Int J Clin Exp Pathol 2017;10(7):7873-7881
www.ijcep.com /ISSN:1936-2625/IJCEP0056519

Original Article

Overexpression of Sox3 is associated with promoted tumor progression and poor prognosis in hepatocellular carcinoma

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Received May 1, 2017; Accepted June 27, 2017; Epub July 1, 2017; Published July 15, 2017

Abstract: Hepatocellular carcinoma (HCC) is a common malignant tumor lacking sensitive biomarkers for prognosis. Sox3, a member of the Sex determining region Y box gene superfamily, has been demonstrated to be an oncogene in many cancers. However, the expression and clinical importance of Sox3 in HCC remains elusive. In this study, fifty pairs of HCC tissues with adjacent non-tumor samples were collected for detecting Sox3 expression by qPCR and immunoblotting analyses. A total of 104 HCC tissues were included for immunohistochemistry assay and analyzed by immunostaining scores. The correlation of Sox3 expression with clinicopathological factors and prognosis of HCC patients were calculated. Sox3 expression in HCC tissues was significantly higher than that in the non-tumor counterparts at the mRNA and protein levels. High staining scores of Sox3 was detected in 75.96% of HCC tissues. Statistical analyses demonstrated that highly expressed Sox3 was significantly correlated with low tumor capsule formation, advanced tumor stage and poor tumor differentiation. Moreover, patients with high Sox3 expression showed worse recurrence-free survival and overall survival than those with low Sox3 expression, and multivariate analyses further indicated that status of Sox3 expression is an independent prognostic factor in HCC patients. Therefore, our results suggested that overexpression of Sox3 in HCC tissues is correlated with increased tumor development and poor prognosis in HCC.

Keywords: Sox3, hepatocellular carcinoma, clinicopathological features, tumor prognosis

Introduction

Liver cancer is one of the most common malignancies and the second leading cause for cancer-related death in humans [1]. Nowadays, hepatocellular carcinoma (HCC) accounts for approximately 80% in all primary liver cancers. The development of HCC is presently considered a multistep process, though the underlying pathogenesis remains unclear [2]. Despite tumor resection and liver transplantation being curative treatments for early-stage patients suffering from HCC [3, 4], many patients are diagnosed in advanced stages and cannot benefit from the surgery. What is worse, as the only approved drug available for the treatment of unresectable HCC, Sorafenib reports extending survival only from 7.9 months to 10.7 months [5]. Therefore, if we want to improve the outcome of HCC, one of the utmost needs is to elucidate sensitive biomarkers for prognosis.

Sox3 (sex determining region Y [SRY] related high-mobility group box 3) belongs to SRY box gene superfamily of DNA-binding proteins [6]. It has been reported that Sox3 is evolutionally conserved and plays a key role in many developmental processes, such as neurogenesis, pituitary, craniofacial and testis development [7-10]. While Sox3 expresses high in the development of central nervous system, its expression decreases when tissue matures, correlating with the conversion from cell proliferation to differentiation [11]. Moreover, several studies suggested that Sox3 behaves as an oncogene. Yan and his colleagues reported that Sox3 expression increased from benign and borderline to malignant ovarian tumors and its overexpression promoted proliferation, migration and invasion, while restrained apoptosis and adhesion of ovarian cancer cells [12]. Another study indicated that Sox3 overexpression was critically involved in the pathogenesis of choriocar-

Table 1. Clinicopathological features of hepatocellular carcinoma patients with high or low Sox3 expression

Clinicopathological features	Sox3 expression		Case	*P* value
	High	Low		
Age (years)				
≤ 60	60	14	74	0.076
> 60	19	11	30	
Gender				
Male	68	18	86	0.131
Female	11	7	18	
Symptom				
Yes	28	4	32	0.084
No	51	21	72	
HBV infection				
Yes	67	19	86	0.365
No	12	6	18	
Cirrhosis				
Yes	56	16	72	0.620
No	23	9	32	
Serum AFP (μg/L)				
≤ 400	70	18	88	0.059
> 400	9	7	16	
TBIL (μmol/L)				
≤ 20	74	22	96	0.395
> 20	5	3	8	
Platelet ($\times 10^9$/L)				
≤ 100	49	17	66	0.641
> 100	30	8	38	
Tumor number				
Single	75	23	98	0.629
Multiple	4	2	6	
Tumor size (cm)				
< 5	57	15	72	0.321
≥ 5	22	10	32	
Tumor capsule				
Yes	33	3	36	0.007
No	46	22	68	
Tumor thrombi				
Yes	5	2	7	0.673
No	74	23	97	
Microvascular invasion				
Yes	16	10	26	0.064
No	63	15	78	
TNM stage				
T1	39	19	58	0.028
T2	19	5	24	
T3	21	1	22	
Tumor grade				
G1-G2	39	19	58	0.005
G3-G4	40	6	46	

Abbreviation: HBV, hepatitis B virus; AFP, alpha fetoprotein; TBIL, total bilirubin; TNM, tumor-node-metastasis.

cinoma [13]. And Li et al. inferred that Sox3 played an important role in tumor development and served as a potential prognostic biomarker for esophageal squamous cell carcinoma [14]. However, the correlation of Sox3 expression with tumor progression and prognosis in HCC remains elusive.

In this study, we performed real-time PCR, immunoblotting and immunohistochemistry assays to detect the expression level and pattern of Sox3 in HCC tissues and corresponding non-tumor counterparts, and then analyzed the association of its expression with clinicopathological features and prognosis. Interestingly, we found that the mRNA and protein expression levels of Sox3 were significantly increased in HCC tissues compared to adjacent non-tumor regions. Furthermore, elevated expression of Sox3 was significantly associated with higher tumor progression and worse prognosis in HCC.

Patients and methods

Patients and tissue samples

The study protocol was approved by the Ethics Committee of Eastern Hepatobiliary Surgery Hospital, Shanghai, China and an informed consent on the use of removed surgical samples was given to each patient.

A total of 104 HCC samples and 50 pairs of tumor tissues and corresponding non-tumor liver samples of HCC patients were collected from Eastern Hepatobiliary Surgery Hospital. These samples came from the patients who underwent hepatectomy between January 2009 and December 2011 and received neither chemotherapy nor radiotherapy before surgery. HCC diagnosis was determined according to the criteria of World Health Organization (WHO). Tumor differentiation was based on the Edmondson grading system and tumor stage was designated according to the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. The detailed features of 104 patients are summarized in **Table 1**. All 104 cases of HCC specimens used for immunostaining were stored in paraffin blocks. Another 50 pairs of HCC and adjacent non-tumor liver tissues used for real-time PCR and immunoblotting were snap-frozen in liquid nitrogen and stored at -80°C.

Quantitative real-time PCR

Quantification of Sox3 gene expression levels were performed by quantitative real-time PCR (qPCR). Total RNA was extracted with RNeasy Microarray Tissue Mini Kit (Qiagen, Hilden, Germany) following the manufacturer instruction. For reverse transcription, 2 μg of total RNA was used to synthesize cDNA by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA) according to the manufacture specification. For real-time PCR, 4 μl of cDNA template was used in a 10 μl reaction system containing 1 μl of primer and 5 μl of SYBR Green Mix (Thermo Scientific, Massachusetts, USA). All reactions were performed in triplicate using the following cycling conditions: 7 minutes at 95°C, followed by 40 cycles of 95°C for 10 seconds and 60°C for 22 seconds using a real-time PCR gradient system (Stratagene, California, USA). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T). The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for Sox3 gene and the mean C_T value of that sample for the endogenous control (glyceraldehyde-3-phosphate-dehydrogenase, GAPDH). Relative expression was calculated as the difference ($\Delta\Delta C_T$) between the ΔC_T values of the tumor tissues group and paired non-tumor specimen group. The relative level of expression was measured as $2^{-\Delta\Delta CT}$. The primers for Sox3 and GAPDH were as follows: Sox3 forward primer, 5'-AACGCCTTCATGGTATGGTC-3'; Sox3 reverse primer, 5'-GTCGGTCAGCAGTTTCCAGT-3'; GAPDH forward primer, 5'-CAAGGCTGTGGGCAAGGTCATC-3'; GAPDH reverse primer, 5'-CGTCAAAGGTGGAGGAGTGGGT-3'.

Western blot analysis

The whole liver tissue lysates were extracted with RIPA buffer as reported [15]. Protein amounts were assessed with BCA reagent from Bio-Rad. Forty micrograms protein of tissue lysates were loaded in each lane of SDS-polyacrylamide electrophoresis gel. Then resolved proteins were transferred to polyvinylidene difluoride membrane, which was sequentially blocked with 5% bovine serum albumin (Sigma, Missouri, USA) for 1 hour at room temperature and incubated with anti-Sox3 antibody (ab104248, Abcam, Cambridge, UK, dilution 1:100) or anti-β-actin antibody (A1536, Sigma, Missouri, USA, dilution 1:300) overnight at 4°C. Next, the blot membrane was washed three times with TBST for 5 minutes each time and subsequently incubated with specific secondary antibodies according to the primary antibody for 1 hour at room temperature. At last, the blot signal was revealed with a chemiluminescence kit (Bio-Rad, California, USA).

Immunohistochemical staining analysis

Paraffin sections were deparaffinized, rehydrated, submerged in antigen retrieval solution (citrate buffer, 10 mM, pH 6, 95°C, 30 minutes), blocked with 3% bovine serum albumin for 30 minutes at room temperature, and then incubated at 4°C overnight with goat polyclonal antihuman Sox3 antibody (ab104248, Abcam, Cambridge, UK, dilution 1:50). Then sections were incubated with second antibody for 1 hour at room temperature, and subsequently with ABC complexes coupled to peroxidase (Beyotime, Jiangsu, China). Antigen-antibody complexes were revealed using 3-3' diaminobenzidine (DAB kit, Beyotime, Jiangsu, China) and sections were dehydrated and mounted. As negative controls, sections were treated with the same procedure except for the presence of primary antibody.

For quantification of antibody staining, ten randomly selected areas of liver tissue were observed and measured under 400× magnification with an image analysis software (ImageJ, Media Cybernetics, California, USA). The percentage scores of Sox3 positive cells were counted as follows: 0 (0%), 1 (1-10%), 2 (11-50%) and 3 (> 50%). The staining intensity of Sox3 positive cells was visually scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Then a final score of each section was obtained by multiplying the percentage and the intensity score. Therefore, tumors with a multiplied score exceeding 5 (median of total scores for Sox3) were classified as low expressions of Sox3; the other scores were considered as high expressions of Sox3 [16]. All assessments were analyzed by two independent observers without prior knowledge of the clinical status of the patients.

Statistical analysis

Statistical analysis was carried out using SPSS 22 software (SPSS Inc., California, USA). Data were presented as mean ± SD. Fisher's exact test and the Chi-square test were performed

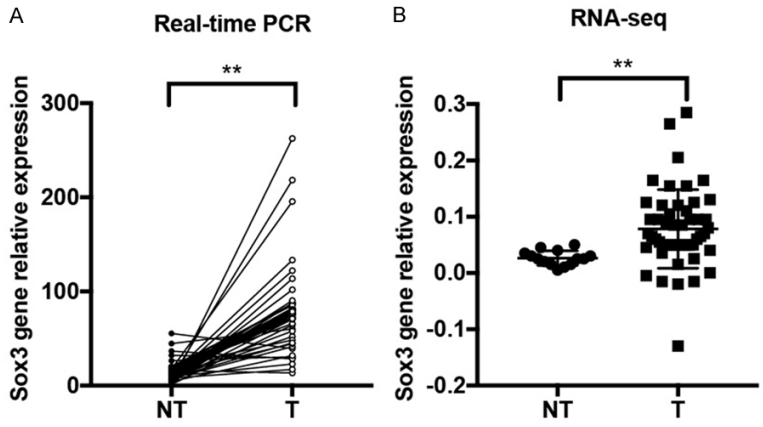

Figure 1. Overexpression of Sox3 mRNA level in HCC tissues (T) compared to adjacent non-tumor samples (NT). Quantitative RT-PCR (A) and RNA sequencing (B) analyses respectively revealed Sox3 mRNA expressed significantly higher in HCC tissues. Beta-actin was used as an internal control. **indicates $P < 0.01$.

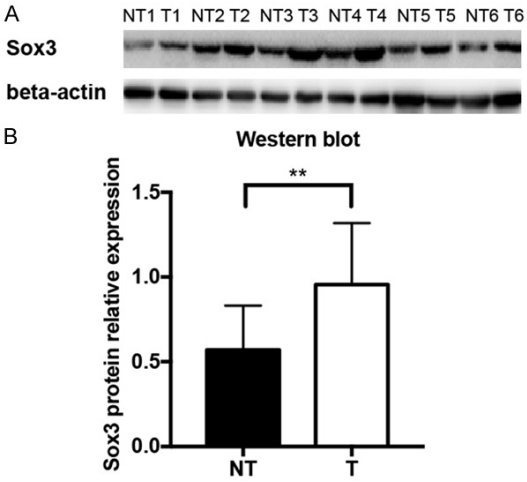

Figure 2. Elevated Sox3 protein levels in HCC tissues (T) compared to adjacent non-tumor samples (NT). A: Representative western blot results showed increased Sox3 expression in HCC tissues. B: Semi-quantitative immunoblotting results showed that expression level of Sox3 were statistically higher in HCC than that in adjacent non-neoplastic liver tissues. Beta-actin was used as an internal control. **indicates $P < 0.01$.

to assess correlations between Sox3 expression and clinicopathological parameters. The Kaplan-Meier method was used to determine survival, and difference was calculated by 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. $P < 0.05$ was considered statistical significant.

Results

Expression of Sox3 is significantly upregulated in HCC tissues

Firstly, the mRNA expression levels of Sox3 were investigated by qPCR in 50 pairs of HCC and corresponding non-tumor liver tissues. There was a significantly higher mRNA expression of Sox3 in the tumor samples compared to non-tumor tissues ($P < 0.01$; **Figure 1A**). To further confirm the overexpressed Sox3 in tumor tissue, we searched the mRNA expression levels from the NCBI Gene Expression Omnibus (GEO) public database. We identified a case of HCC patients containing 50 tumor tissues and 14 adjacent normal samples (GSE65486) examined by RNA sequencing analysis (RNA-seq) [17]. The mRNA expression level was also obviously elevated in the HCC tumor area ($P < 0.05$; **Figure 1B**).

Then we examined the protein expression levels of Sox3 in 50 pairs of HCC and adjacent non-tumor samples. As shown in **Figure 2**, the distinct overexpression of Sox3 protein in HCC tissues compared with corresponding non-tumor liver tissues was detected ($P < 0.01$).

To observe the expression pattern of Sox3, we checked HCC tissues from 104 patients by immunohistochemistry. The results revealed that Sox3 was absent or rare in the non-tumor area (**Figure 3A**) and Sox3 positive signals were located in the nucleus of tumor cells (**Figure 3B-D**). We further quantified the staining scores and found that 79 of 104 (75.96%) HCC samples received high Sox3 scores, while 25 of 104 (24.04%) HCC samples received low Sox3 scores.

Correlation of Sox3 expression with clinicopathological features of HCC patients

Next, we inspected the potential association between Sox3 expression and clinicopathological features of HCC patients listed in **Table 1**. Statistical analyses indicated that patients with high Sox3 expression have a significantly lower

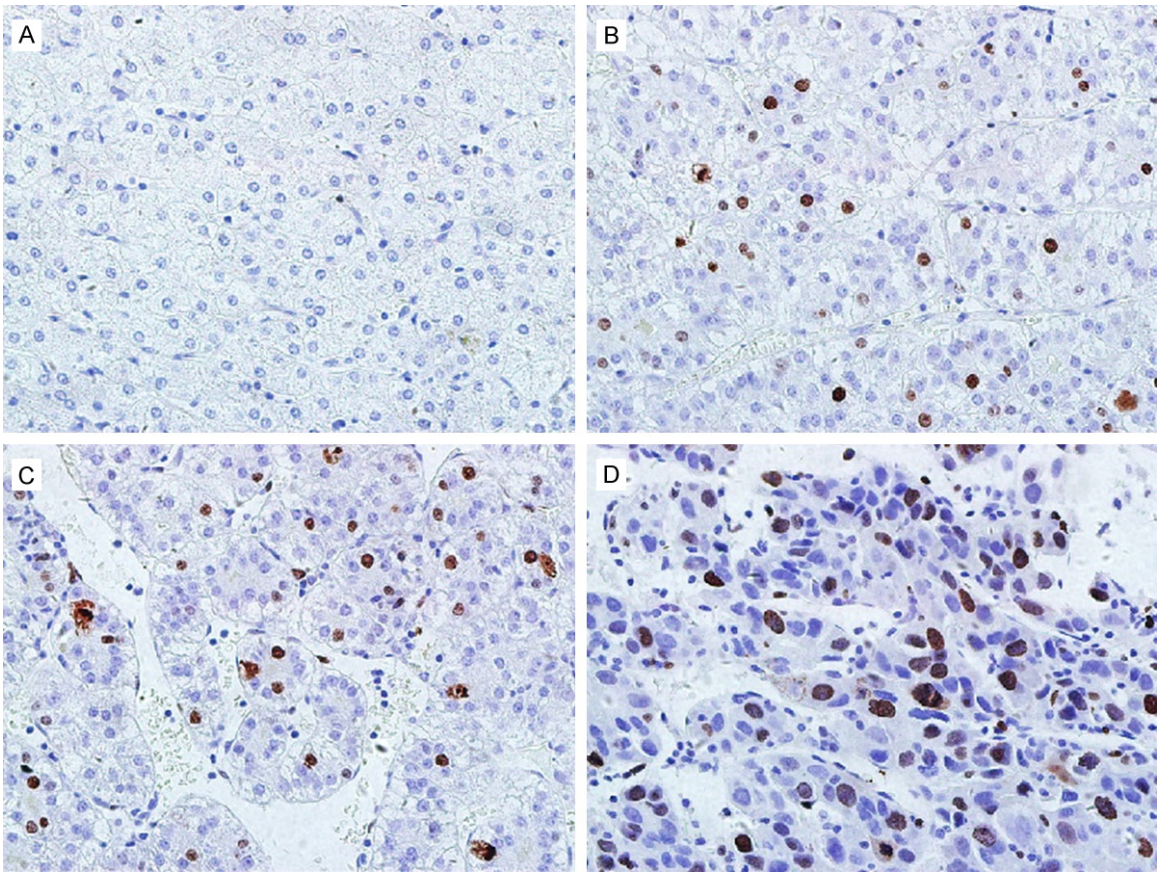

Figure 3. Different expression percentages of Sox3 in HCC and adjacent non-neoplastic tissues. Immunostaining score 0 (A), 1 (B), 2 (C) and 3 (D) of Sox3-positive signals in HCC patients tissues were detected, respectively. Original magnification 400×.

prevalence of tumor capsule formation (P = 0.007), poorer tumor differentiation grades (P = 0.005) and worse TNM classification (P = 0.028). However, the other clinicopathological factors including age, gender, symptom, hepatitis B virus (HBV) infection, cirrhosis, tumor number, tumor size, tumor thrombi, microvascular invasion and the levels of serum alpha fetoprotein (AFP), total bilirubin (TBIL), and platelet counts did not show significantly difference between HCC patients with high and low Sox3 expression (P = 0.076, 0.131, 0.084, 0.365, 0.620, 0.629, 0.321, 0.673, 0.064, 0.0059, 0.395 and 0.641, respectively).

Correlation of Sox3 expression with prognosis of HCC patients

To elucidate the prognostic values of Sox3 expression in HCC patients, we firstly shed light on the recurrence-free survival (RFS) and overall survival (OS) curves based on expression levels of Sox3 by the Kaplan-Meier analysis with the log-rank test. It is clearly demonstrated that the RFS rate from patients with high Sox3 expression was significantly lower than that from patients with low Sox3 expression (24.818% vs. 48.672%; P < 0.01; **Figure 4A**). As for the OS rate, a significant reverse correlation was also found between Sox3 expression and overall survival (30.114% vs. 58.643%; P < 0.01; **Figure 4B**).

Additionally, the univariate and multivariate analyses for RFS and OS by the Cox proportional hazards regression model in HCC patients were assessed. Univariate analysis indicated that TNM stage, tumor grade and Sox3 expression were statistically correlated with RFS of HCC patients (P = 0.009, 0.041 and 0.009, respectively). Multivariate analysis further revealed that Sox3 expression, along with TNM stage, was an independent prognostic factor in HCC patients (hazards ratio [HR]: 2.441; 95%

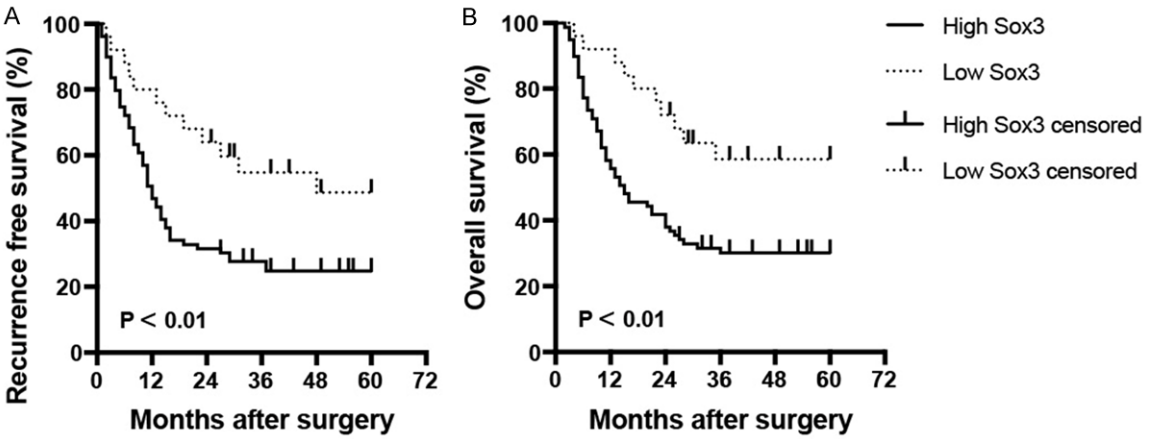

Figure 4. Recurrence-free survival and overall survival curves for Sox3 expression in HCC patients. The HCC patients with high Sox3 expression showed significantly shorter recurrence-free survival ($P < 0.01$, A) and overall survival ($P < 0.01$, B) rates than those with low Sox3 expression.

Table 2. Univariate and multivariate analyses of recurrence-freesurvival in HCC patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	1.346	0.359-3.457	0.201			
Gender	1.207	0.354-3.386	0.125			
HBV infection	1.381	0.621-3.953	0.416			
Cirrhosis	2.073	0.684-4.001	0.073			
Tumor size	1.575	0.551-3.266	0.102			
TNM stage	2.918	1.611-5.515	0.009	2.721	1.482-4.965	0.01
Tumor grade	1.411	0.779-4.308	0.041	1.332	0.698-4.021	0.072
Sox3 expression	2.732	1.485-5.464	0.009	2.441	1.298-4.786	0.01

Abbreviation: HBV, hepatitis B virus; AFP, alpha fetoprotein; TBIL, total bilirubin; TNM, tumor-node-metastasis.

Table 3. Univariate and multivariate analyses of overall survival in HCC patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	1.453	0.381-3.428	0.204			
Gender	1.215	0.359-3.151	0.119			
HBV infection	1.443	0.706-4.114	0.452			
Cirrhosis	2.151	0.657-3.950	0.079			
Tumor size	1.397	0.483-2.872	0.122			
TNM stage	2.938	1.733-5.575	0.008	2.428	1.359-3.221	0.021
Tumor grade	1.379	0.864-4.418	0.052			
Sox3 expression	2.636	1.409-4.945	0.012	2.186	1.364-4.390	0.015

Abbreviation: HBV, hepatitis B virus; AFP, alpha fetoprotein; TBIL, total bilirubin; TNM, tumor-node-metastasis.

confidence interval [CI]: 1.298-4.786; $P = 0.01$, **Table 2**). On the other hand, we observed that TNM stage and Sox3 expression were also significantly correlated with OS ($P = 0.008$ and 0.012, respectively). In a multivariate Cox model we found that Sox3 expression was an independent poor prognostic factor for OS in HCC (HR = 2.186, CI = 1.364-4.390, $P = 0.015$, **Table 3**).

Discussion

The incidence of HCC is growing fast recently, especially in Southeastern Asia and sub-Saharan Africa [18]. Because of the limited treatments for advanced stages of HCC, elucidating efficient prognostic biomarkers for HCC seems very critical.

The first Sox family gene, Sry, was discovered by Goodfellow's group in more than two decades ago [19]. Since then approximately 20 Sox genes have been identified in mammals and classified into eight groups according to the phylogenetic analysis of the HMG box domains: A (Sry), B1 (Sox1, -2 and -3), B2

(Sox14 and -21), C (Sox4, -11 and -12), D (Sox5, -6 and -13), E (Sox8, -9 and -10), F (Sox7, -17 and -18), G (Sox15) and H (Sox30) [20]. Among these Sox genes, Sox3 is located in chromosome Xq27 [21].

It has been recognized that Sox proteins play crucial roles in gene transcription involving in many developmental processes. Recently some researchers suggested that Sox genes such as Sox1 [22], -2 [23] and -9 [24] contribute to tumorigenesis and progression in a variety of cancers, whereas the exact mechanisms were still unknown. As for Sox3, Xia et al. reported that ectopic expression of Sox3 induced oncogenic transformation of chicken embryo fibroblasts [25]. Sox3 was also identified as a proto-oncogene in T-cell lymphomas because it contains retroviral insertion sites and may lead to retroviral DNA integrate into the genome [26]. In addition, some researchers demonstrated that the serum titer of Sox3 autoantibody was increased in patients with small-cell lung cancer (SCLC), and Sox3 expression was detected in 10% of SCLC cell lines [27]. For the underlying mechanisms research, Yan et al. interpreted that overexpression of Sox3 could lead to upregulated phosphorylation of SRC and then promote cell migration and invasion and inhibit cell adhesion [12]. Surprisingly, Sox3 can also act as a tumor suppressor. Sox3 was found to physically interact with beta-catenin and antagonize its activity in *Xenopus* embryos and tissue culture assays [28]. Additionally, Nieto's group reported that in cancer cells, Sox3 repressed Snail which can induce epithelial-mesenchymal transition (EMT), thereby prevented EMT to maintain epithelial integrity [29]. However, there is no research about the correlation of Sox3 expression with HCC in vitro or in vivo.

In our study, qPCR analysis from 50 pairs of HCC and non-tumor samples indicated a dramatic increase in Sox3 from HCC tissues compared to adjacent benign counterparts. RNA-seq result from GEO database further validated the upregulation in Sox3. By western blot assay, we confirmed the protein level of Sox3 was significantly higher in HCC tissues. We also detected the positive signals of Sox3 in the nucleus of HCC tissues by immunohistochemistry analysis. Using immunostaining scores quantification, we demonstrated that Sox3 was highly expressed in 75.96% of HCC samples, whereas 24.04% of HCC samples showed low expression. Therefore, elevated Sox3 expression at both mRNA and protein level might play an important role in HCC progression. Further analysis indicated that overexpression of Sox3 was statistically correlated with less tumor capsule formation, poorer tumor differentiation grades and worse TNM classification. Taken together, these results suggested that Sox3 plays an oncogenic role in HCC. Moreover, the univariate and multivariate analyses demonstrated that increased expression of Sox3 was an independent poor prognostic factor for both RFS and OS in HCC. All of the results above determined the potential prognostic value of Sox3 in HCC.

While our study indicated Sox3 activation in a majority of HCC tissues and further suggested a crucial role for prognosis, it remains elusive as how Sox3 is activated under this disease condition. Very relevant to the present study, it is demonstrated that Sox2 and geminin are Sox3 downstream targets. Sox2 has been commonly acknowledged as an oncogene which can moderate microRNAs in HCC [23]. Geminin can accelerate proliferation and inhibit DNA replication [30], which is enhanced in cancers and leads to dismal prognosis. In addition, Rogers and his colleagues reported that Sox3 can indirectly repress Xvent2 expression to induce progenitor cells formation [31]. Moreover, considering the significant correlation between Sox3 high expression and low tumor capsule incidence, Sox3 maybe promote the HCC progression via inhibiting the formation of tumor capsule.

To the best of our knowledge, this is the first study investigating the Sox3 expression level and pattern in HCC with clinicopathologic features and prognostic values. Since this study's patient samples were specific, the results should be further validated in international populations.

In conclusion, our data indicated that Sox3 expression is enhanced in HCC tumor tissues compared to the non-tumor counterparts. Furthermore, the most significant finding of the study is that Sox3 is a novel and potential biomarker for predicting the poorer prognosis of HCC patients after surgery. Although there are some important results revealed by this study,

further investigation are apparently required to clarify signaling pathways responsible for Sox3 activation in HCC development.

Acknowledgements

We deeply thank Wendy Chen at University of California, San Diego (La Jolla, CA) for providing language help. This work was supported by grants from Science and Technology Commission of Shanghai Municipality (104119-63200 and 14411964000).

Disclosure of conflict of interest

None.

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