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Original Article

Prognostic significance of SOX-1 expression in human hepatocelluar cancer

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Abstract: Sex-determining region Y (SRY)-box 1 (SOX1) as a member of the SOX gene superfamily is reported to function as a tumor suppressor in hepatocelluar cancer (HCC). However, the clinicopathological and prognostic significance of SOX-1 expression in HCC is unclear. First, semi-quantitative RT-PCR and Western blot assays were performed to detect the expression of SOX-1 mRNA and protein in 15 paired of HCC tissues and corresponding nontumor tissues. Next, immunohistochemistry was performed to detect SOX-1 protein expression in another 96 cases of HCC tissues, and analyze its correlation with clincopathological factors of patients. Finally, the survival was evaluated by the Kaplan-Meier method and proportional hazards model. Results showed that the expression levels of SOX-1 mRNA and protein in HCC tissues were significantly lower than that in the corresponding nontumor tissues. Statistical analyses indicated that low SOX-1 expression was significantly correlated with higher incidence of venous or lymphatic invasion and advanced TNM stage. Also, patients with high SOX-1 expression showed better overall survival than those with low SOX-1 expression, and multivariate analysis with the Cox proportional hazards indicated that status of SOX-1 expression might be an independent prognostic factor in HCC patients. Collectively, our results indicated that downregulation of SOX-1 was correlated with poor prognosis and tumor development in HCC.

Keywords: Hepatocelluar cancer (HCC), SOX-1, immunohistochemistry, overall survival, prognosis

Introduction

Hepatocellular carcinoma (HCC) has become the second leading cause of tumor-related death around the world [1]. Despite great advancement in both diagnostic techniques and combined treatments for this disease, the prognosis of HCC patients remains poor, mainly resulting from the high recurrence rate and intrahepatic metastasis after surgery [2]. The progression of HCC is a complicated process which is determined by environmental and genetic factors. Therefore, elucidation of the molecular mechanisms underlying HCC development will contribute to identifying better prognostic markers and developing novel therapeutic strategies for HCC patients.

SOX-1 (sex determining region Y [SRY] related high-mobility group box 1) belongs to the SRY (sex determining region Y) box gene superfamily, which plays a critical role during embryonic development [3]. It has been reported that SOX-1 protein is evolutionarily conserved in many species and implicated as a key regulator of neural cell fate determination and differentiation [4]. Recently, the roles of SOX-1 in tumor development are also reported. By performing a differential methylation hybridization using a CpG island microarray in squamous cell carcinomas (SCC) of the uterine cervix, Lai' et al identified 6 genes (SOX-1, PAX1, LMX1A, NKX6-1, WT1 and ONECUT1) more frequently methylated in SCC tissues than in their normal controls [5]. Likewise, epigenetic inactivation of SOX-1 is found in other human cancers including lung cancer, esophageal squamous cell carcinoma and ovarian cancer [6-8]. In HCC, Shih'et al also showed that concomitant epigenetic silencing of SOX-1 and SFRPs through promoter hypermethylation is frequent [9]. However, the correlation of SOX-1 expression with prognosis and tumor progression in HCC is not still fully understood and remains to be further elucidated.

Table 1. Correlation between SOX-1 expression and clinicopathological factors of HCC patients

Factor	High-SOX-1 (n=38)	Low-SOX-1 (n=58)	*P*-value
	Number (%)	Number (%)	
Age (years)			0.186
≥55	20 (52.6)	32 (55.2)	
<55	18 (47.4)	16 (44.8)	
Gender			0.399
Male	28 (73.7)	38 (65.6)	
Female	10 (26.3)	20 (34.4)	
HBV infection			0.422
No	12 (31.6)	14 (24.1)	
Yes	26 (68.4)	44 (75.9)	
Cirrhosis			0.745
No	17 (44.7)	24 (41.4)	
Yes	21 (55.3)	34 (58.6)	
Serum AFP (μg/l)			0.955
≤400	26 (68.4)	40 (49.0)	
>400	12 (31.6)	18 (51.0)	
Liver function			0.875
Child-Pugh A	21 (55.3)	33 (56.9)	
Child-Pugh B	17 (44.7)	25 (43.1)	
T-factor (cm)			0.242
≤5.0	19 (50.0)	22 (37.9)	
>5.0	19 (50.0)	36 (52.1)	
V/Ly factor			0.001
No	31 (81.6)	17 (29.3)	
Yes	7 (18.4)	21 (70.7)	
TNM stage			0.030
I	23 (60.5)	22 (37.9)	
II+III	15 (39.5)	36 (62.1)	

Abbreviation: HBV, hepatitis B virus; V/Ly factor, venous or lymphatic invasion; TNM, tumor-node-metastasis.

In this study, we performed Western blotting and immunohistochemistry assays to detect the expression of SOX-1 protein in HCC tissues and corresponding nontumor tissues, and then analyze the correlation of SOX-1 expression with clinicopathological factors and prognosis of HCC patients. Taken together, our data suggest that reduced SOX-1 correlates with poor prognosis and tumor progression in HCC.

Materials and methods

Patients and tissue samples

Ninty-six cases of HCC tissues and another 15 paired of HCC tissues and corresponding nontumor liver tissues were collected from Shanghai Outdo Biotech Co. Ltd. between Aug. 2006 and Jan. 2010 (Outdo Biotech, Shanghai) and the Liver Disease Center of the 81th Hospital of PLA with informed consent in accordance with the requirements of the Ethics Committee of Chinese Medical Association. None of the participants received preoperative treatment. The tumor type and the grade of cell differentiation were designated based on the criteria of World Health Organization (WHO) and tumor pathological stage was determined based on the International Union Against Cancer (UICC) TNM classification. The detailed characteristics of patients were shown in **Table 1.** Ethical approval was obtained from the hospital and fully informed consent from all patients prior to tumor collection. Except tissues used for RNA extraction, the remnant tissues were rapidly frozen in liquid nitrogen and stored at -80°C.

Immunohistochemistry

Resected HCC tissues were fixed in 10% formaldehyde and embedded in paraffin. Sections (4 μm), cut from the original paraffin blocks, were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. After inhibition of endogenous peroxidase activity for 30 min with methanol containing 0.3% H_2O_2, the sections were blocked with 10% normal goat serum (Invitrogen, USA) for 20 min and incubated overnight with rabbit anti-human-Aurora-A (diluted 1:150, Santa Cruz Biotechnology, CA) at 4°C. The sections were then incubated with biotinylated anti-rabbit IgG for 30 min at room temperature, followed by incubation with peroxidase-conjugated avidin/biotin complexes and stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB). Finally, the sections were counter-stained with hematoxylin. Normal rabbit serum was used as a negative control for the staining reactions. The number of positive-staining cells showing immunoreactivity in the nucleus for SOX-1 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). All slides were scored in the absence of any clinical data simultaneously assessed by two observers

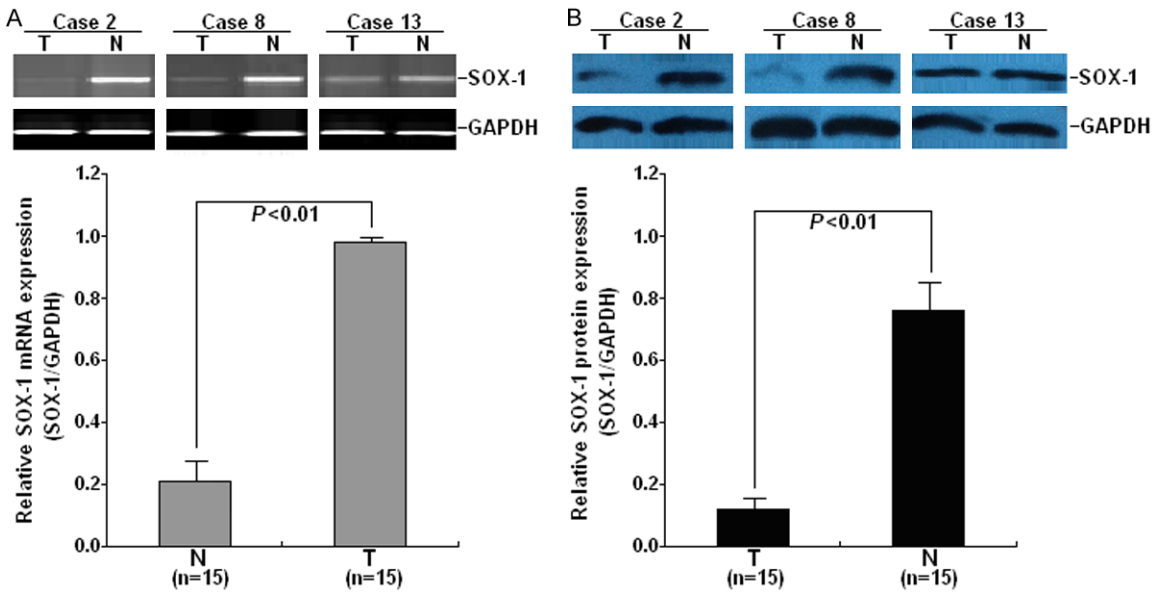

Figure 1. Semi-quantitative RT-PCR (A) and Western blot (B) detection of SOX-1 mRNA and protein expression in 15 paired of HCC tissues (T) and corresponding nontumor liver tissues (N). GAPDH was used as an internal control. Each experiment was performed at least in triplicate.

using a double-headed discussion microscope. In a final discussion round all slides were reviewed and the results were confirmed by a third observer. The final immunostaining score reported was the average of three observers. Therefore, tumors with a multiplied score exceeding 5 (median of total scores for SOX-1) were deemed to be low SOX-1 expression and all other scores were considered to be high SOX-1 expression.

Semi-quantitative RT-PCR assay

Total RNAs were extracted from tissues using TRIzol reagent (Invitrogen, USA). RNA was reverse-transcribed into cDNA in a 20 μL reaction system using Superscript First-Strand Synthesis Kit for RT-PCR according to the manufacturer's instruction. First strand cDNAs were synthesized and analyzed by PCR to detect the expression of SOX-1 and GAPDH. The primers were as follows: SOX-1, forward: 5'-*GGCCAAGCGGCTGCGCGCGCTG*-3'; reverse: 5'-*GGCCAGCTGCGCCTCCTGCAT*-3' (328 bp). GAPDH, forward: 5'-*CACCATCTTCCAGGAGCGAG*-3', reverse: 5'-*TCACGCCA-CAGTTTCCCGGA*-3' (372 bp). Then RT-PCR products were electrophoresed through a 2% agarose gel with ethidium bromide and signals were quantified by densitometric analysis using the Labworks Image Acquisition (UVP, Inc., Upland, CA).

Western blot assay

Total protein extracts from tissues were separated on SDS (10%-12%) polyacrylamide gel electrophoresis (20-50 g/lane), and electrotransferred to a PVDF (polyvinylidene fluoride) membrane. Anti-SOX-1 and anti-GAPDH antibody (Santa Cruz Biotechnology) were diluted in TBST (Tris-buffered saline/Tween) (5% milk powder) and incubated at 4°C overnight. The appropriate secondary antibody was applied (1:2000; horseradish peroxidase anti-mouse and horseradish peroxidase anti-rabbit) at room temperature for 1 hour. Visualization was performed by enhanced chemiluminescence (ECL; Amersham).

Statistical analysis

All statistical analyses were performed using the SPSS 17.0 statistical software. Data were expressed as the mean ± SD of at least three independent assay. Statistical analyses were carried out using one-way ANOVA and Student's *t* test to evaluate the continuous variables. The Kaplan-Meier method was used to determine survival, and statistical significance was calculated using the log-rank test. Both univariate and multivariate analyses of survival were conducted using the Cox proportional hazards model. Statistical differences were considered significant when $P<0.05$.

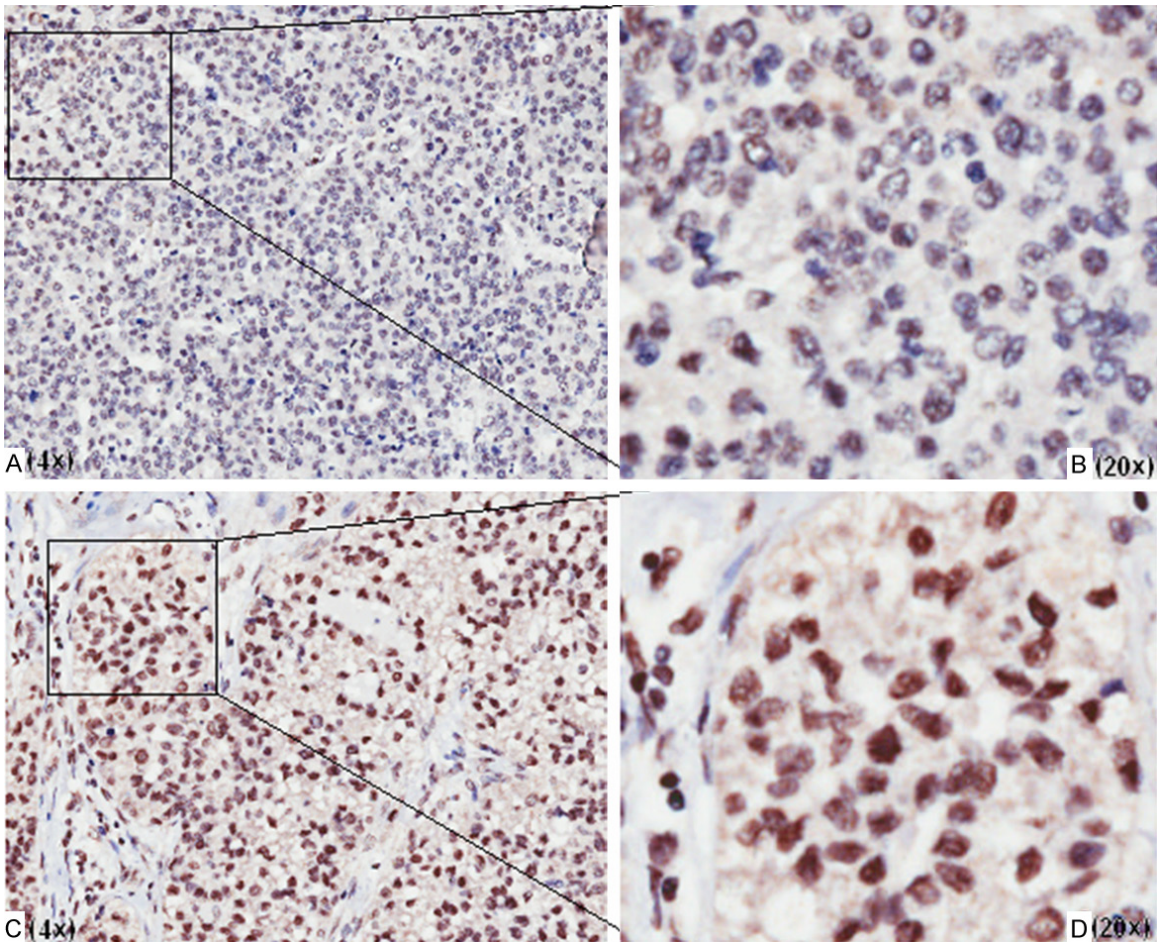

Figure 2. Immunostaining of SOX-1 protein in 96 cases of HCC tissues. The staining of SOX-1 protein was mainly localized in the nucleus of HCC cells. (A) (4×) and (B) (20×) showed low SOX-1 protein expression, while (C) (4×) and (D) (20×) showed high SOX-1 protein expression.

Results

Expression of SOX-1 protein is significantly downregulated in HCC tissues

First, qRT-PCR was performed to detect the expression of SOX-1 mRNA in 15 paired of HCC tissues and corresponding nontumor tissues. As shown in **Figure 1A**, the relative expression level of SOX-1 mRNA in HCC tissues was significantly lower than that in the corresponding nontumor tissues ($P<0.01$). Then, we further performed Western blotting to detect the expression of SOX-1 protein in above tissues, and showed that the relative expression level of SOX-1 protein in HCC tissues was significantly lower than that in the corresponding nontumor tissues ($P<0.01$; **Figure 1B**). Thus, reduced SOX-1 might play a role in HCC progression.

Correlation of SOX-1 expression with clinicopathological factors of HCC patients

Immunohistochemistry was performed to detect the expression of SOX-1 protein in another 96 cases of HCC tissues, and it was revealed that the staining of SOX-1 protein was mainly localized in the nucleus of HCC cells (**Figure 2A**, **2B**). Moreover, we showed 75.38% of HCC tissues with high SOX-1 expression (n=38) and (24.62%) of HCC tissues with low SOX-1 expression (n=58). Then, we further analyzed the correlation of SOX-1 protein expression with clinicopathological factors of HCC patients (**Table 1**). Statistical analyses indicated that low SOX-1 expression was closely correlated with higher incidence of venous or lymphatic invasion and advanced TNM stage ($P=0.001$ and 0.030, respectively), but not with other clinicopatho-

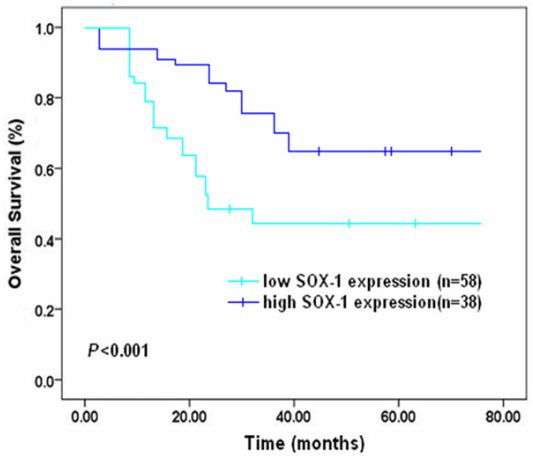

Figure 3. Kaplan-Meier survival curve of HCC patients according to the status of SOX-1 expression. The 5-year OS rate of patients with low SOX-1 expression was significantly lower than that of those with high SOX-1 expression (P<0.001). Corresponding P values analyzed by log-rank tests are indicated.

logical factors including age, gender, HBV infection, cirrhosis, serum AFP, liver function and T-factor (P=0.186, 0.399, 0.422, 0.745, 0.955, 0.875 and 0.242, respectively).

Correlation of SOX-1 protein expression with prognosis of HCC patients

The correlation between SOX-1 expression and prognosis of HCC patients was analyzed by the Kaplan-Meier method with the log-rank test. **Figure 3** showed the results of the Kaplan-Meier analyses for overall survival (OS) of patients based on status of SOX-1 expression were shown in **Figure 3**. The OS rate of patients with high SOX-1 expression was significantly higher than that of those with low SOX-1 expression (5-year OS rate, 65.0 *vs.* 44.2%; P<0.001). Next, the results of univariate and multivariate analyses for OS using the Cox proportional hazards regression model in HCC patients were shown in **Table 2**. Univariate analysis indicated that these factors (V/Ly factor, TNM stage and status of SOX-1 expression) were observed to be significantly correlated with OS of HCC patients (P=0.018, 0.007 and 0.015, respectively). Furthermore, multivariate analysis with the Cox proportional hazards indicated that the status of SOX-1 expression, along with TNM stage, was an independent prognostic factor in HCC patients (HR: 2.65; 95% CI: 1.18-4.06; P=0.006).

Discussion

HCC carcinogenesis is an incompletely understood process which is determined by the aberrant gene expression, including oncogene upregulation and tumor suppressor downregulation [10]. However, the molecular mechanisms underlying the development of HCC are still poorly understood. Thus, it is necessary to identify novel molecular markers that can accurately represent biological features of HCC and predict the clinical outcome, which will help us to perform tailored therapy for individual cases in clinic.

Sox family proteins are a conserved group of transcriptional regulators (see Box 1) defined by the presence of a highly conserved HMG domain that mediates DNA binding [11]. Up to date, there have been at least 20 members divided into 8 groups (from A to H), based on their HMG sequence identity in humans. Accumulating evidence indicates that Sox genes play important roles in sex determination, chondrogenesis, hematopoiesis, neural crest development and neurogenesis [12-14]. Recently, dysregulation of Sox factors has been further implicated in a variety of human diseases, including cancer [15]. For example, SOX2 is highly expressed in several human cancers, including lung and esophageal squamous cell carcinomas and central nervous system tumors [16-18]. Additionally, SOX9 has also been demonstrated to be a proto-oncogene in many human cancers, such as bladder cancer, breast cancer and hepatocelluar cancer [19-21]. Meanwhile, other Sox members (SOX3, SOX7, SOX8 and SOX17) are also reported to function as oncogenes or tumor suppressors in different types of human malignancies [22-25]. However, up to date, there are only a few studies on the SOX1 protein. SOX1 has been reported to be a tumor suppressor in a variety of cancers. Lin and his colleagues reported that SOX1 can function as a tumor suppressor partly by interfering with Wnt/β-catenin signaling in cervical cancer [26]. Also, it was reported that SOX1 decreases the expression of β-catenin in a proteasome-independent manner and reverses the malignant phenotype in nasopharyngeal carcinoma [27]. Li' et al showed that SOX1 is epigenetically silenced in the majority of lung cancer and restoration of SOX1 inhibited cell migration by regulating actin cytoskeletal remo-

Table 2. Univariate and multivariate analysis of OS rates in HCC patients

Clinicopathological factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (≥55 years/<55 years)	1.37 (0.44-3.12)	0.283		
Gender (Male/Female)	0.87 (0.36-2.47)	0.108		
HBV infection (Yes/No)	1.59 (0.81-4.16)	0.518		
Cirrhosis (Yes/No)	2.38 (0.52-3.68)	0.095		
Serum AFP (>400 μg/l/≤400 μg/l)	0.95 (0.42-1.39)	0.337		
Liver function (Child-Pugh B/Pugh A)	1.73 (0.89-2.68)	0.198		
T-factor (>5.0 cm/≤5.0 cm)	0.84 (0.35-1.47)	0.181		
V/Ly factor (Yes/No)	2.15 (1.12-4.37)	0.018	1.55 (0.79-1.85)	0.154
TNM stage (II+III/I)	3.19 (2.08-5.34)	0.007	1.47 (1.12-2.07)	0.022
SOX-1 expression (low/high)	1.75 (1.27-2.95)	0.015	2.65 (1.18-4.06)	0.006

Abbreviation: OS, overall survival; HBV, hepatitis B virus; V/Ly factor, venous or lymphatic invasion; TNM, tumor-node-metastasis; HR, hazard ratio; 95% CI, 95% confidence interval.

deling in lung cancer [28]. Likewise, Lin and his colleagues also showed that SOX1 functions as a tumor suppressor in HCC [29]. In their report, it was found that epigenetic downregulation of SOX-1 lead to aberrant activation of Wnt/β-catenin signaling and restoration of SOX-1 repressed β-catenin/TCF-responsive transcriptional activity by interacting with β-catenin and restraining the expression of downstream genes. Although these data indicate that SOX-1 might play a role during the development of HCC, the clinicopathological and prognostic significance of SOX-1 in HCC is still unclear.

In the present study, we first detected the expression of SOX-1 mRNA and protein in 15 paired of HCC tissues and corresponding nontumor liver tissues by semi-quantitative RT-PCR and Western blotting, and it was observed that the expression level of SOX-1 mRNA and protein in HCC tissues was significantly higher than that in the corresponding nontumor tissues Furthermore, immunohistochemistry was performed to detect the expression of SOX-1 protein in 96 cases of HCC tissues and analyze its correlations with clinicopathological factor or prognosis of patients. It was observed that low SOX-1 expression in tissues was significantly correlated with higher incidence of venous or lymphatic invasion and advanced TNM stage. These results indicated that SOX-1 could play an important role in the process of HCC development. Meanwhile, patients with low SOX-1 expression showed poorer OS than those with high SOX-1 expression. Furthermore, Cox's multivariate analyses proved that status of SOX-1 protein expression was an independent factor in predicting the OS of HCC patients, which provided theoretical basis for judging prognosis of HCC patients. To the best of our knowledge, this is the first report about the prognostic significance of SOX-1 in HCC. Of course, since the size of tissue samples in this study is smaller, further investigation of a larger patient population is necessary to confirm prognostic evaluation of SOX-1 in human HCCs.

Taken together, this study demonstrated that reduced SOX-1 expression was significantly poor prognosis and tumor progression of HCC patients. Therefore, the status of SOX-1 protein expression may also become a novel prognostic biomarker for HCC patients. However, the biological functions and clinical significance of SOX-1 in HCC is urgently needed for further investigation in future.

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Disclosure of conflict of interests

None.

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