

SOX10 is a novel oncogene in hepatocellular carcinoma through Wnt/ β -catenin/TCF4 cascade

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Received: 24 February 2014 / Accepted: 25 March 2014 / Published online: 8 July 2014
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Abstract SOX (high mobility group) genes play an important role in a number of developmental processes. Potential roles of SOXs have been demonstrated in various neoplastic tissues as tumor suppressors or promoters depending on tumor status and types. The aim of this study was to investigate the function role of SOXs in the human hepatocellular carcinoma (HCC). The gene expression changes of SOXs in HCC tissues compared with those in noncancerous hepatic tissues were detected using real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) analysis and immunohistochemistry. In addition, we identified the gene SOX10 that was significantly upregulated in HCC by QRT-PCR analysis and immunohistochemistry. Furthermore, we discovered that SOX10 promoted cancer cell proliferation *in vitro*, and SOX10 expression correlated with elevated β -catenin levels in HCC, and β -catenin function was required for SOX10's oncogenic effects. Mechanistically, SOX10 facilitates TCF4 to bind to β -catenin and form a stable SOX10/TCF4/ β -catenin complex and trans-activate its downstream target gene. SOX10 mutations that disrupt the SOX10- β -catenin

interaction partially prevent its function in tumor cells. All in all, SOX10 is a commonly activated tumor promoter that activates Wnt/ β -catenin signaling in cancer cells of HCC.

Keywords SOX10 · Oncogene · HCC · β -catenin

Introduction

Hepatocellular carcinoma (HCC) is one of the major malignant neoplasms, affecting more than half a million people worldwide each year and has a multifactorial etiology including hepatitis B or hepatitis C infections and alcoholism [1–3]. Several studies have shown that there is an association between hepatitis B infection and HCC, but precise molecular mechanisms that regulate the proliferation in these cells remain unknown [4–6].

Wnt/Wingless signaling transduction pathway regulates cell proliferation, cell polarity, and cell fate during embryonic development and tissue homeostasis. Aberrant activation of this pathway is linked to various human diseases, including cancer (1, 2). A critical and heavily studied Wnt pathway is the canonical Wnt pathway, which functions by regulating the amount of the transcriptional coactivator β -catenin, which controls key developmental gene expression programs. For a more comprehensive and historic perspective, we refer readers to earlier reviews [7]. In the absence of Wnt, cytoplasmic β -catenin protein is constantly degraded via the ubiquitin–proteasome system by a degradation complex composed of the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli (APC) gene product, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). When Wnt binds to cell surface receptors, it initiates a transduction cascade leading to the stabilization of transcription coactivator β -catenin. As a result, cytosolic β -catenin accumulates and translocates into the nucleus, where it interacts with TCF/LEF

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transcription factors (T cell factor/lymphoid enhancer factors), further binding directly to various target gene promoters such as CCND1, c-Myc, and MMP7 via the high mobility group (HMG) domain [8].

SRY-related HMG box (SOX) genes belong to the HMG superfamily, and the SOX family of transcription factors is well-established regulators of cell fate decisions and are expressed in a tissue-specific manner during development [9–11]. SOX family proteins contain a highly conserved HMG domain similar to that of SRY. Based on HMG sequence similarities, SOX family can be classified into seven groups termed A to H [12–15]. SOX10 belongs to SOX group E. SOX proteins generally need to cooperate with specific partner factors to regulate gene transcription through binding to specific DNA sequence motifs and causing the DNA to bend—a property of so-called architectural transcription factors [16, 17].

Members of the SOX gene family code for transcription factors either activate or repress transcription of target genes which participate in important biological processes during embryonic development [18]. Based on HMG box homology and intron-exon structure, SOX/Sox genes are divided into ten distinct groups, designated from A to J [19]. Recently, the SOX family of transcription factors has emerged as critical modulators of canonical Wnt/ β -catenin signaling in diverse development and disease contexts [20]. Dysregulation of SOX factors has been further implicated in multiple diseases, including various cancers [12].

However, perplexing expression patterns of SOXs in HCC have not been clear. To address this problem, in our studies, SOX10, SOX9, and SOX10 were identified as target genes because of their statistically differential expression in HCC tissues evaluated further by real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) analysis and semiquantitative immunohistochemistry in a large series of HCC and adjacent benign tissues, and we described our data examining the expression and functional role of SOX10 in HCC through Wnt/ β -catenin signaling.

Results and discussion

Real-time PCR was employed to validate the SOX gene RNA expression levels of the candidate SOX genes (Fig. 1) in 57 HCC and 57 adjacent benign hepatic tissues. Significant up-regulation in HCC tumor tissues of group E genes SOX10, SOX9, and SOX10 was observed ($p < 0.01$). Thus, the expression and localization of group E SOX gene (SOX8, SOX9, and SOX10) in the 213 HCC and 67 adjacent benign hepatic tissues were examined using immunohistochemical analysis. Among the group E SOX gene, the expression levels of SOX10 [immunoreactivity score (IRS): HCC=9.62 \pm 0.412 vs benign=1.51 \pm 0.332, $p = 0.007$] in HCC tissues (169/213)

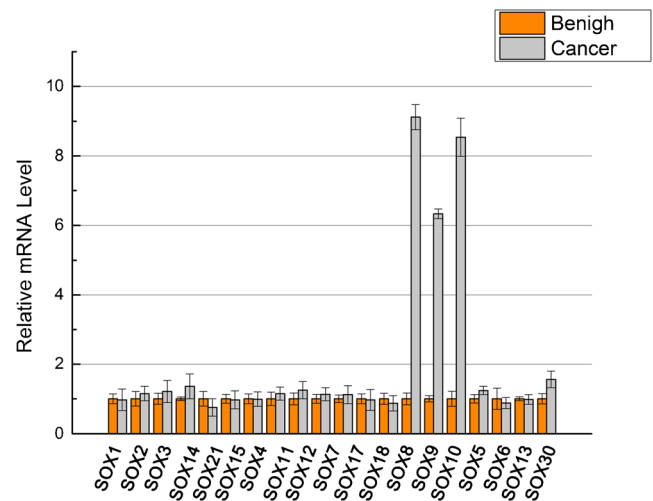


Fig. 1 Validation of SOX gene mRNA levels using quantitative real-time RT-PCR analysis

were significantly higher than those in adjacent benign hepatic tissues (Fig. 2), while the expression levels of SOX9 (IRS: HCC=6.29 \pm 0.441 vs benign=6.23 \pm 0.147, $p = 0.691$) and SOX8 (IRS: HCC=3.447 \pm 0.198 vs benign=3.514 \pm 0.271, $p = 0.351$) (Fig. 2) in HCC tissues seem to have no statistically significant difference than those in adjacent benign hepatic tissues. In addition, the expression level of SOX10 protein in HCC tissues was significantly negatively correlated with that of SOX8 protein ($r_s = 0.669$, $p < 0.001$) and positively correlated with that of SOX9 protein ($r_s = 0.414$, $p < 0.001$), respectively; the expression level of SOX9 protein was also significantly negatively correlated with that of SOX10 protein ($r_s = -0.789$, $p = 0.002$), these data were analyzed with the Spearman correlation analysis.

To further investigate function of SOX10 genes in HCC, we first explored the effect of SOX10 ectopic expression on cellular growth in vitro in multiple cell lines with differing

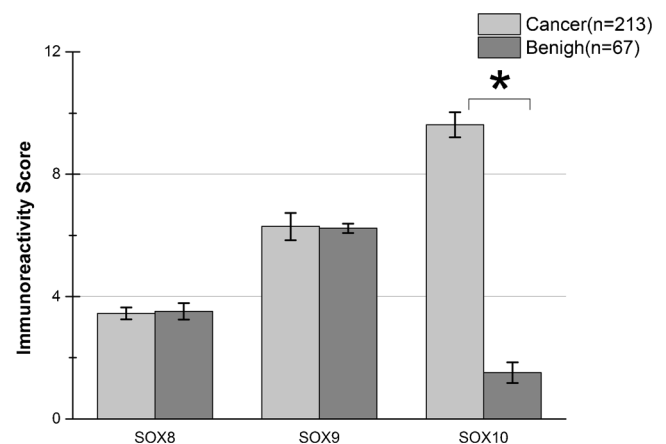


Fig. 2 Immunoreactivity scores of SOXs and SOX10 immunostaining of representative samples of normal liver and hepatic carcinoma. Immunoreactivity scores (IRS) of SOXs in hepatic and benign tissues. Asterisk denotes significance at $p < 0.05$ in comparison between the IRS of SOXs in HCC and Benign tissues

levels of endogenous SOX10 expressions. Following transfection with an SOX10 complementary DNA (cDNA) expression construct, HEK 293 cells, which normally do not express SOX10, and huh7 (HCC cells), which express low endogenous levels of SOX10, demonstrated a significant increase in cellular proliferation (Fig. 3a, b). Conversely, cellular proliferation was attenuated when endogenous SOX10 expression was silenced by stable transfection with short hairpin RNA (shRNA) vectors targeting distinct regions of SOX10 in hepg2 (HCC), which have high endogenous levels of SOX10 (Fig. 3c). Expression of SOX10 shRNA did not alter basal cell proliferation rates in HEK 293 cells, verifying the specificity of the inhibitory function of the SOX10 shRNAs on SOX10's function.

Sox genes activate the Wnt/ β -Catenin/TCF signaling cascade

Next, we asked the mechanism of possible downstream mediators of SOX10's growth-promoting effects, thus, we noted that a significant increase of β -catenin levels in Huh7 cells was found after the overexpression of SOX10 (Fig. 3d).

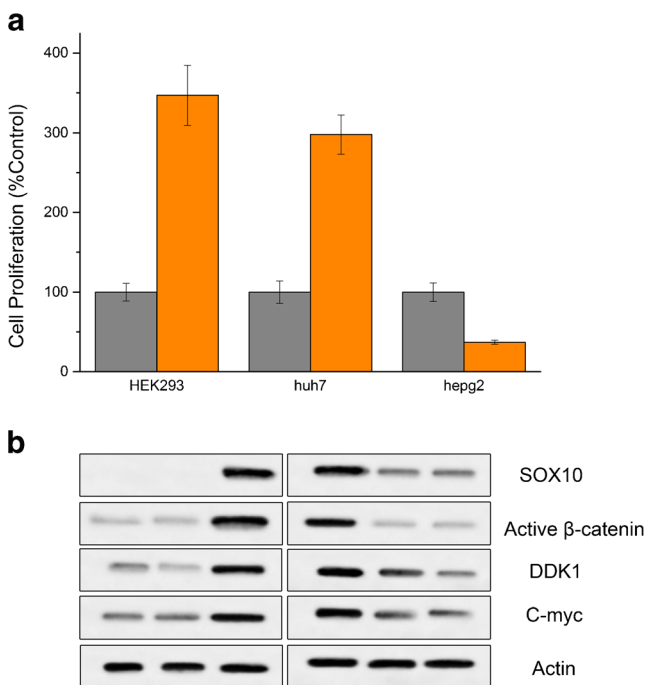


Fig. 3 SOX10 promotes cell proliferation and upregulates β -catenin levels and TCF transcriptional activity. **a** MTS proliferation assay in SOX10-transfected HEK 293 cells (mean \pm SE, $n = 5$). SOX10-transfected huh7 cells. SOX10 shRNA-transfected hepg2 cells (mean \pm SE, $n = 3$). **b** Representative western blots of wild-type, empty vector, and SOX10-transfected huh7 cells (*bottom panel*). Overexpression of SOX10 results in upregulation of β -catenin, active β -catenin, and the TCF target genes DDK1 and c-Myc. β -actin was used as a loading control. Hepg2 cells expressing control shRNA, shRNA1, or shRNA2 (*right panel*). Silencing of SOX10 decreases levels of active β -catenin, DDK1, and c-Myc. β -Actin serves as a loading control

Conversely, SOX10 shRNA-mediated silencing of endogenous SOX10 expression significantly decreased total β -catenin and active β -catenin levels in hepg2 cells (Fig. 3d). The active form of β -catenin exerts its growth-promoting effects by translocating from the cytoplasm to the nucleus, where it binds to transcription factors such as the TCF/lymphoid enhancer binding factor and thereby stimulates the transcription of Wnt target genes. Overexpression of SOX10 in Huh7 cells increased expression of the Wnt/ β -catenin target genes c-Myc and DKK1 (Fig. 3a, b), while c-Myc and DKK1 levels were significantly reduced in hepg2 cells expressing SOX10-targeting shRNA1 or shRNA2 (Fig. 3d). Together, these data strongly suggest that SOX10 enhances β -catenin levels and activates β -catenin/TCF target gene expression in HCC cells.

SOX10 facilitates the interaction of TCF4 with β -catenin

Studies describing the mechanisms of SOX proteins regulating β -catenin/TCF activity have been reported [20]. Thus, we explored whether SOX10 could interact with β -catenin; coimmunoprecipitation analyses were performed using cell lysates from huh7 cells transfected with SOX10 and found that endogenous β -catenin could bind to SOX10 (Fig. 4a). In reciprocal IP using anti- β -catenin antibody, SOX10 specifically precipitated with β -catenin (Fig. 4b). Furthermore, in *in vitro* pull-down assay, SOX10 binds to a GST fusion of β -catenin, but not GST alone, indicating that SOX10 and β -catenin directly associate with each other (Fig. 4c).

β -catenin can form a transcriptional complex with T cell factor (TCF) or lymphoid enhancer factor (LEF) to activate target gene expression. Since both TCF4 and SOX10 belong to HMG superfamily and bind to similar DNA sequences specifically via a single HMG domain [20, 21], we investigate whether SOX10 form a ternary complex with TCF4 and β -catenin. Coimmunoprecipitation assay revealed that TCF4 was detected in immunoprecipitated complexes with β -catenin in 293T cells transfected with GFP and was significantly increased in cells transfected with SOX10 (Fig. 4d), suggesting that SOX10 facilitates TCF4 to bind to β -catenin. Moreover, both TCF4 and β -catenin were detected in immunoprecipitated complexes with SOX10 in 293T cells transfected with SOX10, indicating a ternary complex.

Discussion

In this study, we found that SOX upregulated β -catenin levels in HCC cell lines. A large body of data supports the contribution of activation of the canonical (β -catenin-dependent) Wnt signaling pathway in the development of HCC. Sustained β -catenin pathway activation independent of APC, Axin1, or β -catenin mutations has been demonstrated in a subset of breast

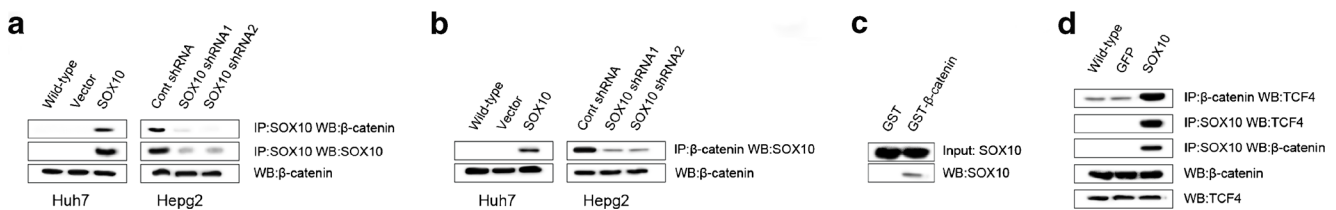


Fig. 4 SOX10 activates Wnt signaling via facilitating TCF4 to bind with β -catenin through a direct interaction to SOX10/TCF4/ β -catenin complex. **a** Representative western blots of wild-type, empty vector, and SOX10-transfected huh7 cells (*left panel*). SOX10 was immunoprecipitated and β -catenin and SOX10 were blot. Hepg2 cells expressing control shRNA, shRNA1 or shRNA2 (*right panel*). Silencing of SOX10 decreases the interaction of β -catenin with SOX10. **b** Representative western blots of wild-type, empty vector, and SOX10-

transfected huh7 cells (*left panel*). β -catenin was immunoprecipitated and β -catenin and SOX10 were blot. Hepg2 cells expressing control shRNA, shRNA1 or shRNA2 (*right panel*). Silencing of SOX10 decreases the interaction of β -catenin with SOX10. **c** GST pull-down assay was employed to confirm SOX10/ β -catenin complex. **d** Representative western blots of wild-type, GFP vector, and SOX10-transfected HEK293 cells. β -catenin or SOX10 was immunoprecipitated and the TCF4/ β -catenin/SOX10 complex was assayed

and ovarian cancers [22, 23]. Although the mechanisms by which SOX10 levels are upregulated in HCC remain unclear, interestingly, our data show strong connection that the dysregulation of SOX10 is associated with the canonical Wnt signaling pathway.

β -catenin plays a central role in Wnt signaling pathway. A growing number of SOX proteins have been revealed to interact with β -catenin through a highly evolutionarily conserved domain (DXXEFDQYL), including SOX7 and SOX17 [13, 24]. Interestingly, SOX10 also contains a short 9-amino acid motif which is essential for SOX10- β -catenin direct interaction. Mutations or deletion of this motif not only disrupted the SOX10- β -catenin direct interaction but also significantly decreased the abilities of SOX10 to both bind and trans-increase target gene promoters. Importantly, deletion or mutation of this motif also abolished the tumor promotion function of SOX10, indicating that its mediated SOX10- β -catenin interaction is critical to SOX10's tumor suppressor functions.

β -catenin has no DNA-binding capacity and is required to interact directly with TCF/LEF proteins and be recruited to promoters of Wnt/ β -catenin target genes. SOX and TCF proteins bind similar DNA sequences [21]. We found that SOX10 facilitate TCF4 to bind β -catenin and formed a functional complex on the promoters of target genes. A DNA-binding-negative mutant of SOX10 maintained the ability to bind β -catenin but failed to bind target gene promoters but still have the tumor promotion function. These data indicate that SOX10 falls to be an important DNA-binding transcription factor for recruiting β -catenin in Wnt/ β -catenin pathway during cancer progression.

The functions of SOX10 may not be limited to the regulation of β -catenin transcriptional activity, because overexpression of SOX10 mutants lacking the DNA-binding domain retained same promotion activity on tumor cell proliferation. Additional signaling pathways required for SOX10 tumor transformed function are interesting questions that need further investigations.

In summary, our study identifies SOX10 as a functional oncogene and an important regulator of the Wnt/ β -catenin signaling pathway. These findings improves our understanding of the molecular mechanisms underlying β -catenin activation and tumor progression and provide a potential target for cancer therapy.

Materials and methods

Cells and culture condition

Tumor cell lines HEK293, Huh7, and Hepg2 were obtained from American Type Culture Collection and were grown with either 0.4 mg/ml genecitin or 0.05 mg/ml hygromycin.

Real-time quantitative reverse transcriptase PCR

Quantitative PCR was used to examine the expression status of the three candidate genes which have statistically differential expression in HCC tissues compared with that in noncancerous tissues. The cDNA templates for qRT-PCR were synthesized from RNA samples. The primers 5'-GGTG GCTTTTAGGATGGCAAG-3' and 5'-ACTGGAACGG TGAAGGTGACA G-3' were used to amplify 161-bp transcripts of β -actin. Gene expression was determined using SYBR Green PCR mix (Toyoko) and 1 μ g of template. Real-time PCR was performed on a MyiQ 2 two-color real-time PCR detection system (Bio-Rad), using the following amplification conditions: 5 min, 95 $^{\circ}$ C; followed by 40 cycles of 10 s, 95 $^{\circ}$ C; 20 s, 60 $^{\circ}$ C; and 20 s, 72 $^{\circ}$ C. All assays were carried out in triplicate. Cycle threshold (CT) values were determined using the IQ5 software (Bio-Rad). Gene expression in each sample was normalized with the housekeeping gene (β -actin) expression. Relative quantification of target gene expression was evaluated using the comparative CT method.

Immunohistochemistry analysis

Normal adult and fetal tissue RNA samples were purchased commercially (Stratagene, La Jolla, CA, USA or Millipore-Chemicon, Billerica, MA, USA). DNA samples of primary carcinomas (T) and matched surgical margin normal tissues (N) have been described previously. For IHC staining, human normal tissue TMA composed of 24 different tissue types (Superchip Co., Ltd., Shanghai, China) and Human HCC tissue TMA (US Biomax, Rockville, MD) were utilized. Paired tumor tissues were collected in Chinese PLA General Hospital. Tissue samples were collected with informed consent, and the procedure was approved by and the Clinical Ethics Committee of Chinese PLA General Hospital.

Glutathione-S-transferase pull-down assay

Glutathione-S-transferase (GST) and GST-tagged β -catenin were expressed in *Escherichia coli* and purified by glutathione-sepharose beads (GE). We incubated SOX10 protein extracted from 293T cells transfected with SOX10 with GST or the GST proteins for 1 h at 4 °C. After washing, we resolved the adsorbed proteins by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and analyzed them by western blotting.

Coimmunoprecipitation assays

Protein interaction was evaluated in a coimmunoprecipitation assay, as describe [25–27]. Transfected cells were lysed in 1 ml RPMI lysis buffer (1 % v/v DevelopTriton X-100, 1 % w/v CHAPS, 10 mM HEPES (pH 7.2), in RPMI medium containing 0.2 mM PMSF, 10 μ g/ml leupeptin, 10 U/ml aprotinin, and 1 mM Na_3VO_4). Targeted proteins were immunoprecipitated from 500 μ l of lysate using mouse anti-V5 (AbD Serotec) antibody, covalently coupled to agarose (Pierce ultralink) at 4 °C for 2 h. Complexes were washed two times in lysis buffer and resuspended in SDS sample buffer and boiled. Samples were separated on a 4–12 % polyacrylamide gel (Novex) and electroblotted onto nitrocellulose membranes. Blots were blocked in 10 % BSA/TBST and probed with different antibodies. To verify and normalize for expression of transfected constructs between experimental conditions, 50 μ g of cell lysate was checked by western blotting with anti-V5 antibody (AbD Serotec) or anti-Flag antibody (Sigma). Anti-mouse HRP-conjugated secondary antibody was used for visualization by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Western blot analysis

For western blot analysis, 40 μ g of whole cell extracts were fractioned by SDS-PAGE and transferred onto Hybond

nitrocellulose membranes (GE Healthcare). Filters were blocked in PBS-Tween 20/5 % skim milk and probed with antibodies against respective target proteins (SOX10 Ab., Santa Cruz #sc-20093; SOX9 Ab., Santa Cruz #sc-20093; SOX10 Ab., Santa Cruz #sc-17342) at a dilution of 1:50 (SOX10 and SOX10) and 1:100 (SOX9) or probed with anti- β -catenin antibodies, which were visualized by SuperSignal West PICO chemiluminescent detection system (Pierce Biotechnology). β -actin was used as equal protein loading control.

Statistical analysis

All in vitro assay results represent the arithmetic mean \pm SE of triplicate determinations of three independent experiments done under the same conditions. Student's *t* test was used to determine the differences between groups and $P < 0.05$ was considered as statistically significant. All statistical tests were two-sided. Calculations were done with the Statistical Package for the Social Sciences version 11.0 (SPSS, Inc., Chicago, IL).

Acknowledgments We thank Dr. Yumin Chen, of Institute of Shanghai Springermedia, for the critical review of the manuscript.

This work was supported by AfterTumor.com Grant (No. CN1324V)

Conflicts of interest None.

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