGX6 in the presence or absence of BML284 treatment (20 μM). (B) Levels of apoptosis of U2OS cells transfected with pcDNA-NGX6 in the presence or absence of BML284 treatment (20 μM) were analyzed using flow cytometry. (C) Migration rate of U2OS cells transfected with pcDNA-NGX6 in the presence or absence of BML284 treatment (20 μM) was analyzed using a wound healing assay (magnification, x200). (D) Invasion rate of U2OS cells transfected with pcDNA-NGX6 in the presence or absence of BML284 treatment (20 μM) was analyzed using a Transwell Matrigel assay (magnification, x200). *P<0.05, **P<0.01 vs. pcDNA-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. pcDNA-NGX6. NGX6, nasopharyngeal carcinoma-associated gene 6; NC, negative control; OD, optical density; PI, propidium iodide.

downregulated in OS cell lines (MG-63, Saos-2, U2OS and HOS) compared with the human osteoblastic cell line hFOB1.19, but especially in U2OS and HOS cells (P<0.01; Fig. 1B). Therefore, U2OS and HOS cells were selected for subsequent experiments. These findings suggested that NGX6 expression levels may be downregulated in OS tissues and cell lines.

Overexpression of NGX6 inhibits the viability, while promoting the apoptosis of OS cells. To determine the effect of NGX6 on OS progression, NGX6 was overexpressed by the transfection of pcDNA-NGX6 into U2OS and HOS cells. RT-qPCR illustrated that the NGX6 expression levels were significantly upregulated in the pcDNA3-NGX6 groups compared with the BLANK groups in both cell lines (P<0.01; Fig. 2A). The results of the MTT assay revealed that the optical density (OD)450 values of the pcDNA-NGX6 groups were significantly decreased at 48 h in both cell lines compared with the BLANK groups (P<0.05; Fig. 2B). The levels of apoptosis of U2OS and HOS cells in the pcDNA-NGX6 groups were significantly increased compared with the BLANK groups (**P<0.01; Fig. 2C). Conversely, the transfection of cells with pcDNA-NC did not influence the viability or apoptotic rate of U2OS and HOS cells (Fig. 2B and C). These results indicated that the overexpression of NGX6 may impede the viability and promote the apoptosis of U2OS and HOS cells.

Overexpression of NGX6 suppresses the migration and invasion of OS cells. The relative migration and invasion rates were significantly decreased in the pcDNA-NGX6 groups compared with the BLANK groups in both cell lines (P<0.01; Fig. 3A and B). The transfection of cells with pcDNA-NC did not influence the migration and invasion rates of U2OS and HOS cells. Taken together, these data indicated that the overexpression of NGX6 may repress the migration and invasion of U2OS and HOS cells.

NGX6 overexpression inhibits the Wnt/β-catenin signaling pathway. To determine the potential mechanism of NGX6 in OS, transfected U2OS and HOS cells were treated with BML284 (Wnt/β-catenin signaling pathway activator). In both cell lines, the expression levels of β-catenin, c-Jun and c-Myc were all significantly downregulated in the pcDNA-NGX6 groups compared with the pcDNA-NC groups (P<0.01; Fig. 4). Conversely, BML284 treatment significantly reversed

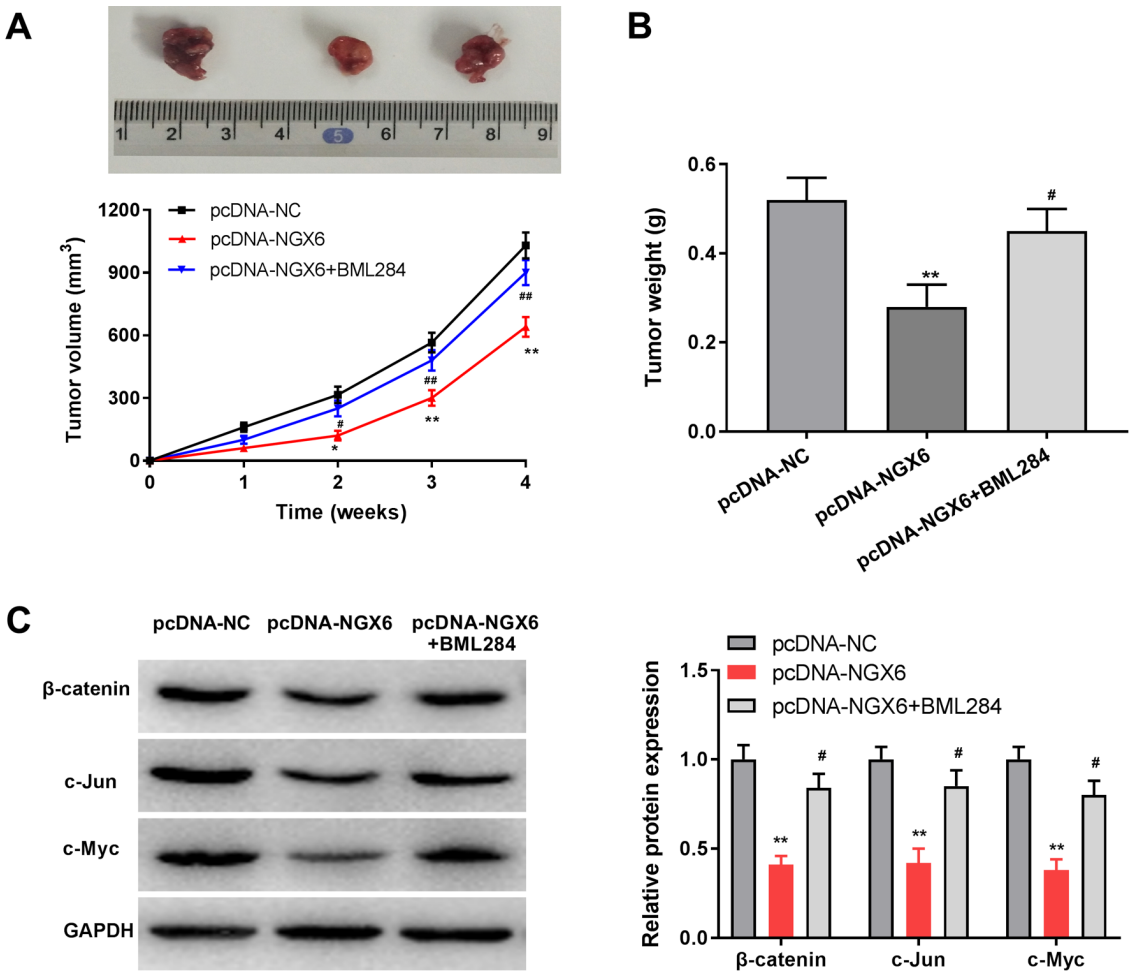

Figure 6. NGX6 represses the growth of xenograft tumors *in vivo*. (A) Tumor morphology at the 4th week post-injection and the tumor volume of xenograft mice in the different groups at different time points. (B) Tumor weight at the 4th week post-injection in the different groups. (C) Expression levels of β-catenin, c-Jun and c-Myc in the different groups were analyzed using western blotting. *P<0.05, **P<0.01 vs. pcDNA-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. pcDNA-NGX6. NGX6, nasopharyngeal carcinoma-associated gene 6; NC, negative control.

the inhibitory effects of pcDNA-NGX6 on the expression levels of β-catenin, c-Jun and c-Myc in U2OS and HOS cells ($^{\#\#}$P<0.01; Fig. 4). These results indicated that the transfection of pcDNA-NGX6 may block the Wnt/β-catenin signaling pathway, while the intervention with BML284 may reverse the inhibitory effect of pcDNA-NGX6 on the Wnt/β-catenin signaling pathway.

NGX6 overexpression inhibits the viability, migratory and invasive abilities, while facilitating the apoptosis of OS cells by blocking the Wnt/β-catenin signaling pathway. To explore the possible effect of the Wnt/β-catenin signaling pathway on the occurrence and development of OS *in vitro*, U2OS cells were treated with an activator of the Wnt/β-catenin signaling pathway (BML284). The results indicated that treatment with BML284 reversed the inhibitory effects of pcDNA-NGX6 on the viability, migration and invasion, and the promoting effect on the apoptosis of U2OS cells (P<0.05, P<0.01; Fig. 5A-D). Collectively, the results implied that treatment BML284 could reverse the effect of NGX6 overexpression on the progression of OS *in vitro*.

Overexpression of NGX6 inhibits the growth of xenograft tumors in vivo by blocking the Wnt/β-catenin signaling pathway. To verify the inhibitory effect of pcDNA-NGX6 on the tumorigenesis of OS, xenograft tumor model mice were established. A significant decrease was observed in both the tumor volume (P<0.05; Fig. 6A) and tumor weight (P<0.01; Fig. 6B) in the pcDNA-NGX6 group compared with the pcDNA-NC group. However, the addition of BML284 treatment partially rescued the inhibitory effect of pcDNA-NGX6 on the tumor growth and weight of mice (P<0.05, P<0.01). In addition, the expression levels of β-catenin, c-Jun and c-Myc in the tumor tissues were significantly downregulated in the pcDNA-NGX6 group compared with the pcDNA-NC group (P<0.01; Fig. 6C), while the addition of BML284 partially rescued this inhibitory effect of pcDNA-NGX6 on the expression levels of β-catenin, c-Jun and c-Myc in the tumor tissues of mice (P<0.05; Fig. 6C). The results indicated that treatment with BML284 reversed the suppressive effect of NGX6 overexpression on the growth of xenograft tumors *in vivo*.

Discussion

NGX6 expression levels were discovered to be downregulated in colorectal (22), gastric (11) and liver cancers (23). Liu *et al*

also reported that NGX6 expression levels were downregulated in gastric cancer tissues, and these low expression levels of NGX6 facilitated the progression of gastric cancer (11). In addition, Zhang *et al* (9) reported that the expression levels of NGX6 were downregulated in colorectal cancer tissues, and the downregulation of NGX6 exerted a promoting effect on the occurrence and metastasis of colorectal cancer. The findings of the present study were similar to those of previous studies; for instance, the results revealed that NGX6 expression levels were significantly downregulated in OS tissues and cell lines.

Peng *et al* (24) previously revealed that NGX6 inhibited the proliferation and migration of NPC cells. NGX6 was discovered to suppress cell proliferation, invasion and metastasis, regulate the cell cycle and inhibit tumor angiogenesis in colon cancer (10). Similar to the effects of NGX6 observed in other types of cancer, the results of the present study demonstrated that the overexpression of NGX6 inhibited the cell viability, migration and invasion, while promoting the apoptosis of U2OS and HOS cells. These findings indicated that NGX6 may function as a tumor suppressor gene during OS progression.

The Wnt/β-catenin signaling pathway serves a crucial role in tumorigenesis and the abnormal activation of the Wnt/β-catenin signaling pathway has been observed in various types of human malignancies, such as HCC, CRC and OS (16,17,25,26). Numerous genes have been identified to exert their tumor suppressive roles in OS through blocking the Wnt/β-catenin signaling pathway, including TraB domain containing 2B (27), bone morphogenetic protein 9 (28) and forkhead box protein O1 (29). Notably, NGX6 is involved in the regulation of the Wnt/β-catenin signaling pathway in CRC (25,30). Liu *et al* (31) demonstrated that NGX6 overexpression downregulated the expression levels of the downstream target genes of the Wnt/β-catenin signaling pathway, cyclin D1, c-Jun and c-Myc, in colon cancer cells. Guo *et al* (10) illustrated that NGX6 suppressed the translocation of β-catenin from the nucleus and cytoplasm to the plasma membrane, thereby inhibiting the activity of transcription factor 4 and downregulating the expression levels of the Wnt-target genes, c-Myc, cyclin D1 and cyclooxygenase-2 in colon cancer cells. In the present study, the overexpression of NGX6 significantly downregulated the expression levels of β-catenin, c-Jun and c-Myc in OS cells. These findings were consistent with previous studies, which further suggested that the Wnt/β-catenin signaling pathway may be blocked by NGX6 in OS. Thus, the blocked Wnt/β-catenin signaling pathway may directly contribute to the antitumor effect of NGX6 in colon cancer. Guo *et al* (30) also discovered that NGX6 inhibited the proliferation, invasiveness and extracellular matrix adhesion, in addition to promoting the apoptosis, of colon cancer cells through suppressing the Wnt/β-catenin signaling pathway. Consistent with these findings, the current study revealed that NGX6 suppressed the viability, migration and invasion, and promoted the apoptosis of OS cells, through blocking the Wnt/β-catenin signaling pathway. In addition, the migration of OS cells was inhibited by the NGX6-mediated suppression of the Wnt/β-catenin signaling pathway. The verification experiments further illustrated the regulatory relationship between NGX6 and the Wnt/β-catenin signaling pathway in OS; the BML284-induced activation of the Wnt/β-catenin signaling pathway reversed the antitumor effects of NGX6 on the viability, apoptosis, migration and invasion of OS cells.

Furthermore, the present study also discovered that NGX6 inhibited tumor growth *in vivo* through blocking the Wnt/β-catenin signaling pathway. These findings indicated that NGX6 may inhibit the tumorigenesis of OS both *in vitro* and *in vivo* through blocking the Wnt/β-catenin signaling pathway.

In conclusion, the findings of the present study revealed that the expression levels of NGX6 were downregulated in OS tissues and cell lines. The overexpression of NGX6 significantly inhibited the viability, migration and invasion, and induced apoptosis in OS cells *in vitro*. The growth of xenograft tumors *in vivo* was also suppressed by NGX6 overexpression. Additionally, the inhibitory effect of NGX6 on OS progression was suggested to possibly occur through the suppression of the Wnt/β-catenin signaling pathway. These data implied that NGX6 may function as a potential therapeutic target for the treatment of OS.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

LL made substantial contributions to the conception and design of the study. RW, ZZ and XW made substantial contributions to the acquisition, analysis and interpretation of data, as well as the drafting and revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Qingdao West Coast Hospital (approval no. QYFYWZLL25779A), and written informed consent was obtained from all patients. The animal experiments were approved by the ethics committee of Qingdao West Coast Hospital (approval no. QYFYWZLL25779) and performed in accordance with the Guide for the Care and Use of Laboratory Animals (version 1996).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Biermann JS, Chow W, Reed DR, Lucas D, Adkins DR, Agulnik M, Benjamin RS, Brigman B, Budd GT, Curry WT, *et al*: NCCN Guidelines Insights: Bone Cancer, Version 2.2017. J Natl Compr Canc Netw 15: 155-167, 2017.
2. Mirabello L, Troisi RJ and Savage SA: Osteosarcoma incidence and survival rates from 1973 to 2004: Data from the Surveillance, Epidemiology, and End results program. Cancer 115: 1531-1543, 2010.
3. Siegel HJ and Pressey JG: Current concepts on the surgical and medical management of osteosarcoma. Expert Rev Anticancer Ther 8: 1257-1269, 2008.
4. Mialou V, Philip T, Kalifa C, Perol D, Gentet JC, Marec-Berard P, Pacquement H, Chastagner P, Defaschelles AS and Hartmann O: Metastatic osteosarcoma at diagnosis: Prognostic factors and long-term outcome-the French pediatric experience. Cancer 104: 1100-1109, 2010.
5. Friebele JC, Peck J, Pan X, Abdel-Rasoul M and Mayerson JL: Osteosarcoma: A meta-analysis and review of the literature. Am J Orthop (Belle Mead NJ) 44: 547-553, 2015.
6. Ma J, Li J, Zhou J, Li XL, Tang K, Zhou M, Yang JB, Yan Q, Shen SR, Hu GX and Li GY: Profiling genes differentially expressed in NGX6 overexpressed nasopharyngeal carcinoma cells by cDNA array. J Cancer Res Clin Oncol 128: 683-690, 2002.
7. He S, Zhong Y, Shuai C, Gao D, Wei P, Li G and Peng S: Tumor suppressor NGX6 inhibits the growth and metastasis of multiple cancers. Tumour Biol 37: 5751-5760, 2016.
8. Wang L, Ma J, Li J, Li X, Zhang Q, Peng S, Peng C, Zhou M, Xiong W, Yang J, *et al*: NGX6 gene inhibits cell proliferation and plays a negative role in EGFR pathway in nasopharyngeal carcinoma cells 95: 64-73, 2005.
9. Zhang XM, Wang XY, Sheng SR, Wang JR and Li J: Expression of tumor related genes NGX6, NAG-7, BRD7 in gastric and colorectal cancer. World J Gastroenterol 9: 1729-1733, 2003.
10. Guo Q, Shen S, Liao M, Lian P and Wang X: NGX6 inhibits cell invasion and adhesion through suppression of Wnt/beta-catenin signal pathway in colon cancer. Acta Biochim Biophys Sin (Shanghai) 42: 450-456, 2010.
11. Wang YT, Sun XY, Wang H, Huang TT, Wang Y and Yang MX: Effects of NGX6 expression on proliferation and invasion of nasopharyngeal carcinoma cells and survival of patients. Eur Rev Med Pharmacol Sci 21: 5378-5385, 2017.
12. Liu J, Zhu X, Xu X and Dai D: DNA promoter and histone H3 methylation downregulate NGX6 in gastric cancer cells. Med Onco 31: 817, 2014.
13. Lin ZF, Shen XY, Lu FZ, Ruan Z, Huang HL and Zhen J: Reveals new lung adenocarcinoma cancer genes based on gene expression. Eur Rev Med Pharmacol Sc 16: 1249-1256, 2012.
14. Nusse R and Clevers H: Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell 169: 985-999, 2017.
15. Lin CH, Ji T, Chen CF and Hoang BH: Wnt signaling in osteosarcoma. Adv Exp Med Biol 804: 33-45, 2014.
16. Anastas JN and Moon RT: WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer 13: 11-26, 2013.
17. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX and Ivy SP: Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: Clinical update. Nat Rev Clin Oncol 12: 445-464, 2015.
18. Zou J, Zhang W and Li XL: Effects of SOST gene silencing on proliferation, apoptosis, invasion, and migration of human osteosarcoma cells through the Wnt/β-catenin signaling pathway. Calcif Tissue Int 100: 551-564, 2017.
19. Tang L, Wang D and Gu D: Knockdown of Sox2 inhibits OS cells invasion and migration via modulating Wnt/beta-catenin signaling pathway. Pathol Oncol Res 24: 907-913, 2018.
20. Clark DJ, Gebhart FG, Gonder CJ, Keeling ME and Kohn DF: Special Report: The 1996 Guide for the Care and Use of Laboratory Animals. ILAR J 38: 41-48, 1997
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
22. Liu M, Peng Y, Wang X, Guo Q, Shen S and Li G: NGX6 gene mediated by promoter methylation as a potential molecular marker in colorectal cancer. Bmc Cancer 10: 160-160, 2010.
23. Xiao JD and Shen SR: Expression and significance of NGX6 gene in human hepatocellular carcinoma. Zhong Nan Da Xue Xue Bao Yi Xue Ban 33: 937-941, 2008 (In Chinese).
24. Peng SP, Li XL, Wang L, Ou-Yang J, Ma J, Wang LL, Liu HY, Zhou M, Tang YL, Li WS, *et al*: The role of NGX6 and its deletion mutants in the proliferation, adhesion and migration of nasopharyngeal carcinoma 5-8F cells. Oncology 71: 273-281, 2006.
25. Logan CY and Nusse R: The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781-810, 2004.
26. Ma Y, Zhu B, Liu X, Yu H, Yong L, Liu X, Shao J and Liu Z: Inhibition of oleandrin on the proliferation show and invasion of osteosarcoma cells in vitro by suppressing Wnt/β-catenin signaling pathway. J Exp Clin Cancer Res 34: 115, 2015.
27. Li R, Liu J, Wu H, Liu L, Wang L and Zhang S: TIKI2 suppresses growth of osteosarcoma by targeting Wnt/β-catenin pathway. Mol Cell Biochem 392: 109-116, 2014.
28. Lv Z, Wang C, Yuan T, Liu Y, Song T, Liu Y, Chen C, Yang M, Tang Z, Shi Q and Weng Y: Bone morphogenetic protein 9 regulates tumor growth of osteosarcoma cells through the Wnt/β-catenin pathway. Oncol Rep 31: 989-994, 2014.
29. Guan H, Tan P, Xie L, Mi B, Fang Z, Li J, Yue J, Liao H and Li F: FOXO1 inhibits osteosarcoma oncogenesis via Wnt/β-catenin pathway suppression. Oncogenesis 4: e166, 2015.
30. Guo Q, Wu M, Lian P, Liao M, Xiao Z, Wang X and Shen S: Synergistic effect of indomethacin and NGX6 on proliferation and invasion by human colorectal cancer cells through modulation of the Wnt/beta-catenin signaling pathway. Mol Cell Biochem 330: 71-81, 2009.
31. Liu F, Sheng S, Wang X, Peng Y, Guo Q, Xiao Z and Liu J: Role of NGX6 in Wnt/β-catenin signaling pathway in colon cancer. Zhong Nan Da Xue Xue Bao Yi Xue Ban 36: 235-242, 2011 (In Chinese).