

Original Article

Negative regulation of SOX11 in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is the sixth most common tumor and the third most leading cause of cancer-related death worldwide. Sex-determining region Y box (SOX) family proteins play important roles in a variety of developmental processes. The transcription factor SOX11, a member of the SOX family, is emerging as a vital factor in the regulation of oncogenesis. Whether SOX11 plays a role in the pathogenesis of HCC is unknown. The present study was conducted to detect the SOX11 expression in HCC patients and to determine the role of SOX11 in regulating HCC cell malignant behaviors. Immunohistochemistry, Western Blot, and Real-time PCR results revealed that SOX11 decreased in human HCC tissues compared with that in normal tissues. Ectopic overexpression of SOX11 in HCC cell lines inhibited cell proliferation. Transwell and Matrigel assays manifested that SOX11 overexpression inhibited HCC cell migration and invasion *in vitro*. Moreover, SOX11 expression related to the clinicopathologic parameters of clinical stage and histologic grade in HCC patients. In conclusion, these results demonstrated that SOX11 plays an important role in regulating HCC cell proliferation, migration and invasion. Therefore, SOX11 may provide a novel strategy for HCC prognosis and therapy.

Keywords: Hepatocellular carcinoma, SOX11, proliferation, migration, invasion

Introduction

Liver cancer is a common malignancy worldwide. It has been reported an estimated number of 782,500 new liver cancer cases and 745,500 deaths globally in 2012 [1]. In the year of 2016, the estimated numbers of liver cancer new cases and new deaths are 39,230 and 27,170 respectively in USA [2]. In particular, 70%-90% of primary liver cancer is hepatocellular carcinoma (HCC) [1], which is the sixth most common tumor and the third most leading cause of cancer-related death [3]. Currently, surgical resection and liver transplantation are still the primary and most efficient treatments for HCC. However, 70-80% of HCC patients were diagnosed at an advanced stage, in another term, they have lost the opportunity to receive complete resection at diagnosis [4]. The 5-year survival rate of HCC patients is very low worldwide [5, 6] and, it is reported only 15% in the USA [7, 8]. To improve the HCC patient survival, new diagnostic and therapeutic strategies are imperative.

Sex-determining region Y box (SOX) 11 is a member of SOX family, which includes a group of transcription factors that are defined by a highly conserved high-mobility group DNA-binding domain [9, 10]. The best known function of SOX proteins is its regulation of embryonic development and cellular differentiation [11, 12]. However, SOX family has been found in a variety of cancer tissues [13-16] and, perform important roles in cell proliferation, migration and invasion. In particular, SOX2 is a cancer stem cells marker and plays a crucial role in inducing cellular reprogramming [17, 18]. SOX7 [19, 20] and SOX17 [21] have been reported that suppress biological cellular behaviors in breast cancer and lung cancer. Moreover, SOX11 is downregulated in prostate cancer tissue and inhibits the proliferation, migration and invasion of prostate cancer cells [22]. SOX11 decreases cell proliferation in epithelial ovarian cancer *in vitro* and correlates to the survival in patients with high grade epithelial ovarian cancer [23].

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In the light of these studies that SOX11 is related to cancer prognosis and cancer cell biological behaviors, we detected the SOX11 expression in HCC tissues and investigate the role of SOX11 in HCC cell malignant behavior in the present study.

Materials and methods

Clinical samples

The tissue microarray, including 70 different cases of HCC and normal hepatocellular tissues, were purchased from Shanghai outdo biotech co., LTD (outdo biotech co., LTD, Shanghai, China). Tissue samples used for Western Blot and Real-time PCR detection were from Peking University People's Hospital. The protocol was approved by the Institutional Review Board of Peking University People's Hospital.

Cell lines

The HCC cell lines (HepG2, BEL-7404) were purchased from Cell Bank of Shanghai Biology Institute, Chinese Academy of Science (Shanghai, China). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco). BEL-7404 cells were cultured with Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone) supplemented with 10% FBS and 1% penicillin-streptomycin. All cultures were maintained at 37°C in 5% CO₂ atmosphere.

Overexpression of SOX11

Overexpression of SOX11 in HepG2 and BEL-7404 cells was achieved by transient transfection of plasmids containing Myc-DDK-tagged ORF clone of Homo sapiens SOX11 (Origene Technologies, MD, USA). Cells were seeded in culture plates at a density of $6 \times 10^4/\text{cm}^2$ and incubated overnight to reach 70-90% confluence. Plasmids were transfected into HepG2 and BEL-7404 cells by using Lipofectamine 3000 (Invitrogen, CA, USA) following the manufacturer's instruction. In parallel, vectors (pCMV6-Entry) were transfected at same conditions as control.

Immunohistochemistry

The Envision TM ABC Kit (Dako, Denmark) was used to detect SOX11 expression in HCC tissues. The immunohistochemistry procedure was performed according to manufacturer's protocol with modifications. Briefly, after blocking with peroxidase block solution, the tissue sections were incubated with SOX11 primary antibody (1:200, Abcam, Cambridge, UK) overnight at 4°C. Followed by washing with PBS, the peroxidase labeled polymer was applied to tissue sections for 30 min at room temperature for staining. SOX11 reactivity was visualized by adding substrate-chromogen to tissue sections for 5 min. Tissues were then counterstained with hematoxylin and mounted and coverslipped.

The immunohistochemistry analysis was performed based on the frequency and intensity of SOX11 immunoreactivity. The frequency scores were defined as the following: 0, negative; 1, 1-25%; 2, 26-50%; and 3, 51-100% of immunoreactivity positive cells per field. The intensity was evaluated based on a 4-tiered scale as follows: 0, absence of signal; 1, low-intensity signal (light brown); 2, moderate-intensity (brown); and 3, high-intensity signal (dark brown). The frequency score and intensity score were multiplied to obtain the score for each field.

Western blot

Cells or liver tissues were washed twice with cold PBS. The whole cell lysates were prepared in RIPA buffer (BioRad, Hercules, CA) while liver tissues were lysed in tissue extraction reagent (Invitrogen), followed by sonication for complete lysis. After quantification of protein concentrations with BCA protein assay kit (ThermoFisher Scientific, Waltham, MA), 20 µg of protein was loaded onto 10% SDS-polyacrylamide gels. After electrophoresis, the separated proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was then blocked with 5% dry milk dissolved in TBS, followed by incubation of primary antibody SOX11 (1:1000, Abcam) at 4°C. Next day, membranes were washed in TBS with 0.1% Tween for 5 min \times 4 times and incubated for 1.5 h at room temperature with HRP-conjugated goat-anti-mouse secondary antibody (1:10,000, Jackson ImmunoResearch

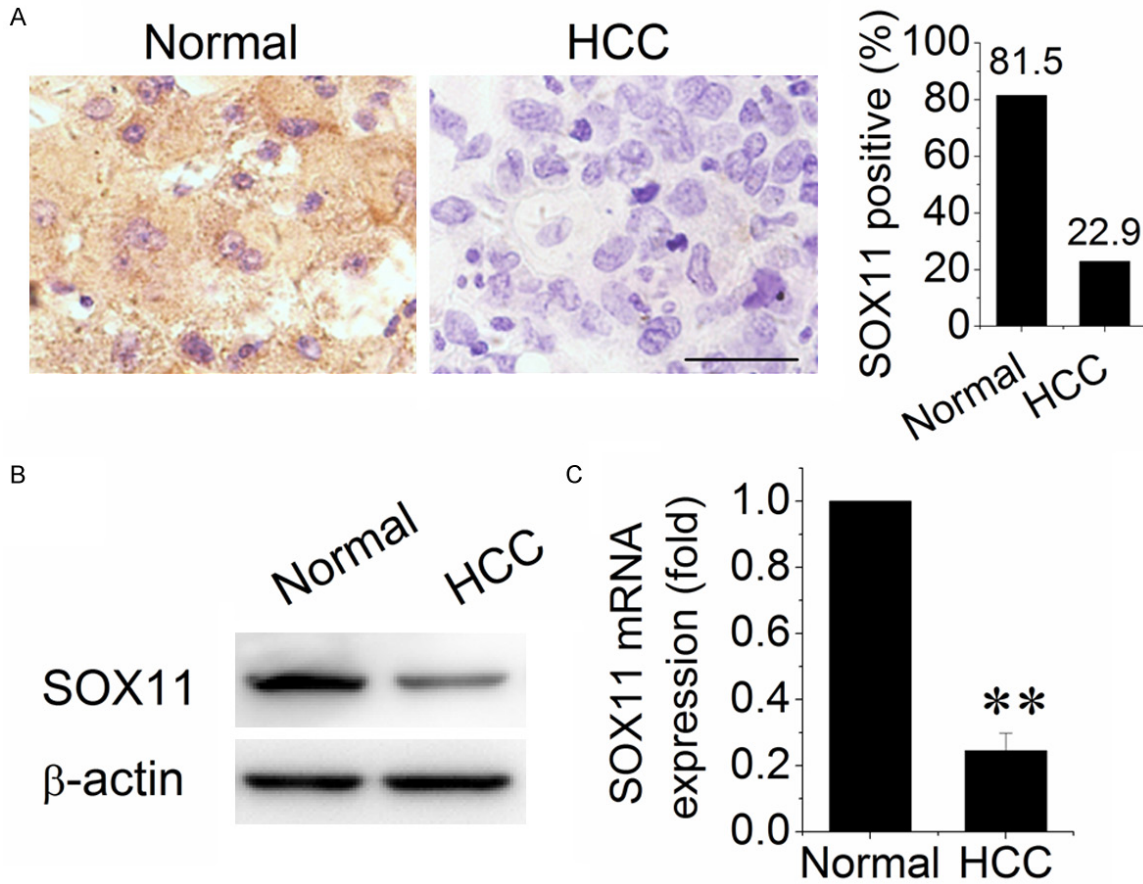


Figure 1. Downregulation of SOX11 in human HCC tissues. A: Representative fields of SOX11 immunohistochemistry staining (brown) of normal and HCC tissues. Right bar graph shows the percentage of SOX11 immunohistochemistry positive cases in tissue microarray. B: Representative blot of Western blot shows SOX11 protein expression in normal and HCC tissue samples. C: Real-time PCR results of SOX11 mRNA expression in normal and HCC tissue samples (relative to normal, n = 6). ** $P < 0.01$ vs Normal. Scale bar equals 50 μ m.

Laboratories, West Grove, PA). After washing, membranes were finally incubated with Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL) and imaged by the FluorChem M system (ProteinSimple, Santa Clara, CA).

Real-time PCR

RNA extraction was achieved by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was accomplished on 1 μ g of the RNA sample in 20 μ L reaction volume with a SuperScript III first-strand synthesis kit (Invitrogen) using oligo-dT primers according to instructions. Primer sequences were designed according to the human cDNA: SOX11 (forward) 5'-GGT GGA TAA GGA TTT GGA TTC G-3' and (reverse) 5'-GCT CCG GCG TGC AGT AGT-3'. GAPDH (forward)

5'-TGG TAT CGT GGA AGG ACT C-3' and (reverse) 5'-AGT AGA GGC AGG GAT GAT G-3' [22]. Reactions containing cDNA, primer pairs and SYBR green PCR supermix (Bio-Rad, CA) were projected to PCR amplification. PCR cycles were performed as follows: initial 5 min at 95°C, then 40 cycles of 95°C (10 s) and 60°C (30 s). The comparative threshold cycle (Ct) method was used to calculate the relative fold changes in gene expression.

Cell proliferation assay

The cell proliferation was detected by cell counting kit-8 assay (CCK8, Sigma). Cells were counted and then seeded in 96-well plates at a density of 1.0×10^3 cells/well. After transfection, WST-8 solution was added to the culture medium. After 2 h incubation at 37°C, the cell viability was evaluated by examination of the

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Table 1. Correlation between SOX11 expression and clinicopathologic parameters in HCC patients

	No. of Patients	SOX11 immunoreactivity score		P-value
		(-)	(+)	
Age (years)				0.8792
> 60	8	6	2	
≤ 60	62	48	14	
Sex				0.2207
Male	66	52	14	
Female	4	2	2	
Clinical stage				< 0.0001
I-II	11	3	8	
III-IV	59	51	8	
Histologic grade				0.0089
I-II	49	34	15	
III-IV	21	20	1	

absorption at 450 nm. All experiments were performed in triplicate, and the results were reported as mean of absorption \pm SD.

Cell migration and invasion assay

The migration and invasion of HCC cells were detected by Transwell and Matrigel assay respectively. For Transwell assay, cells were suspended in serum-free medium after transfection and then seeded in the upper chamber of 24-well plate culture insert with 8 μ m-pore polycarbonate membrane (Corning, NY, U.S.) at a density of 5×10^5 /well. The upper chamber contained serum-free medium while the bottom chamber was filled with 10% FBS culture medium. After 24 h, the cells stayed on the upper side of culture insert membrane were carefully removed by using a cotton swab. The migrated cells on the bottom side of membrane were fixed with methanol and stained with hematoxylin. In order to evaluate the HCC cell invasion *in vitro*. Matrigel assay was performed in a similar fashion with Transwell assay except the culture insert membrane was pre-coated with 100 μ l matrigel. The numbers of cells in both assays were counted in five randomized fields of each membrane under a microscope. Results were obtained from at least three individual experiments.

Statistical analyses

Data are presented as values of mean \pm SEM unless otherwise stated. Statistical analysis

was carried out with *Chi*-square test and student *t* test. Values of $P < 0.05$ were considered as statistically significant difference. All statistical evaluations were performed with SPSS 17.0 (SPSS, Chicago, IL).

Results

Downregulation of SOX11 in HCC tissues

We first detected the SOX11 expression in human HCC tissues. Immunohistochemistry was performed to determine the SOX11 expression in tissue microarray sections. Western Blot and Real-time PCR were used to examine protein and mRNA levels of SOX11 in fresh frozen HCC tissues and normal adjacent tissue samples collected from HCC patients. The results showed that the rate of SOX11 positive immunostaining in HCC tissues was 22.9% (16/70), while the rate in normal tissues was 81.5% (53/65) (**Figure 1A**). The SOX11 protein expression was decreased in HCC tissues compared with that in normal tissues (**Figure 1B**). In agreement, SOX11 mRNA expression was downregulated by approximately 75% in HCC tissues compared with that in normal liver tissues (**Figure 1C**).

Correlations between SOX11 expression and clinicopathologic parameters in HCC patients

After analysis of the SOX11 expression, we analyzed the correlations of SOX11 expression with HCC clinical parameters. The results showed that the SOX11 expression related to the clinical stage and histologic grade. The SOX11 immunoreactivity positive rate of the I-II clinical stage was 72.7%, while the positive rate of III-IV was 13.6%. The SOX11 positive rate of histologic grade I-II was 30.6%, while the positive rate of III-IV was 4.8%. However, there was no evidence showing the correlation between SOX11 expression and age or sex (**Table 1**).

SOX11 reduction of HCC cell viability

Given that SOX11 expresses lower in HCC tissues and relates to clinical stage and histologic grade in HCC patients, we next sought to investigate whether SOX11 affect the HCC cell growth. Plasmids containing SOX11 gene (pCMV6-SOX11) were transfected into HepG2 and BEL-7404 cells to overexpress SOX11 in these HCC cell lines. Achievements of overexpression were confirmed by Western Blot as shown in **Figure 2A**. CCK8 assay was used to

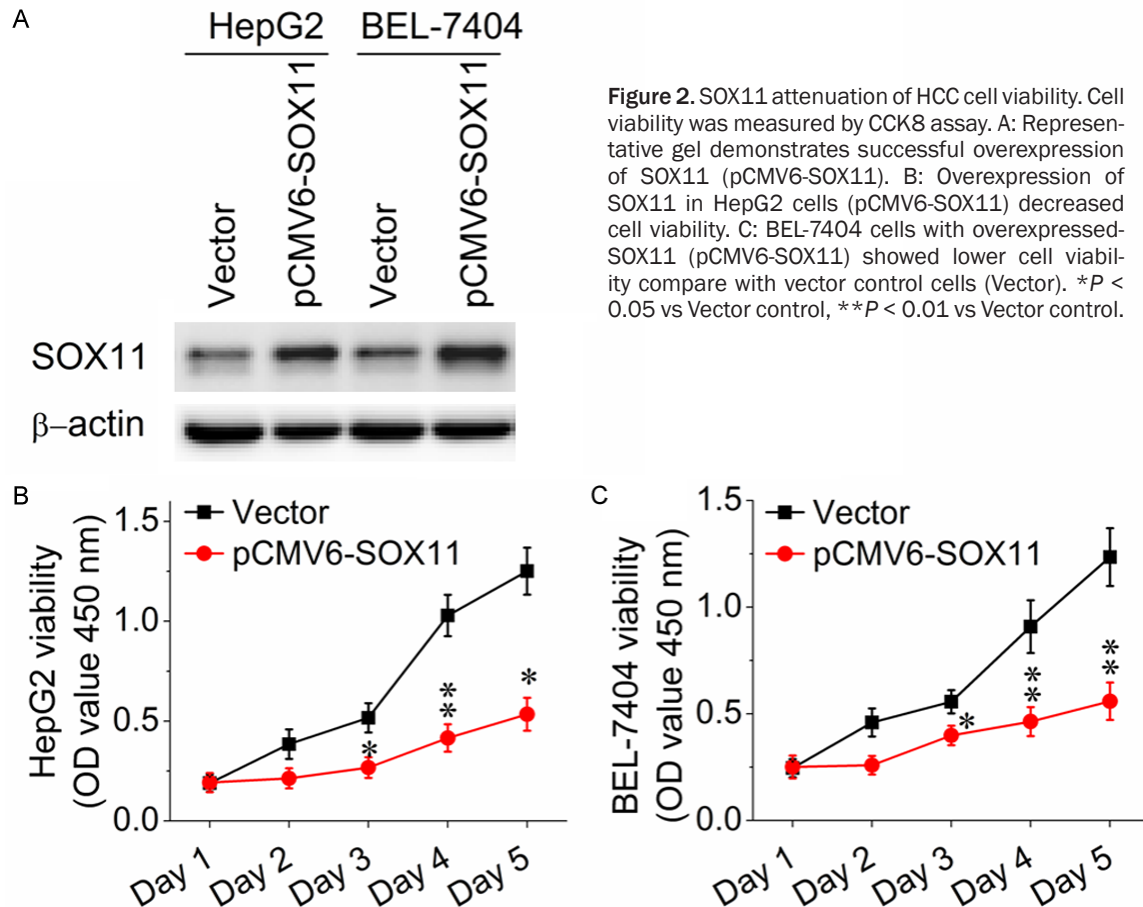


Figure 2. SOX11 attenuation of HCC cell viability. Cell viability was measured by CCK8 assay. A: Representative gel demonstrates successful overexpression of SOX11 (pCMV6-SOX11). B: Overexpression of SOX11 in HepG2 cells (pCMV6-SOX11) decreased cell viability. C: BEL-7404 cells with overexpressed-SOX11 (pCMV6-SOX11) showed lower cell viability compare with vector control cells (Vector). * $P < 0.05$ vs Vector control, ** $P < 0.01$ vs Vector control.

examine the cell viability at different days (after transfection) as indicated in **Figure 2B** and **2C**. The results showed that SOX11 ectopic overexpression significantly decreased the cell viability in both HepG2 and BEL-7404 cells (**Figure 2B** and **2C**), suggesting SOX11 inhibits the HCC cell proliferation or/and survival.

Overexpression of SOX11 suppresses HCC cell migration and invasion in vitro

To investigate the effects of SOX11 on HCC cell's malignant behavior, migration and invasion of HCC cells (HepG2 and BEL-7404) were detected with or without SOX11 overexpression by Transwell (**Figure 3**) and Matrigel assay (**Figure 4**). Ectopic overexpression of SOX11 in HepG2 cells decreased the migrated cells from 78.6 ± 5.3 to 8.3 ± 2.4 of each field (**Figure 3A**). The migrated BEL-7404 cells were also decreased from 87.5 ± 5.6 to 9.0 ± 2.5 of each field with SOX11 overexpression (**Figure 3B**). Similar results were found in Matrigel assay that SOX11 overexpression in HepG2 cells

inhibited the cell invasion (79.8 ± 7.7 of Vector-transfected cells vs. 4.2 ± 1.8 of pCMV6-SOX11-transfected cells, **Figure 4A**). The invaded BEL-7404 cells were reduced from 53.8 ± 6.8 to 5.0 ± 1.9 with SOX11 overexpression (**Figure 4B**). These data suggest that SOX11 strongly suppressed the HCC cell migration and invasion (**Figures 3** and **4**).

Discussion

In the present study, we showed that the SOX11 expression decreases in HCC tissues compare with in adjacent normal tissues and, manifests correlations with clinical stage and histologic grade. Overexpression of SOX11 in HCC cell lines inhibits cell proliferation, migration and invasion, suggesting SOX11 may perform an anti-oncogenic function in HCC.

As mentioned, SOX family has been found in many cancer tissues and, SOX11 is reported as a tumor suppressor in the prostate cancer [22]. Expression of SOX11 is downregulated in pros-

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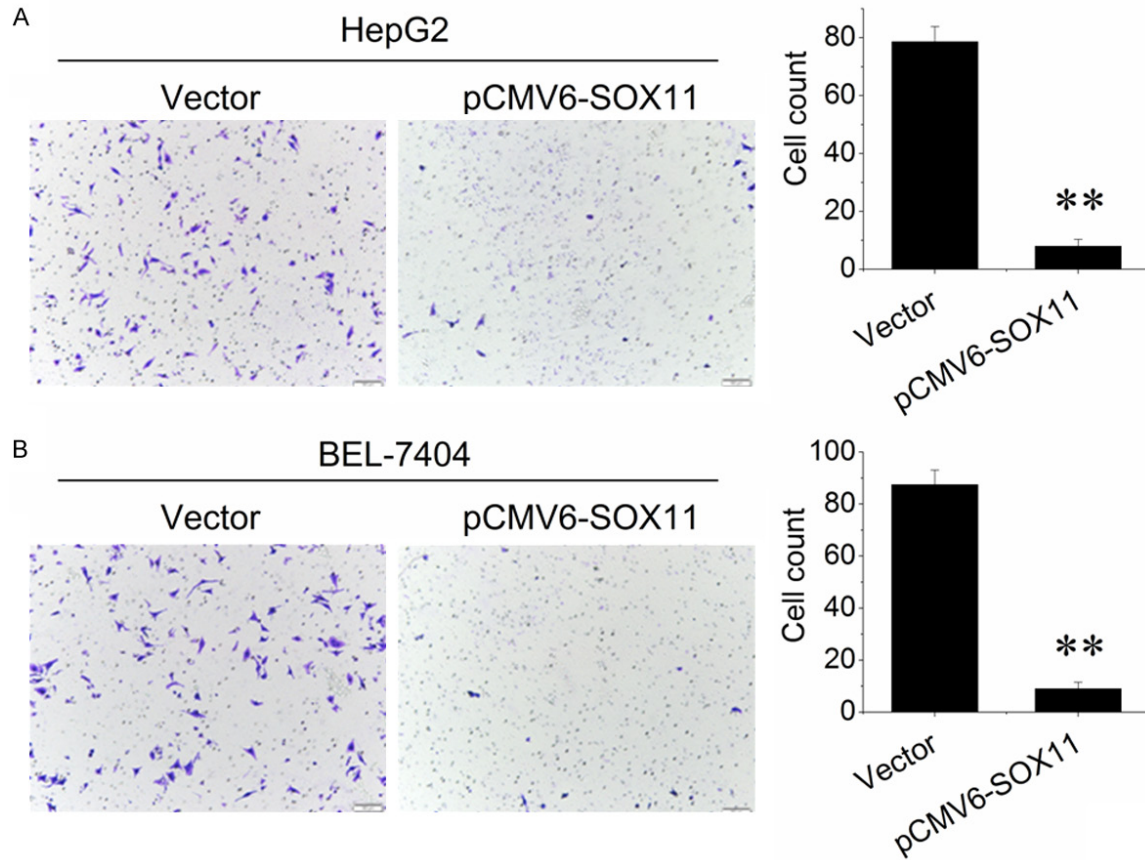


Figure 3. Overexpression of SOX11 inhibited HCC cell migration *in vitro*. Cell migration was measured by Transwell assay in HepG2 cells (A) and BEL-7404 cells (B) without (Vector) or with (pCMV6-SOX11) SOX11 overexpression. (A) HepG2 cells with ectopic overexpression of SOX11 showed less migrated cells. (B) Overexpression of SOX11 in BEL-7404 cells decreased cell migration. ** $P < 0.01$ vs Vector control. Scale bar equals 100 μm .

tate cancer tissue and cell lines [22]. In agreement, our results showed that the SOX11 mRNA and protein expressions were lower in human HCC tissues (Figure 1), indicating SOX11 may involve in regulation of HCC oncogenesis. To further detect whether SOX11 participates in regulating HCC, we tested cell proliferation, migration and invasion in SOX11-overexpressed HCC cell lines HepG2 and BEL-7404. The results showed that the SOX11 overexpression significantly inhibits the cell proliferation and/or survival (Figure 2), migration (Figure 3) and invasion ability (Figure 4) in both HepG2 and BEL-7404 cells. These data suggest that SOX11 may act as an important negative regulator in HCC, which is consistent with previous findings in prostate cancer and epithelial ovarian cancer [22, 23]. However, discrepancies are recently reported in breast cancer. High expression or activity of SOX11 is critical for basal-like breast cancer growth, invasion and migration.

It is also demonstrated that the roles of SOX11 are likely to be dependent on the context of the tumor and cell type [24]. Another research group recently showed that high grade breast tumors express lower levels of nuclear SOX11 protein, and that nuclear SOX11 is associated with improved clinical outcome [25].

SOX family proteins play crucial roles in the regulation of embryonic development and cellular differentiation [11, 12]. There are emerging evidence show the participation of SOX proteins in oncogenesis. SOX1 has been found that inhibits cell proliferation and invasion in breast cancer cell and laryngeal squamous cell carcinoma by suppressing Wnt/ β -catenin pathway [14, 26]. Similarly, SOX7 functions as a tumor suppressor in glioma and ovarian cancer through negatively regulating Wnt/ β -catenin pathway [27, 28] and, SOX15 may perform tumor suppressive effects in pancreatic cancer through

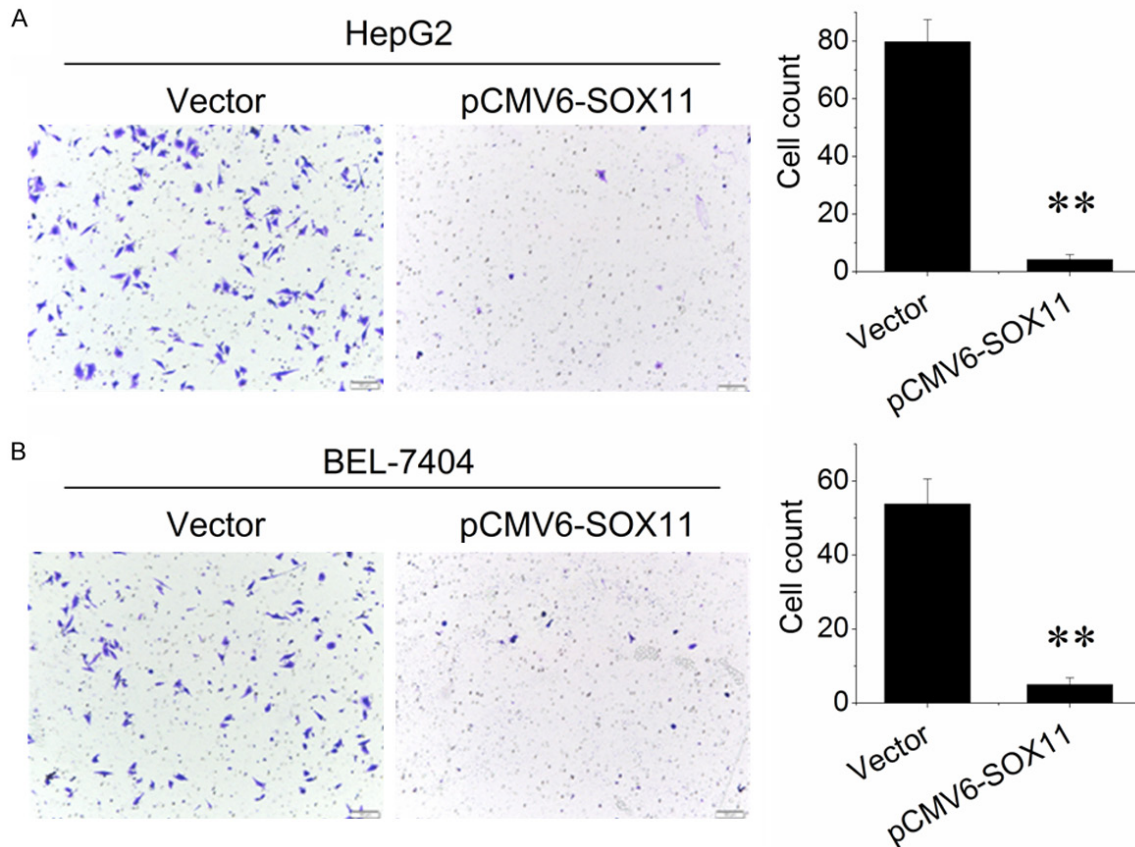


Figure 4. Inhibition of invasion in HCC cells by SOX11 overexpression. Matrigel assay was used to detect HCC cell invasion *in vitro*. A: HepG2 cells with ectopic overexpression of SOX11 (pCMV6-SOX11) showed less migrated cells compare with control vector-transfected cells (Vector). B: Overexpression of SOX11 in BEL-7404 cells (pCMV6-SOX11) decreased cell migration compare with control vector-transfected cells (Vector). $**P < 0.01$ vs Vector control. Scale bar equals 100 μ m.

Wnt/ β -catenin pathway. SOX4, 11, and 9 belong to SOXC subfamily. SOX4 and SOX9 cooperate to regulate signals of TGF- β , Notch and Hippo-Yap [29]. In particular, SOX4 may activate Notch pathway and regulate Wnt signal in prostate cancer [30]. The mechanisms of how SOX11 inhibit cancer cell growth, migration and invasion remain largely unknown. However, SOX11 may act on aforementioned signaling pathways to attain tumor suppression, of which Wnt/ β -catenin may be a strong candidate. The mechanism studies are needed in the future to further reveal SOX11 functions.

In conclusion, the SOX11 expression was downregulated in HCC tissues at both mRNA and protein levels. SOX11 overexpression decreased HCC cells proliferation, migration and invasion *in vitro*. More importantly, the SOX11 expression was related to the clinical stage and histologic grade, which are closely

associated with HCC patients outcomes. Therefore, SOX11 can be a potential tool to assess the HCC prognosis and, may also provide a novel strategy for HCC therapy.

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Disclosure of conflict of interest

None.

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