Int J Clin Exp Pathol 2016;9(10):9879-9888
www.ijcep.com /ISSN:1936-2625/IJCEP0026011

Original Article

Increased expression of SOX4 contributes to ovarian cancer progression

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Received February 15, 2016; Accepted June 24, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: SOX4 is suggested as a critical factor in many types of cancer progression. However, the function of SOX4 in ovarian cancer is poorly reported. This study is aim at revealing the expression pattern and biological function of SOX4 in ovarian cancer. We examined the SOX4 protein level in ovarian cancer tissues. Further, HO-8910 and SKOV3 were selected to carry out RNA interference. Cell proliferation, apoptosis, metastasis and relative protein levels of EMT pathway were analyzed respectively. Our data implicated that the SOX4 protein level is significantly higher in ovarian tumor tissues as comparing to adjacent normal tissues. And silencing of SOX4 in ovarian cancer cell lines inhibited cell proliferation and metastasis, but induced cell apoptosis. Moreover, depletion of SOX4 depressed epithelial-to-mesenchymal transition (EMT) process by up-regulating the protein expression of E-cadherin, while down-regulating the protein expression of Snail, MMP9 and Twist. In summary, this study demonstrates that SOX4 is highly expressed in ovarian cancer tissues and functions as an oncogene in ovarian cancer progression. And this result may identify SOX4 as a new and useful therapeutic target for ovarian cancer treatment.

Keywords: SOX4, ovarian cancer, apoptosis, metastasis, EMT pathwany

Introduction

SOX4 is a member of SOX (sex-determining region Y-related HMG box) family, that is characterized by a conversed high-mobility group (HMG) domain mediating the DNA binding, and divided into six subgroups on protein identify [1, 2]. SOX4, together with SOX11 and SOX12 belong to the SOXC subgroup sharing a highly conversed C-terminal region with different transactivation efficiencies [3]. SOX4 has been suggested as an essential factor in many developmental processes, such as the development of lung, thymus and nervous system [4]. And SOX4 is also involved in the determination of cell fate during organogenesis of heart, brain and pancreas [5]. Recent studies demonstrate that the abnormal expression of SOX4 is associated with many types of human cancers. Study on breast cancer suggested that over expressed SOX4 promotes the progression of breast cancer by orchestrating epithelical-mesenchymal transition (EMT) [6]. SOX4 is highly expressed and facilitates cell proliferation in colon carcinoma. In hepatocellular carcinoma, SOX4 is also expressed and promotes cell apoptosis by regulating p53 level [7]. Moreover, depletion of SOX4 inhibits metastasis of human lung cancer cells [8]. All these researches implicate that SOX4 may function as a critical factor in cancer progression.

Ovarian cancer ranks the third in genital cancer incidence, but is identified as the leading cause of death among guneacological malignancies [9, 10]. Ovarian cancer is frequently combined with a late diagnosis and a poor prognosis. Approximately 75% of ovarian cancer patients are diagnosed with advanced disease [11], and the cancer survival ratio just stands at 40% to 45% at 5 years post diagnosis. Most patients ultimately die of recurrent disease which is hardly cured. Ovarian cancer can be influenced and regulated by multiple genetic alterations or aberrant hormone secretion. Whereas, the precise pathogenesis and effective therapy for ovarian cancer is still missing understood. Therefore, it is essential to explore useful bio-

markers that can serve as novel targets for ovarian cancer therapy.

In this study, to reveal the expression pattern and biological function of SOX4 in ovarian cancer, we analyzed the SOX4 expression in ovarian tumor tissues. We then picked up two ovarian cancer cell lines, and examined the effects of SOX4 on cell proliferation, apoptosis, migration and invasion by transfection of SOX4-siRNA. We found that SOX4 protein is highly expressed in ovarian cancer tissue. Over expression of SOX4 may promote cell proliferation and metastasis, and suppressed cell apoptosis by up regulation of Bcl-2 level but down regulation of Bax level. Taken together, these data suggest that SOX4 may function as an oncogene in ovarian cancer progression.

Materials and methods

Tissue samples and cell culture

Tissue samples were obtained from debulking surgery from 20 patients with ovarian cancer at Women's Hospital School of Medicine Zhejiang University. All the samples were divided into two parts, tumor samples and adjacent normal samples, to make tissue slides for further experiments. Informed and written consent was obtained from all patients according to the ethics committee guidelines.

Human ovarian cancer cell lines A2780, HO-8910, CaOV3, SKOV3 and OVCAR3 were obtained from Shanghai Cell Bank, Chinese Academy of Science (Shanghai, China). A2780, CaOV3 and SKOV3 were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin. And HO8910 and OVCAR3 were cultured in RPMI-1640 (Hyclone) with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin supplemented. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Immunohistochemistry (IHC)

Sample slides were used for IHC assay. High pressure pretreatment was performed in 0.01 M citrate buffer (pH=6.0) for 15 min to antigen retrieval. Then tissue sections were blocked by incubation with peroxide block and goat serum, and sequentially incubated with rabbit polyclonal anti-SOX4 antibody (1:100, Abcam) following with HRP-labeled secondary antibody (1:300, Beyotime). Visualization of IHC was done using commercial DAB color developing reagent kit (Beyotime) according to the manufacture's procedure. Nuclei were countered stained with hematoxylin. Positive areas of sample slides were analyzed under microscope to evaluate the expression of SOX4 in ovarian cancer.

siRNA transfection

SOX4 siRNA and a non-specific scramble siRNA were designed and synthesized, and then transfected into HO8910 and SKOV3 cell lines. Silencing of SOX4 mediated by SOX4 siRNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. Simultaneously, non-specific siRNA were transfected into negative control (NC) cells. The targeting position of SOX4 siRNA was: 1330-1322; with sequence: 5'-CAAGATCCCTTTCATTCGA-3'. Proliferation, apoptosis, real-time PCR, western blot and metastasis assays were then preformed at 48 h post siRNA transfection.

Reverse transcription and real-time PCR (qRT-PCR)

RNA from ovarian cancer cell lines was isolated with TRIzol (Invitrogen) reagent according to the instruction of manufacture. Reverse transcription was applied with cDNA Synthesis Kit (Fermentas). To quantify the expression of SOX4 in cells, amplification was performed in an ABI-7300 Real-Time PCR System (ABI) and visualization was conducted with SYBR Green PCR Kit (Fermentas). The internal control was served with GADPH. Primer sequences were: SOX4 (NM_003107.2) Primer Forward: 5'-TGTAGGAGAGGGACTAAGTG-3', Primer Reverse: 5'-GAGACCGTGCTAAAGTAGAG-3'; GADPH (NM_001256799.1) Primer Forward: 5'-CACCCACTCCTCCACCTTTG-3', Primer Reverse: 5'-CCACCACCCTGTTGCTGTAG-3'.

Western blot

Cells were harvested after conditional treatment for western blot assay. Briefly, cells were lysed in ice-cold radio immunoprecipitation assay buffer (Solarbio) containing 0.01% protease inhibitor cocktail (Sigma), separated by SDS-PAGE and then detected by incubation

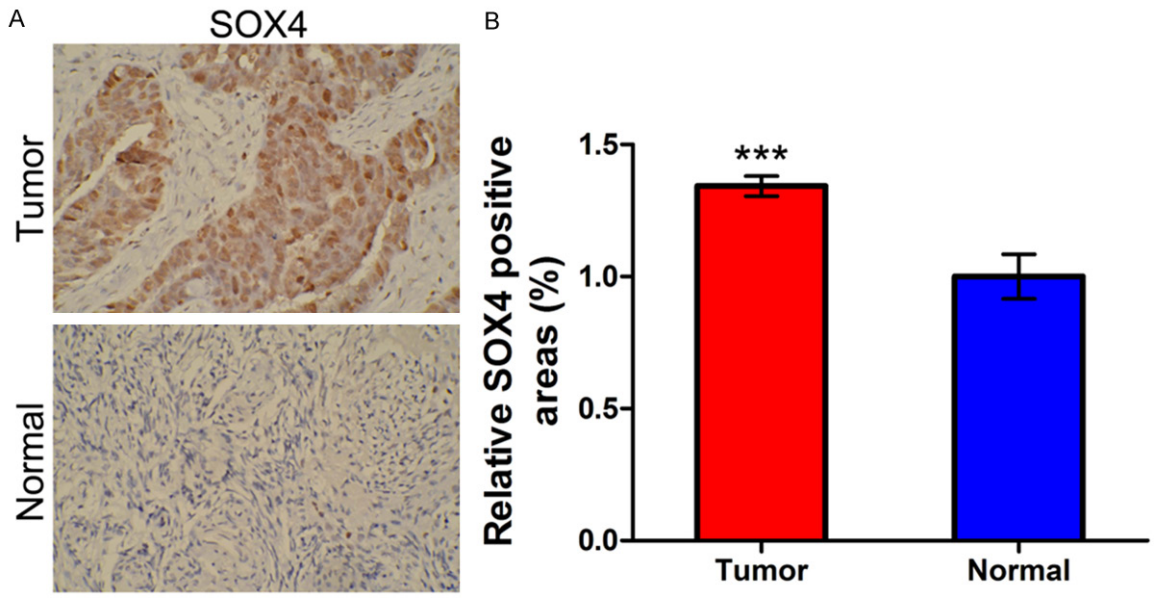

Figure 1. Protein level of SOX4 in ovarian cancer tissues. A. IHC staining of SOX4 in ovarian tumor and adjacent normal tissues (n=10, original magnification ×200). B. SOX4 positive areas of ovarian tumor and adjacent normal tissues (n=10). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

with appropriate antibodies respectively. The antibody list was as follow: SOX4 (1:50, Abcam), Bcl-2 (1:400, Santa), Bax (1:400, Santa), E-cadherin (1:1000, CST), MMP9 (1:500, Abcam), Twist (1:500, Abcam), Snail (1:1000, CST), GADPH (1:1500, CST) and HRP-labeled secondary antibodies (1:1000, Beyotime). The internal control was served with GADPH.

Proliferation assay

After SOX4 siRNA transfection, HO-8910 and SKOV3 were seeded into 96-well plates and examined at 0 h, 24 h, 48 h and 72 h using commercial Cell Counting Kit-8 (7seabiotech) according to the manufacture's procedure. Absorbance of treated cells was measured with excitation at 450 nm to evaluate cell proliferation.

Cell apoptosis assay

HO8910 and SKOV3 cells were harvested at 48 h post RNA interference, and then double stained with Annexin V-fluorescein isothiocyanate (FITC, Sigma) and propidium iodide (PI, Sigma). The double staining was performed using commercial Annexin V Apoptosis Detection Kit APC (eBioscience). And stained cells were then detected by flow cytometry (BD Biosciences). Three independent experiments were carried out for each cell lines.

Migration and invasion assay

To evaluate the migration of ovarian cancer cells, HO-8910 and SKOV3 were collected after siRNA transfection with serum starvation for 24 h and then seeded in upper chamber of a 24-well transwell chamber (Trueline) with a pore size of 8 μm. And the medium supplemented with 30% fetal bovine serum was placed in the lower chamber beforehand. After 24 h incubation, the cells migrating from upper side to the surface of lower membrane were fixed and stained with 0.05% crystal violet (Solarbio). And then, the stained cells were counted under the microscope.

In cell invasion assay, the transwell chambers contained a polycarbonate filter, of which the inserts of upper chambers were coated with 50 μl Matrigel (1:2 dilution, BD Biosciences). And the rest of invasion assay proceeded as migration assay described above.

Statistical analysis

At least three independent experiments were carried out for every assay and data were analyzed by SPSS 13.0 statistical package (SPSS, Inc, Chicago, IL). All data were expressed as mean (± SD) and analyzed with t-test for multi-

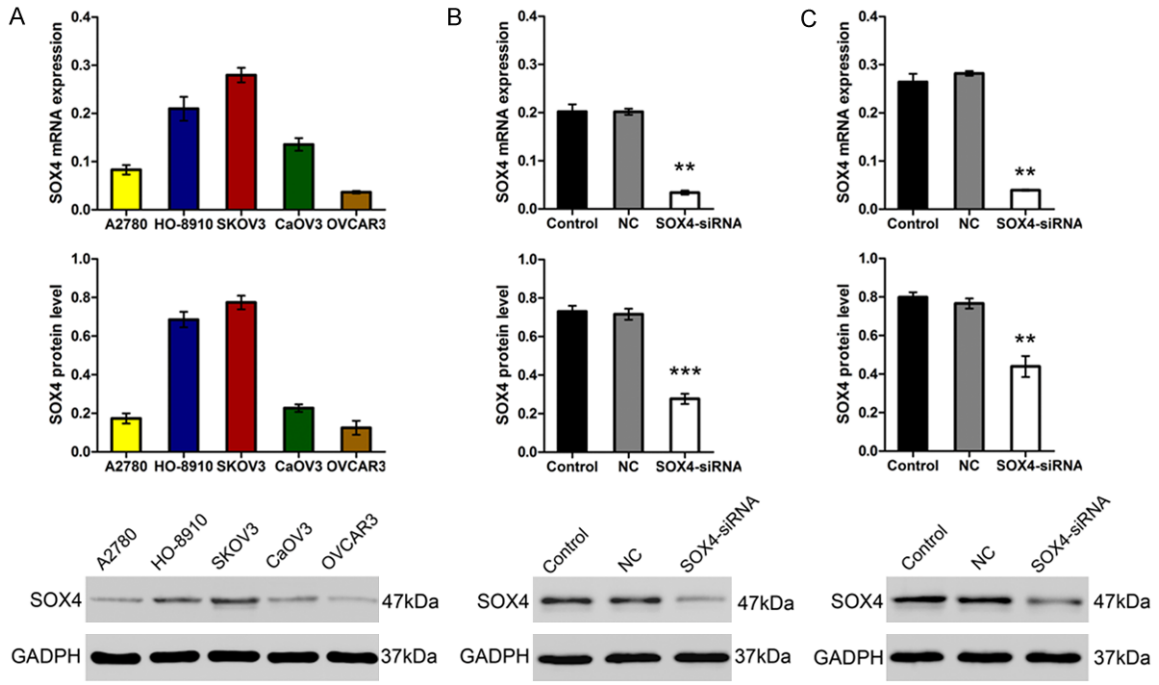

Figure 2. Expression of SOX4 in ovarian cancer cell lines and depletion of SOX4. A. SOX4 expression level in five ovarian cell lines examined by qRT-PCR and western blot (n=3). And HO-8910 and SKOV3 were selected to carry out further experiments. B and C. Depletion of SOX4 in HO-8910 and SKOV3 after SOX4-siRNA transfection (n=3). Data were analyzed by qRT-PCR and western blot. Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

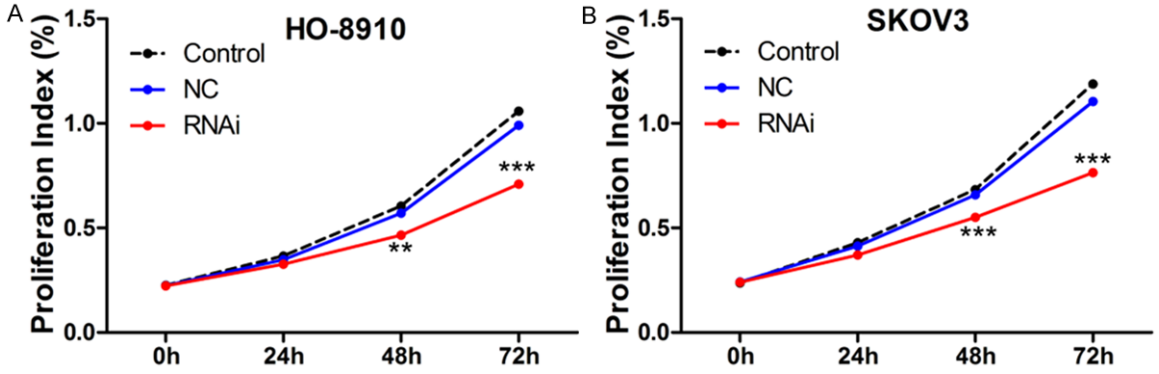

Figure 3. Silencing of SOX4 inhibited proliferation of ovarian cancer cells. A and B. Relative proliferation rates of HO-8910 and SKOV3 were detected at 0 h, 24 h, 48 h and 72 h post RNA interference (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

ple comparisons. *P*-value less than 0.05 was considered statistically significant.

Results

High expression level of SOX4 protein in ovarian cancer tissues

To verify the abnormal expression of SOX4 in ovarian cancer, we examined SOX4 protein expression level in paraffin-embedded ovarian tissue from 20 patients diagnosed as ovarian cancer by IHC staining. As shown in **Figure 1A**, SOX4 protein levels were significantly higher in tumor tissues as comparing to adjacent normal tissues. And the positive staining of SOX4 was mainly in cytoplasm of cancer cells. We then calculated the SOX4 positive staining areas in sample slides of tumor and corresponding non-

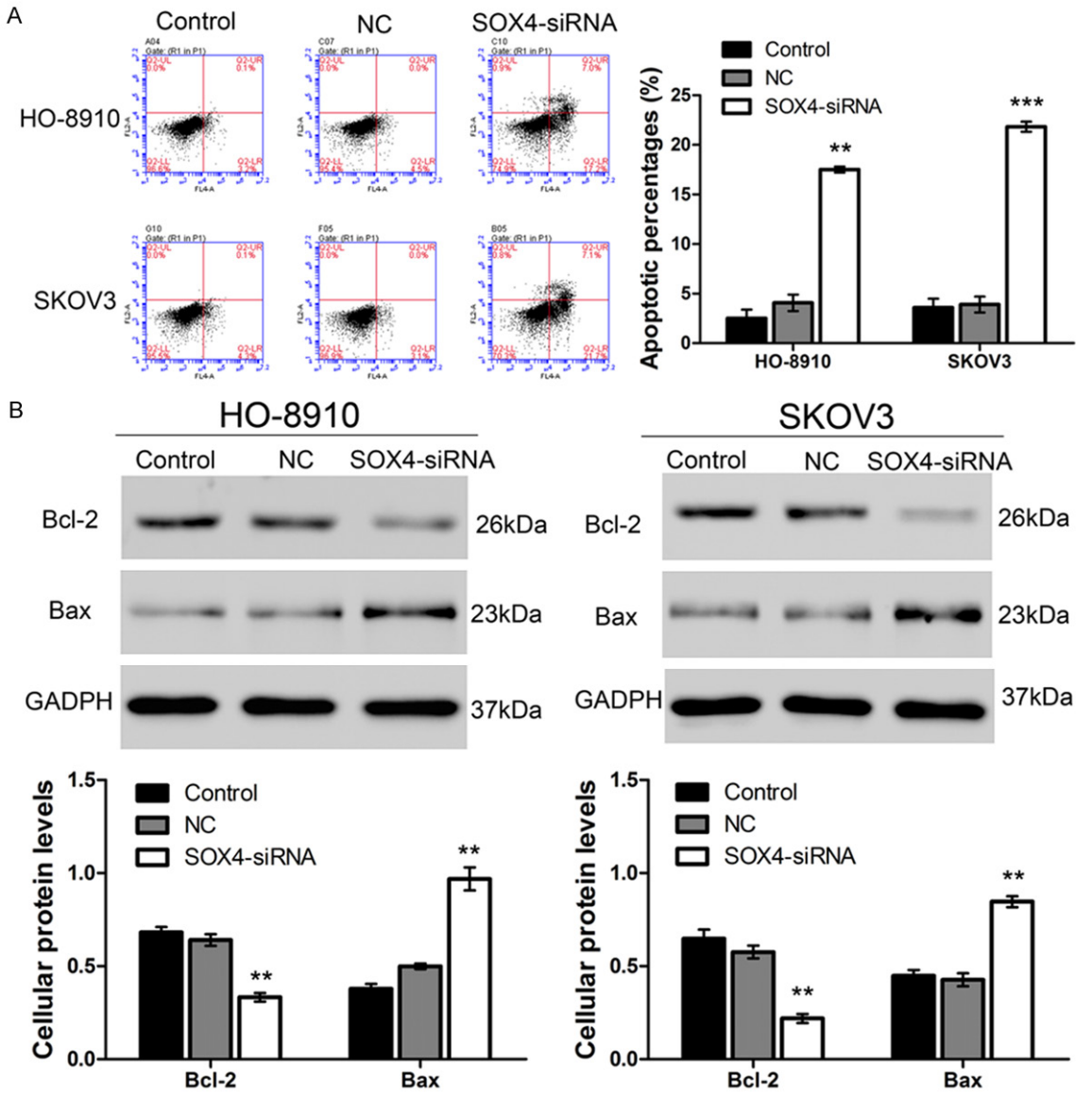

Figure 4. Silencing of SOX4 induced cell apoptosis of ovarian cancer. A. Apoptosis rates of HO-8910 and SKOV3 were analyzed by flow cytometry after double stained with annexin V-FITC/PI (n=3). Silencing of SOX4 obviously promoted apoptosis in ovarian cancer cells. B. Cellular protein levels of Bcl-2 and Bax in ovarian cancer cells were examined by western blot at 48 h post RNA interference (n=3). Expression of Bcl-2 protein was depressed, while the expression of Bax protein was elevated. Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

tumor tissues. The increase rate of SOX4 positive area is 34.29% in ovarian tumor relative to adjacent non-tumor tissues. All the data showed that SOX4 may over expressed in ovarian cancer.

Silencing of SOX4 in ovarian cancer cell lines

To further detected the biological function of SOX4 in ovarian cancer, we examined the expression of SOX4 mRNA and protein in five ovarian cancer cell lines, containing A2780, HO-8910, CaOV3, SKOV3 and OVCAR3, using qRT-PCR and western blot respectively. As shown in **Figure 2A**, both SOX4 mRNA expression and endogenous protein level were notably higher in HO-8910 and SKOV3 compared to other three cell lines. According to the result, HO-8910 and SKOV3 were picked up to carry out further experiments.

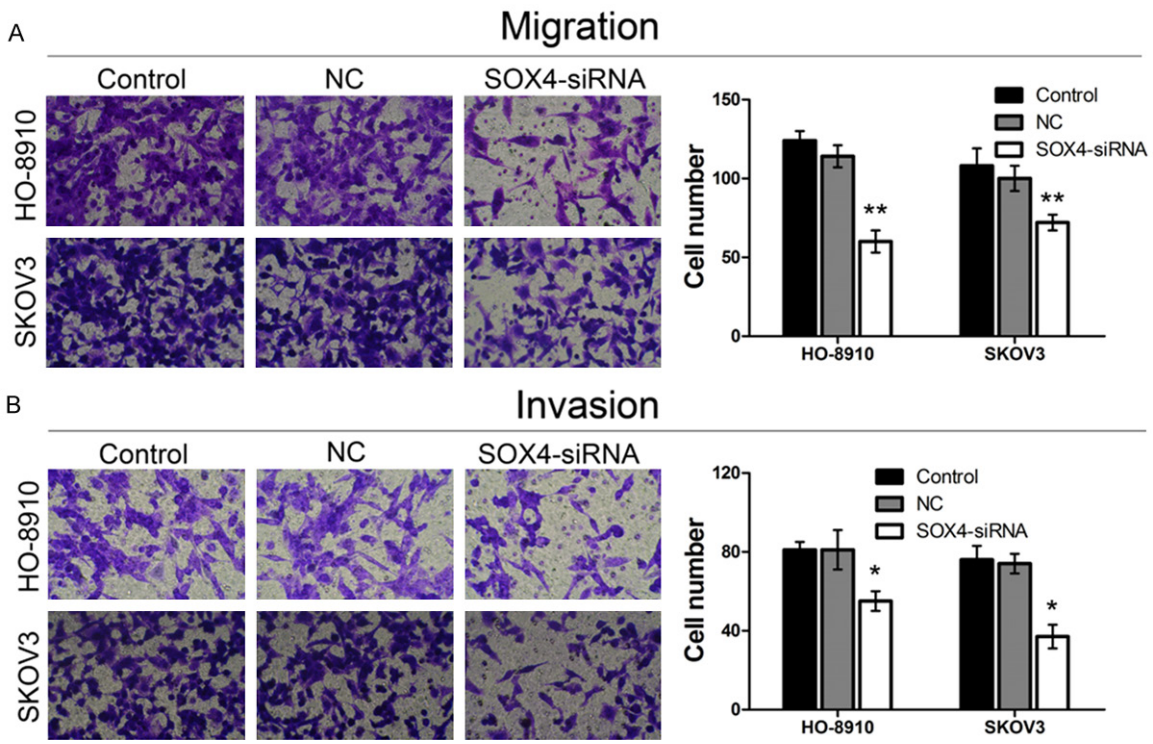

Figure 5. Silencing of SOX4 suppressed metastasis of ovarian cancer cells. HO-8910 and SKOV3 were analyzed using transwell assay. The numbers of migrated and invaded cancer cells were calculated at 48 h post RNA interference. A and B. Migrated and invaded ovarian cancer cells were examined under the microscope (n=3). And metastasis of ovarian cancer cells was significantly inhibited by depletion of SOX4. Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

RNA interference was performed to silence the expression of SOX4 in ovarian cancer cells. SOX4-siRNA and non-specific siRNA were synthesized and transfected into HO-8910 and SKOV3 cells. As **Figure 2B** and **2C** showed, SOX4 expression in two cell lines was suppressed obviously post SOX4-siRNA transfection. The expression inhibition rates of mRNA and protein were 82.04% and 62.12% in HO-8910, 85.11% and 45.00% in SKOV3 respectively. Whereas, endogenous SOX4 expression showed little affect after non-specific siRNA transfection.

Inhibition of cell proliferation by SOX4 knockdown

In an effort to reveal the effects of SOX4 on proliferation of ovarian cancer cells, we analyzed relative growth rate of HO-8910 and SKOV3 using CCK-8 assay at 0 h, 24 h, 48 h and 72 h after siRNA transfection. As shown in **Figure 3A** and **3B**, knocking down of SOX4 inhibited cell proliferation of HO-8910 and SKOV3 significantly. And obvious proliferation repression was detected during 48 h post RNA interference. These data revealed that SOX4 may promote ovarian cancer cell growth.

Effect of SOX4 silencing on cancer cell apoptosis

As silencing of SOX4 expression inhibited proliferation of ovarian cancer cells, SOX4 may involved in the regulation of cell apoptosis. We then analyzed cell apoptosis of HO-8910 and SKOV3 at 48 h post RNA interference. Cell apoptosis ratio was measured by flow cytometry. And as **Figure 4A** showed, cell apoptosis was obviously promoted by knockdown of SOX4 compared to control. 7-fold and 6-fold increase rates were examined in RNA interference HO8910 and SKOV3 cells respectively.

Sequentially, to investigate the activation mechanism of cell apoptosis, we measured relative protein expression levels of Bcl-2 and Bax using western blot at 48 h after siRNA transfection. As shown in **Figure 4B**, cellular Bcl-2 protein expression in cancer cells was

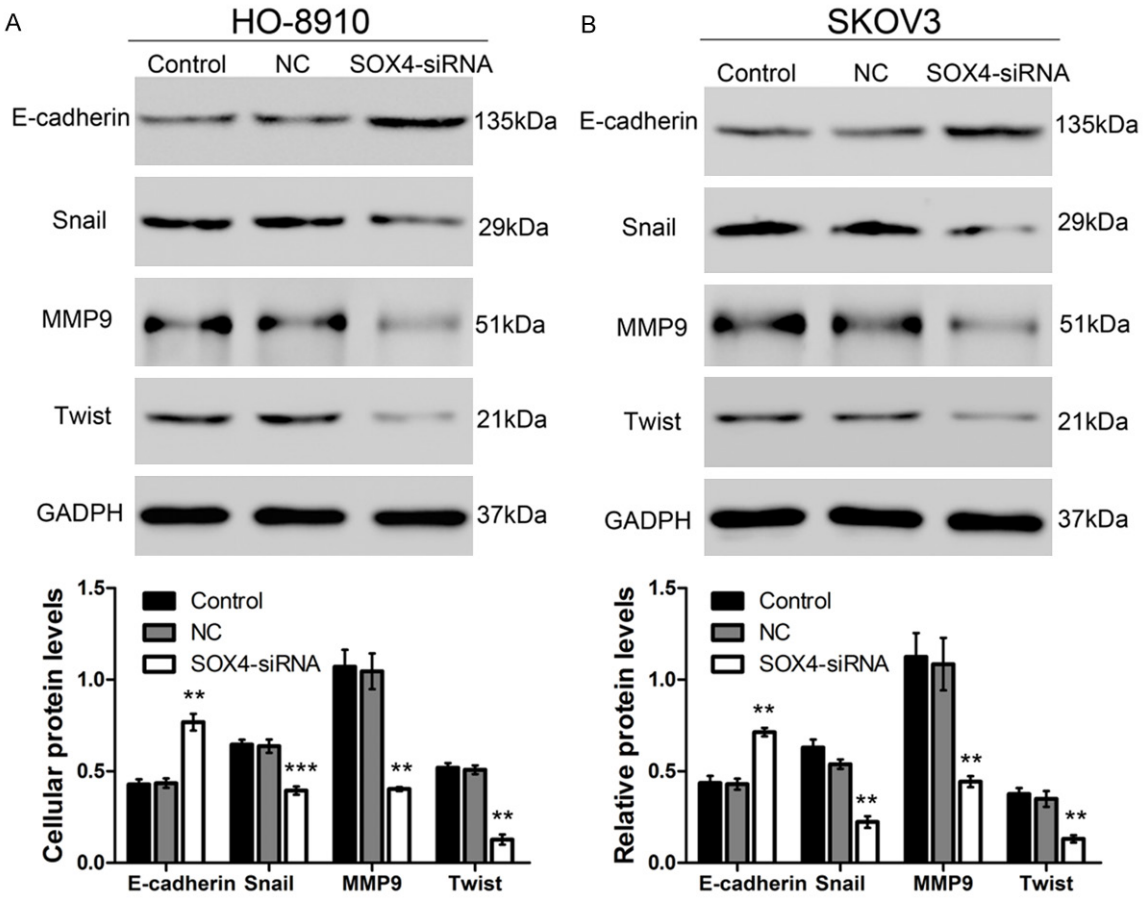

Figure 6. Knockdown of SOX4 affected inducer factors of EMT pathway in ovarian cancer cells. Relative protein levels were examined at 48 h after RND interference using Western blot. A and B. SOX4 knockdown increased cellular E-cadherin level, but decreased the levels of cellular Snail, MMP9 and Twist obviously in HO-8910 and SKOV3 respectively (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (as comparing to controls).

notably repressed by depletion of SOX4, with decrease ratios 51.18% in HO-8910 and 68.33% in SKOV3. Whereas, endogenous Bax protein level was significantly higher in RNA interference cells as comparing to controls (increase ratios: HO-8910, 155.92% and SKOV3, 88.97%). NC siRNA transfection slightly decreased Bcl-2 protein level and promoted Bax expression which did not reach statistical significant. Summarily, silencing of SOX4 facilitated ovarian cancer cell apoptosis with regulation of Bcl-2 and Bax protein expression.

Inhibition of metastasis caused by SOX4 knocking down

To reveal the biological function of SOX4 on tumor metastasis, we analyzes cell migration and invasion by transwell assay. Significant inhibition was induced in both HO-8910 and SKOV3 by depletion of SOX4. As shown in **Figure 5**, migrating cell numbers were: HO8910, Control 124 ± 6, NC 114 ± 7, SOX4-siRNA 60 ± 7; and SKOV3, Control 108 ± 11, NC 100 ± 8, SOX4-siRNA 75 ± 5. And the numbers of invasive cells were: HO8910, Control 81 ± 4, NC 80 ± 10, SOX4-siRNA 55 ± 5; and SKOV3, Control 76 ± 7, NC 74 ± 5, SOX4-siRNA 37 ± 6. All these data suggested that SOX4 may facilitate metastasis of ovarian cancer cells.

Effects of SOX4 knockdown on EMT pathway

As SOX4 may promote migration and invasion of ovarian cancer cells, we then examined cellular protein levels of essential factors in EMT pathway, including E-cadherin, MMP9, Snail and Twist, using western blot during 48 h post RNA interference. As showed in **Figure 6**, protein expression of E-cadherin was obviously

facilitated by SOX4 silencing with nearly 1-fold increase ratio in HO-8910 and SKOV3 respectively. However, endogenous protein levels of MMP9, Snail and Twist were obviously decreased in siRNA transfected cells compared to controls.

Discussion

As the carcinoma of highest death rate among gynecologic malignancies, ovarian cancer needs to explore novel targets and effectively biomarkers for therapeutic approaches. Increasing evidence has demonstrated that SOXC subgroup is involved in many types of human cancer processes, the role of which in cell proliferation, apoptosis and metastasis are still remain controversial [4]. Overexpression of SOX11 is reported in epithelial ovarian cancer. And highly expressed SOX11 is identified as a regulator of cell growth and a new prognostic marker in epithelial ovarian cancer [12, 13]. Abnormal expression of SOX4 is also found in varieties cancer types. In prostate cancer [14, 15], breast cancer [16], liver cancer [17] and colon carcinoma, SOX4 is highly expressed in vivo and in vitro. Whereas, the expression level of SOX4 is decreased in gallbladder carcinoma and melanoma [18, 19]. In ovarian cancer, SOX4 is just suggested as a target of miRNA-138 to depress invasion and metastasis of ovarian cancer cells [20]. The expression pattern and biological function of SOX4 in ovarian cancer have been poorly reported. In our study, we examined the protein level of SOX4 in ovarian cancer tissues using IHC staining, and evaluated the SOX4 positive areas. We found protein level of SOX4 was significantly higher in ovarian tumor as comparing to adjacent normal tissues. We investigated the effects of SOX4 on cancer process in selected two ovarian cancer cell lines. SOX4-siRNA was transfected into HO-8910 and SKOV3 to silence SOX4 expression. The data showed that cell proliferation was obviously inhibited by depletion of SOX4. Therefore, overexpression of SOX4 may contribute to malignant growth of ovarian cancer cell.

Analysis on apoptosis in this study implicated that suppressing of SOX4 promoted cell apoptosis in ovarian cancer. Further western blot assay revealed that, cellular protein level of Bcl-2 was promoted, which of Bax was decreased post RNA interference. Bcl-2 is an anti-apoptotic factor that is classified as an oncogene. Up-regulated Bcl-2 can specifically assumed stable heterodimer with Bax to prevent cell apoptosis caused by Bax, which is identified as apoptosis activator inducing the release of cytochrome *c* [21]. Induced apoptosis by over expressed SOX4 is suggested in human prostate cancer [14] and hepatocellular carcinoma [7]. In these studies, nuclear expression of SOX4 inhibited the transcriptional activity of p53 specifically without p53 somatic mutations. And the expression of Bax can be suppressed by down-regulation of p53 protein [22, 23]. We conjecture the promoted apoptosis may be involved in the regulation of p53down regulation caused by highly expressed SOX4 in ovarian cancer. And the relationship between SOX4 and p53 in ovarian cancer will be considered in our future works.

Metastasis is a critical factor affecting survival rate of ovarian cancer. We analyzed cell migration and invasion of HO8910 and SKOV3. The data suggested silencing of SOX4 depressed metastasis of ovarian cancer cells. Researchers have reported SOX4 can promote metastasis by inducing EMT progression in colorectal cancer, prostate cancer and breast cancer [15, 24, 25]. So we further examined the protein levels of EMT factors. We found SOX4 knocking down notably increased the level of E-cadherin, but decreased the protein levels of Snail, Twist and MMP9. E-cadherin acts as a pivotal factor in epithelial cell-cell adhesion. Loss of E-cadherin is implicated in many metastasis of cancer [26, 27]. Inducers of EMT containing Snail and Twist are able to transcriptionally inhibit the expression of E-cadherin to promote EMT progression in cancer cells [28, 29]. MMP9 belongs to MMPs family that is involved in tumor metastasis. MMP9 causes the degradation of IV collagen in basement membranes [30]. And recent studies up-regulated MMP9 is related to growing malignant potential and poor prognosis of ovarian cancer [31, 32]. Summarily, overexpression of SOX4 promoted metastasis by inducing EMT in ovarian cancer.

In conclusion, our study implicates that SOX4 is over expressed in ovarian cancer tissues, and the abnormal expression promotes malignant proliferation of ovarian cancer cells in vitro. Highly expressed SOX4 in ovarian cancer cell depresses cell apoptosis through regulating

cellular protein levels of Bcl-2 and Bax. Moreover, metastasis of ovarian cancer cell is facilitated with up-regulating the inducers' protein level of EMT pathway caused by SOX4 over expressed. Therefore, we propose that SOX4 may be a useful therapy target for the treatment of ovarian cancer.

Acknowledgements

This work was supported by research fund from the Zhejiang traditional Chinese medical science and technology program (No. 2014ZA073).

Disclosure of conflict of interest

None.

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