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Original Article

Nuclear expression of SOX18 in cancer cells indicates a poor prognosis in patients with hepatocellular carcinoma

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Abstract: Transcription factor SOX18 is involved in the development of many tumors, but SOX18 protein expression in hepatocellular carcinoma (HCC) has not been studied. Thus, we measured SOX18 protein expression in HCC and correlated its significance for patients with HCC. After measuring expression and identifying the location of SOX18 in 153 paired HCC and adjacent non-tumor tissues, we found that SOX18 protein was chiefly expressed in the HCC cell nucleus, but was mainly expressed in normal liver cell cytoplasms. Also, nuclear expression of SOX18 protein was associated with TNM stage ($P<0.01$) and vascular invasion ($P<0.01$) of HCC. Furthermore, greater nuclear expression of SOX18 protein indicated shorter 3-year overall and disease-free survival of patients with HCC. Thus, SOX18 may be a prognostic marker for HCC.

Keywords: SOX18, nuclear expression, Hepatocellular carcinoma, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer-associated deaths worldwide [1, 2]. Approximately, 675,000 new HCC cases are diagnosed annually globally and although treatments including hepatic resection, chemotherapy and radiotherapy offer hope, the 5-year survival for liver cancer patients is only 16.6% [3]. Thus, better understanding of underlying molecular mechanisms of hepatocarcinogenesis is need.

The SOX (Sex-determining region on the Y chromosomerelated high mobility group box) genes are a well-conserved gene family that encodes a group of transcription factors and SOX18 belongs to the subgroup F of SOX family [4, 5]. Previous studies indicate that SOX18 contributes to neonatal and postnatal vascularization [6, 7], and SOX18 loss function contributes to cardiovascular and hair follicle defects in ragged mice and hypotrichosis-lymphodema-teleangiectasia (HLT) syndrome in humans [8, 9].

Recently, SOX18 has been reported to be involved in tumor development: over-expression of SOX18 promotes migration and invasion of osteosarcoma and cervical carcinoma cell [10, 11]. Also, over-expression of SOX18 indicates poor prognosis for non-small cell lung cancer and gastric cancer patients [12, 13]. However, SOX18 protein in HCC has not been reported.

We measured the expression and location of SOX18 in HCC tissues and analyzed an association between SOX18 location in HCC cells and clinicopathological features of HCC patients. We observed that the SOX18 protein mainly localized to the nucleus of HCC cells, but was chiefly in the cytoplasm of non-tumor liver cells. Also, nuclear SOX18 protein expression indicated poor prognosis for HCC patients.

Materials and methods

Patients and tissue samples

Tumor and matched non-tumor liver tissues were obtained from 153 HCC patients who underwent hepatic resection and were diagnosed his-

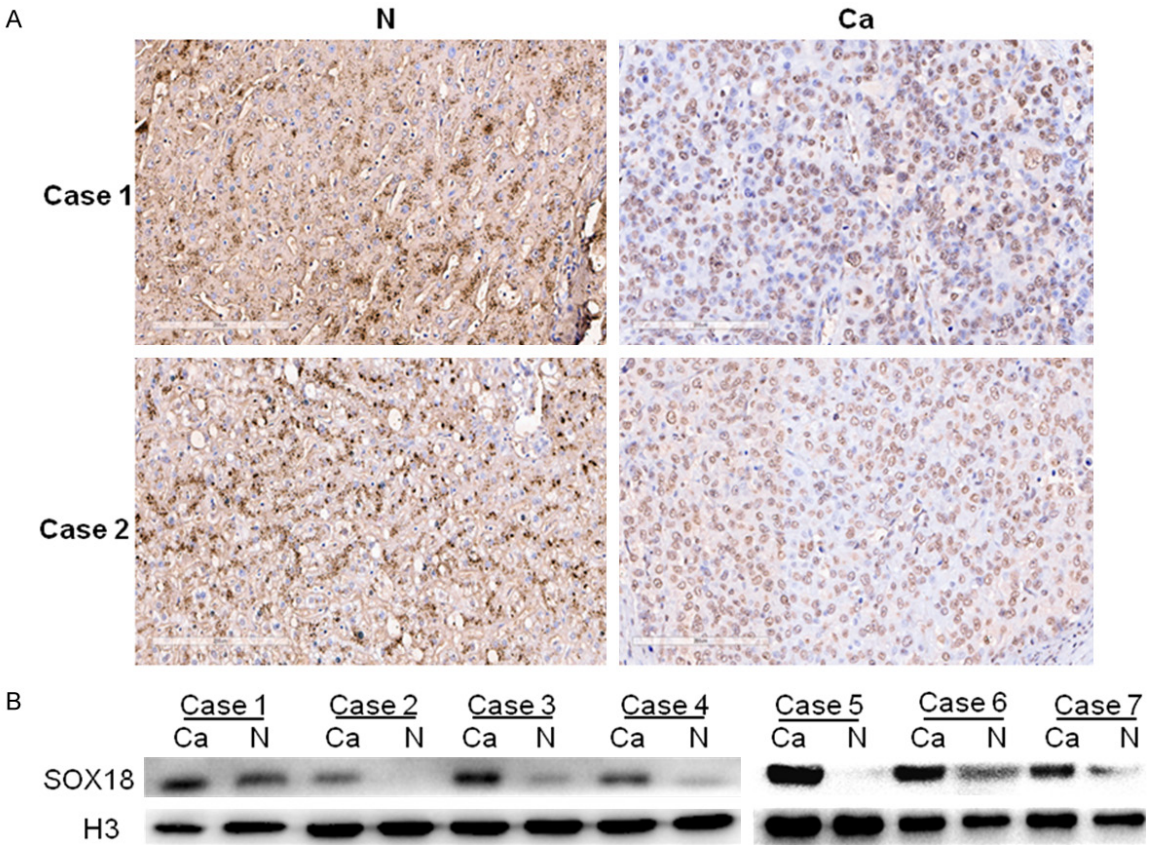

Figure 1. Expression of SOX18 in hepatocellular carcinoma (HCC) and adjacent non-tumor liver tissues. A. SOX18 protein mainly localized to the nucleus of HCC cells, but was chiefly in the cytoplasm of non-tumor liver cells. B. Western Blot analysis of 7 representative cases showed that the nuclear expression of SOX18 was increased in HCC cells compared with non-tumor liver cells. (Ca, HCC tissues; N, non-tumor liver tissues).

Table 1. SOX18 expression in HCC cells and non-tumor liver cells

Tissues	SOX18 expression in nucleus		*P* value	SOX18 expression in cytoplasm		*P* value
	Low	High		Low	High	
Tumor	88 (57.52%)	65 (42.48%)	*<0.01*	138 (90.2%)	15 (9.8%)	*<0.01*
Non-tumor	141 (92.16%)	12 (7.84%)		70 (45.75%)	83 (54.25%)	

topathologically at Peking University People's Hospital from 2009 to 2012. Samples were snap-frozen in liquid nitrogen and then stored at -80°C or fixed in formaldehyde solution. The clinicopathological features of patients and samples were collected from the patient information management system of our hospital. The TNM system of the International Union Against Cancer and American Joint Committee on Cancer was used to judge the tumor stages and the Edmondson-Steiner grading system was used to assess the histological grade [14, 15]. This study was conducted with the approval of the Ethics Committee of Peking University People's Hospital.

Immunohistochemistry

HCC tissues were fixed in 10% formaldehyde solution after resection and then embedded in paraffin. Next, 5-μm-thick sections were cut from the paraffin blocks and placed on poly-L-lysine-coated slides. Slides were deparaffinized through xylene and rehydrated in graded ethyl alcohols and then rinsed in distilled water. After quenching endogenous peroxidase activity with 0.3% H_2O_2 for 30 min at room temperature, slides were autoclaved for 8 min in sodium citrate buffer (10 mM, pH 6.0) for antigen retrieval. Slides were blocked with 10% normal goat serum (Beyotime Biotechnology, Beijing, China) for 30 min and incubated with anti-

Table 2. Nuclear expression of SOX18 and clinicopathological features in 153 hepatocellular carcinoma patients

Features		Low sox18 expression	High sox18 expression	*P* value
Age (year)	<50	48 (31.37%)	35 (22.88%)	0.531
	≥50	40 (26.14%)	30 (19.61%)	
Sex	Female	8 (5.22%)	7 (4.58%)	0.787
	Male	80 (52.29%)	58 (37.91%)	
Tumor size (cm)	<5	40 (26.14%)	25 (16.34%)	0.412
	≥5	48 (31.37%)	40 (26.14%)	
Vascular invasion	None	42 (27.45%)	17 (11.11%)	*0.007*
	Present	46 (30.07%)	48 (31.37%)	
Tumor grade	G1-G2	14 (9.15%)	11 (7.19%)	0.867
	G3-G4	74 (48.37%)	54 (35.30%)	
TNM stage	I-II	72 (47.06%)	35 (22.88%)	*<0.001*
	III-IV	16 (10.46%)	30 (19.61%)	
Recurrence	No	42 (27.45%)	22 (14.38%)	0.099
	Yes	46 (30.07%)	43 (28.10%)	
AFP(μg/L)	<400	45 (29.41%)	34 (22.22%)	1.000
	≥400	43 (28.10%)	31 (20.26%)	

SOX18 antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Next, the slides were incubated with biotin-conjugated secondary antibody (1:100, Zhongshan Jinqiao Biology Technology Company, Beijing, China) for 45 min at room temperature, and the 3, 3-diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen. Finally, slides were counterstained with hematoxylin. Each slide was assessed by 2 double-blinded pathologists in 2 respects: the proportion and the staining intensity of positive cells. The proportion of positive cells was graded as follows: grade 0, no positive cells; grade 1, 1%-25% positive cells; grade 2, 25%-50% positive cells; grade 3, >50% positive cells. The staining intensity, ranging from light brown to dark brown, was graded as follows: 0, negative; 1, weak intensity; 2, moderate intensity; 3, strong intensity. The final score of each slide was generated by multiplying the two values. A score of <6 was regarded as low, while a score of ≥6 was regarded as high [16].

Nuclear protein extraction and Western blotting

Nuclear protein of each clinical specimen was extracted with a nuclear extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's protocol, and the concentration was measured by taking bovine serum albumin (BSA) as a standard. Equal amount of nuclear protein was separated by 10% SDS-PAGE and then transferred to PVDF membranes (PALL life science, New York, USA). After blocked with 5% skim milk, the membranes were incubated with anti-human SOX18 antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-human Histone H3 antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was taken as an internal control, overnight at 4°C. Next, the membranes were incubated with the corresponding secondary antibody (1:2000, Zhongshan Jinqiao Biology technology Company, Beijing, China) conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. Finally, the ECL Western Blotting system (Thermo, South Logan, UT, USA) was used to detect the immunolabeled proteins.

Statistical analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The χ^2 test was used to estimate correlations between expression of SOX18 and clinicopathologic features, and the χ^2 test was also used for assessing SOX18 in HCC and non-tumor liver tissues. A Kaplan-Meier method and a log-rank test were used to calculated 3-year overall survival (OS) and disease-free survival (DFS). A multivariate Cox model was used to for multivariate

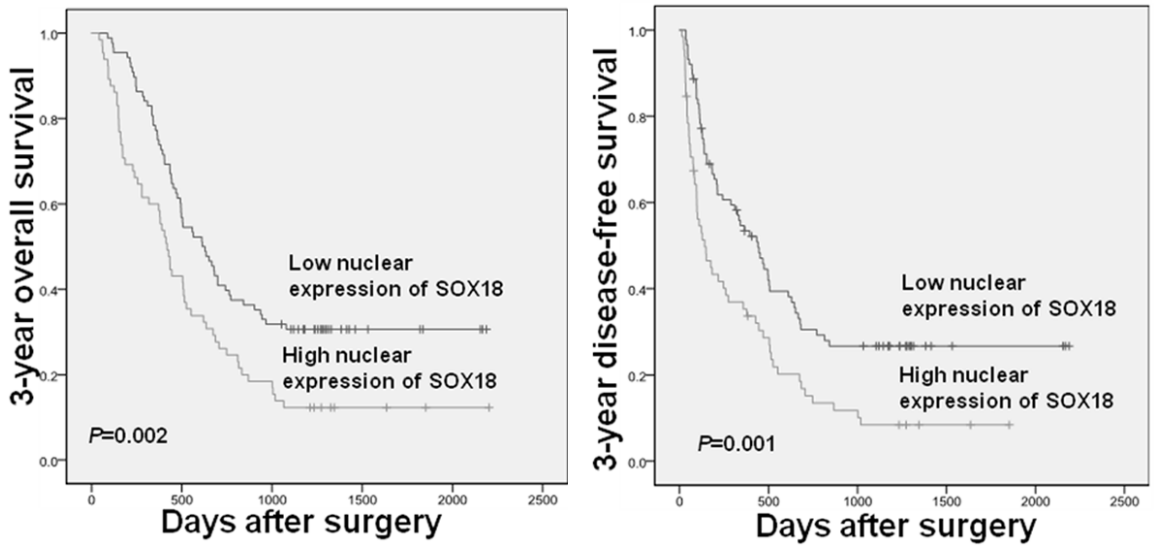

Figure 2. Kaplan-Meier survival curve results showed that HCC patients with more nuclear expression of SOX18 in HCC cells had significantly shorter 3-year overall survival (P<0.01) and 3-year disease-free survival (P<0.01) rates than those with less nuclear expression of SOX18.

Table 3. Multivariate survival analysis of 3-year overall survival and disease-free survival in 153 HCC patients

features	3-year overall survival			3-year disease-free survival		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Tumor size	1.424	0.964-2.104	0.076	1.546	1.049-2.278	0.028
TNM stage	1.100	0.725-1.668	0.655	0.996	0.656-1.513	0.985
Tumor grade	1.730	0.938-3.191	0.079	2.086	1.145-3.800	0.016
Vascular invasion	1.663	1.051-2.632	*0.030*	1.921	1.216-3.034	*0.005*
Serum AFP	1.072	0.730-1.573	0.724	1.305	0.885-1.926	0.179
Nuclear expression of SOX18	1.640	1.123-2.395	*0.010*	1.716	1.170-2.518	*0.006*

analysis, and results are presented with 95% confidence intervals (CI) and hazards ratio (HR). P<0.05 was considered to be statistically significant.

Results

Expression of SOX18 protein in HCC

Immunohistochemistry (IHC) results showed that SOX18 was expressed in HCC tissues and in adjacent non-tumor tissues. In HCC cells, SOX-18 expression was chiefly nuclear and in adjacent non-tumor cells, SOX18 staining was mainly localized to the cytoplasm (**Figure 1A**; **Table 1**).

To verify IHC, we extracted nuclear protein from HCC cells and matched non-tumor liver cells and use Western blot to confirm that HCC cells had more nuclear SOX18 expression compared with non-tumor liver cells (**Figure 1B**).

SOX18 protein nuclear expression and HCC clinicopathological features

Using a χ^2 test, we assessed patient age, sex, tumor size, vascular invasion, TNM stage, tumor grade, recurrence and serum AFP (**Table 2**), and we noted that nuclear expression of SOX18 protein was significantly correlated to vascular invasion (P<0.01) and TNM stage (P<0.01).

Prognostic value of nuclear expression of SOX18 in HCC

The Kaplan-Meier method was used to investigate nuclear expression of SOX18 protein and overall survival (OS) and disease-free survival (DFS). **Figure 2** showed that patients with more

nuclear expression of SOX18 tended to have prolonged OS (P<0.01, a) and DFS (P<0.01, b) compared to those with low nuclear SOX18 expression. Then, a multivariate Cox model was used for multivariable analysis, and nuclear SOX18 expression was an independent poor prognostic factor for both 3-year OS and DFS (**Table 3**).

Discussion

Recently, SOX18 has been shown to be over-expressed in many tumors. Wang's group reported that the SOX18 mRNA was over-expressed in HCC compared with non-tumor tissues [17]. However mRNA does not always approximate protein. Recent studies in non-small cell lung cancer indicated non-small cell lung cancer tissues had more SOX18 protein but less mRNA expression compared with paired normal lung tissues [12, 18, 19].

Thus, we measured the expression and location of SOX18 protein in cells, and HCC cells had the greatest SOX18 protein in the nucleus, but non-tumor liver cells had more cytoplasmic SOX18 protein expression. Nuclear expression of SOX18 protein was associated with TNM stage and vascular invasion, and high nuclear expression of SOX18 protein indicated a poor 3-year OS and DFS according to Kaplan-Meier analysis. A multivariate Cox model confirmed that nuclear expression of SOX18 protein was an independent poor prognostic factor for 3-year OS and DFS.

Nuclear expression of SOX18 in HCC cells may be explained by the specific structure of this transcription factor. SOX18 has a high-mobility group domain, which can bind to the 5'-CAAG-3' DNA sequence motif specifically [20, 21]. Specifically, SOX18 protein can bind to the minor groove of DNA to modify its conformation [22], thus regulating expression of other genes. Therefore, we speculate that nuclear expression of SOX18 in HCC cells is an activated state of SOX18, which may regulate another gene or cell signaling pathway. For example, in human endothelial cells, SOX18 regulates the expression of MMP-7 by binding to the proximal site in the *MMP-7* promoter [23].

SOX18, SOX7 and SOX17 are all subgroup F members of SOX family, and studies show that SOX7 and SOX17 are involved in Wnt/β-catenin signaling pathway [24, 25]. Therefore, the underlying association between nuclear expression of SOX18 in HCC cells and Wnt/β-catenin signaling pathway activity will be the focus of future studies. We have shown for the first time that nuclear expression of SOX18 may be a new prognostic marker for HCC outcomes, but more intensive studies are needed to reveal the role of SOX18 in HCC.

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Disclosure of conflict of interest

None.

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