Retraction

Retraction: Transcription Factor SOX9 Promotes Osteosarcoma Cell Growth by Repressing Claudin-8 Expression

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We reported "Transcription Factor SOX9 Promotes Osteosarcoma Cell Growth by Repressing Claudin-8 Expression" in Tohoku J. Exp. Med., 2017, 241 (1), 55-63; doi: 10.1620/tjem.241.55. After publication of the paper, we have become aware of the ethical violation in collecting the samples from some patients. We therefore wish to retract the paper and apologize for any inconvenience caused by the retraction.

Transcription Factor SOX9 Promotes Osteosarcoma Cell Growth by Repressing Claudin-8 Expression

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Osteosarcoma is a malignant bone cancer that mainly affects children. SOX9 plays a key role in bone formation and osteosarcoma, and Claudin-8 (CLDN8), a tight junction protein, contributes to proliferation of osteosarcoma cells. The aim of this study was to investigate the relationship between SOX9 and CLDN8 in osteosarcoma. The expression levels of SOX9 and CLDN8 were determined in osteosarcoma specimens (n = 25) by qRT-PCR and Western blot analyses. The levels of SOX9 mRNA and protein were significantly higher in osteosarcoma tissues than those in adjacent non-tumor tissues, whereas the expression levels of CLDN8 mRNA and protein were significantly lower in osteosarcoma tissues. Immunohistochemical analysis showed the high expression of SOX9 in 56 out of 97 osteosarcoma tissues (57.7%). By contrast, the low expression of immunoreactive CLDN8 was observed in 62 of 97 osteosarcoma tissues (63.9%). There was the inverse correlation between the expression levels of SOX9 and CLDN8 proteins (R = -0.633, P < 0.001). The overall survival was poorer in the patients with high SOX9 expression. Subsequently, using two human osteosarcoma cell lines, we showed that knockdown of SOX9 inhibited cell proliferation and migration but promoted cell apoptosis. Importantly, knockdown of SOX9 increased the expression levels of CLDN8 protein. The transient luciferase-reporter assays suggest that SOX9 may inhibit the promoter activity of the CLDN8 gene in osteosarcoma cells. In conclusion, we provide the evidence demonstrating that SOX9 may promote cell growth by repressing the expression of CLDN8. Thus, SOX9 may be a therapeutic target for osteosarcoma.

Keywords: Claudin-8; migration; osteosarcoma; proliferation; SOX9 Tohoku J. Exp. Med., 2017 January, **241** (1), 55-63. © 2017 Tohoku University Medical Press

Introduction

Osteosarcoma (OS) is a highly malignant bone tumor in children and young adult (Luetke et al. 2014). About 0.02% adults and 0.06% children develop osteosarcoma (Siclari and Qin 2010). OS often occurs in long bone, like distal femur and proximal tibia (Saha et al. 2013). In addition, a high rate of tumor metastasis from bones to the lungs was observed in osteosarcoma (Geller and Gorlick 2010). Patients who received standard chemotherapy before and after surgery and then radiation had the 5-year survival rate of 60-70% (Sampson et al. 2015). Moreover, patients with metastatic and advanced tumors have only 23 months of median survival time (Fagioli et al. 2002). Current clinical trials have not improved the overall survival of osteosarcoma patients, and the understanding of molecular pathogenesis of osteosarcoma is limited. Hence, there is an urgent need to identify diagnostic molecular markers and develop novel approaches for treating osteosarcoma.

The transcription factor, sex determining region Y (SRY) related high-mobility group box 9 (SOX9) is a member of the SRY box gene superfamily (Foster et al. 1994). SOX9 plays a key role in the regulation of sex determination, intestinal differentiation, cartilage development and adult progenitor cell pool maintenance (Jakob and Lovell-Badge 2011). During endochondral bone formation, SOX9 expression is down-regulated in growth plate chondrocytes (Leung et al. 2011). The exact role of SOX9 in carcinogenesis and cancer progression is controversial because both oncogenic and tumor-suppressing functions of SOX9 have been described (Passeron et al. 2009; Cai et al. 2013; Yu et al. 2013). Furthermore, SOX9 expression is up-regulated in several tumor types, including lung adenocarcinoma, esophageal squamous cell carcinoma and breast cancer (Müller et al. 2010; Hong et al. 2015; Wang et al. 2015). It has been demonstrated that expression levels of SOX9

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mRNA and protein are significantly higher in osteosarcoma tissues (Zhu et al. 2013). However, the functional consequences of SOX9 overexpression remain unclear in osteosarcoma.

Tight junctions (TJ) are the main components of the intestinal epithelial barrier and mainly control cellular polarity and adhesion (Tsukita et al. 2001). The large Claudin family is one of the components of tight junction proteins (Günzel and Yu 2013). The Claudins consist 24-27 members in mammalians and each is expressed in a tissue-specific manner (Yu 2003). Besides, Claudins have been described as essential modulators of paracellular permeability (Matter and Balda 2007; Günzel and Yu 2013). Claudin-8 is restricted to the proximal tubule (PST), distal tubule and collecting duct in the mouse nephron (Li et al. 2004). In addition, Claudin-8 plays an important role in the paracellular cation barrier of the distal renal tubule (Hou et al. 2014).

Tumor cells exhibit abnormal tight junction function and decreased cell differentiation (Morin 2005). Several studies have shown the function of claudins in various cancers. For example, the role of Claudin-1 in tumorigenesis has been found, demonstrating that Claudin-1 may be an oncogenic or tumor-suppressing protein (Morohashi et al. 2007; Blanchard et al. 2009). Claudin-7 overexpression promotes the loss of tumor cell polarization and contributes to tumorigenesis (Darido et al. 2008). Moreover, a recent study has demonstrated that Claudin-8 (CLDN8) contributes to cell proliferation in human osteosarcoma cells (Xu et al. 2015).

The objectives of present study were to identify the relationship between SOX9 and CLDN8 in OS cell proliferation and migration and high expression of SOX9 on the clinical significance.

Materials and Methods

Cell culture

Human osteosarcoma cell lines (Saos-2 and U2OS cells) were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). The cell lines were cultured in DMEM supplemented with 10% FBS and incubated at 37°C in a humidified chamber with 5% CO₂.

Patients and tissue samples

Tumor tissues and adjacent non-tumor normal tissues were obtained from routine therapeutic surgery in Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University. This study was approved by the Ethics Committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University. Written informed consent was obtained from all the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

A total of 25 OS and corresponding non-cancerous tissue samples were snap-frozen in liquid nitrogen and stored at -80°C for qRT-PCR and Western blot analyses. A total of 97 OS and corresponding non-cancerous tissue samples were collected for immunohistochemistry analysis. These patients were diagnosed as osteosarcoma between 2005 and 2010. No patients in this study had radiotherapy or chemotherapy before the surgery. Clinical stages of these osteosarcoma patients were determined according to the sixth edition of the tumornode-metastasis (TNM) classification of the International Union against Cancer. The clinic pathological features of 97 patients are summarized in Table 1.

All 97 osteosarcoma patients received follow-up. The median follow-up was 36 months (range: 10-72 months).

Immunohistochemistry (IHC)

The expression of SOX9 and CLDN8 proteins was detected by IHC. Formalin-fixed, paraffin-embedded sections (5 μ m) were used. Then the sections were incubated with anti-SOX9 or anti-CLDN8 antibody (1:100, Santa Cruz Biotechnology, USA) followed by incubation with the corresponding secondary antibody conjugated with horseradish peroxidase. After that, the protein was visualized by DAB staining. Moreover, the degree of immunostaining was evaluated based on the percentage of positive tumor cells and the intensity of staining by two observers independently according to the methods described previously (Zhang et al. 2013). The percentage scoring of immunoreactive tumor cells was: 0 (no positive tumor cells), 1 (< 10% positive tumor cells), 2 (10% to 50% positive tumor cells) and 3 (> 50% positive tumor cells). In addition, the intensity of staining was scored as: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellowish brown) and 3 (strong staining, brown). A final immunoreactivity score was obtained for each case by multiplying the percentage and intensity score. Then, the expression level of SOX9 was categorized with the immunoreactivity score as "low" (< 5) or "high" (> 5).

Quantitative real-time PCR (qRT-PCR) assay

The expression levels of SOX9 and CLDN8 mRNAs in osteosarcoma tissues and adjacent non-tumor normal tissues were detected by qRT-PCR assays according to the methos described previously (Tang et al. 2013). Total RNA was extracted from tissue samples and cell lines using TRIzol (Invitrogen, Carlsbad, CA). M-MLV (Promega, USA) was used to reversely transcript RNA to cDNA, according to the manufacturer's protocols. The following primers were used to amplify equal cDNA amounts from each sample: SOX9,

Forward: 5'-CGAAATCAACGAGAAACTGGAC-3' Reverse: 5'-ATTTAGCACACTGATCACACG-3' CLDN8,

Forward: 5'-CCGTGATGTCCTTCTTGGCTTTC-3' Reverse: 5'-CTCTGATGATGGCATTGGCAACC-3' Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Forward: 5'-CCCACTCCTCCACCTTTGAC-3' Reverse: 5'-ATGAGGTCCACCACCCTGTT-3'

ABI 7900 thermo cycler (Applied Biosystems, USA) was used to perform qRT-PCR. Each cDNA sample was examined in triplicate. Quantification of the relative expression of mRNA was calculated by the 2^{-ddCt} method. GAPDH was used as an internal reference.

Western blot analysis

The expression of SOX9 and CLDN8 proteins was detected by Western blot analysis according to the methods described previously (Hollande et al. 2003). Cells or tissues were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 2% w/v SDS, 10% Glycerol, 32 mM 2-ME). After that, proteins were separated by 10% SDS-PAGE, and transferred to NC membranes (Amersham Bioscience, UK). The membranes were incubated with primary antibodies against SOX9, CLDN8 or GADPH (Cell signaling, USA) overnight. The transferred proteins were visualized with the ECL Detection Reagents (Amersham Biosciences). GAPDH was used to verify equal protein loading as a control. Each protein sample was examined in triplicate.

Lentivirus-delivered short hairpin RNA transfection

The short hairpin RNA (shRNA) against SOX9 (sh-SOX9) and its negative control shRNA (sh-NC) were designed and synthesized by QIAGEN (Shanghai, China). Saos-2 and U2OS cells were seeded into six-well plates at a density of 4×10^4 cells per well. Subsequently, the cells were transfected with shRNA-expressing lentivirus by using LipofectamineTM 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. After transfection for 24 h, the cells were used for further assay.

Cell proliferation assay

The effect of SOX9 on the proliferation of osteosarcoma cell lines was evaluated by the MTT (Sigma, USA) methods according to the protocols of the previous study (Zhang et al. 2012). Cells were seeded in 96-well plates. After transfected for 48 h, the cells were incubated with MTT (20 μ l, 5mg/ml) for 4 h at 37°C. Then 150 μ l DMSO was added to dissolve the formazan product. The formazan absorbance was analyzed with a microplate reader (Multiskan Mk3, Thermo Labsystems, Finland) at 595 nm.

Cell migration assay

Cell migration was detected using a transwell chamber $(8-\mu m)$ pore size, Millipore, USA); 5×10^4 cells (200 μ l) were seeded into the upper chamber. DMEM containing 10% FBS was added into the lower chamber as a chemoattractant. After 24 h of incubation, migrated cells on the lower membrane were counted under a microscope (Olympus, Japan).

TUNEL-positive cells assessment

To identify cell apoptosis after SOX9 knockdown, TUNEL was performed using an *in situ* cell death detection kit (Fluorescein dUTP Kit, Roche Inc., USA), according to the manufacturer's instructions. The number of TUNEL-positive cells (green) and the total number of cells (blue) in each field were calculated. After that, Image-Pro Plus 5.0 software was used to figure the ratio of two values automatically.

Luciferase reporter assay

To generate a CLDN8 gene promoter-luciferase reporter, the CLDN8 genomic DNA fragment containing a putative binding site for SOX9, as predicted by the MatInspector program (Genomatix, http://www.genomatix.de/matinspector.html), was amplified by PCR using the following primers: sense 5'-GGGTTTCTATGTCCTACT-3' and reverse 5'-TATACTTCTCGGTCTTCC-3'. A potential SOX9binding motif was found in the CLDN8 gene promoter region (-199 to -207). Then, the CLDN8 promoter sequence (792 bp) was inserted into the 5'-untranslated region of pGL3 luciferase reporter (Promega Corporation, USA). Total cDNA from Saos-2 cells was used to amplify full-length SOX9 cDNA by PCR. The full-length SOX9 cDNA fragment was cloned into pcDNA 3.1, generating pcDNA 3.1-SOX9. After transfection with the indicated constructs, the luciferase activity was determined by the dual-luciferase reporter assay system (Promega Corporation, USA) according to the manufacturer's instructions. Human embryonic kidney cells (HEK293T) cells were also used for transfection.

Statistical analysis

The software of SPSS 16.0 (SPSS Inc., USA) was used for statistical analysis. All data were expressed as the mean \pm SD Student's *t*-test was used to analyze differences between two groups. Survival analysis and their differences were estimated by Kaplan-Meier method and log-rank test respectively. Univariate and multivariate analysis of prognostic factors were analyzed by Cox regression (Proportional hazard model). Spearman rank correlation was used to analyze the correlation between CLDN8 protein expression and SOX9 protein expression. P < 0.05 was considered statistically significant.

Results

Upregulation of SOX9 and downregulation of CLDN8 at mRNA and protein levels in osteosarcoma tissues

The statistical analysis demonstrated that the expression levels of SOX9 mRNA were higher in osteosarcoma tissues than those in adjacent non-tumor tissues (Fig. 1A). Similarly, the expression levels of SOX9 protein were significantly higher in osteosarcoma tissues than those in adjacent non-tumor tissues (Fig. 1B). Conversely, the expression levels of CLDN8 mRNA and protein were lower in osteosarcoma tissues compared with those in adjacent nontumor tissues (Fig. 1C, D).

We also analyzed the expression patterns of SOX9 and CLDN8 in osteosarcoma tissues by IHC (Fig. 2), showing that the expression level of SOX9 was classified as "high" (immunoreactivity score > 5) in 56 of 97 (57.7%) osteosarcoma tissues. By contrast, the expression level of CLDN8 was classified as "low" in 62 of 97 (63.9%) osteosarcoma tissues. Importantly, as shown in Fig. 3A, there was the significant inverse correlation between zthe expression levels of SOX9 protein and CLDN8 protein (R = -0.633, P < 0.001).

Upregulation of SOX9 protein is related to the advanced clinic pathological features of osteosarcoma

To evaluate whether SOX9 was related to clinic pathological features of patients with osteosarcoma, we analyzed the association of SOX9 protein expression levels with sex, age, tumor size, clinical stages and distant metastasis (Table 1). High SOX9 expression was more frequently occurred in osteosarcoma with advanced clinical stage (P = 0.001) and positive distant metastasis (P = 0.003). However, no significant differences between the expression of SOX9 and sex, age, tumor size or histological subtype were observed.

Upregulation of SOX9 is associated poor prognosis of patients with osteosarcoma

Using Kaplan-Meier method and log-rank test, the overall survival of osteosarcoma patients with high SOX9 expression was significantly poorer than those with low SOX9 expression (Fig. 3B). Besides, as shown in Table 2, in univariate and multivariate regression analyses, includJ. Qi et al.



Fig. 1. The expression levels of SOX9 and CLDN8 mRNAs and proteins in osteosarcoma and corresponding non-cancerous tissues.

(A) Representative photographs and statistical analysis of SOX9 mRNA expression levels. (B) Representative photographs and statistical analysis of SOX9 protein expression levels. (C) Representative photographs and statistical analysis of CLDN8 mRNA expression levels. (D) Representative photographs and statistical analysis of CLDN8 protein Expression levels. ***P < 0.001 was considered as statistical significance. 'N' refers to adjacent non-tumor tissues, and 'O' refers to osteosarcoma tissues. Representative gels were derived from two different samples out of 25 samples. Note that the same tissue specimens were used to detect the expression levels of SOX9 and CLDN8 mRNAs and proteins.



Fig. 2. IHC images of SOX9 and CLDN8 expression in in osteosarcoma. The representative images show the immunohistochemical expression of SOX9 and CLDN8 in one chondroblastic osteosarcoma (Osteosarcoma) and its adjacent non-cancerous tissue (Normal) (magnification, ×100). Note that a total of 97 osteosarcoma and adjacent non-cancerous tissue samples were analyzed.

ing sex, age, tumor size, clinical stages, distant metastasis and SOX9 expression, we found clinical stages (hazard ratio [HR] = 3.335, 95% confidence interval [CI] = 1.952-

5.517, P < 0.001), distant metastasis status (HR = 2.907, 95% CI = 1.677-5.183, P < 0.001) and SOX9 expression (HR = 3.153, 95% CI = 1.824-5.299, P < 0.001) were inde-



Fig. 3. Kaplan-Meier curves for patients with osteosarcoma and the inverse correlation in the expression levels of SOX9 and CLDN8 proteins.

(A) The correlation between SOX9 protein expression and CLDN8 protein expression (R = -0.633, P < 0.001, Spearman rank correlation analysis, n = 97). (B) Overall survival curves for two groups defined by low and high expression of SOX9 in patients with osteosarcoma (n = 97).

 Table 1. Correlation between clinic pathological factors and SOX9 expression levels in patients.

Variable	Number of patients	SOX9 Low	SOX9 High	P value
		(%)	(%)	
Sex				0.605
Male	61	27 (44.3)	34 (55.7)	
Female	36	14 (38.9)	22 (61.1)	
Age (years)				0.819
< 50	46	20 (43.5)	26 (56.5)	
≥ 50	51	21 (41.2)	30 (58.8)	
Tumor size (cm)	•			0.443
< 5	62	28 (45.2)	34 (54.8)	
≥5	35	13 (37.1)	22 (62.9)	
Clinical stages				0.001
Ι	45	11(24.4)	34 (75.6)	
II/III	52	30 (57.7)	22 (42.3)	
Distant metastasis				0.003
Negative (N)	38	23 (60.5)	15 (39.5)	
Positive (P)	59	18 (30.5)	41 (69.5)	
Histological subtype				0.286
Osteoblastic	54	24 (44.4)	30 (55.6)	
Chondroblastic	22	9 (40.9)	13 (59.1)	
Fibroblastic	18	7 (38.9)	11 (61.1)	
Not specified	3	1 (33.3)	2 (66.7)	

Characteristics	Subset	Univariate analysis		Multivariate analysis	
		Hazard ratio	P value	Hazard ratio	P value
		(95% CI)		(95% CI)	
Sex	Male/Female	1.187 (0.689-1.746)	0.862		
Age	< 50/≥ 50	1.151 (0.706-1.685)	0.885		
Tumor size (cm)	< 5/≥ 5	1.416 (0.997-2.125)	0.373		
Clinical stages	I/(II/III)	3.335 (1.952-5.517)	< 0.001	2.363 (1.358-3.726)	< 0.001
Distant metastasis	N/P	2.907 (1.677-5.183)	< 0.001	2.257 (1.178-4.132)	0.004
SOX9	Low/High	3.153 (1.824-5.299)	< 0.001	2.067 (1.148-3.527)	< 0.001

Table 2. Univariate and multivariate regression analyses of parameters associated with prognosis of patients.

pendent prognostic markers for univariate analysis. For multivariate analysis, SOX9 expression (HR = 2.067, 95% CI = 1.148-3.527, P < 0.001), distant metastasis status (HR = 2.257, 95% CI = 1.178-4.132, P = 0.004) and clinical stages (HR = 2.363, 95% CI = 1.358-3.726, P < 0.001) were also independent prognostic markers.

Knockdown of SOX9 suppresses cell proliferation and migration but promotes cell apoptosis of osteosarcoma cells

To explore the effect of SOX9 knockdown on cell proliferation and migration, Saos-2 and U2OS cells were transfected with sh-SOX9 to inhibit the expression of endogenous SOX9. As shown in Fig. 4A, the mRNA expression of SOX9 significantly decreased in the Saos-2 and U2OS cells transfected with sh-SOX9 in contrast to the cells that transfected with sh-NC. Western blot analysis confirmed the downregulation of SOX9 expression at the protein levels (Fig. 4B). As shown in Fig. 4C and D, the relative cell number of Saos-2 and U2OS cells transfected with sh-SOX9 was much lower than those transfected with sh-NC. However, TUNEL assays showed the relative positive cells increased dramatically in the Saos-2 and U2OS cells transfected with sh-SOX9 than those in the cells transfected with sh-NC (Fig. 4E). Furthermore, we investigated the effect of SOX9 knockdown on cell migration, transwell migration assay revealed that Saos-2 and U2OS cells transfected with sh-SOX9 dramatically inhibited cell migration as compared with that in sh-NC group (Fig. 4F). These results indicated that knockdown of SOX9 could reduce cell proliferation and migration but could promote cell apoptosis of the Saos-2 and U2OS cells.

SOX9 directly targets CLDN8 gene promoter in osteosarcoma cells

To explore the role of SOX9 in CLDN8 expression, we knock-downed the SOX9 expression in Saos-2 and U2OS cells using sh-SOX9 (see Fig. 4B). Western blot analysis showed the expression level of CLDN8 was increased in the cells transfected with sh-SOX9 compared with those transfected with sh-NC (Fig. 5A). Thus, SOX9 may down-regulate CLDN8 expression in Saos-2 and U2OS cells. We then analyzed the effect of SOX9 on the CLDN8 promoter activity. The luciferase reporter assay showed the promoter activity of the CLDN8 promoter region in HEK293T, Saos-2 and U2OS cells (Fig. 5B). Importantly, the relative luciferase activity obtained with the CLDN8 promoter region was significantly decreased in cells that were co-transfected with SOX9 cDNA (Fig. 5B).

Discussion

SOX9 was originally considered as a chondrogenic transcription factor which was involved in bone formation (Südbeck et al. 1996). Moreover, SOX9 belongs to the SOX family of transcription factors which have a homologous high-mobility group (HMG) box DNA binding domain (Schepers et al. 2002). Recent studies found that SOX9 functioned as an oncogene in a variety of human cancers. For example, Liu et al. (2016) suggested SOX9 promoted cell proliferation and invasion in hepatocellular carcinoma. Wang et al. (2016) illustrated SOX9 was associated with cell proliferation, migration and invasion and SOX9 was an important functional mediator of miR-124 in lung adenocarcinoma cells. Zhang et al. (2012) found miR-101 suppressed SOX9-dependent tumorigenesis in human hepatocellular carcinoma. Together, these findings suggest that SOX9 may be a molecular marker that is involved in tumorigenesis.

It has been demonstrated that the expression levels of SOX9 mRNA and protein were higher in osteosarcoma tissues (Zhu et al. 2013). We also obtained the similar results in the present study and have provided the evidence that SOX9 may promote growth and metastasis of osteosarcoma. Zhu et al. (2013) reported the SOX9 was upregulated in aggressive osteosarcoma and associated with tumorigenesis (Zhu et al. 2013). Xu et al. (2014) found upregulation of SOX9 protein expression levels elevated cell







(A) SOX9 targets CLDN8 expression in Sa0s-2 and 020S cens.
(A) SOX9 knockdown increases the expression levels of CLDN8 protein. Western blot analysis showed the increased levels of CLDN8 protein in Saos-2 and U2OS cells transfected with sh-SOX9 or sh-NC (negative control). (B) Luciferase reporter assays in HEK293T, Saos-2 and U2OS cells. *P < 0.05 compared with the activity in cells transfected with pcDNA3.1 + pGL3; #P < 0.05 compared with the activity in cells transfected with pcDNA3.1 + pGL3-CLDN8.</p>

proliferation and invasion in osteosarcoma cells. In our study, knockdown of endogenous SOX9 expression by shRNA-expressing lentivirus significantly decreased the proliferation and migration of Saos-2 and U2OS cells. Conversely, TUNEL positive cell analysis showed that knockdown of SOX9 induced a significant increase in cell apoptosis. To the best of our knowledge, the current study provides the first evidence demonstrating that SOX9 may promote osteosarcoma cell growth by down-regulating CLDN8 expression.

Bioinformatic analysis demonstrated that CLDN8 was decreased in inflammatory bowel disease (Clark et al. 2012). Moreover, the expression of CLDN8 mRNA is down-regulated in tumor tissues (Gröne et al. 2007), and CLDN8 has been revealed as a candidate biomarker for the diagnosis in renal cell carcinoma and renal oncocytoma (Kim et al. 2009; Osunkoya et al. 2009). Recent research demonstrated CLDN8 functioned as an oncogenic factor and was up-regulated in OS cells (Xu et al. 2015). In contrast, our research showed the tumor-suppressing function of CLDN8 and the expression of CLDN8 was down-regulated in OS tissues. It has been suggested that the claudin expression is frequently altered based on the expression profiling of protein and gene in other cancers (Swisshelm et al. 2005; Hewitt et al. 2006). Hence, we speculated CLDN8 had both tumor-suppressing and oncogenic functions in osteosarcoma.

Recent results show that SOX9 is an essential mediator of the Tcf-4-driven down-regulation of Claudin-7 in colorectal cancer cells (Darido et al. 2008). Our work presented the first demonstration that CLDN-8 was negatively regulated by SOX9. The protein expression of CLDN8 was dramatically up-regulated in the Saos-2 and U2OS cells transfected with sh-SOX9. In addition, luciferase reporter assay proved CLDN8 promoter harbored a potential SOX9 binding site. Taken together, SOX9 promoted osteosarcoma cell growth by regulating CLDN8.

In the present study, we show the four major points. Firstly, the expression levels of CLDN8 mRNA and protein were lower in human osteosarcoma tissues than those in noncancerous bone tissues, while the expression of SOX9 was up-regulated in osteosarcoma. Secondly, the up-regulation of SOX9 expression in osteosarcoma tissues is associated with aggressive clinic pathological features. The Kaplan-Meter analysis suggests that osteosarcoma patients with high SOX9 expression had poorer overall survival, which is in agreement with previous study (Zhu et al. 2013). Thirdly, the univariate and multivariate analyses of regression clearly reveal SOX9 as an independent prognostic marker for osteosarcoma. Lastly, SOX9 may promote osteosarcoma cell proliferation and migration by targeting CLDN8 expression.

In conclusion, our data provide a novel insight into the function of SOX9 in human osteosarcoma and indicate that SOX9 promotes cell growth by repressing the expression of CLDN8. More importantly, SOX9 may be a potential prog-

nostic marker for osteosarcoma, which may provide an effective treatment strategy for osteosarcoma patients.

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Conflict of Interest

The authors declare no conflict of interest.

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