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Expression features of SOX9 associate with tumor progression and poor prognosis of hepatocellular carcinoma

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Abstract

Background: SOX9 as a member of the SOX (SRY [sex determining region Y] box) gene superfamily has been previously demonstrated to be a proto-oncogene in a variety of malignancies. However, the clinical significance of SOX9 expression in hepatocellular carcinoma (HCC) remains unclear. The aim of this study was to investigate the expression of SOX9 in HCC and determine its correlation with tumor progression and prognosis.

Methods: One-hundred and thirty HCC patients who had undergone curative liver resection were selected and immunohistochemistry, Western blotting, and quantitative real time polymerase chain reaction (Q-PCR) were performed to analyze SOX9 expression in the respective tumors.

Results: Immunohistochemistry, Western blotting, and Q-PCR consistently confirmed SOX9 overexpression in HCC tissues compared with their adjacent nonneoplastic tissues ($P < 0.01$). Additionally, immunostaining showed more SOX9 positive cells in the higher tumor stage (T3~4) and tumor grade (G3) than in the lower tumor stage (T1~2, $P = 0.03$) and tumor grade (G1~2, $P = 0.01$), respectively. Moreover, HCC patients with high SOX9 expression were significantly associated with lower 5-year overall survival ($P < 0.01$) and lower 5-year disease-free survival ($P < 0.01$), respectively. The Cox proportional hazards model further showed that SOX9 over-expression was an independent poor prognostic factor for both 5-year disease-free survival (hazards ratio [HR] = 2.621, 95% confidence interval [CI] = 1.548-5.829, $P = 0.01$) and 5-year overall survival (HR = 3.825, CI = 1.638-7.612, $P = 0.003$) in HCC.

Conclusion: Our data suggest for the first time that the overexpression of SOX9 protein in HCC tissues is of predictive value on tumor progression and poor prognosis.

Virtual slides: The virtual slide(s) for this article can be found here: <http://www.diagnosticpathology.diagnomx.eu/vs/9029740396926377>.

Keywords: Hepatocellular carcinoma, SOX9, Expression, Tumor progression, Prognosis

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies worldwide. Especially in China, it has become a major cause of cancer-related death [1]. As a highly aggressive solid tumor, HCC is characterized by fast infiltrating growth, early metastasis, high-grade malignancy, and poor prognosis. It is often secondary to hepatitis B virus (HBV) and hepatitis C virus (HCV)

infections, both of which increase the risk of HCC 20-fold [2]. Curative therapies of surgical treatment, including hepatic resection and liver transplantation, improve the 2 short-term survival of HCC patients greatly. However, the prognosis for most patients remains poor because of multicentric recurrence and intrahepatic metastasis. The progression of HCC is a complicate process that associated with cumulative genomic alterations [3,4]. The aberrant gene expression, including oncogene upregulation and tumor suppressor downregulation, is responsible for the development of HCC. However, the molecular pathogenesis of HCC still remains unclear.

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SOX9 (sex determining region Y [SRY] related high-mobility group box 9) is a member of the SRY (sex determining region Y) box gene superfamily [5]. As a transcriptional regulator, its expression has been observed in multiple tissues during embryogenesis, including cartilage, neural crest, notochord, kidney, pancreas, and endocardial cushions of the heart [6,7]. SOX9 takes an important part in chondrogenesis, male sex gonad or respiratory epithelium development, melanocyte differentiation, and the differentiation of Paneth cells in the gut [8,9]. Recently, SOX9 has also been demonstrated to be a proto-oncogene in a variety of malignancies [10-13]. For example, Wang et al. [14] detected the expression of SOX9 in prostate cancer cells contributes to tumor growth and invasion; In primary bladder tumours, Aleman et al. [15] found that SOX9 hypermethylation was present more than half of the cases and SOX9 hypermethylation was significantly associated with tumour grade and overall survival; Malki et al. [16] shown that the embryonic male prostaglandin D synthase/SOX9 pathway was expressed at both the RNA and protein levels in different types of human ovarian tumors, pointing to SOX9 as a possible diagnostic marker for ovarian carcinomas. However, the clinical significance of SOX9 expression in HCC remains unclear. The aim of this study was to investigate the expression of SOX9 in HCC and determine its correlation with tumor progression and prognosis.

Materials and methods

Patients and tissue samples

The study was approved by the Research Ethics Committee of 302nd Hospital of PLA, Beijing, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

A total of 130 patients with primary HCC who underwent a curative liver resection at the 302nd Hospital of PLA, Beijing, China, were included in this retrospective study. Tissues used in the study were retrieved from the tissue bank of the Department of Pathology in the 302nd Hospital of PLA. These patients were diagnosed as HCC between 2001 and 2006. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. HCC diagnosis was based on World Health Organization (WHO) criteria. Tumor differentiation was defined according to the Edmondson grading system. Liver function was assessed using the Child-Pugh scoring system. Tumor staging was determined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. The clinicopathological features of 130 patients are summarized in Table I. In addition, 30 self-pairs of HCC specimens (5 TNM stage I, 8 TNM stage II, 12 TNM stage

III, and 5 TNM stage IV) and adjacent nonneoplastic liver tissues were snap-frozen in liquid nitrogen and stored at -80°C following surgery for real-time quantitative RT-PCR assay and western blot analysis.

The median follow-up period was 8.6 years. Postoperative surveillance included routine clinical and laboratory examinations every third month, computed tomography scans of the abdomen, and radiographs of the chest every third month. After 5 years, the examination interval was extended to 12 months.

Immunohistochemistry analysis

Immunohistochemical staining was carried out following the protocol of our previous study [17-19]. The primary antibody against SOX9: rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc. USA), dilution 1:50. The specificity of the primary antibody has been validated by the previous studies of Müller et al. [20] and Lü et al. [21]. Secondary antibody for the detection of primary antibody: anti-rabbit IgG (Sigma, St. Louis, MO, USA). The negative controls were processed in a similar manner with PBS instead of primary antibody. Further, positive SOX9 expression confirmed by western blotting was used as positive controls for immunostaining.

Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells showing immunoreactivity in the nucleus for SOX9 in ten representative microscopic fields was counted and the percentage of positive cells was calculated. The percentage scoring of immunoreactive tumor cells was as follows: 0 (0%), 1 (1-10%), 2 (11-50%) and 3 (>50%). The staining intensity was visually scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). A final score was obtained for each case by multiplying the percentage and the intensity score. Therefore, tumors with a multiplied score exceeding 5 (median of total scores for SOX9) were deemed to be low expressions of SOX9; all other scores were considered to be high expressions of SOX9.

Western blot

The Western blot protocol and semiquantitative analysis were carried out following the protocol of Xu et al [22]. SOX9 antibody (rabbit polyclonal antibody, dilution 1:50, Santa Cruz Biotechnology, Inc. USA) was used, and GAPDH antibody (CW0266, dilution 1:1,000, CoWin Biotech) was used as internal control.

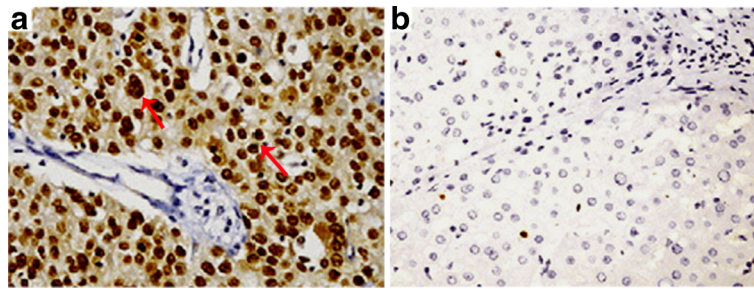


Figure 1 SOX9 expression in hepatocellular carcinoma (HCC) and adjacent nonneoplastic liver tissues (Original magnification $\times 400$). **a**, SOX9 positive staining was indicated by numerous yellowish granules in the nucleus of HCC cells; **b**, SOX9 negative staining was seen in adjacent nonneoplastic liver tissues.

Quantitative RT-PCR

To measure the mRNA expression levels of SOX9, total RNA was extracted from frozen liver tissues using TriZol reagent (Invitrogen) following the manufacturer's instructions. Two micrograms of total RNA was subjected to reverse transcription to synthesize cDNA using the ProtoScript M-MuLV Taq RT-PCR Kit (New England Biolabs), according to the manufacturer's instruction, followed by

real-time PCR using the TransStart Green qPCR SuperMix (TransGen Biotech). The primer sequences of SOX9 were forward primer, 5'-CGA ACG CAC ATC AAG ACG A-3', reverse primer, 5'-AGG TGA AGG TGG AGT AGA GGC-3'. The transcription of GAPDH was used as an internal control for normalization. SOX9 expression levels were calculated relative to GAPDH using the delta-delta computed tomography method [23].

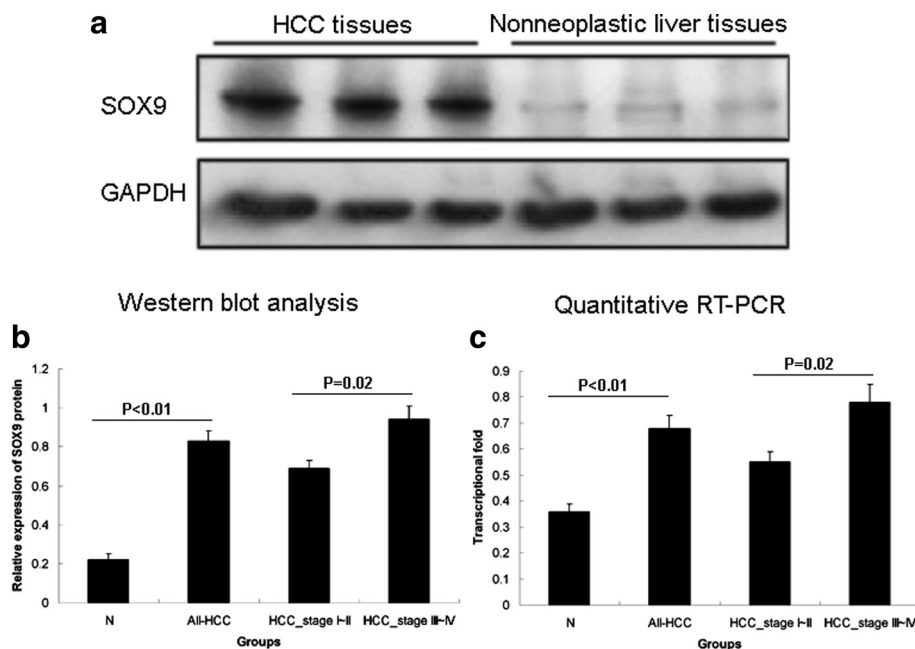


Figure 2 Increased SOX9 protein and mRNA levels in hepatocellular carcinoma (HCC) with different TNM stages and adjacent nonneoplastic liver tissues. **(a)** Representative Western blotting of SOX9 protein levels in HCC tissues and adjacent nonneoplastic liver tissues. **(b)** Semiquantitative Western blotting showed that the expression levels of SOX9 protein were significantly higher than those in adjacent nonneoplastic liver tissues ($P < 0.01$). Additionally, the expression levels of SOX9 protein were increased with ascending tumor TNM stages. GAPDH was used as internal control. Means, standard deviation (SD), and P values were given (T test). **(c)** Quantitative RT-PCR assay showed significantly increased SOX9 mRNA level in HCC tissues compared with adjacent nonneoplastic liver tissues ($P < 0.01$). Additionally, the expression levels of SOX9 mRNA were increased with ascending tumor TNM stages. GAPDH was used as internal control. Means, standard deviation (SD), and P values were given (Mann-Whitney test).

Statistical analysis

The software of SPSS version13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Fisher's exact test and the χ^2 test were performed to assess associations between SOX9 expression and clinicopathological parameters. The Kaplan-Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. A multivariate survival analysis was performed for all parameters that were significant in the univariate analyses using the Cox regression model. Differences were considered statistically significant when P was less than 0.05.

Results

Expression of SOX9 protein and mRNA in HCC

Immunohistochemical analysis revealed that SOX9 staining was mainly localized in the nucleus of HCC cells (Figure 1a). SOX9 expression was absent or sporadic in adjacent nonneoplastic liver tissues (Figure 1b). In addition, we found 98 (75.38%) of 130 HCC tissues with high SOX9 expression and 32 (24.62%) of 130 HCC tissues with low SOX9 expression, while 6 (4.62%) of 130 adjacent nonneoplastic liver tissues with high SOX9 expression and 124 (95.38%) of 130 adjacent nonneoplastic liver tissues with low SOX9 expression. Thus, the SOX9 immunostainings in HCC tissues were significantly higher than those in the adjacent nonneoplastic liver tissues ($P < 0.01$).

To confirm SOX9 protein expression by an independent method, Western blot analysis was performed using 30 self-pairs of HCC and adjacent nonneoplastic liver tissues. The distinct overexpression of SOX9 protein in HCC tissues compared with adjacent nonneoplastic liver tissues was also detected ($P < 0.01$, Figure 2a and b), as well as significantly increased mRNA level by quantitative RT-PCR ($P < 0.01$, Figure 2c). The expression levels of SOX9 protein and mRNA in HCC tissues with high stage (III-IV) were both significantly stronger than those with low stage (I-II; for protein and mRNA: both $P = 0.02$; Figure 2b and c).

Association of SOX9 expression with the clinicopathological features of HCC

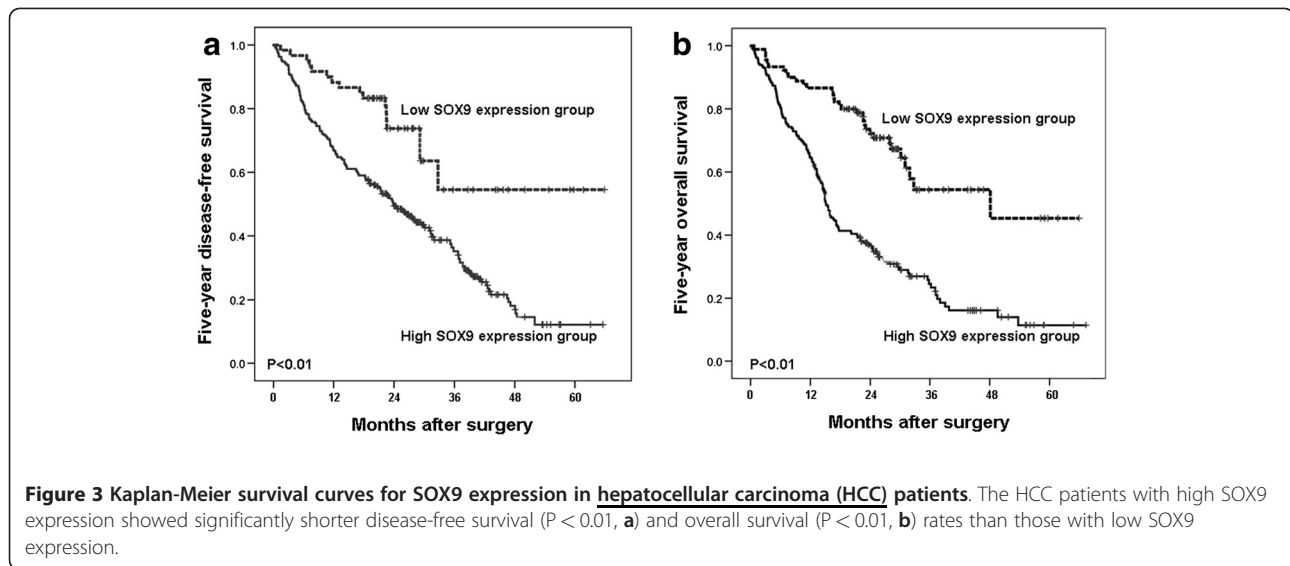
To evaluate whether SOX9 protein expression was associated with clinicopathological features of patients with HCC, we correlated immunohistochemical SOX9 staining results with T stage, tumor grade, presence of cirrhosis, underlying liver disease including alcohol abuse, viral hepatitis B and C, sex, and age (Table 1). As the results, we found that more SOX9 positive cells in the higher tumor stage (T3~4) and tumor grade (G3) than in the lower tumor stage (T1~2, $P = 0.03$) and tumor grade (G1~2, $P = 0.01$), respectively.

Table 1 Clinicopathological features and the expression of SOX9 in 130 hepatocellular carcinoma patients

Clinicopathological Features	Case	SOX9 expression frequency (n,%)		P
		High	Low	
Age (years)				
≤50	72	55 (76.39)	17 (23.61)	NS
>50	58	43 (74.14)	15 (25.86)	
Gender				
Male	96	73 (76.04)	23 (23.96)	NS
Female	34	25 (73.53)	9 (26.47)	
Tumor stage				
T1	23	8 (34.78)	15 (65.22)	0.03
T2	40	25 (62.50)	15 (37.50)	
T3	52	50 (96.15)	2 (3.85)	
T4	15	15 (100.00)	0 (0)	
Tumor grade				
G1	31	18 (58.01)	13 (41.99)	0.01
G2	76	58 (76.32)	18 (23.68)	
G3	23	22 (95.65)	1 (4.35)	
Growth pattern				
Trabecular	101	78 (77.23)	23 (22.77)	NS
Nontrabecular	29	20 (68.97)	9 (31.03)	
Cirrhosis				
Yes	86	62 (72.09)	24 (27.91)	NS
No	44	36 (81.82)	8 (18.18)	
Underlying liver disease				
Alcoholic	25	18 (72.00)	7 (28.00)	NS
Hepatitis B	49	40 (81.63)	9 (18.37)	
Hepatitis C	35	28 (80.00)	7 (20.00)	
Unknown	21	12 (57.14)	9 (42.86)	

Prognostic values of SOX9 expression in HCC

Five-year disease-free survival was observed in 30 (23.08%) patients, whereas in 100 (76.92%) patients, disease recurred, and 88 (67.69%) even died during a 5-year follow-up period. We observed a trend that 5-year disease-free survival in the group with high SOX9 expression was significantly poorer than that in the group with low SOX9 expression ($P < 0.01$, log-rank test; Figure 3a). Additionally, the Kaplan-Meier plot of 5-year overall survival curves stratified by SOX9 expression was shown in Figure 3b. A significant relationship was found between SOX9 expression and 5-year overall survival ($P < 0.01$, log-rank test, Figure 3b). Furthermore, in a multivariate Cox model, including tumor size, tumor stage, tumor grading, presence of cirrhosis, gender, age, and SOX9 staining, we found that SOX9 expression was an independent poor prognostic factor for both 5-year



disease-free survival (hazards ratio [HR] = 2.621, 95% confidence interval [CI] = 1.548-5.829, $P = 0.01$, Table 2) and 5-year overall survival (HR = 3.825, CI = 1.638-7.612, $P = 0.003$, Table 2) in HCC.

Discussion

In the present study, we provide the first analysis of SOX9 protein and mRNA expression in human HCC tissue and its association with patient clinical outcome. SOX9 immunoreactivity was significantly increased in a substantial proportion of HCC cases compared with their adjacent nonneoplastic liver tissue. The overexpression of SOX9 was observed in tumor tissues with higher tumor stage and higher tumor grade. Additionally, our investigation reveals that high SOX9 expression is associated with a significant trend toward both poorer disease-free survival and poorer overall survival. Our study further confirms that high SOX9 expression independently predicts a higher risk of disease relapse or death after multivariate adjustment for other prognostic factors.

Members of SOX gene family share homology with the high-mobility group box of the sex-determining region Y (SRY), which encodes transcription factors that bind to high-mobility group domains of DNA [24]. SOX9 belongs to the subgroup of SOX E genes, which play vital roles in the regulation of the differentiation of astrocytes, oligodendrocytes, and Schwann cells [25]. SOX9 is involved in the development of multiple tissues and in maintaining the stem cell compartments in adult tissues [26]. Mutations in the SOX9 gene may result in autosomal XY sex reversal and in campomelic dysplasia, a syndrome with severely malformed skeleton [27]. Recent studies have demonstrated the direct roles for SOX9 in tumorigenesis. In digestive system tumors, Jiang et al. [28] found that SOX9-transfected cells injected into severe combined immunodeficient mice show markedly stronger tumorigenicity, whereas SOX9-knockdown cells injected into severe combined immunodeficient mice show significantly attenuated tumorigenicity in mice. Sashikawa et al. [29] then detected the expression of SOX9 in human intestinal metaplasia and gastric

Table 2 Multivariate survival analysis of five-year overall and disease-free survival in 130 patients with **hepatocellular carcinoma**

Features	Five-year overall survival			Five-year disease-free survival		
	HR	95% CI	P	HR	95% CI	P
Age	1.132	0.316-3.516	0.192	1.536	0.322-3.736	0.125
Gender	1.191	0.345-3.857	0.136	1.559	0.357-3.831	0.131
Tumor size	1.931	0.685-4.056	0.063	1.953	0.615-4.273	0.062
Tumor stage	2.879	1.366-5.196	0.009	2.686	1.386-6.009	0.01
Tumor grade	1.563	0.609-4.088	0.081	1.551	0.607-4.466	0.086
Presence of cirrhosis	1.919	0.738-4.102	0.063	1.921	0.793-4.219	0.062
SOX9 expression	3.825	1.638-7.612	0.003	2.621	1.548-5.829	0.01

carcinoma. Liu et al. [30] further demonstrated that SOX9 expression significantly increased from nonneoplastic lesions to gastric neoplastic lesions, which might promote the tumor progression of gastric carcinoma. On the other hand, Jay et al. [31] found that the overexpression of SOX9, a novel intestinal crypt transcription factor, may inhibit carcinoembryonic antigen expression and may induce apoptosis in a human colon carcinoma cell line. In human colorectal cancer tissues, Lü et al. [21] also detected the overexpression of SOX9, and further demonstrated that the detection of SOX9 expression might contribute to predicting clinical outcomes for patients with this tumor. However, the role of SOX9 in HCC remains to be elucidated. In this study, our data may offer new insight into SOX9 that is potentially important in the progression of HCC, as well as new prognostic factor for HCC. As the 130 cases of the present study were all Chinese population, the results reported here should be further confirmed in other populations.

In conclusion, our study suggests that SOX9 is overexpressed in HCC tissues compared with their benign counterparts. To the best of our knowledge, this is the first study evaluating the expression levels of SOX9 mRNA and protein in HCC tissues and its association with clinicopathologic parameters. Especially, the most important finding of this study is that SOX9 also is a novel and potential factor for predicting the poorer prognosis of HCC patients after surgery. Further studies are needed to investigate the precise function of SOX9 in the progression of HCC.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Guo XD, Xiong L, Li HW and Zhao JM: participated in study design and coordination, analysis and interpretation of data, material support for obtained funding, and supervised study; Sun T, Peng RY, and Zou L: help to translated and edit the paper; Zhu HY and Zhang J: carry out part of the experiments. All authors read and approved the final manuscript.

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