Carcinogenesis vol.31 no.7 pp.1298–1307, 2010
doi:10.1093/carcin/bgq072
Advance Access publication April 16, 2010

SOX4 overexpression regulates the p53-mediated apoptosis in hepatocellular carcinoma: clinical implication and functional analysis *in vitro*

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Background and aims: The underlying molecular mechanisms of hepatocellular carcinoma (HCC) remain poorly understood due to its complex development process. The human T cell-specific transcription factor sex-determining region Y-related high-mobility group (HMG) box 4 (*SOX4*) has been linked to development and tumorigenesis. In this study, we characterized the roles of SOX4 in regulation of the p53 transcription activity and evaluated the expression patterns and prognostic value of the transcription factor SOX4 in HCC. Methods: The expression levels of human SOX4 were examined in HCC samples obtained from 58 patients having curative partial hepatectomy. The interaction and effects of SOX4 on the p53 pathway were assessed in HCC cell lines. Luciferase reporter assay to examine p53-mediated transcription of target genes was performed. The association of SOX4 expression level with tumor recurrence and overall survival was evaluated. Results: We showed that the HMG box domain of SOX4 interacted with p53, resulting in the inhibition of p53-mediated transcription by the Bax promoter. More importantly, SOX4 overexpression led to a significant repression of p53-induced Bax expression and subsequent repression of p53-mediated apoptosis induced by γ-irradiation. In clinicopathological analysis, nuclear overexpression of SOX4 was observed in 37 out of 58 (63.8%) HCC samples, and this correlated with diminished risk of recurrence ($P = 0.014$) and improved overall survival time ($P = 0.045$) in HCC patients. Conclusion: These results suggest that SOX4 contributes to hepatocarcinogenesis by inhibiting p53-mediated apoptosis and that its overexpression might be a useful prognostic marker for survival after surgical resection.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third leading cause of cancer-related death globally. Chronic hepatitis B virus (HBV) and hepatitis C virus infections contribute to HCC development in >80% of the HCC cases worldwide (1). Other factors associated with HCC include heavy alcohol drinking, exposure to aflatoxin B1 (AFB_1), non-alcoholic fatty liver disease, hemochromatosis, diabetes and obesity (2). Although the risk factors for HCC are well defined, the underlying molecular mechanisms remain unclear because hepatocarcinogenesis is a complex process associated with the accumulation of genetic and epigenetic changes that pass through steps of initiation, promotion and progression. These molecular events are accompanied by enhanced expression of several factors that influence cancer cell survival by regulating the cell cycle and apoptosis. Over the past decade, extensive research has focused on the identification of molecular biomarkers and cellular signaling pathways implicated in HCC, including Wnt/β-catenin, p53, retinoblastoma protein, mitogen-acivated protein kinase, Janus kinases/signal transducers and activators of transcription, stress response signaling, epidermal growth factor receptor and transforming growth factor-β pathways (2). Although the reason why these different signaling pathways are independently involved in the carcinogenesis of HCC is unclear, it might be due to a unique manner of each risk factor for HCC in signal transduction.

The *SOX* gene family (sex-determining region Y-related high-mobility group [HMG] box) plays a key role in regulating transcription in diverse developmental processes (3,4). The SOX family shares the highly conserved HMG box (5), which binds DNA directly in the minor helix groove of the DNA helix (6). The SOX proteins show diverse functions in mammals, because the residues outside the HMG box domain are variable and may influence the selection of cellular partner proteins and subsequently lead to DNA-binding stabilities (3,7,8). Among the many SOX members, *SOX4* belongs to the C subgroup of the family (5,7,9). The *SOX4* gene encodes a protein of 474 amino acids (aa) with three distinguishable domains: an HMG box (aa 57–135), a glycine-rich region (aa 152–227) and a serine-rich region (SRR, aa 333–397). The HMG box serves as a DNA-binding region, whereas the SRR domain serves as a transactivation domain. Moreover, the central domain (CD) containing the glycine-rich region located between the HMG box and SRR domains serves as a novel functional region for promoting apoptotic cell death (10–12).

Previous studies have shown that SOX4 proteins play essential roles in endocardial ridge development and in the regulation of lymphocyte development and differentiation (11,13–16). Recently, the overexpression of SOX4 has been reported in several tumors, including bladder carcinoma, medulloblastoma, prostate cancer, colon cancer, breast cancer and HCC cell lines (17–23). Although the precise mechanisms by which SOX proteins contribute to tumorigenesis are poorly understood, they regulate their target genes by pairing off with specific partner factors. This partnering might allow SOX proteins to act in a cell-specific manner, which would be an essential role in cell differentiation or tumorigenesis (9). SOX4 does not affect normal liver functions due to its lack of expression in normal adult liver (24). To date, little is known about the molecular and clinical involvement of SOX4 in HCC. However, Liao *et al.* (25) demonstrated that SOX4 has an important function in liver tumor metastasis, as RNA interference knock-down reduced HCC cell migration, invasion and intrahepatic metastasis in an orthotopic liver cancer model.

The tumor suppressor p53 is a cellular transcription factor involved in a number of cellular signaling pathways (26,27) and the loss of its function is known to be a common feature of many human cancers (28). Particularly, a mutant in codon 249 of the p53 gene in human HCC has been associated with exposure to AFB_1 (29). Regarding the relationship between SOX4 and p53, the induction of SOX4 in response to DNA damage is critical for p53 stabilization and function (30). To the best of our knowledge, the expression of SOX4 in human HCC has not been demonstrated. Hence, in the present study, we evaluated SOX4 expression in human HCC samples. We found SOX4 overexpression in human HCC, which prompted us to investigate the relationship between SOX4 overexpression and p53 in the regulation of apoptosis and in hepatocarcinogenesis. Furthermore, we assessed the clinical prognostic value of SOX4 protein expression as a biomarker for HCC.

Abbreviations: aa, amino acids; AFB_1, aflatoxin B1; CD, central domain; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HEK, human embryonic kidney; HMG, high-mobility group; IgG, immunoglobulin G; Mdm2, murine double minute 2 homolog; SOX, sex-determining region Y-related high-mobility group (HMG) box; SRR, serine-rich region; WT, wild-type.

Materials and methods

Clinical samples

Fifty-eight paired samples of HCC and their corresponding non-tumorous liver tissues were obtained from HCC patients who had undergone curative partial hepatectomy at Kangnam St Mary's Hospital at the Catholic University of Korea (Seoul, Korea) between January 2000 and December 2002. Informed consent was obtained for specimen collection in all cases and the study protocol was approved by the Ethics Committee of the Catholic University of Korea.

The liver specimens were prepared for histopathological evaluation using conventional paraffin embedding, sectioning and hematoxylin and eosin staining. Fresh tissue specimens were also frozen in liquid nitrogen and stored until use. The histopathological grade of tumor differentiation was assessed according to the Edmondson–Steiner grade criterion (31).

Tissue microarray generation

Representative tissue areas were marked on standard hematoxylin- and eosin-stained sections, punched out of the paraffin block with a 2.0 mm punch and inserted into a recipient paraffin block, resulting in 5 × 6 arrays for each of the 30 cases. Additionally, three control tissue specimens were inserted near the 5 × 6 arrays on the same recipient block. Duplicate tissue cores per specimen were arrayed on a recipient paraffin block to minimize the error introduced by sampling and to minimize the impact of tissue loss during processing.

Immunohistochemical staining

Five micrometer-thick sections were cut from the completed array block and transferred to silanized glass slides. The tissue sections were deparaffinized by incubation in xylene and rehydrated in a graded series of ethanol–water solutions. Antigen retrieval was performed by heating the sample in 0.01 M citrate buffer (pH 6.0) using a microwave vacuum histoprocessor (RHS-1, Milestone, Bergamo, Italy) at a controlled final temperature of 121°C for 15 min. The endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in methanol for 10 min. The slides were incubated with rabbit polyclonal antibody to SOX4 (Sigma, St Louis, MO), which had been diluted 1:800 in Dako antibody diluent (Dako, Carpinteria, CA) with background-reducing components, at room temperature for 30 min. After washing, we detected antibody binding using a Dako EnVision Plus system. The immunoreaction was developed with diaminobenzidine (Dako) for 5 min, and hematoxylin counterstaining was used. The nuclear and cytoplasmic staining with SOX4 was evaluated for the tumor cells. The immunostaining was considered positive when >10% of the tumor cells were immunoreactive. The intensity of staining was graded semiquantitatively as negative, weak, moderate or strong positivity. The two pathologists independently reviewed all core biopsies.

Clinicopathological analysis

The clinicopathological characteristics of HCC patients analyzed included age, sex, tumor size, Edmondson–Steiner grade, pathological tumor-node-metastasis stage, vascular invasion, intrahepatic metastasis, microscopic involvement of resection margin, serum alpha-fetoprotein levels, presence of HBV infection and accompanying liver cirrhosis.

Cell culture

HepG2, Hep3B and human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen) at 37°C in a humidified environment with 5% CO_2.

Plasmids and cell transfection

The cDNA encoding the N-terminal region of human SOX4 (aa residues 1–173 encompassing the HMG box domain) was generated by polymerase chain reaction amplification with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using the pCDM7-SOX4 plasmid as a template (11). The amplified fragment was inserted into the pGEX-4T-1 plasmid (Amersham Biosciences Corp., Cardiff, UK) and designated as pGST-HMG. A plasmid encoding the wild-type (WT) SOX4 with the C-terminal FLAG epitope tag was generated by modifying the pGST-HMG and pCDM7-SOX4 plasmids (12), designated as pFLAG-SOX4 (WT). The following truncated SOX4 constructs of various sizes were generated by digesting with appropriate unique restriction enzymes within the SOX4 cDNAs and inserting the appropriate fragment into pFLAG-CMV5a (Sigma) or modified pFLAG plasmids: HMG (aa 1–173), CD (aa 166–342) and SRR (aa 343–474) (12). The pDS332-p53 plasmids were kindly provided by Dr Shin (Dan-kook University, Korea). The verification of sequence integrity and expression of each plasmid construct was achieved by DNA sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster City, CA) and by immunoblot analyses with specific antibodies, respectively.

For all transfections, 1×10^6 cells were plated on a 100 mm culture dish and 1.5 μg of the indicated plasmid was transfected into each cell line using Lipofectamine™ reagent (Invitrogen) according to the manufacturer's instructions.

Co-immunoprecipitation

For the co-immunoprecipitation experiments, HepG2 and HEK293 cells transfected with expression plasmids were lysed by incubation with lysis buffer [0.2% digitonin, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.5), 100 mM KCl, 10 mM $CaCl_2$ and 50 mM $MgCl_2$] containing protease inhibitor tablets (Roche, Mannheim, Germany). The total protein extracts (1 mg) were incubated with anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-FLAG beads (Sigma) and then Protein A agarose (Invitrogen) was added. After 1 h, the beads were washed with ice-cold phosphate-buffered saline, and the bound immunoprecipitates were eluted from the beads by boiling in sample buffer [62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 144 mM β-mercaptoethanol and 0.0005% bromophenol blue].

Western blot analysis

Immunoblot analysis was carried out for frozen liver tissues and harvested cells. The frozen liver tissue samples were pulverized in liquid nitrogen suspended in RIPA cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS] containing protease inhibitors and further dispersed using a dounce homogenizer. The protein extracts and immunoprecipitated pellets were resolved by 12% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked in 5% skim milk and then incubated with the following primary antibodies: polyclonal rabbit anti-SOX4 (1:500; Sigma), monoclonal mouse anti-FLAG (1:1000; Sigma), polyclonal rabbit anti-p53 (1:1000; Santa Cruz), polyclonal rabbit anti-Bax (1:1000; Santa Cruz), monoclonal mouse anti-enhanced green fluorescent protein (1:2000; Santa Cruz) and monoclonal mouse anti-β-actin (1:2500; Sigma). The blots were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000; Amersham) and developed using the enhanced chemiluminescence system (Amersham). Densitometry values were determined for the SOX4 and β-actin bands, and the ratio of the SOX4 and β-actin values was calculated for each sample. These values are reported as the mean ratio from three separate blots. To compare the expression levels of SOX4 between the tumorous and surrounding non-tumorous liver tissues, the protein bands were quantified using a Kodak molecular imaging system and standardized relative to the β-actin expression level.

Cell fractionation and immunofluorescence staining

Transfected HepG2 and Hep3B cells were fractionated into nuclear and cytoplasmic fractions using an NE-PER kit (Pierce Biotechnology, Rockford, IL). Transfected cells were incubated with anti-FLAG (1:500; Sigma) and anti-p53 antibodies (1:500; Santa Cruz), followed by incubation with Cy3-coupled anti-mouse immunoglobulin G (IgG) (1:500; Jackson Laboratory, Harbor, ME) or Alexa 488-labeled anti-rabbit IgG (1:500; Jackson Laboratory), respectively. Cells were stained with 4′,6-diamidino-2-phenylindole (Sigma) to counterstain the nuclei and were then examined by fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

Luciferase reporter assays

Luciferase reporter plasmids for Bax, murine double minute 2 homolog (Mdm2) and $p21^{waf1}$ were kindly provided by A. Fusco (Università degli Studi di Napoli Federico II, Italy). Cells were transfected with various plasmids, as indicated. Two days after transfection, the luciferase activity was measured using a Veritas microplate luminometer (Tuner Biosystems, Sunnyvale, CA). The firefly luminescence signal was normalized to the *Renilla* luminescence signal. The results are reported as the mean ± SD of at least three independent experiments.

Chromatin immunoprecipitation assays

SOX4 overexpression was shown to suppress p53 transcriptional activity on the Bax promoter in a dose-dependent manner. To determine if SOX4 is present in the p53 transcription complex, we performed chromatin immunoprecipitation assays on the Bax gene promoter with anti-FLAG, anti-p53 or a control IgG. HepG2 cells were transfected with 2 μg pFLAG-SOX4 (WT) expression plasmid in 100 mm dish. Cells were then treated with the cross-linking reagent formaldehyde (1% final concentration) for 10 min at 37°C, rinsed twice with cold phosphate-buffered saline and swollen on ice in SDS lysis buffer (1% SDS, 10 mM ethylenediaminetetraacetic acid and 50 mM Tris–HCl, pH 8.1) supplemented with protease inhibitors (Roche). Nuclei were collected and sonicated on ice. Supernatants were diluted 5-fold in chromatin immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM ethylenediaminetetraacetic acid, 16.7 mM Tris–HCl, pH 8.1, and 167 mM NaCl) and incubated with 2 μl of anti-FLAG M2 antibody, anti-p53 or control IgG on a rotator at 4°C overnight.

Immunoprecipitation was performed using protein A/G-agarose for 1 h at 4°C with rotation. Cross-linked, immunoprecipitated chromatin complexes were then recovered and reversed according to Upstate's protocol (Upstate, Chicago, IL). Final DNA pellets were recovered and analyzed by polymerase chain reaction using a pair of primers that encompass the Bax promoter region. The primers for the Bax gene promoter were (5′-TAATCCCAGCGCTTTGGAAG-3′ and 5′-GTCCAATCGCAGCTCTAATG-3′) and the glyceraldehyde 3-phosphate dehydrogenase gene promoter were (5′-AAAAGCGGGGAGAAAGTA-3′ and 5′-CTAGCCTCCCGGGTTTCTCT-3′).

Induction and measurement of apoptosis

To investigate whether SOX4 can modulate the biological activity of p53, we treated HepG2 and Hep3B cells with γ-irradiation to induce p53-mediated apoptosis. The transfected cells with pFLAG-SOX4 (WT) were treated with 7.5 Gy γ-irradiation (^{132}Cs, 2.875 Gy/min) using a Gammacell irradiator (Atomic Energy of Canada Ltd, Ottawa, Canada). To determine the radiation-induced apoptosis, the cells were washed once with phosphate-buffered saline and stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (BD Biosciences, San Jose, CA) for 15 min according to the manufacturer's instructions. Stained cells were detected using the FACSCalibur flow cytometer (BD Biosciences).

Genomic DNA extraction and DNA sequencing analysis

In order to isolate genomic DNA from the paraffin-embedded 12 HCC tissues for the determination of p53 mutation, tumor foci were identified and five 10 μm-thick sections were cut with standard microtome from every paraffin wax block and transferred into a microtube. The microtome blade was washed with xylene and ethanol. After paraffin removal, genomic DNA was extracted using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. All DNA samples were stored at −20°C. Polymerase chain reaction amplification of genomic DNA was performed using primers specific for human p53. Ten different sets of 20mer oligonucleotide primers were designed using the genomic sequence of p53 (GenBank accession numbers NM_005228.3). Their sequences have been reported elsewhere (32–34). The primers were also used for sequencing analysis.

Statistical analysis

To analyze the correlation intensity of SOX4 expression with clinicopathological factors, the unpaired Student's *t*-test, Fisher's exact test or chi-square test, depending on categorical or numerical date, were applied. Cumulative survival rate was calculated using the Kaplan–Meier method. $P < 0.005$ was considered statistically significant. All statistical analyses were conducted using the SPSS version 13.0 software package (SPSS Inc., Chicago, IL).

Results

SOX4 expression in HCC

To investigate whether the SOX4 protein is expressed in HCC tissues or not, western blot analysis was conducted in 20 paired HCCs and corresponding non-tumorous liver tissues. As shown in Figure 1A, the SOX4 was expressed at high levels in 14 of 20 (70%) HCC tissues and only at low levels in the corresponding non-tumorous liver tissues. The optical density of each band from the tumorous (T) and non-tumorous (N) tissues in the same patient was measured using TINA image analysis software. The ratio between the optical density of proteins of interest and β-actin in the same sample was calculated as the relative content.

An immunohistochemical study using anti-SOX4 polyclonal antibodies was performed to determine whether SOX4 is expressed in HCC samples. Tumor cells showed nuclear and/or cytoplasmic expression of SOX4 (Figure 1B): (i) weak cytoplasmic, (ii) strong cytoplasmic and (iii) strong nuclear SOX4 expression. Nuclear SOX4 staining was negative for 4 (6.9%) patients, weak for 17 (29.3%) patients, moderate for 31 (53.4%) patients and strong (10.3%) for 6 patients. Cytoplasmic SOX4 staining was negative for 2 (3.4%) patients, weak for 7 (12.1%) patients, moderate for 29 (50.0%) patients and strong for 20 (34.5%) patients. In normal liver tissue, hepatocytes were negative but lymphocytes were positive for SOX4 (Figure 1B, d). This finding is consistent with prior evidence that B and T lymphocytes express SOX4 (11).

Correlation between SOX4 expression and clinicopathological parameters in HCC

We analyzed the correlation between the SOX4 expression pattern and clinicopathological characteristics of the tumors. By statistical analysis, there were no significant correlation between SOX4 expression and any clinicopathological characteristics, such as age, gender, tumor size, tumor grade, vascular invasion, intrahepatic metastasis, serosal invasion, resection margin, serum alpha-fetoprotein level, hepatitis B surface antigen status or cirrhosis (Table I). However, we found that patients with nuclear SOX4 overexpression (moderate or strong immunopositivity) had a better prognosis than those with negative or weak nuclear SOX4 expression (disease-free survival rate, $P = 0.014$; overall survival rates, $P = 0.045$; as shown in Figure 2). In contrast, there was no significant correlation between the cytoplasmic SOX4 expression and recurrence or survival rates. These results suggest the nuclear location of SOX4 as a prognostic indicator of SOX4 expression-related HCC patients.

SOX4 interacts with p53 in HCC cells

Although recent studies have demonstrated SOX4 to be an important developmental transcription factor that is often overexpressed in several types of cancers, little is known about how SOX4 is regulated in cancer cells. SOX4 overexpression has been reported in an HCC cell line (18). More recently, it has been shown that SOX4 regulates p53 stability (30) and p53 target genes such as Bcl 2 binding component 3 (21). In this study, we sought to understand whether human SOX4 binds to p53 in HCC cells. To this end, we conducted co-immunoprecipitation studies in HEK293 and HepG2 cells. We used HEK293 cells because it can be efficiently transfected with expression vector. In brief, HEK293 and HepG2 cells were transiently co-transfected with pFLAG-SOX4 (WT) and/or pDS332-p53, followed by immunoprecipitation with anti-FLAG beads and immunoblotting with anti-p53 polyclonal antibody. The FLAG-immunoprecipitated complex revealed an interaction between SOX4 and p53 in both the HEK293 and HepG2 cells (Figure 3A). In the reverse experiment, HepG2 cells were transfected with the pFLAG-SOX4 (WT) expression plasmid and immunoprecipitated with anti-p53 polyclonal antibody followed by immunoblotting with anti-FLAG monoclonal antibody. The immunoprecipitated complex of SOX4 with endogenous p53 is shown in Figure 3A. These results demonstrate that SOX4 interacts with p53 in HCC cells.

Next, we assessed the subcellular co-localization of these two proteins by cell fractionation and fluorescence immunocytochemistry in HepG2 cells transfected with pFLAG-SOX4 (WT). The results revealed the exogenous expression of SOX4 in the cytoplasmic and nuclear compartments of HepG2 cells and endogenous expression of p53 throughout the nucleus (Figure 3B). This finding indicates that SOX4 and p53 proteins are co-localized and thus can potentially interact in the nucleus.

HMG domain of SOX4 is required for the interaction with p53

To identify the region of SOX4 required for the nuclear p53 interaction, we used a series of SOX4 deletion mutants to express the HMG domain, SRR domain or CD containing the glycine-rich region motif (12). Each of these SOX4 mutants was expressed at a level similar to that of the WT (Figure 3C). To examine the ability of the SOX4 mutants to interact with p53, we transiently co-transfected HEK293 cells with pDS332-p53 expression plasmid and the expression plasmid for SOX4 (WT), the HMG domain, the SRR domain or the CD. The anti-FLAG immunoprecipitation from these cells were immunoblotted with anti-p53 antibody. As shown in Figure 3C, SOX4 (WT) and HMG formed complexes readily with p53, whereas the mutants expressing only the CD or SRR domain failed to bind p53. These findings indicate that the SOX HMG domain is necessary for the interaction with p53 but the CD and the extreme C-terminal transactivation region of SOX4 are not involved in the interaction with p53. These results are consistent with previous finding reported by Pan *et al.* (30).

SOX4 suppresses p53-mediated transactivation of p53-responsive promoters

To further define the effects of SOX4 on p53-mediated transcription, we transfected HepG2 cells harboring WT p53 and Hep3B cells deficient in p53 with pFLAG-SOX4 (WT) and with reporter vectors

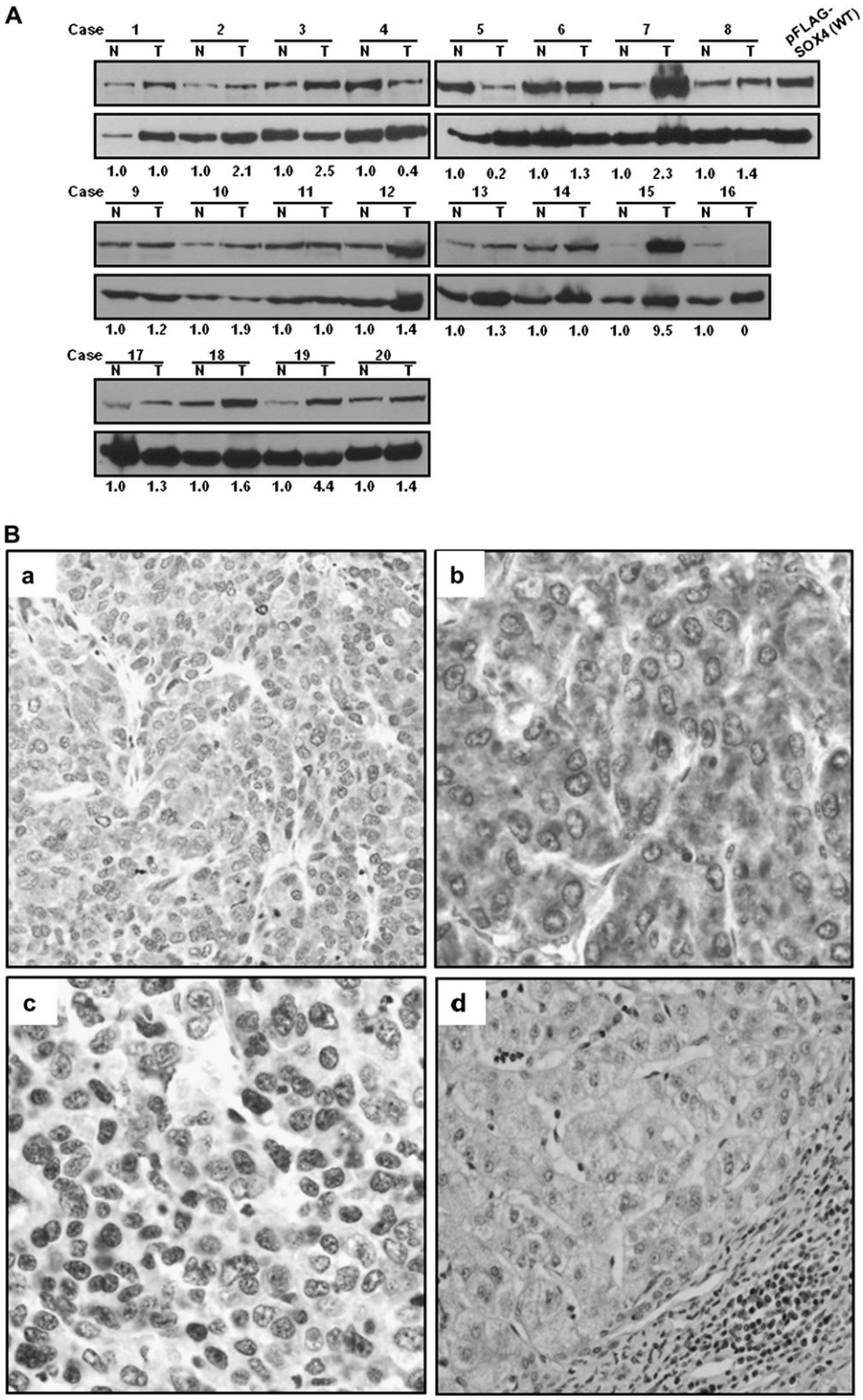

Fig. 1. SOX4 expression in HCC. (**A**) Expression of SOX4 by immunoblot analysis in matched tumorous (T) and non-tumorous (N) tissues from 20 HCC patients. The results shown are from one representative experiment among three replicates. The band densities were quantified with TINA image analysis software using β-actin as a reference. The data are expressed relative to the density of the control. HEK293 cells that were transiently transfected with the pFLAG-SOX4 (WT) expression vector were used as a positive control. (**B**) Immunohistochemical staining pattern of SOX4 in HCC. The tumor cells show weak cytoplasmic (a), strong cytoplasmic (b) or strong nuclear (c) SOX4 expression. SOX4 expression was not observed in normal liver and in non-tumor liver cells (d), whereas most of the lymphocytic cells are positive for SOX4. Original magnification ×400.

Table I. Association of SOX4 expression with clinicopathological characteristics in human HCCs

Variable	No. of cases	Nuclear SOX4 expression		*P*	Cytoplasmic SOX4 expression		*P*
		Negative (%)	Positive (%)		Negative (%)	Positive (%)	
Mean age ± SD (years)		55.1 ± 11.1	54.3 ± 11.5	0.723	56.6 ± 11.8	54.2 ± 11.2	0.927
Gender				0.451			0.603
Male	46	16 (34.8)	30 (65.2)		7 (15.2)	39 (84.4)	
Female	12	5 (41.7)	7 (58.3)		2 (16.7)	10 (83.3)	
Tumor size				0.256			0.581
≤5 cm	37	11 (29.7)	26 (70.3)		6 (16.2)	31 (83.8)	
>5 cm	21	10 (47.6)	11 (52.4)		3 (14.3)	18 (85.7)	
Edmondson grade				0.940			0.454
Grade 1, 2	30	11 (36.7)	19 (63.3)		4 (13.3)	26 (86.7)	
Grade 3, 4	28	10 (35.7)	18 (64.3)		5 (17.9)	23 (82.1)	
Stage				0.560			0.071
I, II	36	12 (33.3)	24 (66.7)		8 (22.2)	28 (77.8)	
III, IV	22	9 (40.9)	13 (59.1)		1 (4.5)	21 (95.5)	
Vascular invasion				0.328			0.053
Positive	15	7 (46.7)	8 (53.3)		0 (0)	15 (100)	
Negative	43	14 (32.6)	29 (67.4)		9 (20.9)	34 (79.1)	
Intrahepatic metastasis				0.504			0.632
Positive	11	5 (45.5)	6 (54.6)		2 (18.2)	9 (81.8)	
Negative	47	16 (34.0)	31 (66.0)		7 (17.5)	33 (82.5)	
Serosal invasion				0.143			0.424
Positive	18	9 (50.0)	9 (50.0)		2 (11.1)	16 (88.9)	
Negative	40	12 (30.0)	28 (70.0)		7 (17.5)	33 (82.5)	
Resection margin				0.504			0.128
Positive	11	5 (45.5)	6 (54.5)		0 (0)	11 (100)	
Negative	47	16 (34.0)	31 (66.0)		9 (19.1)	38 (80.9)	
Alpha-fetoprotein				0.139			0.581
≤300 ng/ml	37	16 (43.2)	21 (56.8)		6 (16.2)	31 (83.8)	
>300 ng/ml	21	5 (23.8)	16 (76.2)		3 (14.3)	18 (85.7)	
Hepatitis B surface antigen status				0.926			0.527
Positive	41	15 (36.6)	26 (63.4)		6 (14.6)	35 (85.4)	
Negative	17	6 (35.3)	11 (64.7)		3 (17.6)	14 (82.4)	
Cirrhosis				0.282			0.500
Positive	25	11 (44.0)	14 (56.0)		5 (20.0)	20 (80.0)	
Negative	33	10 (30.3)	23 (69.7)		4 (16.0)	21 (84.0)	

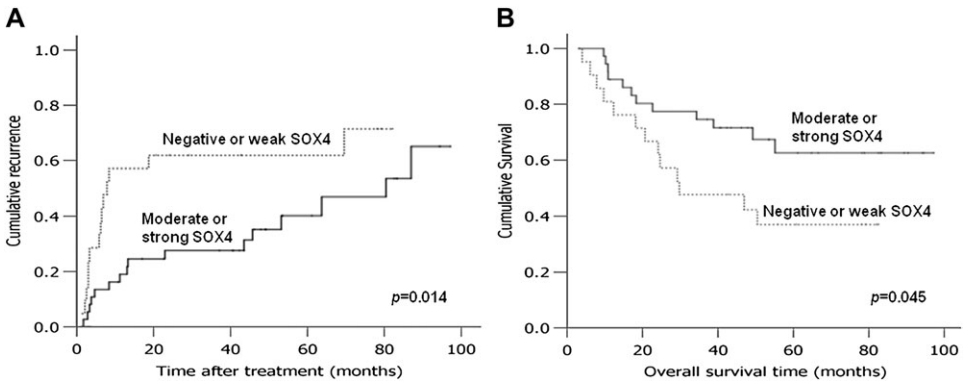

Fig. 2. Nuclear overexpression of SOX4 correlates with diminished risk of recurrence (**A**) and improved overall survival time (**B**) in patients with HCC. Solid and dotted lines present moderate to strong nuclear expression of SOX4 ($n = 37$) and negative or weak nuclear expression of SOX4 ($n = 21$) cases, respectively. Strong nuclear expression of SOX4 was significantly associated with better prognosis (A: $P = 0.014$; B: $P = 0.045$).

carrying the luciferase gene under the control of the p53-responsive Bax, Mdm2 or $p21^{waf1}$ promoter. The expression of SOX4 reduced p53 transcriptional activity on the Bax promoter in a dose-dependent manner in HepG2 cells (Figure 4A) but did not lead to significant difference between the levels mediated by the Mdm2 nor $p21^{waf1}$ promoter (data not shown).

Furthermore, ectopically expressed p53 in p53-deficient Hep3B cells activated the transcription of the Bax-luc vector (Figure 4B). However, increasing the level of SOX4 protein greatly reduced this p53-mediated Bax-luc transcription in a dose-dependent manner. These results suggest that tumor suppressor p53 transcriptional activity may be inhibited in HCC cells overexpressing SOX4.

To clarify the mechanism by which SOX4 overexpression represses the p53-mediated Bax-luc transcription, we performed a chromatin immunoprecipitation analysis. Figure 4C demonstrates that the precipitation of SOX4 protein cross-linked to DNA under these conditions resulted in successful amplification of a p53-response element region in the Bax promoter and the precipitation of p53 protein was specifically recruited to the Bax promoter as a positive control. No precipitation was observed with anti-IgG precipitates, and

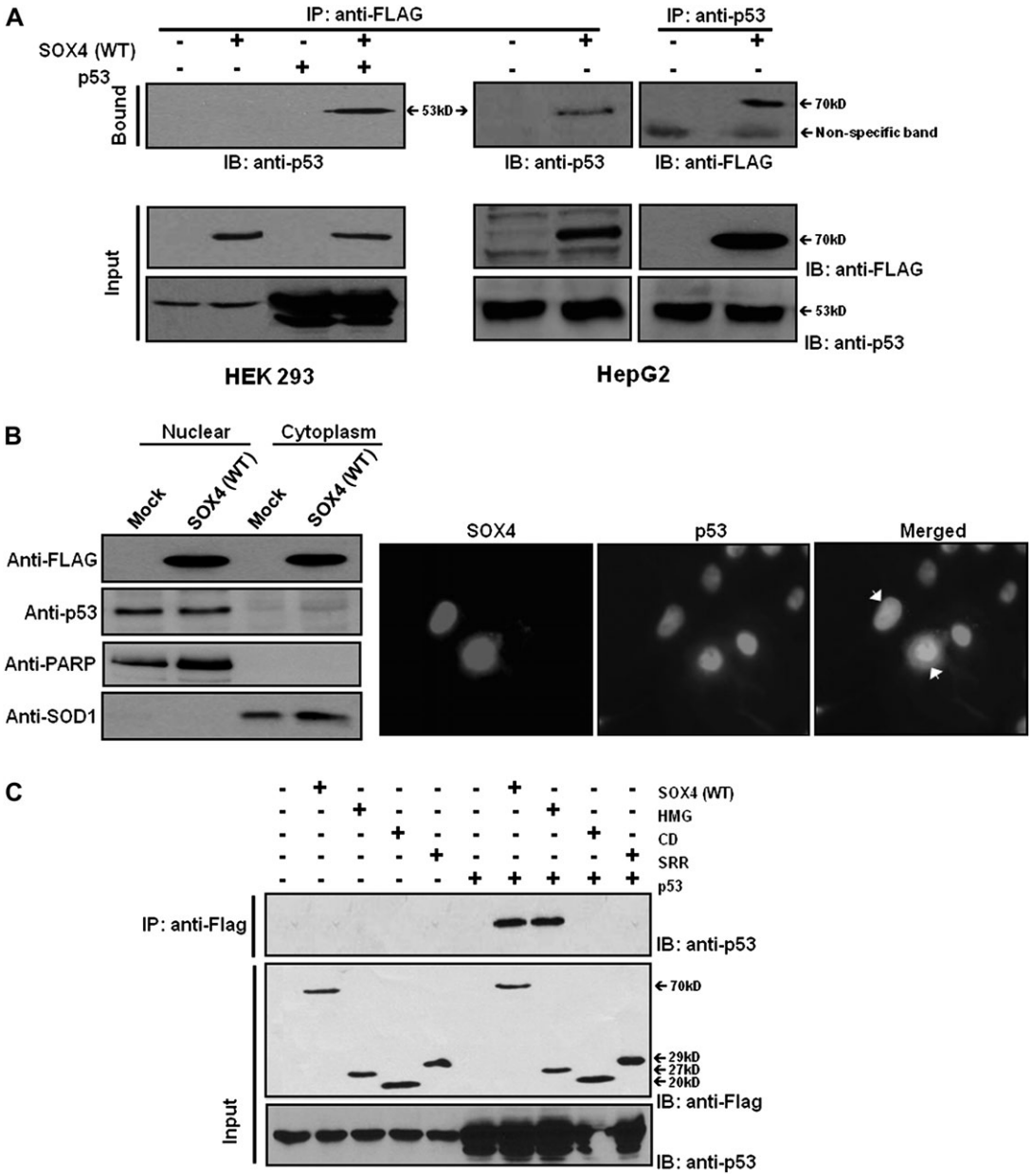

Fig. 3. SOX4 protein interacts with p53 in nucleus. (**A**) HepG2 and HEK293 cells were transfected with the indicated plasmid pairs. Equal amounts of whole-cell lysates were precleared with the A/G-agarose beads and used for immunoprecipitation (IP) with anti-FLAG beads. SOX4 was co-immunoprecipitated with p53. The blot was stripped and probed with FLAG antibody to detect immunoprecipitated SOX4 protein. The *in vivo* interaction between SOX4 and endogenous p53 was assessed in HepG2 cells. The cell lysates were also immunoprecipitated with anti-FLAG beads followed by immunoblot analysis with anti-p53 antibody. The reverse experiment was performed with immunoprecipitation using p53 antibody and western blot using anti-FLAG antibody. The blot was stripped and probed with p53 antibody to detect immunoprecipitated p53 protein. (**B**) SOX4 is distributed in the nuclear and cytoplasmic compartments of HepG2 cells, and p53 is localized predominantly in the nucleus. Nuclear and cytoplasmic compartments were prepared as described in the Materials and Methods. Transfected HepG2 cells were seeded on a slip for dual immunofluorescence analysis. Cells were stained with monoclonal anti-FLAG and polyclonal anti-p53 antibodies. The detection of SOX4 with Cy3-conjugated secondary antibody (red) shows its distribution in the cytoplasm and nucleus of HepG2 cells; the nuclear localization of p53 was detected with Alexa 488-labeled secondary antibody (green). An overlay of the images reveals the co-localization of both proteins in the nucleus (yellow; arrowhead). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole. (**C**) Mapping of the p53 interaction domain of SOX4. Various truncated SOX4 constructs were assessed for the ability to interact with p53 by co-immunoprecipitation. HEK293 cells were transiently co-transfected with pDS332-p53 and the indicated plasmid carrying the pFLAG-SOX4 (WT) vector or a deletion vector. After 24 h, total cell extracts were prepared, and equal amounts of proteins were immunoprecipitated with anti-FLAG antibody beads, followed by immunoblot analysis of the immunocomplexes using anti-p53 antibody. The relative inputs are the total cell extracts derived from HEK293-transfected cells with the indicated plasmid pairs.

glyceraldehyde 3-phosphate dehydrogenase promoter-specific primers were used to confirm specificity of recruitment of SOX4/p53 onto the Bax promoter (Figure 4C). These results therefore confirm that SOX4 is certainly bound to the Bax promoter in HepG2 cells and may thus act by associating with p53 once it is bound to the promoter.

SOX4 suppresses p53-mediated apoptosis induced by γ-irradiation in HCC cells

It is well established that p53 plays a critical role in DNA damage-induced apoptosis (35). Based on our previous study that demonstrated that SOX4 overexpression in HEK293 cells results in increased cell death (12), we examined p53-mediated apoptosis induced by

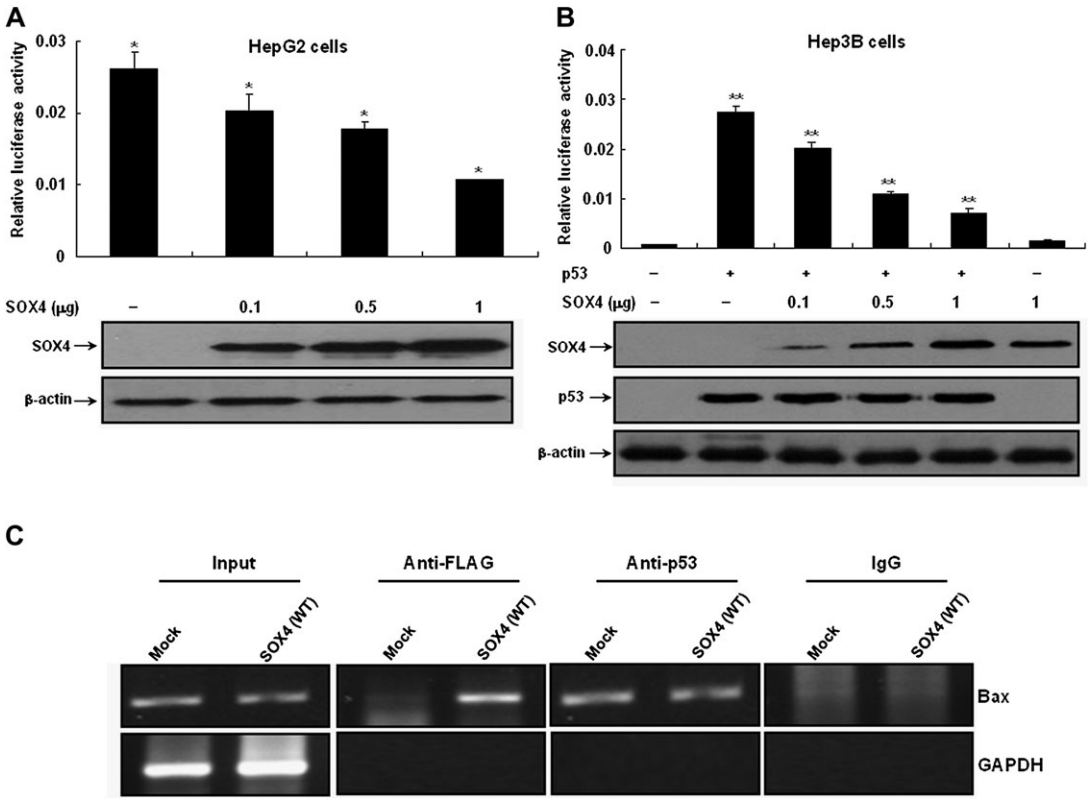

Fig. 4. SOX4 modulates p53-mediated transcription activity. (**A**) A dose–response analysis of SOX4 on the Bax promoter containing p53-responsive elements. The indicated promoter-luciferase plasmid and *Renilla* luciferase plasmid (pRL-TK) were transiently transfected into HepG2 cells expressing WT p53. (**B**) The p53-deficient Hep3B cells were transiently co-transfected with the Bax-luciferase plasmid, pRL-TK reporter plasmid and 25 ng of pDS332-p53 in combination with increasing amounts of pFLAG-SOX4 (WT) plasmid. At 24 h after transfection, the cells were lysed, and luciferase activity was analyzed. The firefly luminescence signal was normalized to the *Renilla* luminescence signal. All transfections were performed in triplicate; the data are presented as means ± SDs. $^{*}P < 0.05$ versus groups expressing SOX4. $^{**}P < 0.01$ versus groups expressing SOX4. (**C**) Chromatin immunoprecipitation with anti-FLAG antibody beads or anti-p53 antibody or an immunoprecipitation control IgG in HepG2 cells or anti-p53 antibody or an immunoprecipitation control IgG in HepG2 cells transfected with mock vector or SOX4 (WT) vector. The DNAs were then amplified by polymerase chain reaction using primers that cover a region of human Bax promoter (-250/-530), which contains the p53-binding sites. The panel shows polymerase chain reaction amplification of the immunoprecipitated DNA using primers for the glyceraldehyde 3-phosphate dehydrogenase gene promoter.

γ-irradiation in cells expressing SOX4 (WT) in order to evaluate the effects of SOX4 on the biological activity of p53 and then quantified by the annexin V–fluorescein isothiocyanate. We observed apoptosis rates of ∼160 and 135% in SOX4 (WT)-transfected HepG2 and Hep3B cells versus in the cells transfected with mock vector alone (Figure 5A and B). Ionizing radiation increased the apoptotic population to >140% in mock vector-transfected HepG2 and Hep3B cells, respectively. The γ-irradiation-induced apoptotic cell death was markedly lower (84%) in SOX4 (WT)-transfected HepG2 but not in SOX4 (WT)-transfected Hep3B cells (Figure 5A and B). We next examined whether reduced apoptosis in the SOX4-expressing cell population was caused by p53 transcription repression, as determined indirectly by measuring the endogenous Bax expression level, in irradiated SOX4 (WT)-expressing HepG2 and Hep3B cells. The Bax expression level was not significantly different between the pFLAG-SOX4 (WT)- and mock vector-transfected HepG2 and Hep3B cells in the absence of irradiation (Figure 5C). It decreased significantly in HepG2 cells expressing SOX4 following irradiation as compared with the control cells (Figure 5C). However, Bax expression decreased markedly in SOX4-expressing HepG2 cells following irradiation, as compared with the control cells (Figure 5C). These results suggest that the inhibition of γ-irradiation-induced p53-mediated apoptosis occurs through a SOX4 (WT)-mediated repression of p53 transactivational activity.

Discussion

Although various risk factors for HCC are well recognized, the mechanisms of hepatocarcinogenesis are not completely understood due to the complexity of the process. However, in general, HCC develops in the setting of cirrhosis or chronic hepatitis in which continuous exposure to injurious stimuli such as hepatitis viruses elicits chronic inflammation and hepatocyte regeneration. However, little is known about what leads to hepatocarcinogenesis. Numerous studies have shown that cellular signaling pathways involved in HCC differ in different settings based on various risk factors: the p53 pathway is affected by HBV, AFB_1 and hemochromatosis; Wnt/β-catenin is affected by HBV, hepatitis C virus, AFB_1 and alcohol; the mitogen-acivated protein kinase pathway is affected by HBV and hepatitis C virus and the retinoblastoma protein pathway is affected by HBV (2).

Mounting evidences suggest that the SOX4 protein is involved in the development of several tumor types (17,18,20–23,25); however, the way in which SOX4 exerts its oncogenic effects during the malignant transformation of normal cells remains unclear. In particular, there has been no report regarding the role of SOX4 on hepatocarcinogenesis, to date. In this study, we analyzed SOX4 expression by western blotting and immunohistochemical staining to obtain evidence that SOX4 is overexpressed significantly in human HCC compared with the corresponding non-tumorous tissue (in 63.8 and 70% of HCC cases by immunohistochemistry and western blot, respectively). We also evaluated the correlation between the SOX4 expression pattern (cytoplasm and nuclear) and clinicopathological features in HCC after surgical resection. Interestingly, the nuclear expression of SOX4 in HCC was associated significantly with better prognosis in both disease-free survival and overall survival rates ($P = 0.014$ and $P = 0.045$, respectively), indicating that patients with SOX4-related HCC have a better

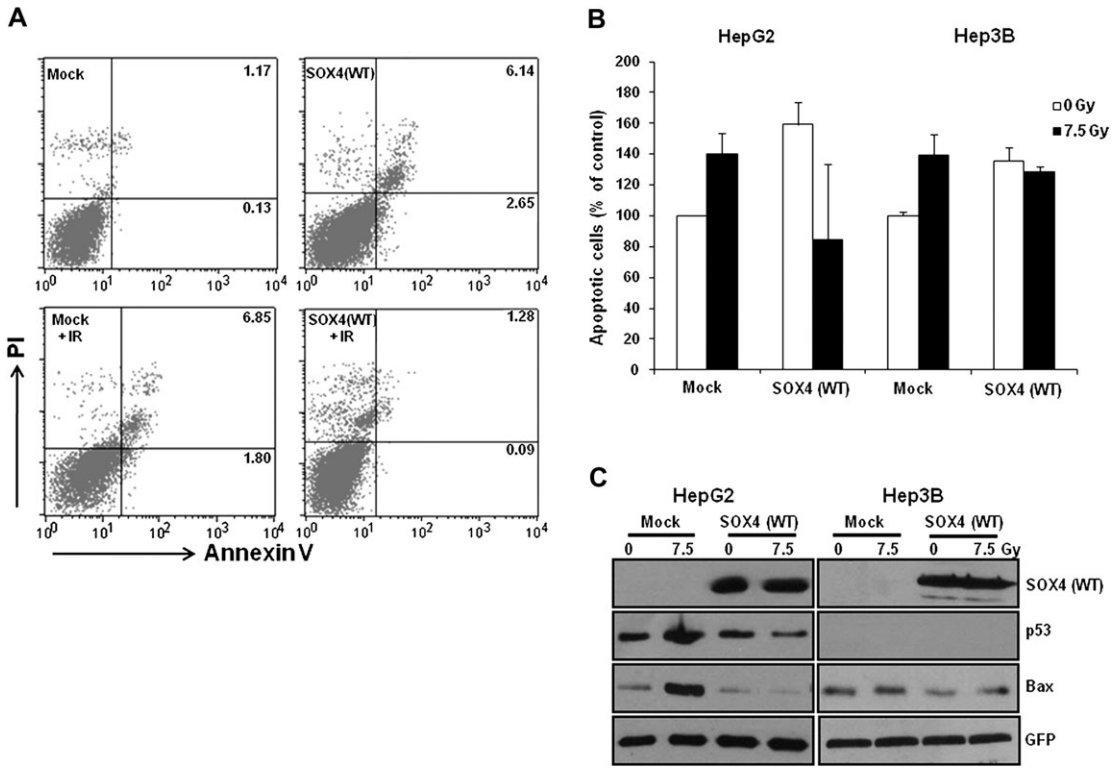

Fig. 5. SOX4 inhibits the pro-apoptotic activity of p53. (**A**) Flow cytometric analysis of annexin V–fluorescein isothiocyanate/propidium iodide double-stained cells. HepG2 cells transfected with mock vector or SOX4 (WT) vector were treated with 7.5 Gy γ-irradiation to activate endogenous p53. Apoptosis was assessed 24 h after γ-irradiation. The percentage of apoptotic cells was expressed as the percentage of annexin V-positive cells divided by total cells in the gate. (**B**) Quantitative analysis of the percentage of apoptotic cells in HepG2 and Hep3B cells transfected with mock vector or SOX4 (WT) vector. The results are presented as percentage of apoptotic cells relative to mock-transfected cells. Data are representative of three independent experiments. (**C**) SOX4 regulates Bax protein expression. Total homogenates of HepG2 and Hep3B cells transfected with mock vector or SOX4 (WT) vector, which had been treated with γ-irradiation at doses of 7.5 Gy, were analyzed by immunoblotting for the presence of SOX4, p53, Bax and green fluorescence protein (GFP). The expression level was normalized to that of GFP. Values are reported as means ± SDs from three separate experiments.

prognosis than those with SOX4-unrelated HCC. Thus, SOX4 protein expression could be a potentially useful prognostic indicator of HCC patient outcome. To the best of our knowledge, this is the first study to report such a relationship between SOX4 expression and survival rates in human HCC. Recently, Liao *et al.* (25) have showed that intrahepatic metastasis was significantly associated with SOX4 expression in messenger RNA level. However, the expression level of SOX4 was not verified by immunoblot or immunohistochemistry. This contradictory finding to our result may be explained by different methodology for confirmation of SOX4 expression because protein expression may be modified by posttranscriptional modification. In addition, it is known that SOX4 exerts its effect on transcription via cooperative binding to DNA with transcription factor partner (8). Therefore, the overexpression of SOX4 on tumor initiation and metastatic progression may be involved in regulating tumor microenvironment.

Previous microarray analyses have revealed SOX4 up-regulation in pineoblastoma (36), medulloblastoma (20), lung (37), bladder cancers (17), prostate cancer (38) and colorectal cancer (39). In bladder cancer patients, the overexpression of SOX4 protein in the nucleus and cytoplasm is significantly correlated with longer overall survival rates (17). In colorectal cancer, patients with high SOX4 transcript levels have a higher recurrence rate (39); however, they do not demonstrate the association of SOX4 protein level and recurrence in colorectal cancer. As mentioned previously, our results indicate that low nuclear SOX4 expression might reflect the malignant potential of HCC. These clinicopathological findings prompted us to investigate the role of SOX4 in human hepatocarcinogenesis. To clarify the underlying mechanism that connects SOX4 expression to HCC development, we performed an *in vitro* functional characterization of SOX4 in HCC cells. The mutation and inactivation of p53 have been reported for >50% of human cancers. In particular, it has been reported that the most common mutation associated with HCC occurs at codon 249 of p53, which is causally related to high AFB_1 (40). The inactivation of p53 owing to point mutation or allelic deletion is a crucial step during carcinogenesis and a critical event during all stages of HCC development (41,42). In many cases, these genetic alterations have contributed to the progression but not the initiation of HCC (43). Although HCC has been associated with the somatic mutation and inactivation of the p53 gene, no p53 mutation has been identified in the majority of HCC cases (44,45), suggesting that another mechanism may be involved in the development or progression of HCC. The functions of p53 are regulated by protein stabilization, posttranscriptional modifications and protein subcellular localization through interactions with numerous proteins (46). Pan *et al.* (30) were the first to report that SOX4 is a novel mediator for p53 activation in response to DNA damage, and it interacts with and stabilizes p53 protein by blocking Mdm2-mediated p53 ubiquitination and degradation. Their findings, together with our results demonstrating the overexpression of SOX4 in human HCC, led us to examine the ability of SOX4 to regulate p53 transcriptional activity in HCC cells. Furthermore, in order to determine whether SOX4 nuclear overexpression is correlated with the inactivation of p53 gene function through p53 somatic mutations, we performed p53 gene sequencing in the HCC collection. As shown in Table S1 (Supplementary data are available at *Carcinogenesis* Online), 5 of our 12 samples showed WT p53 and the other (7 out of 12) showed Arg72Pro polymorphisms without mutation, which together shows functionally competent p53 in our samples. These results support the notion that SOX4 nuclear overexpression inhibits WT p53 transcriptional activity without p53 somatic mutations.

In this study, we identified p53 as a SOX4-interacting protein and demonstrated that the SOX4–p53 interaction binds onto the Bax promoter and inhibits p53 transcriptional activity on the Bax promoter. The transcription factor SOX4 regulates SOX4-mediated transcription activity through multiple protein interactions (22,47–49). Usually, these interactions are mediated by the HMG domain of SOX4; this domain is highly conserved among all SOX proteins (10), binds DNA in a site-specific manner and facilitates protein dimerization and other protein–protein interactions (50). Here, we demonstrated that the HMG domain of SOX4 is required for the interaction between SOX4 and p53.

Although SOX4 has been well characterized in many different developmental processes, the proteins that interact with SOX4 and their target gene promoters are not well defined. Among the known SOX4-interacting proteins, syntenin is associated with interleukin 5-mediated activation via its interaction with SOX4 in B cell development (47). In addition, Pan *et al.* (48) demonstrated that human ubiquitin-conjugating enzyme 9, which regulates Bcl-2 expression, interacts with the HMG domain of SOX4 to repress SOX4 transcriptional activity. Moreover, SOX4 forms complexes with the predicted binding motifs of at least 31 unidentified target genes (25), and among the identified SOX4 target genes, *semaphorin* (*SEMA3C*) and *neuropilin-1* (*NRP1*) play important roles in tumorigenesis or tumor progression (51,52). Various SOX4 target genes may to be associated with tumorigenesis or tumor progression in other cancer types; however, only their role in HCC progression is known (25). This identification of SOX4-interacting proteins and SOX4 target genes in HCC may provide clues to the identities and roles of SOX4-binding partners in other cancer types.

The depletion of SOX4 messenger RNA via small interfering RNA can induce apoptosis in cancer cells and can regulate p53 stability (21,30), suggesting that SOX4 may regulate p53-mediated apoptosis, as indicated by the interaction between SOX4 and p53 proteins demonstrated in the present study.

In luciferase reporter assays using the p53-responsive promoters of Bax, Mdm2 and $p21^{waf1}$, we demonstrated that overexpressed SOX4 protein regulated p53-mediated transcription from the Bax promoter. The heterogeneity of p53-mediated transcription activity on p53-responsive genes (53) indicates that individual pathways are transactivated via different p53 target promoters at different sequence-specific DNA-binding motifs to accomplish the various biological functions of p53. Thus, our results suggest that p53-mediated transcriptional regulation of Bax and apoptosis could be further modulated by SOX4–p53 interaction.

In the present study, we found a significant reduction in ionizing radiation-induced apoptosis in the cells that had been transfected with SOX4 (WT), compared with cells transfected with mock vector, and this was accompanied by a reduction of Bax expression in the γ-irradiated SOX4-expressing cells (Figure 5C). These results suggest that the modulation of p53 transcriptional activity by SOX4 may be responsible for the observed decrease in apoptosis, which may lead to tumorigenesis. In addition, decreased apoptosis in the SOX4-expressing cells following irradiation indicates that SOX4 expression in any cancer cells may be related to radioresistance in a clinical setting. Although little is known about the biological functions of SOX4 in apoptosis or tumorigenesis, the outcome of SOX4 expression would be expected to differ depending on the protein that dimerizes with SOX4 and the target gene promoter that is affected. Additional studies are required to elucidate the molecular mechanisms underlying the many possible functions of SOX4.

In conclusion, our data indicate that the overexpression of SOX4 protein is closely associated with hepatocarcinogenesis in human HCC. Thus, the positivity or negativity in SOX4 expression in surgically excised HCC tissues might help to predict prognosis such as disease-free survival or overall survival. Notably, our findings suggest that SOX4 interacts with p53 and that this association in turn modulates p53-mediated transcription at the Bax promoter, leading to the inhibition of apoptosis via the suppression of Bax gene expression. These results offer a novel mechanism of SOX4 in the p53 signaling pathway that can be explored to identify new target drugs for HCC.

Funding

21C Frontier Functional Human Genome Project from the Ministry of Science and Technology, Korea (FG-08-12-05).

Supplementary material

Supplementary material can be found at http://carcin.oxfordjournals.org/

Acknowledgements

Conflict of Interest Statement: None declared.

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Received November 11, 2009; revised March 10, 2010; accepted April 2, 2010