Prostate Cancer and Prostatic Disease (2013) 16, 301–307

www.nature.com/pcan

ORIGINAL ARTICLE

SOX4 is associated with poor prognosis in prostate cancer and promotes epithelial–mesenchymal transition *in vitro*

L Wang[1,2,6], J Zhang[3,6], X Yang[1], YWY Chang[4], M Qi[1], Z Zhou[5], J Zhang[5] and B Han[1,5]

BACKGROUND: The SOX4 transcription factor is involved in the development and cell fate decision. Although upregulation of SOX4 has been described in human prostate cancer (PCa), the prognostic value of SOX4 and its exact role in PCa progression remain unclear.
METHODS: Three tissue microarrays were constructed from 241 Chinese PCa patients who underwent TURP. Immunohistochemistry (IHC) was used to detect the expression of SOX4. Genetic aberrations of epidermal growth factor receptor and HER2 were detected by fluorescence *in situ* hybridization. The effect of SOX4 on proliferation was evaluated by MTT (methyl thiazolyl tetrazelium), and cell migration and invasion were evaluated by transwell and wound-healing assays. The distribution of cell-cycle phase was analyzed by flow cytometry. Real-time PCR and western blot were used to study transcript and protein levels.
RESULTS: Using tissue microarray, we found that SOX4 was overexpressed in 33.0% (76/230) Chinese PCa patients by IHC. SOX4 overexpression was significantly associated with high Gleason scores ($P = 0.009$) and the presence of distant metastasis ($P = 0.023$). Additionally, SOX4 overexpression was significantly correlated with high Ki67 labeling index ($P = 0.005$) and tended to associate with amplification of HER2 ($P = 0.052$) in our cohort. Notably, SOX4 was correlated with cancer-specific mortality of PCa patients by Kaplan–Meier analysis ($P = 0.001$). Multivariate Cox regression analysis indicated that SOX4 was an unfavorable independent prognostic factor in Chinese PCas ($P = 0.017$). SOX4 overexpression enhanced proliferation of Vcap cells and siRNA knockdown of SOX4 significantly decreased Vcap cell migration and invasion, suggesting a role of SOX4 in cancer metastasis. Additionally, flow cytometry DNA analysis revealed that siRNA SOX4 leads to significant accumulation of cells in the S phase and marked decrease of cells in the G2/M phase. Further *in vitro* study revealed that SOX4 silencing could inhibit TGF-β (transforming growth factor-β)-induced epithelial–mesenchymal transition (EMT) in Vcap cells. Overexpression of SOX4 could promote the EMT phenotype in Vcap cells.
CONCLUSIONS: Our results define an important role for SOX4 in the progression of PCa by orchestrating EMT and may serve as a prognostic marker for PCa patients.

Prostate Cancer and Prostatic Disease (2013) **16,** 301–307; doi:10.1038/pcan.2013.25; published online 6 August 2013

Keywords: SOX4; epithelial–mesenchymal transition; prognosis; metastasis

INTRODUCTION

Prostate cancer (PCa) is a common heterogeneous disease with marked variability in progression.[1] Currently, the established prognostic factors (Gleason score, pathological stage and serum PSA) cannot precisely distinguish indolent PCas from highly aggressive cancers.[1,2] Thus, novel prognostic biomarkers are urgently needed for PCa management.

The SOX4 (sex-determining region Y-box 4) gene, a member of the SOX family, has been shown to have important roles in the development and cell fate decision.[3] It is overexpressed in many types of human cancers, including leukemias,[4] glioblastomas[5] and cancers of the liver,[6] lung,[7] urinary bladder[8] and prostate.[9] Emerging data, although preliminary, suggested that SOX4 is an oncogene in PCa. Liu *et al.*[9] reported that SOX4 can induce anchorage-independent growth in non-malignant RWPE cells. In addition, SOX4 was one of the most common retroviral integration sites, resulting in increased mRNA.[10] Furthermore, genome-wide promoter analyses indicated that SOX4 directly regulates a number of genes that are important for metastasis,[11] such as epidermal growth factor receptor (*EGFR*), Rac1, Paxillin and Integrin,[11] suggesting a potential link between SOX4 and PCa metastasis. Although correlation of SOX4 overexpression with a high Gleason score or tumor stage was identified in patient samples, outcome analysis of SOX4 has not yet been performed in PCa.

Epithelial–mesenchymal transition (EMT), an early embryonic development program in which cells convert from the epithelial to the mesenchymal state, has been shown to have a critical role during cancer progression and metastasis.[12] During this process, the epithelial cells acquire mesenchymal cell morphology through the downregulation of epithelial markers and the upregulation of mesenchymal markers.[13,14] It has been reported that the upregulation of SOX4 may produce an EMT phenotype in liver and breast cancer.[6,15] However, the link between SOX4 and the EMT process is unclear in the context of PCa progression. In the current study, we systematically characterized the expression and prognostic significance of SOX4 in a large cohort of Chinese PCa patients ($n > 200$) for the first time. Furthermore, the roles of SOX4 in EMT processes of PCa, as well as the relationship between

[1]Department of Pathology, Shandong University Medical School, Jinan, China; [2]Research Center for Medicinal Biotechnology, Shandong Academy of Medicinal Sciences, Jinan, China; [3]Department of Pharmacology, Shandong Provincial Hospital, Jinan, China; [4]Department of Health and Disease and Psychology, University of Tornoto, Markham, Ontario, Canada and [5]Department of Pathology, Shandong University Qilu Hospital, Jinan, China. Correspondence: Dr B Han, Department of Pathology, Shandong University Medical School, #44, Wenhua Xi Road, Jinan 250012, China.
E-mail: boh@sdu.edu.cn
[6]These authors contributed equally to this work.
Received 16 April 2013; revised 8 June 2013; accepted 27 June 2013; published online 6 August 2013

SOX4 expressions with other molecular markers, were also investigated.

MATERIALS AND METHODS

Patients and tissue microarray construction

Our study consisted of 241 PCA patients with TURP between 2001 and 2012 at the Qilu Hospital of Shandong University (Jinan, China), Shandong Provincial Hospital (Jinan, China) and General Hospital of Linyi (Linyi, China). All of these patients were hospitalized due to symptoms of lower tract urinary obstruction. A total of 25.2% PCa patients in the current study had transrectal ultrasound-guideds biopsy. None of the patients received preoperative hormone or radiation therapy. The patients with incidental PCas were excluded from the cohort. Androgen-deprivation therapy was followed, and follow-up data were available for 210 patients, ranging from 1 to 127 months (mean 37 months). A total of three tissue microarrays were constructed. Two cores (1.0 mm in diameter) were taken from each representative tumor focus, and the morphology was confirmed by three pathologists (MQ, BH and XY). Detailed clinical and pathological profile were obtained from medical records and maintained in a secure relational database with tissue microarraydata. Patient demographics are shown in Table 1. This study was approved by the Institutional Review Board at the School of Medicine of Shandong University.

Table 1. Clinicopathological demographics of the 241 Chinese prostate cancer patients

Parameters	*Count*	*Percentage (%)*
Age (years)		
<60	23	10
60–69	56	24
$\geqslant 70$	155	66
Gleason scores		
<7	30	12
$=7$	86	36
>7	125	52
Tumor stage at diagnosis		
$\leqslant$T2	168	77
T3	26	12
T4	25	11
Preoperative PSA level (ng ml^{-1})		
$\leqslant 4$	22	10
4–10	27	13
10–20	26	13
>20	137	64
Distant metastasis		
No	165	79
Yes	45	21
Cancer-specific death		
No	157	75
Yes	53	25

Fluorescence *in situ* hybridization (FISH)

FISH analysis for *EGFR* and *HER2* gene aberrations was performed according to the manufacturer's protocol using the GLP *EGFR*/CSP 7 probe and GLP *HER2*/CSP17, respectively (GP Medical Technologies, Beijing, China). Slides were examined using an ImagingZ1 microscope (Carl Zeiss, Oberkochen, Germany). FISH signals were scored manually ($\times 100$ oil immersion) in morphologically intact and non-overlapping nuclei by a pathologist (BH), and a minimum of 50 cancer cells from each site were recorded. Cancer sites with very weak or no signals were recorded as insufficiently hybridized. Cases lacking tumor tissue in all the three cores were excluded. A previously documented method was utilized to validate genetic aberrations of EGFR and HER2.[16,17] Briefly, specimens were considered to carry amplification of EGFR gene when $>10\%$ of tumor cells displayed either EGFR: CEP 7 ratio >2 or countless tight clusters of signals of the locus probe (3–5 copies). Specimens were considered to carry amplification of HER2 gene when $>10\%$ of tumor cells displayed either HER2: CEP 17 ratio >2 or countless tight clusters of signals of the locus probe (3–5 copies).

Immunohistochemistry (IHC)

IHC was performed as previously described.[17] IHC staining was done using the standardized labeled streptavidin biotin (LSAB) kit (DakoCytomation, Carpinteria, CA, USA) according to the manufacturer's instructions. Antigen retrieval was performed by microwave pretreatment in 0.01 M citrate buffer (pH 6.0) for 15 min. The slides were incubated with rabbit polyclonal anti-SOX4 antibody (1:100 dilution, Abcam, Cambridge, MA, USA) or mouse monoclonal anti-Ki67 antibody (1:100 dilution, Dako, Carpinteria, CA, USA).

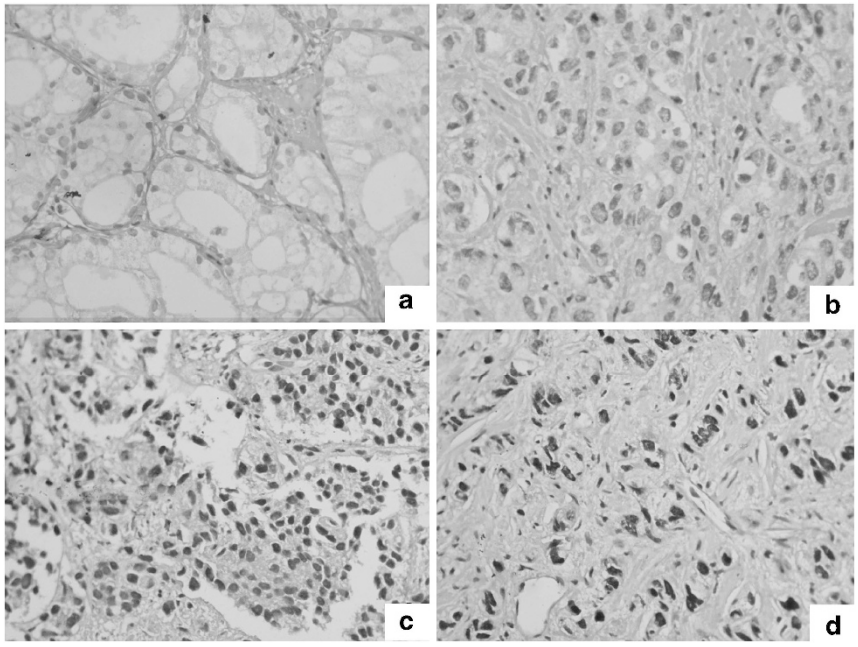

Figure 1. Expression of sex-determining region Y-box 4 (SOX4) protein in Chinese prostate cancer (PCa) patients by immunohistochemistry (PV-9000 two-step method). (**a**) Negative staining, $\times 400$; (**b**) weak staining, $\times 400$; (**c**) moderate staining, $\times 400$; and (**d**) strong staining, $\times 400$.

Table 2. Relation of SOX4 expression with clinicopathological parameters in Chinese prostate cancer patients

Clinicopathological variables	*Number of cases*	*SOX4 protein expression (%)*		P
		Negative or weak positive	*Moderate and strong*	
Age (years)				0.114
⩽65	42	24 (57)	18 (43)	
>65	179	125 (70)	54 (30)	
Gleason scores				0.009
<7	29	24 (83)	5 (17)	
=7	81	59 (73)	22 (27)	
>7	119	72 (61)	47 (39)	
Tumor stage at diagnosis				0.085
⩽T2b	167	114 (68)	47 (32)	
⩾T3a	47	27 (57)	20(43)	
Preoperative PSA level (ng ml^{-1})				0.965
⩽4	21	15 (71)	6 (29)	
4–10	22	15 (68)	7 (32)	
>10	150	108 (72)	44 (28)	
Distant metastasis				0.023
M0	165	111 (67)	54 (33)	
M1	45	22 (49)	23 (51)	
HER2 amplification				0.056
No	187	128 (68)	59 (32)	
Yes	12	5 (42)	7 (58)	
EGFR amplification				0.224
No	196	130 (66)	66 (34)	
Yes	2	0 (0)	2 (100)	
Ki67 LI (%)				0.005
⩽10	204	143 (70)	61 (30)	
>10	24	10 (42)	14 (58)	

Abbreviations: EGFR, epidermal growth factor receptor; LI, labeling index; SOX4, sex-determining region Y-box 4.

The slides were evaluated blindly by two independent observers (BH and XY), and the following scoring system was utilized to validate SOX4 expression.[18] The nuclear SOX4 staining was scored into four grades, which were classified by its staining intensity: 0, 1+, 2+, and 3+. The percentages of SOX4-positive cells were scored into five categories: 0 (0%), 1(1–25%), 2(26–50%), 3(51–75%), and 4 (76–100%). A final score was built by multiplying the scores of these two parameters, which are defined as follows: 0, negative; 1–3, weak; 4– 6, moderate; and 8–12, strong. The Ki67 labeling index was defined as the fraction of tumor cells showing any nuclear Ki67 immunoreactivity and was considered high if ⩾10% of the tumor nuclei were stained. For this purpose, 100–200 tumor cells were analyzed for each case.

Cell culture and treatment

Human PCa cell lines Vcap, PC3 and DU-145 were from the American Type Culture Collection (Rockville, MD, USA) and were cultured following the manufacturer's recommendations. For experiments, Vcap cells were stimulated with 5 ng ml^{-1} of transforming growth factor-β (TGF-β; RD Biosciences, San Diego, CA, USA) for 72 h and then harvested and lysed.

In vitro overexpression of SOX4

Human SOX4 cDNA (NM_003107.2) was subcloned into the pcDNA3.1 eukaryotic expression vectors. SOX4 and empty control plasmids were independently transfected into Vcap cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

siRNA-mediated SOX4 knockdown

Three SOX4-specific siRNAs were designed and synthesized by GenePharma (Shanghai, China), and the most effective single siRNA (sense strand: 5′-GGACAGACGAAGAGUUUAA-TT-3′ and anti-sense strand: 5′-UUAAACUCUUCGUCUGUCC-TT-3′) was used for further experiments, Non-specific negative control siRNAs were also designed and synthesized (sense strand: 5′-UUCUCCGAACGUGUCACG-3′ and anti-sense strand: 5′-ACGUGACACGUUCGGAGAATT-3′). The mock group was defined as the ones supplemented with the transfection reagent only.

Analysis of cell-cycle phase distribution by flow cytometry

After 24 h of siRNA SOX4, Vcap cells were stained with 1 ml propidium iodide (0.1 mg ml^{-1} with 0.1% TritonX-100) and incubated in the dark for 30 min. The samples were analyzed by flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ, USA).

Reverse transcription PCR (RT-PCR) and qRT-PCR analyses

Total RNA was extracted using Trizol (Invitrogen) and reverse transcription was done as described before.[19] The primers for each gene in RT-PCR and qRT-PCR were the same and are as listed in Supplementary Table S1. GAPDH was used as internal loading controls. Three independent experiments were completed; each reaction was performed in triplicate.

Western blot analysis

Western blotting was performed as previously described.[20,21] The membrane was incubated with primary antibodies for SOX4 (Abcam, Cambridge, Cambridgeshire, UK), β-catenin (Cell Signaling Technology, Danvers, MA, USA), Vimentin (Cell Signaling Technology) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase; Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The signals were detected with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK).

MTT (methyl thiazolyl tetrazelium) assay, migration and invasion assays

Cell viability was measured by MTT assays as described previously.[22] Briefly, 1×10^4 cells per well in 200 μl medium were seeded in a 96-well plate and were cultured for 24–48 h. For each assay, 20 μl MTT was added to each well and incubated for 4 h. After incubation, culture medium was discarded and 150 μl dimethyl sulfoxide was added. The absorbance was determined at 490 nm. All experiments were performed in triplicates.

Wound-healing experiments were performed to investigate the migratory properties of the cells. A wound was created by a p200 pipette tip on cells grown to confluence using six-well plates, and the cell-free space was measured on photographs taken at both 0 and 48 h. The invasion assays were performed by plating 10^4 cells per well into a 24-well transwell chamber with 8.0–l0-um pore polycarbonate filter inserts (Costar, Cambridge, MA, USA) in serum-free medium. Inserts were placed in a well containing normal growth medium with 10% fetal bovine serum. After 24 h, the cells in the upper compartment were removed completely by gentle swabbing. The migrated cells attached to the bottom side were fixed in 100% methanol and stained by the crystal violet method. For quantification, cells were counted in five randomly selected microscopic fields ($\times$ 200). Three independent experiments were performed.

Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences, version 19.0 (SPSS, Chicago, IL, USA). Two-sided Student's *t*-test and Mann–Whitney test were used for statistical comparisons; correlations between SOX4 overexpression with clinicopathological parameters were evaluated by the Spearman's test. The Kaplan–Meier method and Cox regression hazard tests were applied for the analysis of follow-up data, and hazard ratio (HR) with 95% confidence intervals (CI) were calculated. A *P*-value of <0.05 was considered significant.

RESULTS

SOX4 protein expression in Chinese PCas and relation to clinicopathological variables

Predominantly nuclear staining of SOX4 was observed. SOX4 protein levels were greater in cancer cells than in adjacent benign prostatic epithelia (Supplementary Figure S1). For analysis, we combined both negative and weakly SOX4-positive tumors into one group and compared it with moderately and strongly SOX4-positive PCas. Overall, SOX4 overexpression was identified in 76 (33.0%) of the 230 interpretable cases, demonstrating moderately or strongly positive staining, whereas 154 (67.0%) were SOX4

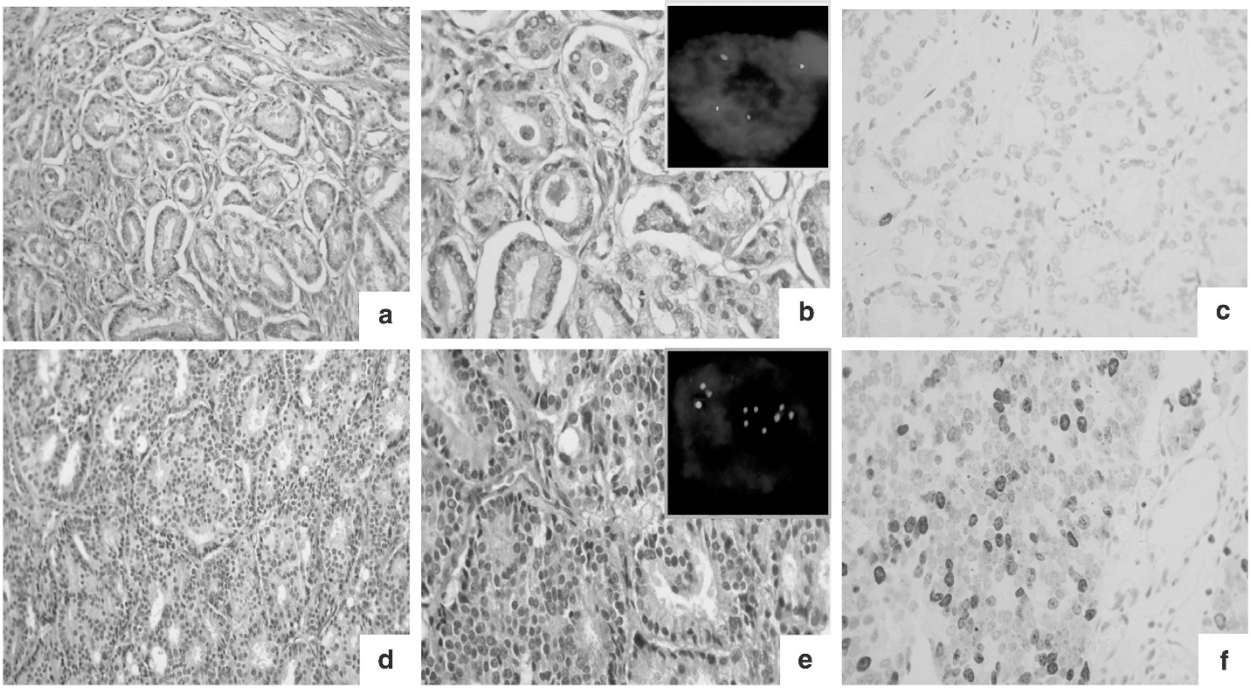

Figure 2. Examples of HER2 fluorescence in situ hybridization (FISH) images and Ki67 by immunohistochemistry (IHC) in prostate cancer (PCa). A Gleason 3 + 3 tumor was illustrated in **a**–**c**; (**a**) HE staining, × 200; (**b**) HE staining, × 400, yellow box area shows representative FISH image of HER2 without amplification. The ratio of red to green signal was 1. (**c**) IHC staining of Ki67, × 400; a Gleason 4 + 4 tumor was shown in **d**–**f**; (**d**) HE staining, × 200; (**e**) HE staining, × 400, green box area shows representative FISH image of HER2 with amplification. The ratio of red to green signal was > 2. (**f**) IHC staining of Ki67, × 400.

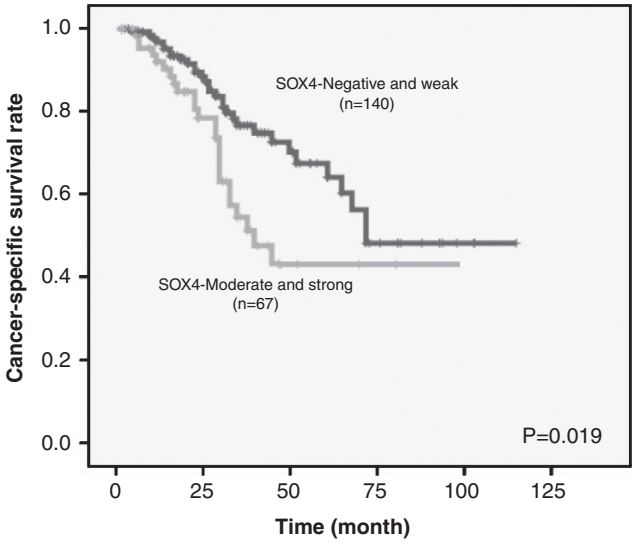

Figure 3. Kaplan–Meier survival analysis of Chinese prostate cancer patients with and without sex-determining region Y-box 4 (SOX4) overexpression using cancer-specific death as the end point.

negative or weakly positive. Representative IHC images of SOX4 are shown in Figure 1.

The relationship between SOX4 expression level and clinicopathological variables are summarized in Table 2. Notably, SOX4 overexpression was significantly associated with a high Gleason score ($P = 0.009$) as well as the presence of distant metastasis ($P = 0.023$). Although it was not found to be statistically significant, PCa cases with SOX4 overexpression tend to show high T (tumor) stage ($P = 0.085$). No significant association was identified between SOX4 expression levels and patients' age ($P = 0.114$) or preoperative PSA levels ($P = 0.965$).

Table 3. Univariable and multivariable Cox regression analysis

	Hazard ratio (95% CI)	P-*value*
Univariate analysis		
Preoperative PSA level	0.61 (0.42–0.90)	0.031
Clincial tumor stage (⩾T3 vs⩽T2)	2.15 (1.18–3.94)	0.013
HER2 amplification	7.10 (2.32–12.26)	<0.001
SOX4 overexpression	2.27 (1.37–3.76)	0.001
Multivariate analysis		
Clincial tumor stage (⩾T3 vs⩽T2)	1.87 (1.11–3.18)	0.02
HER2 amplification	4.62 (1.34–15.95)	0.016
SOX4 overexpression	2.07 (1.14–3.78)	0.017

Abbreviations: CI, confidence interval; SOX4, sex-determining region Y-box 4.

Association between SOX4 expression with other molecular markers

Genetic aberrations of EGFR and HER2 are relevant genomic aberrations in PCa, and EGFR has been suggested to be a direct transcriptional target of SOX4.[11] In our study, amplification of HER2 was present in 6.0% (12/199) of PCa cases. As shown in Table 2, HER2 amplification occurred more frequently in cases with SOX4 overexpression (10.8%, 7/65) as compared with those PCa cases without SOX4 overexpression (3.8%, 5/133; $P = 0.056$). EGFR amplification was only found in 2 of the 203 analyzed cases (0.99%), and both of them showed SOX4 overexpression. Ki67 was a classic proliferation biomarker, and previous studies have shown Ki67 to be an independent predictor of outcome in PCa patients.[23] In the current study, high Ki67 labeling index (>10% nuclei staining) was present in 10.5% (24/228) of PCa cases. Notably, a significant association was identified between Ki67 positivity and SOX4 overexpression. ($P = 0.005$, Table 2). Representative FISH images of HER2 and IHC image of Ki67 are shown in Figure 2.

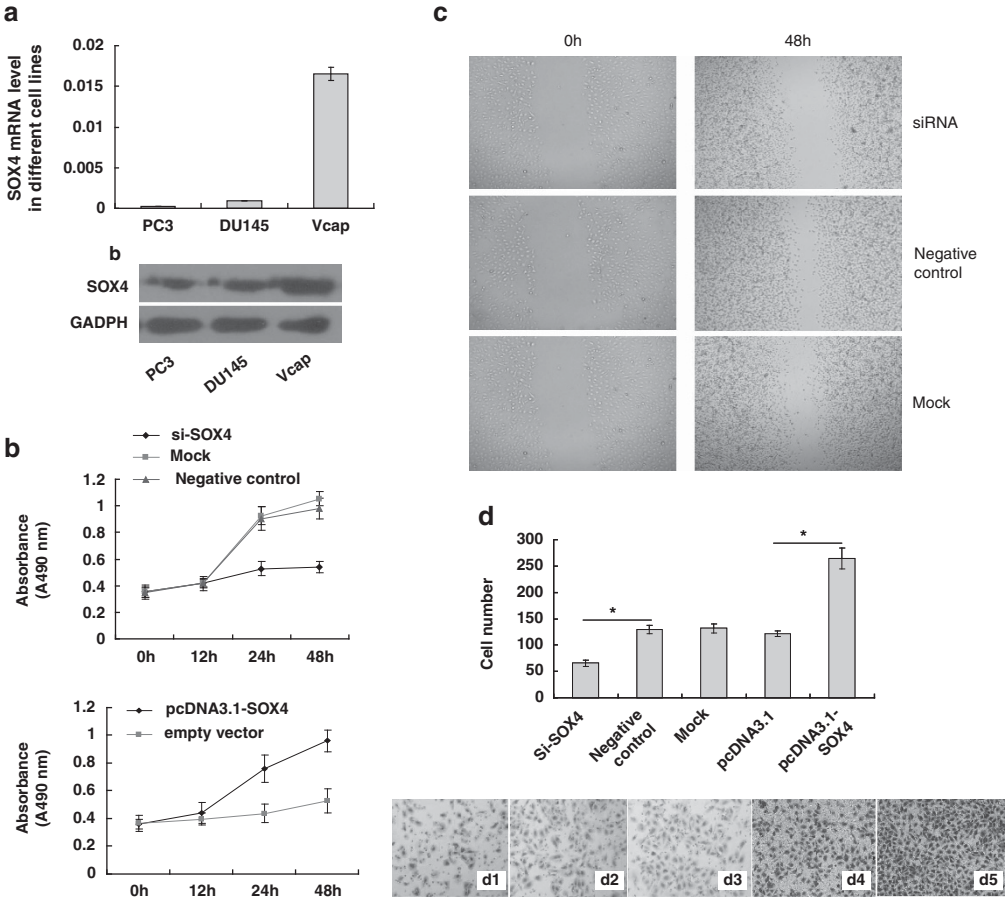

Figure 4. Sex-determining region Y-box 4 (SOX4) promoted cellular proliferation, migration and invasion of prostate cancer (PCa) cells. (**a**) mRNA and protein expression levels of SOX4 (relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) in different PCa cells; upper panel, quantitative reverse transcriptase–PCR (qRT-PCR); lower panel, western blot. (**b**) Cell viability as assessed by MTT (methyl thiazolyl tetrazelium) assay at different time points ranging from 0 to 48 h in Vcap cells. Points, mean of triplicate experiments; bars, s.d. (**c**) Effects of siRNA SOX4 on Vcap cell migration by wound-healing assay. Original magnification, × 100. (**d**) Cellular invasive capacity of Vcap cells (d1–d5) as assessed by invasion chambers; (d1) siRNA-SOX4; (d2) Mock; (d3) negative control; (d4) empty vector; and (d5) pcDNA3.1-SOX4. The data shown were the means ± s.d. of triplicate experiments; $^*P<0.01$.

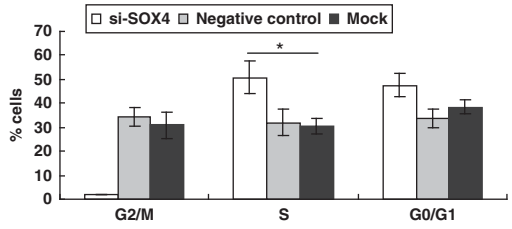

Figure 5. Sex-determining region Y-box 4 (SOX4) knockdown induces S cell-cycle arrest in Vcap cells. Cell-cycle distribution was monitored by flow cytometry using a propidium iodide-staining assay. $^*P<0.01$.

SOX4 overpexression correlates with cancer-specific mortality of PCa patients

We compared cancer-specific survival rates between patients with or without SOX4 overexpression in univariate and multivariate models. The group of patients who were with SOX4 overexpression had a much greater rate of mortality than patients who were without SOX4 overexpression ($P=0.019$; Figure 3). On the basis of the Kaplan–Meier survival estimates, SOX4 overexpression was significantly linked to cancer-specific mortality in our cohort.

In univariate Cox regression analysis, SOX4 overexpression was a strong predictor for cancer mortality (HR = 2.27, 95% CI = 1.37–3.76, $P=0.001$; Table 3). In multivariate analysis using stepwise backward entering of covariates (T stage and HER2 amplification), the independent values of SOX4 to predict time to cancer-specific death was significant (HR = 2.07, 95% CI = 1.14–3.78, $P=0.017$), which follows after HER2 amplification ($P=0.016$), but superior to clinical T stage at diagnosis ($P=0.02$). All of the aforementioned data strongly suggested that SOX4 was an independent and unfavorable prognostic indicator in Chinese PCas.

The expression levels of SOX4 in human PCa cell lines

qRT-PCR and western blot were used to determine the expression levels of SOX4 in a panel of human PCa cell lines. As shown in Figure 4a, mRNA and protein expression levels of SOX4 were significantly higher in Vcap, compared with those in PC3 and DU-145 cells.

SOX4 promotes cellular proliferation, migration and invasion of PCa cell lines

Using the MTT assay, we found that SOX4 overexpression in Vcap cells promoted cell growth after 48 h, whereas knockdown of SOX4 in Vcap cells significantly decreased cell proliferation at 24 and 48 h after treatment, when compared with negative controls (Figure 4b; $n=3$, $P<0.05$).

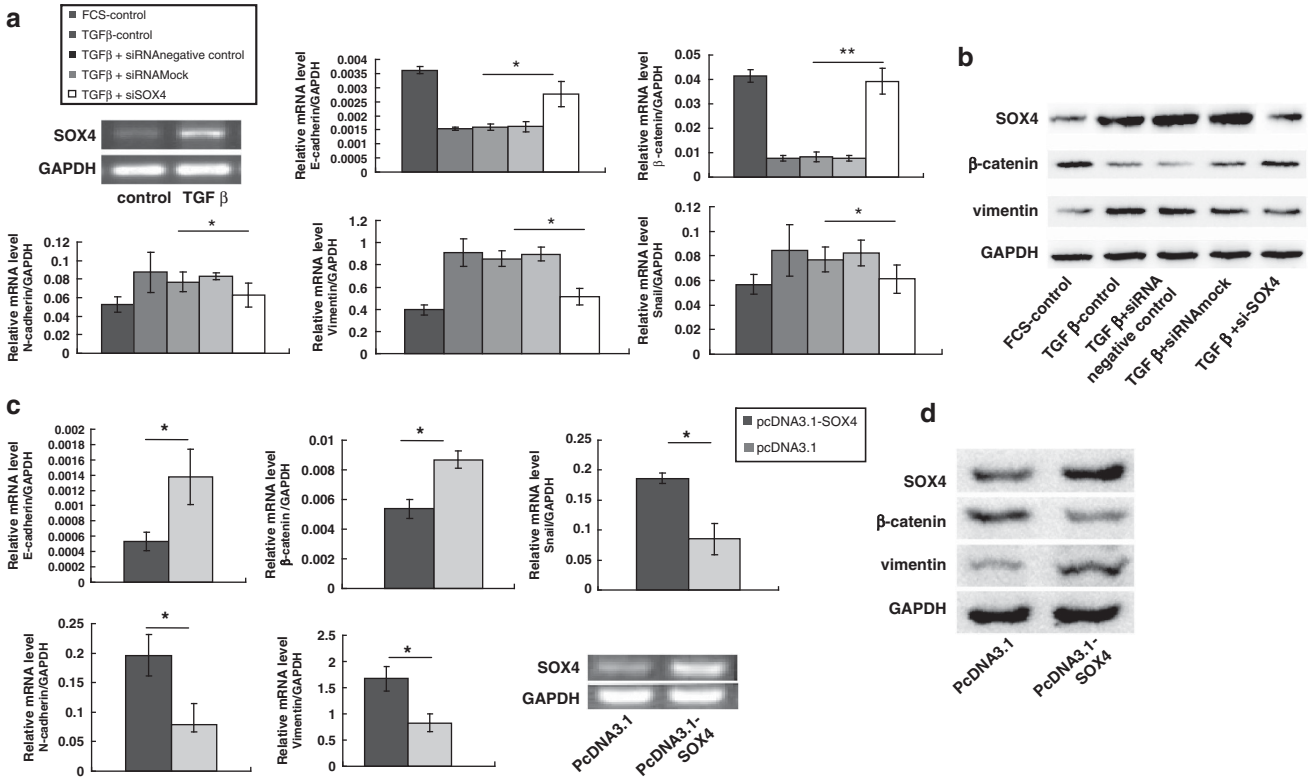

Figure 6. Sex-determining region Y-box 4 (SOX4) promotes epithelial–mesenchymal transition (EMT) regardless of transforming growth factor-β (TGF-β) status in prostate cancer (PCa) cells. (**a**) Expression of mesenchymal markers (Vimentin, N-cadherin and Snail) and the epithelial markers (E-cadherin, β-catenin) was shown after siRNA SOX4 in TGF-β-induced Vcap cells. (**b**) The protein expression levels of SOX4, β-catenin and Vimentin was presented by western blot after siRNA SOX4 in TGF-β-induced Vcap cells. The effects of SOX4 overexpresion on EMT-related markers were detected by quantitative reverse transcriptase–PCR (**c**) and western blot (**d**) after pcDNA3.1-SOX4 transfection in Vcap cells. Representative results from triplicate experiments are shown as mean ± s.d. (*$P<0.05$, **$P<0.01$). All of the relative expression of target genes was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Wound-healing assay found that siRNA-SOX4-transfected Vcap displayed a significant decrease in cell migration ability compared with various control conditions ($P<0.05$, Figure 4c).

To further examine the effect of SOX4 on cell invasion, siSOX4-transfected Vcap cells and PcDNA3.1-SOX4-transfected Vcap cells were cultured on a transwell apparatus. The percentage of migrated cells was significantly less in both siSOX4-treated groups when compared with the negative control groups (for both, $P<0.05$). PcDNA3.1-SOX4-transfected Vcap cells showed increased percentage of migrated cells (for both, $P<0.05$; Figure 4d).

Analysis of cell-cycle phase distribution by flow cytometry

In Vcap cells, flow cytometry DNA analysis revealed that siRNA SOX4 led to significant accumulation of cells in the S phase (50.7%) and marked decrease of cells in the G2/M phase (from 34.3 to 1.8%). This confirmed that siRNA SOX4 blocked DNA replication in the S phase of the cell cycle (Figure 5).

In vitro effect of SOX4 on EMT

TGF-β is known to have a major role in EMT induction, which induce an elongated fibroblast-like morphology with scattered distribution in cultured Vcap cells (see in Supplementary Figure S2). We firstly transiently knocked down SOX4 and found its downregulation suppressed the TGF-β-induced EMT, as shown by inhibition of the increase of N-cadherin ($P=0.043$), Vimentin ($P=0.036$) and Snail ($P=0.027$) and the decrease of E-cadherin ($P=0.015$) and β-catenin ($P=0.0018$) at mRNA levels (Figure 6a). The repression of Vimentin and concomitant induction of β-catenin after silencing SOX4 in TGF-β-induced Vcap cells were also validated by western blot (Figure 6b). By contrast, as shown in Figures 6c and d, in the SOX4 overexpressing Vcap cells, E-cadherin ($P=0.026$) and β-catenin ($P=0.031$), both of which are epithelial markers, were significantly downregulated. Yet the expression levels of mesenchymal markers, N-cadherin ($P=0.027$), Vimentin and Snail ($P=0.019$) were significantly upregulated.

DISCUSSION

To the best of our knowledge, this is the first study to systematically characterize SOX4 expression in a Chinese PCa cohort treated with TURP. All of the study patients had symptoms of lower tract urinary obstruction and therefore represented a select subgroup of clinically recognized PCas. This differed from most Western patients who were found to have PCa due to PSA screening and were treated often with radical prostatectomy. Of note, the patients with incidental PCas were excluded, and our cohort included a subset of patients with high-grade PCa. Here, for the first time, we showed that the overexpression of SOX4 is an independent unfavorable prognostic factor in Chinese PCa patients. However, one limitation of our study is the variable follow-up times, which ranged from 1 to 127 months. It is also limited by the length of time of follow-up. Longer follow-up of the cohort will be required to better assess the prognostic role of SOX4 in Chinese PCa patients.

Although SOX4 may act as a tumor suppressor, many reports suggested that SOX4 has an oncogenic role in multiple cancers.[8,18,24,25] Our study showed that SOX4 knockdown

inhibited Vcap cell proliferation, and this is partly due to the accumulation of cells in the S phase of the cell cycle. Of note, a significant association was identified between high Ki67 labeling index and SOX4 overexpression by IHC in clinical samples.

More importantly, our results suggested overexpression of SOX4 significantly correlated with the presence of distant metastasis. Additionally, siRNA knockdown of SOX4 significantly decreased Vcap cell migration and invasion, which are two of the most critical events in the process of cancer progression toward metastasis. *In vivo* study, Tavazoie *et al.*[26] found that partial knockdown of SOX4 by shRNA resulted in fewer lung metastases by using a xenograft model of breast cancer. Similarly, siRNA SOX4 reduced *in vivo* tumorigenesis and metastasis of liver cancer.[6] Therefore, SOX4 might be a metastasis contributor in PCa progression, and functional characterization of SOX4 in PCa using animal models merits further investigation.

EMT is a developmental program and associated with PCa progression and metastasis.[12] In 2008, Liao *et al.*[6] reported that SOX4 overexpression may produce an EMT phenotype in liver cancer. A recent study has shown that ectopic overexpression of SOX4 in immortalized human mammary epithelial cells positively regulated the expression of known EMT inducers, also activating the TGF-β pathway to contribute to EMT.[15] In our study, SOX4 promotes the TGF-β-induced EMT, and alterations of SOX4 expression could also disturb EMT phenotype in PCa cells. Taken together, these data suggested that SOX4 may contribute to PCa metastasis by initiating a transcriptional program that enables EMT phenotype.

EGFR and HER2 are two EGFR family growth factor receptors and known to regulate tumor progression in many different cancer types.[27,28] Of note, it has been reported that EGFR is one of the direct targets of SOX4.[11] Interestingly, a marginally significant correlation ($P = 0.056$) was noted between HER2 amplification and SOX4 overexpression, which suggested a potential link between SOX4 and HER2 aberrations in a subset of Chinese PCa patients.

In conclusion, we believe this is the first analysis to show that the overexpression of SOX4 is an independent prognostic factor in Chinese PCa patients. Thus, SOX4 overexpression in surgically excised PCa tissues might help predict cancer-specific survival. Notably, our findings suggest that SOX4 may induce EMT and contribute to PCa metastasis. These results offer a novel mechanism of SOX4 associated with EMT that can be explored to identify new target drugs for PCa.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 81072110 and No. 81171951), Independent Innovation Foundation of Shandong University (Grant No. 2010TB012) and Scientific Research Foundation for Returned Scholars, Ministry of Education of China.

REFERENCES

1 Shen MM, Abate-Shen C. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes Dev* 2010; **24**: 1967–2000.
2 Castelli T, Cimino S, Magno C, Morgia G. Molecular markers for prostatic cancer. *Front Biosci (Elite Ed)* 2010; **2**: 641–656.
3 Schepers GE, Teasdale RD, Koopman P. Twenty pairs of sox: extent, homology, and nomenclature of the mouse and human sox transcription factor gene families. *Dev Cell* 2002; **3**: 167–170.
4 Andersson A, Ritz C, Lindgren D, Eden P, Lassen C, Heldrup J *et al.* Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukemic and genetic subtype as well as of minimal residual disease status. *Leukemia* 2007; **21**: 1198–1203.
5 Lin B, Madan A, Yoon JG, Fang X, Yan X, Kim TK *et al.* Massively parallel signature sequencing and bioinformatics analysis identifies up-regulation of TGFBI and SOX4 in human glioblastoma. *PLoS One* 2010; **5**: e10210.
6 Liao YL, Sun YM, Chau GY, Chau YP, Lai TC, Wang JL *et al.* Identification of SOX4 target genes using phylogenetic footprinting-based prediction from expression microarrays suggests that overexpression of SOX4 potentiates metastasis in hepatocellular carcinoma. *Oncogene* 2008; **27**: 5578–5589.
7 Medina PP, Castillo SD, Blanco S, Sanz-Garcia M, Largo C, Alvarez S *et al.* The SRY-HMG box gene, SOX4, is a target of gene amplification at chromosome 6p in lung cancer. *Hum Mol Genet* 2009; **18**: 1343–1352.
8 Aaboe M, Birkenkamp-Demtroder K, Wiuf C, Sorensen FB, Thykjaer T, Sauter G *et al.* SOX4 expression in bladder carcinoma: clinical aspects and in vitro functional characterization. *Cancer Res* 2006; **66**: 3434–3442.
9 Liu P, Ramachandran S, Ali Seyed M, Scharer CD, Laycock N, Dalton WB *et al.* Sex-determining region Y box 4 is a transforming oncogene in human prostate cancer cells. *Cancer Res* 2006; **66**: 4011–4019.
10 Suzuki T, Shen H, Akagi K, Morse HC, Malley JD, Naiman DQ *et al.* New genes involved in cancer identified by retroviral tagging. *Nat Genet* 2002; **32**: 166–174.
11 Scharer CD, McCabe CD, Ali-Seyed M, Berger MF, Bulyk ML, Moreno CS. Genome-wide promoter analysis of the SOX4 transcriptional network in prostate cancer cells. *Cancer Res* 2009; **69**: 709–717.
12 Nauseef JT, Henry MD. Epithelial-to-mesenchymal transition in prostate cancer: paradigm or puzzle? *Nat Rev Urol* 2011; **8**: 428–439.
13 Thompson EW, Newgreen DF, Tarin D. Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? *Cancer Res* 2005; **65**: 5991–5995.
14 Kong D, Banerjee S, Ahmad A, Li Y, Wang Z, Sethi S *et al.* Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells. *PLoS One* 2010; **5**: e12445.
15 Zhang J, Liang Q, Lei Y, Yao M, Li L, Gao X *et al.* SOX4 induces epithelial-mesenchymal transition and contributes to breast cancer progression. *Cancer Res* 2012; **72**: 4597–4608.
16 Korshunov A, Sycheva R, Gorelyshev S, Golanov A. Clinical utility of fluorescence in situ hybridization (FISH) in nonbrainstem glioblastomas of childhood. *Mod Pathol* 2005; **18**: 1258–1263.
17 Han B, Mehra R, Lonigro RJ, Wang L, Suleman K, Menon A *et al.* Fluorescence in situ hybridization study shows association of PTEN deletion with ERG rearrangement during prostate cancer progression. *Mod Pathol* 2009; **22**: 1083–1093.
18 Jafarnejad SM, Wani AA, Martinka M, Li G. Prognostic significance of Sox4 expression in human cutaneous melanoma and its role in cell migration and invasion. *Am J Pathol* 2010; **177**: 2741–2752.
19 Wang L, Zheng Y, Xu H, Yan X, Chang X. Investigate pathogenic mechanism of TXNDC5 in reumatoid arthritis. *PLoS One* 2013; **8**: e53301.
20 Han B, Nakamura M, Zhou G, Ishii A, Nakamura A, Bai Y *et al.* Calcitonin inhibits invasion of breast cancer cells: involvement of urokinase-type plasminogen activator (uPA) and uPA receptor. *Int J Oncol* 2006; **28**: 807–814.
21 Han B, Mehra R, Dhanasekaran SM, Yu J, Menon A, Lonigro J *et al.* A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: identification of DDX5-ETV4 fusion protein in prostate cancer. *Cancer Res* 2008; **68**: 7629–7637.
22 Han B, Zhou G, Zhang Q, Zhang J, Wang X, Tang W *et al.* Effect of arsenic trioxide (ATO) on human lung carcinoma PG cell line: ATO induced apoptosis of PG cells and decreased expression of Bcl-2, Pgp. *J Exp Ther Oncol* 2004; **4**: 335–342.
23 Bubendorf L, Sauter G, Moch H, Schmid HP, Gasser TC, Jordan P *et al.* Ki67 labelling index: an independent predictor of progression in prostate cancer treated by radical prostatectomy. *J Pathol* 1996; **178**: 437–441.
24 Moreno CS. The sex-determining region Y-box 4 and homeobox C6 transcriptional networks in prostate cancer progression: crosstalk with the Wnt, Notch, and PI3K pathways. *Am J Pathol* 2010; **176**: 518–527.
25 Andersen CL, Christensen LL, Thorsen K, Schepeler T, Sorensen FB, Verspaget HW *et al.* Dysregulation of the transcription factors SOX4, CBFB and SMARCC1 correlates with outcome of colorectal cancer. *Br J Cancer* 2009; **100**: 511–523.
26 Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD *et al.* Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* 2008; **451**: 147–152.
27 Yarden Y. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur J Cancer* 2001; **37**: S3–S8.
28 Hernes E, Fossa SD, Berner A, Otnes B, Nesland JM.. Expression of the epidermal growth factor receptor family in prostate carcinoma before and during androgen-independence. *Br J Cancer* 2004; **90**: 449–454.

Supplementary Information accompanies the paper on the Prostate Cancer and Prostatic Diseases website (http://www.nature.com/pcan)