Transcriptional activation of PD-L1 by Sox2 contributes to the proliferation of hepatocellular carcinoma cells

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Received September 27, 2016; Accepted March 3, 2017

DOI: 10.3892/or.2017.5523

Abstract. Hepatocellular carcinoma (HCC) is one of the most common and lethal malignancies in the world. Sox2 is a potential oncogene in the pathogenesis of HCC, however, the actual mechanisms of Sox2 functions in HCC has not emerged yet. In this study, we explored the expression, function and the relationship between Sox2 and PD-L1 in HCC. We found that both Sox2 and PD-L1 were expressed at a markedly higher level in HCC tissues in comparison to adjacent non-tumor tissues. Moreover, the expression levels of both genes were correlated with each other. Knockdown of Sox2 reduced the cell proliferation ability and induces apoptosis of HCC cells, suggesting the function of Sox2 in regulating both the cell proliferation and apoptosis. Noteworthy, the depletion of Sox2 also reduced the expression of PD-L1. Further analysis showed that there is a consensus Sox2 binding site in the promoter region of PD-L1. Through *in vitro* EMSA assay and *in vivo* chromatin immunoprecipitation assays, we demonstrated that Sox2 directly bound to the PD-L1 promoter through the consensus Sox2 motif. Further evidence by luciferase reporter assays revealed that Sox2 promoted the transcription activity of PD-L1 promoter region through the Sox2 motif. Collectively, our data provide a novel insight into the function and the interplay of Sox2 and PD-L1 in HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignant tumors in the world (1,2). During the past decades, plenty of research regarding the genetic markers (i.e. abnormal gene expression as well as the genomic aberrations) have been accumulated for HCC (3-7). The primary risk factors for the pathogenesis of HCC have been elucidated, and the multiple steps involved in hepatocarcinogenesis have been well defined in recent years (8). In spite of these associations between the risk factors and the development of HCC being well-established (4,9), no clear picture of the actual mechanisms of how and what consequences these factors have leading to malignant transformation has emerged yet.

Sox2 is a transcription factor that controls the expression of a number of target genes through forming a trimeric complex with Oct4 on DNA (10). Until now, the relationship between Sox2 and tumorigenesis as well as overall survival (OS) has been well elucidated in several solid tumors, such as breast, esophageal and lung cancer (11-16). In addition, Sox2 has also been implicated in the progression of HCC. For instance, the expression levels of Sox2 and its co-factor Oct4 correlate with an aggressive phenotype and low survival rate of HCC (17). HCC cells overexpressing Sox2 are characterized by increased ability of epithelial-mesenchymal transition (EMT), invasion and clonal formation (18). miR126, which is a tumor suppressor, inhibits cell growth through reducing the expression of Sox2 (19). These studies indicate that Sox2 functions as an oncogene in HCC. However, it is still unclear through which pathway Sox2 promotes tumorigenesis.

PD-L1 (also known as B7-H1), a gene encoding an immune inhibitory receptor ligand, is expressed in various types of cancer and immune cells (i.e. B cells, T cells, and dendritic cells) (20-22). Binding of PD-L1 to its receptor PD-1 (the programmed cell death-1 receptor) leads to tumor evasion from host immune system (23), which is achieved through suppressing T-cell response, migration, proliferation and restricting the tumor cell killing ability of T cells (21,24-26). Immunotherapeutics targeting the PD-L1/PD-1 pathway are currently in clinical trials and have shown impressive response rates in patients, particularly for non-small cell lung cancer (NSCLC), bladder cancer, renal cell carcinoma and melanoma (27,28). It has been reported that the PD-L1 expression in HCC is significantly associated with tumor malignancy and the risk of postoperative recurrence. Therefore, PD-L1 might represent a potential target for HCC immunotherapy as well as a biomarker that aids in determining the prognosis both before and after therapy (29).

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Key words: hepatocellular carcinoma, Sox2, PD-L1

In this study, we investigated the expression and transcriptional regulation of Sox2 and PD-L1 in HCC. We report that the expression level of Sox2 and PD-L1 is higher in HCC tissues and cell lines compared with adjacent non-tumor tissues and normal liver cell lines. Furthermore, the expression of Sox2 and PD-L1 in HCC tissues are positively correlated with each other. Functionally, we also demonstrate that knockdown of Sox2 reduces the cell vitality, arrests the cell growth and induces the apoptosis of HCC cell lines. Moreover, Sox2 regulates the expression of PD-L1 through directly binding to the Sox2 consensus binding site on PD-L1 promoter region and regulating the promoter activity of PD-L1. This study describes the crucial role of Sox2 in HCC, and for the first time we studied the relationship between Sox2 and PD-L1.

Materials and methods

Cell culture. HL-7702, HepG2, SMMC7721, Huh7 and HEK293 cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco).

RNA extraction and reverse transcription-quantitive PCR (RT-qPCR). Total RNA of cell lines (HL-7702, HepG2, SMMC7721, and Huh7) and patient tissues (hepatocellular carcinoma tissue and adjacent non-tumor tissue) were extracted using RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) were used to remove genomic DNA. Reverse-transcription was carried out with the Superscript III Reverse Transcriptase (Invitrogen). QPCR assays were performed with the SYBE Premix Ex Taq™ (Takara Bio, Otsu, Japan) on ABI ViiA 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression level of Sox2 and PD-L1 were calculated as $2^{-[Ct\ (Gene)-Ct\ (GAPDH)]}$ (30). Each assay was performed three times. The primers used for RT-qPCR are shown in Table I.

RNA interference (RNAi). Lipofectamine™ 2000 (Invitrogen) was used for siRNA delivery. SMMC7721 and HepG2 cells ($2.5x10^5$) were seeded in 6-well plate one day before transfection. A total amount of 100 pmol siRNA were diluted with 250 μl of DMEM without serum and mixed gently. A total amount of 5 μl Lipofectamine 2000 were mixed with 250 μl of DMEM and incubated for 5 min at room temperature. Then the diluted DNA and the diluted Lipofectamine 2000 were firstly mixed gently and incubated for 20 min at room temperature. After 20 min of incubation, 500 μl of DMEM/Lipo/siRNA mix were added to each well containing cells, and mixed gently. The cells were then incubated in a CO_2 incubator containing 5% CO_2 at 37˚C. Cells were collected after 72 h of transfection for mRNA and protein extraction. A Sox2 specific siRNA sequence (siSox2: 5'-CGGCUCUGUAUUAUUUGAATTA-3') was used for RNA interference. A mismatch siRNA sequence (siNC: 5'-UUCUCCGAACGUGUCACGUTT-3') was used as negative control.

Cell proliferation assay. Cell proliferation was detected by the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) and BrdU (Roche, Mannheim, Germany) assays. The cell viability of HepG2 and SMMC7721 cells was detected by the CCK-8 (Dojindo Laboratories, Kumamoto, Japan). Briefly, 10 μl of CCK-8 reagent was added at the 24, 48 and 72 h siRNA posttransfection. Then cells were incubated at 37˚C for 4 h, and the number of viable cells was evaluated through measuring absorbance at 450 nm.

Table I. Primer sequences used for mRNA expression level analysis through qPCR.

Name	Primers sequence (5'-3')
Sox2-RT-F:	TACAGCATGTCCTACTCGCAG
Sox2-RT-R:	GAGGAAGAGGTAACCACAGGG
PD-L1-RT-F:	TGGCATTTGCTGAACGCATTT
PD-L1-RT-R:	TGCAGCCAGGTCTAATTGTTTT
GAPDH-RT-F:	TGTTCGTCATGGGTGTGAAC
GAPDH-RT-R:	ATGGCATGGACTGTGGTCAT

EdU staining. HepG2 and SMMC7721 cells transfected with siRNA were used for EdU assay with Cell-Light™ EdU kit (RiboBio, Guangzhou, China) according to the manufacturer's protocol. Images were captured using a Leica DMI6000B microscope (Leica Microsystems, Wetzlar, Germany).

Apoptosis analysis. siRNA-transfected HepG2 and SMMC7721 were collected for apoptosis analysis. Annexin V-FITC and PI (BD Biosciences) were used for detection of apoptotic cells.

Electrophoretic mobility shift assay (EMSA). EMSA was performed using Lightshift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Biotin-labeled double-stranded oligonucleotide (5'-TATGACACCATCGTCTGTCATC-3') containing the consensus Sox2 motif was used as EMSA probe. An unlabeled double-stranded oligonucleotide was used as competitor probe. An unlabeled-mutated oligonucleotide (5'-TATGACACGTAC CACTGTCATC) was used as negative competitor probe. Nuclear protein was extracted from HepG2 cells. Anti-Sox2 antibody (#5024S, Cell Signaling Technology, Inc., Danvers, MA, USA) was used to supershift the DNA-protein complex.

Chromatin immunoprecipitation-quantitative-PCR (ChIP-qPCR). ChIP was performed according to the manufacturer manual (Active Motif, Carlsbad, CA, USA) in HepG2 cells with the anti-Sox2 antibody (#5024S). The qPCR assay was carried out with the SYBE Premix Ex Taq (Takara Bio) on ABI ViiA 7 Real-time PCR System. Normal rabbit IgG was used as negative control. ChIP-qPCR assay was performed in triplicate. The primers used for ChIP-qPCR are as follows: PD-L1 (forChIP-qPCR)-F: AAGAAAAGGGAGCACACAGG, PD-L1 (for ChIP-qPCR)-R: GCCCAAGATGACAGACGATG.

Plasmid. The PD-L1 promoter region was PCR amplified from HepG2 cells and cloned into the pGL3-basic vector (PD-L1-WT). Mutation of the Sox2 binding site on PD-L1-WT was performed by Quik Change Site Mutagenesis kit

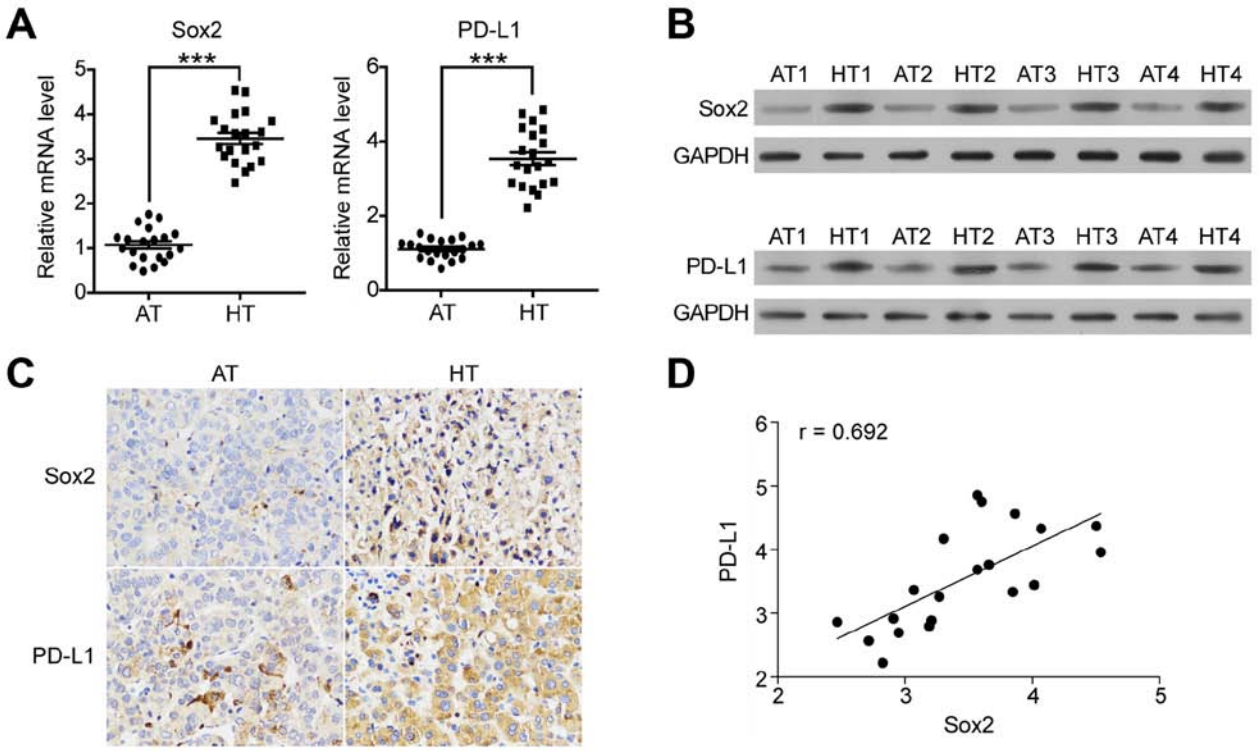

Figure 1. The expression of Sox2 and PD-L1 expression are significantly higher in hepatocellular carcinoma (HCC) tissues adjacent to non-tumor tissues. (A) The mRNA level of Sox2 (upper panel) and PD-L1 (lower panel) are significantly higher in HCC tissues than that in adjacent non-tumor tissues. (B) Western blotting shows that the protein level of Sox2 (upper panel) and PD-L1 (lower panel) are significantly higher in HCC tissues than that in adjacent non-tumor tissues. (C) Immunohistochemistry shows that the protein level of Sox2 and PD-L1 are significantly higher in HCC tissues than that in adjacent non-tumor tissues. (D) Correlation analysis of Sox2 and PD-L1 expression in HCC patients. AT, adjacent non-tumor tissue; HT, hepatocellular carcinoma tissue. Patient samples were retrieved from Department of Hepatobiliary Surgery, Shenzhen Hospital of Southern Medical University. $^{***}p<0.001$.

(Stratagene, La Jolla, CA, USA) according to the manufacturer's manual. Human Sox2 cDNA was amplified from HepG2 cells by RT-PCR and cloned into the pSG5 vector. The primers used for cloning are as follows: PD-L1-Promoter-F: GGGGTACCAGAAGGAAAGGCAAACAAC, PD-L1-Promoter-R: CCGCTCGAGCTTTGGGTTAGTGAATGGG; PD-L1-Mutation-F: TACTTAAGTAAATTATGACATGCAGACGTGTCATCTTGG, PD-L1-Mutation-R: CCAAGATGACACGTCTGCATGTCATAATTTACTTAAGTA; Sox2-cDNA-F: CGGGATCCTCTTCGCCTGATTTTCCTCG, Sox2-cDNA-R: GGAATTCCCTCCCATTTCCCTCGTTTT.

Luciferase. HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen). Luciferase activity was detected by the Dual Luciferase Assay (Promega, Madison, WI, USA) after 24 h of transfection. Co-transfection of *Renilla* luciferase plasmid was used as the internal control for transfection efficiency.

Statistical analysis. Data in this research was presented as mean ±SD, and t-test or one-way ANOVA was used among groups. $p<0.05$ was considered statistically significant. Data were analyzed by using GraphPad Prism 5 for Windows (IBM, USA).

Results

Sox2 and PD-L1 expression are significantly higher in hepatocellular carcinoma (HCC) tissues than in adjacent non-tumor tissues. We initially compared Sox2 and PD-L1 expression in HCC tissue samples and that in adjacent non-tumor tissue samples. We performed RT-qPCR and western blotting to measure Sox2 and PD-L1 expression in HCC tissues versus adjacent non-tumor tissues from HCC patients. As illustrated in Fig. 1A and B, Sox2 expression in HCC tissues (HT) was significantly higher than that in adjacent non-tumor tissues (AT) in both mRNA and protein levels. PD-L1 showed similar expression pattern as Sox2 (Fig. 1A, right panel, Fig. 1B, lower panel). To confirm this expression pattern, we conducted immunohistochemistry (IHC) staining to detect the Sox2 and PD-L1 protein in the HCC tissue and adjacent non-tumor tissue samples. As compared with normal tissue, the Sox2 (Fig. 1C, upper panel) and PD-L1 (Fig. 1C, lower panel) signal is stronger in HCC tissue. Altogether, these analyses indicated that the expression of Sox2 and PD-L1 might be correlated with each other. Thus, we further investigated the relationship between the expression level of Sox2 and PD-L1. As shown in Fig. 1D, the expression level of Sox2 was positively correlated with that of PD-L1 ($r=0.692$). Collectively, these results suggest that both Sox1 and PD-L1 are highly expressed in HCC tissue, and their expression are positive co-related with each other.

Sox2 and PD-L1 expression are significantly higher in HCC cell lines than that of normal liver cell line. We performed RT-qPCR to measure the mRNA level of Sox2 and PD-L1 in HCC cell lines (Huh7, SMMC7721, and HepG2) and normal liver cell line (HL-7702). As shown in Fig. 2A, Sox2 and PD-L1 expression in HCC cell lines were significantly higher than that in the normal liver cell line. We also analyzed the

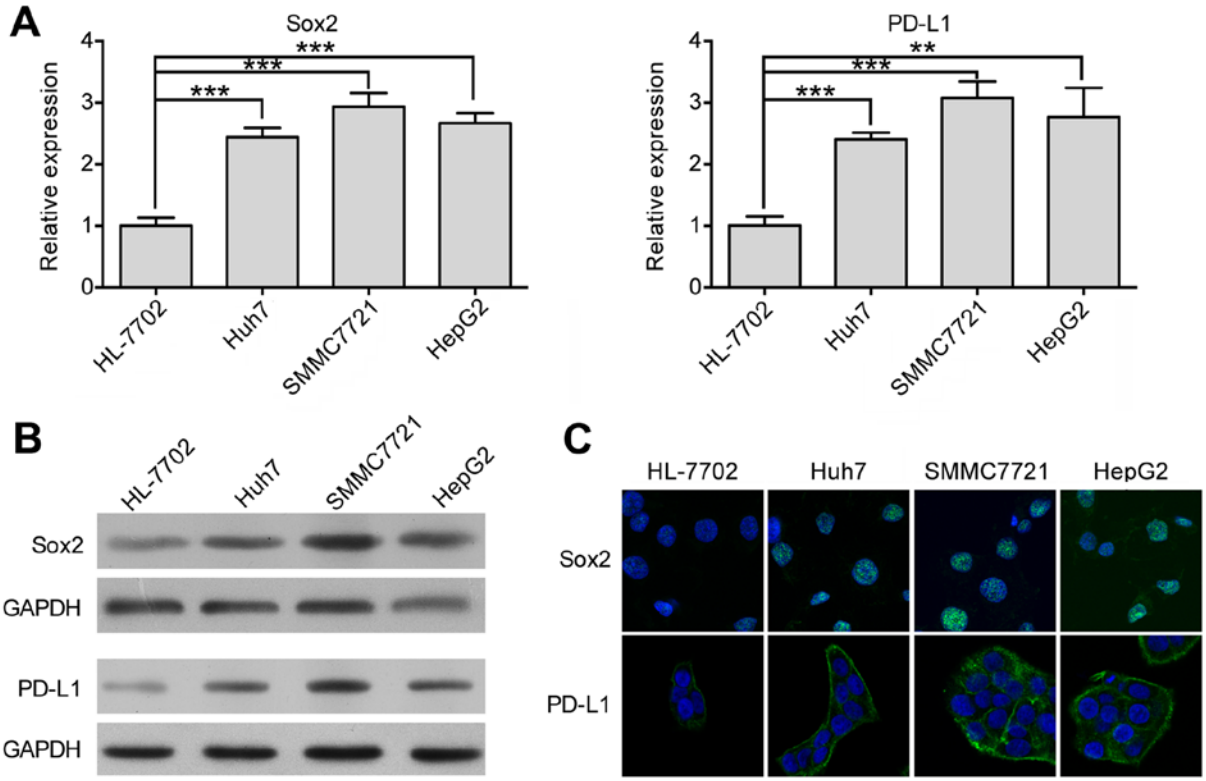

Figure 2. Sox2 and PD-L1 expression are significantly higher in hepatocellular carcinoma cell line than in normal liver cell line. (A) RT-qPCR show the expression level of Sox2 (left panel) and PD-L1 (right panel) are significantly higher in hepatocellular carcinoma cell line, i.e, HepG2, SMMC7721 and Huh7 than that in normal liver cell line. Western blotting (B) and immunofluorescence (C) show the protein level of Sox2 and PD-L1 are higher in three carcinoma cell lines than that in normal liver cell line. $^{**}p<0.01$; $^{***}p<0.001$.

protein level of both proteins through western blot assays. As shown in Fig. 2B, the protein level of Sox2 and PD-L1 were higher in HCC cell lines than that in the normal liver cell line. Moreover, we performed immunofluorescence assays to visualize the expression of Sox2 and PD-L1 globally. As illustrated in Fig. 2C, both Sox2 and PD-L1, even though they localized in the different cellular component, showed higher fluorescence intensity in HCC cell lines than in normal liver cell line. These data are consistent with the aforementioned results shown in Fig. 1 and provide strong evidence that Sox2 and PD-L1 are both highly expressed in HCC.

Knockdown of Sox2 represses the proliferation growth and induces apoptosis of HCC cells. The finding that Sox2 is highly expressed in HCC cells prompted us to elucidate the biological function of Sox2 in HCC. We utilized an siRNA approach to knock down Sox2 expression in Sox2 highly expressed HCC cell lines (HepG2 and SMMC7721), and compared the cell proliferation ability and the status of cellular apoptosis before and after Sox2 knockdown. The reduction of Sox2 expression in Sox2-specific siRNA transfected cells was confirmed by qRT-PCR and western blot assays (Fig. 3A). The effect of Sox2 depletion on cell proliferation was evaluated through using CCK-8 and EdU assays. We observed a significant decrease in proliferation after transfection of siSox2 (Fig. 3B and C) in both HepG2 and SMMC7721 cells. Sox2 knockdown also led to apoptosis of HCC cells, based on the observations of the percentage of the Annexin V-positive and PI-negative cells (increased from 8.3 to 31.0% and 10.0 to 29.4%, in HepG2 and SMMC7221 after Sox2 knockdown, respectively) (Fig. 3D). Taken together, these data indicate that the Sox2 oncogene is required for the proliferation and growth of HCC cells.

Sox2 transactivates PD-L1 through the -757 region of the PD-L1 promoter. Next, we asked whether Sox2 regulates the expression of PD-L1. Through RT-qPCR and western blotting, we found that the expression level of PD-L1 was significantly decreased after Sox2 knockdown (Fig. 4A), which indicated that the expression of PD-L1 might be regulated by Sox2. One important problem that remains to be clarified is whether Sox2 directly target the PD-L1 promoter in HCC cells. To address this question, we first investigated the transcription factor binding sites within the promoter region of PD-L1 using the TRANSFAC database (31). Of note, we found a consensus Sox2 motif upstream of the transcription start site (TSS) at position -757 (Fig. 5A). Then we performed *in vitro* EMSA and *in vivo* ChIP-qPCR assay on Sox2 motif containing promoter regions. As demonstrated in Fig. 5B, when a 22 bp probe contained the Sox2 motif was incubated with nuclear extracts from HepG2, a specific DNA-protein complex was observed (lane 2, GS-Sox2) and was supershifted by the anti-Sox2 antibody (lane 5, SS-Sox2). This indicated that Sox2 bound to the promoter regions of PD-L1 directly through the Sox2 motif. This was further verified by ChIP-qPCR assays in HepG2 cells using primers marked in Fig. 5A and antibodies against Sox2.

To further investigate whether Sox2 transactivates the regulatory region of PD-L1 through the Sox2 binding site identified

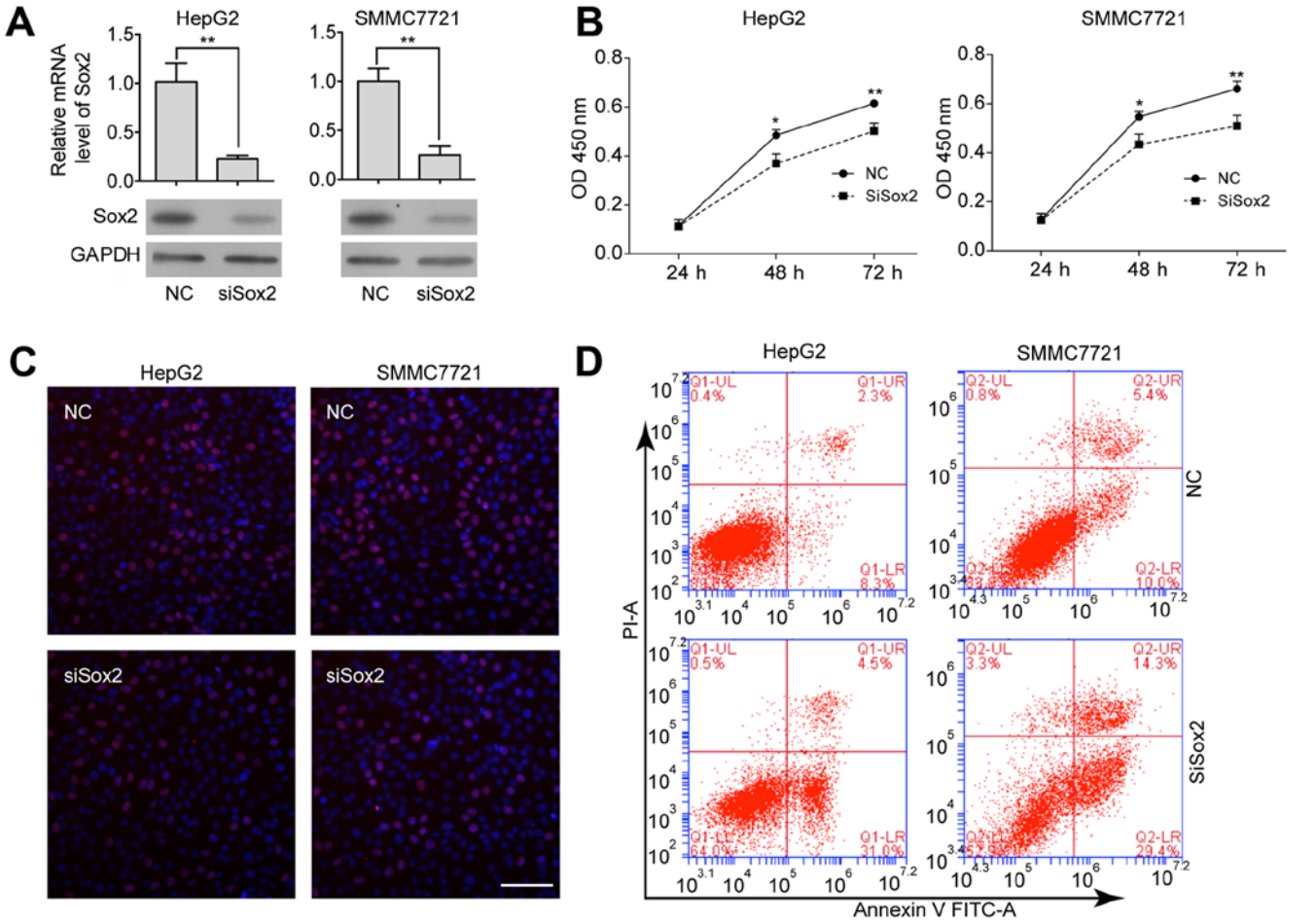

Figure 3. Knockdown of Sox2 repress the growth and induce the apoptosis of HCC cells. (A) RT-qPCR and western blot analysis of Sox2 expression. HepG2 and SMMC7721 cells were harvest after 72 h of after NC or siSox2 transfection. (B) CCK-8 assays showed that Sox2 knockdown repress the growth of HCC cell lines. The CCK-8 assays were performed on HepG2 and SMMC7721 cells after 24, 48 and 72 h after Sox2 knockdown. Error bars represent the SD of triplicate measurements. (C) The BrdU assays showed that Sox2 knockdown repress the proliferation of HCC cells. (D) Sox2 knockdown induced apoptosis of HCC cell lines. Scar bar: 100 μm. *p<0.05; **p<0.01.

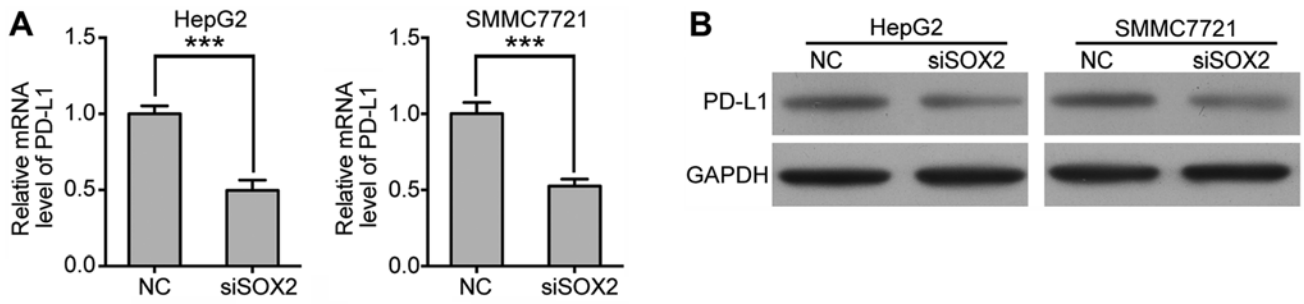

Figure 4. Sox2 knockdown significantly decreases the expression level of PD-L1. The pd-l1 expression is repressed in the Sox2 knockdown cells (HepG2 and SMMC7721). The mRNA (A) and protein levels (B) of PD-L1 were detected by RT-qPCR (A) and western blotting (B). Error bars represent the SD of triplicate measurements. ***p<0.001.

above, we performed luciferase reporter assays in HEK-293T cells. We cloned the promoter region of PD-L1 that contained Sox2 motif and TSS into a luciferase reporter plasmid, which was defined as pGL3-PD-L1. We also constructed a pGL3-PD-L1-mutant plasmid, in which the core sequence of Sox2 motif around -757 bp was mutated from CCATCGTC to TGCAGACG. As shown in Fig. 5D, co-expression of Sox2 resulted in significant activation of the PD-L1 promoter region. In contrast, the transactivation ability of Sox2 on the PD-L1 promoter region was significantly reduced as observed in the PD-L1 mutant promoter. Collectively, the data indicate that Sox2 could transactivate the PD-L1 promoter through the Sox2 motif located at the -757 bp upstream of TSS.

Discussion

In the last few years, numerous studies have been carried out to uncover the molecular markers during the development of

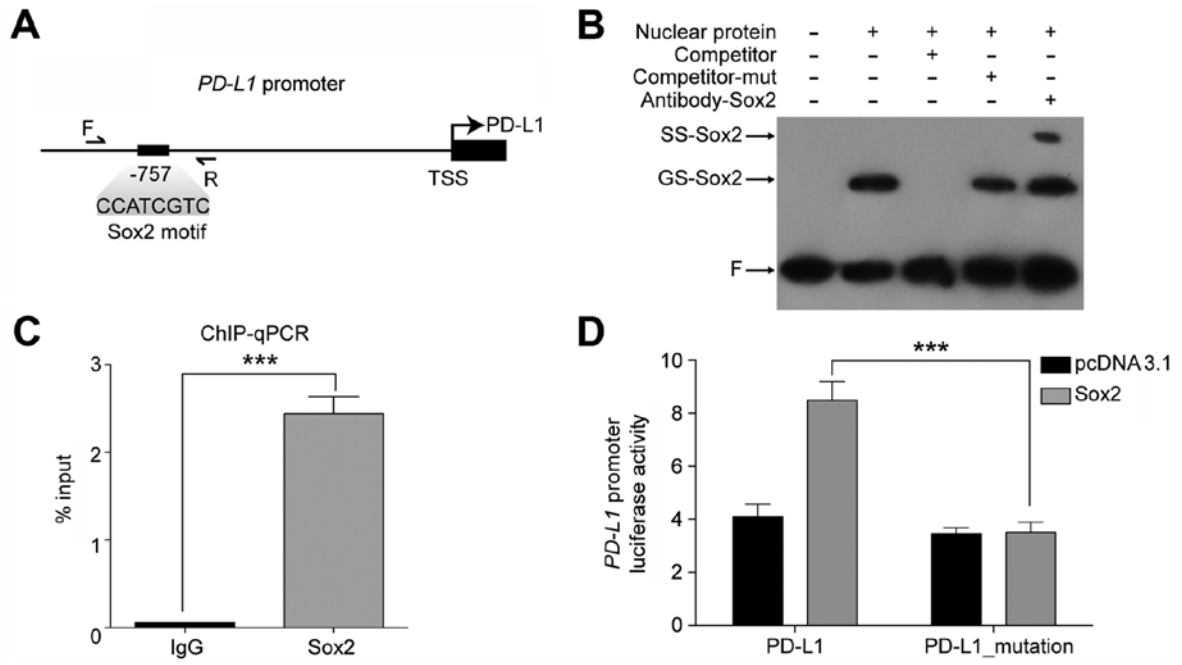

Figure 5. Sox2 activates the expression of PD-L1 through directly binding and activation of the promoter region of PD-L1. (A) Schematic plot represent the promoter region of PDL-L1. Sox2 binding sites are defined using TRANSFAC. Arrows show the primer location of ChIP-qPCR. (B) Demonstration of the binding of Sox2 and DNA probe containing Sox2 motif by EMSA assays. A Sox2 motif-containing probe from the PD-L1 promoter was used. SS-Sox2, super-shifted Sox2-DNA complex with the anti-Sox2 antibody; GS-Sox2, gel-shifted complex formed with Sox2; F, unbound free probe. Nuclear protein was extracted from HepG2 cells. (C) Sox2 bound to the promoter region of PD-L1. ChIP-qPCR was performed in HepG2 cells. Error bars represent the SD of triplicate measurements. (D) Sox2 activates PD-L1 promoter activity through the DNA binding motif. Error bars represent the SD of triplicate measurements. $^{***}p<0.001$.

HCC (32,33); however, the actual mechanisms of how these molecular markers lead to malignant transformation are not yet well understood. Our data indicate that both Sox2 and PD-L1 are highly expressed in hepatocellular carcinoma (HCC). Functional analysis shows the expression of Sox2 is closely related to the proliferation and growth of HCC cells. We also found that PD-L1, a well-qualified immunotherapeutic target in various solid tumors, is transactivated by Sox2. These findings provide novel insight into the function and the interplay between Sox2 and PD-L1 in HCC, and significantly enrich our understanding of the role of Sox2 in HCC malignancy.

Sox2 is a potential biomarker for HCC prognosis (18,34). Previous research including 75 patient samples concluded that high expression of Sox2 in HCC tissues was positively correlated with malignancy and poor survival (34). In our study, we show that the expression of Sox2 is significantly higher in HCC tissue from patient samples and HCC cell lines. This observation is consistent with several previous reports. Moreover, we ivestigated the function of Sox2 in HCC and found that Sox2 is needed for keeping the proliferation ability and prevent HCC cells from apoptosis. Our study not only provides direct evidence (in tissue samples and cell lines) to support the expression pattern of Sox2 but also further explores the Sox2 function. In summary, our finding supports the fact that Sox2 may function as an oncogene in solid tumors especially in HCC.

PD-L1 is an immunotherapeutic target in various solid tumors, such as NSCLC, bladder cancer and renal cell carcinoma (20,21,23,26). We demonstrate here that the expression level of PD-L1 is higher in HCC tissues/cells than that in normal tissues/cells. Recent attention to the role of PD-L1 in the tumor progress of HCC has provided worthy insights into the mechanism of HCC development and associated therapeutic approaches. For example, the PD-L1 expression in HCC is associated with the tumor malignancy and the postoperative recurrence risk (22,35). Accordingly, we tentatively propose that PD-L1 might represent a potential target for HCC immunotherapy.

We observed that the cellular location of PD-L1 and Sox2 are different. In most instances, Sox2 localized only in the nucleus (Fig. 2C). The distribution pattern of Sox2 is due to Sox2 function as a transcriptional factor, and it functions through binding to the promoter regions of its target gene in the nucleus (10,36,37). The location pattern we found here is different from a previous report, which shows that Sox2 locates not only in the nucleus, but also in the cytoplasm of HCC tissue and cell lines, this might be caused by the different method we used for immunofluorescence. On the other hand, as confirmed (38), PD-L1 is located in the cytoplasm and on the cell membrane of tumor cells and immune cells, we observed that PD-L1 indeed localized on the cell membrane and partly in the cytoplasm. It seems that the fluorescence single on the cell membrane is slightly stronger than that in the cytoplasm.

In summary, our findings not only reveal the relationship between Sox2 and PD-L1, but also enrich our understanding of the potential role of Sox2 in HCC malignancy.

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