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Serum *SOX11* promoter methylation is a novel biomarker for the diagnosis of Hepatitis B virus-related hepatocellular carcinoma

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Promoter methylation of tumor suppressor gene *SOX11* has been reported to contribute to the diagnosis and prognosis of various cancerous diseases, including gastric cancer, hematopoietic malignancies and nasopharyngeal carcinoma. However, there is no data on the diagnostic potential of serum *SOX11* promoter methylation in hepatocellular carcinoma (HCC). This study was therefore aimed to investigate the potential role of serum *SOX11* promoter methylation as a noninvasive biomarker in the diagnosis of patients with hepatitis B virus (HBV) associated HCC. A total of 205 subjects were retrospectively included, which consisted of 111 HCC patients, 66 chronic hepatitis B (CHB) and 28 healthy controls (HCs). Methylation of *SOX11* promoter was determined by methylation-specific polymerase chain reaction. The methylation frequency of serum *SOX11* promoter in HCC patients (69.4%, 77/111) was significantly higher than that in CHB patients (13.6%, 9/66; $\chi^2 = 51.467$, $P<0.001$) and HCs (10.7%, 3/28; $\chi^2 = 31.489$, $P<0.001$). There was significant difference of serum *SOX11* promoter methylation in HCC patients with vascular invasion (49/58) and those without vascular invasion (28/53; $\chi^2 = 13.058$, $P<0.001$). Furthermore, the sensitivity of 69% was identified for *SOX11* methylation in discriminating HCC from CHB, which was significant higher than the sensitivity of 57% for serum alpha-fetoprotein (AFP) ($P<0.05$). Notably, *SOX11* promoter methylation plus AFP showed a sensitivity of 85% in discriminating HCC from CHB. These results suggested that serum *SOX11* promoter methylation might serve as a useful and noninvasive biomarker for the diagnosis of HCC.

Key words: SOX11, biomarker, DNA methylation, serum, hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the sixth most prevalent malignancy and the second leading cause of cancer related mortality worldwide [1, 2]. HCC is usually associated with chronic infection of hepatitis B virus (HBV) and hepatitis C virus [3]. Annually, there are nearly 750,000 newly identified HCC patients [4] and most of them die within one year after the onset of diagnosis [5]. Currently, ultrasonography and alpha fetoprotein (AFP) are the main tools for the early diagnosis of HCC [6, 7]. However, ultrasonography is insufficient for the diagnosis of small tumor, particularly in cirrhotic liver [8]. Serum AFP level lacks adequate sensitivity and specificity for early diagnosis of HCC. The prognostic role of AFP may be influenced by the size and number of HCC nodules [9, 10]. Almost one-third of early-stage HCC with small tumors (< 3 cm) were negative for AFP [11]. Therefore, AFP could not be a satisfied biomarker for the early diagnosis of HCC [12]. Therefore, new effective biomarkers for the early detection of HCC are urgently needed [13].

Methylation of gene promoter is a main kind of epigenetic alternations and involves in the development and progression of cancerous diseases [14-16], such as lung, gastric, bladder, prostate and breast cancers [17-19]. DNA methylation is a early cancer event and could be even detected as early as 9 years ahead of the diagnosis onset of cancer [20]. Therefore, methylation of serum gene promoters might act as a potential biomarker for early diagnosis of cancer. Methylations of

Abbreviations: AASLD – American Association for the Study of Liver Diseases; AFP – alpha fetoprotein; AUROC – area under the receiver operating characteristic curve; CHB – chronic hepatitis B; FN – false negative; FP – false positive; HbsAg – hepatitis B surface antigen; HBV – hepatitis B virus; HCC – hepatocellular carcinoma; HCs – healthy controls; MSP – methylation-specific polymerase chain reaction; NPV – negative predictive value; PPV – positive predictive value; SOX – Sry-related HMG box; TN – true negative; TP – true positive.

serum *IGFBP7* [13], *RASSF1A* [20, 21], *P15* [20, 22] and *P16* [23, 24] have been reported to be promising and noninvasive biomarkers for the early detection of HCC.

SOX11 gene encodes a member of the group C *SOX* (SRY-related HMG-box) transcription factor family which involved in the regulation of embryonic development and in the determination of cell fate [25, 26]. *SOX11* has also been identified as a tumor suppressor gene [27] and improve recurrence-free survival rates in epithelial ovarian cancer [28]. *SOX11* could be silenced by methylated modification of *SOX11* gene promoter, and then contributes to the development of tumor [29, 30]. Promoter methylation of tumor suppressor gene *SOX11* has been reported in various cancerous diseases, including gastric cancer [31], hematopoietic malignancies [29] and nasopharyngeal carcinoma [30]. As such, *SOX11* may serve as a biomarker for cancer diagnosis. However, there is no data on the diagnostic potential of serum *SOX11* promoter methylation in HCC. This study was therefore aimed to investigate the potential role of serum *SOX11* promoter methylation as a noninvasive biomarker in the diagnosis of patients with hepatitis B virus associated HCC.

Materials and methods

Study population. A total of 205 subjects were retrospectively collected in this study, including 111 patients with HCC, 66 patients with CHB, and 28 healthy controls (HCs), from March 2013 to June 2015 at the Department of Hepatology, Qilu Hospital of Shandong University. HCC patients were diagnosed according to the 2010 update of the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines for Management of HCC [32]. CHB patients were identified as positive hepatitis B surface antigen (HBsAg) for at least 6 months prior to the beginning of this study [33]. The 111 HCC patients and 66 CHB patients in the study were all HBsAg positive. Exclusion criteria included other tumors, co-infection with hepatitis C virus or human immunodeficiency virus, autoimmune liver diseases, non-alcoholic fatty liver diseases, alcoholic liver diseases and other causes of chronic liver diseases. The selection process of patients is shown in Figure 1. The study protocol was approved by the Ethics Committee of Qilu Hospital of Shandong University and all the subjects have provided informed consents.

Serum DNA extraction and sodium bisulfite modification. Two microliter of serum was isolated from blood by high-speed centrifugal and then stored at -20˚C until use. Serum DNA was extracted from 400 μL of serum with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the DNA Purification from Blood or Body Fluids protocol. According to the manufacturer's instructions, extracted DNA was modified using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, United States).

After bisulfite treatment, the methylated residues could resist to this modification and remained as cytosine, whereas, all unmethylated cytosine residues would be converted to uracil. A final volume of 20 μL modified DNA was obtained and stored at -20 ˚C.

Methylation-specific polymerase chain reaction. The modified DNA samples were amplified with primers specific for both methylated and unmethylated sequences. The primer pairs of *SOX11* for MSP analysis were as described previously [31]. MSP was performed in a total volume of 25 μL containing 1 μL of bisulfite-treated DNA, 0.5 μL each primer (10μmol/L), 12.5 μL Premix Taq (Zymo Research), and 10.5 μL nuclease-free water. The PCR protocol included initial denaturation at 95˚C for 10 min, followed by 45 cycles of a denaturation step at 95°C for 30 sec, an annealing step at 52°C for 40 sec, an extension step at 72°C for 40 sec, and a final extension step at 72°C for 10 min. The expected size of the bisulfite-specific *SOX11* PCR product is 156 bp (Table 1). DNA from normal lymphocytes was used as a negative control. And water without DNA was used as a blank control for contamination. 7 μL of PCR products were electrophoresed on 2% agarose gels, stained with Gel Red, and visualized under UV illumination.

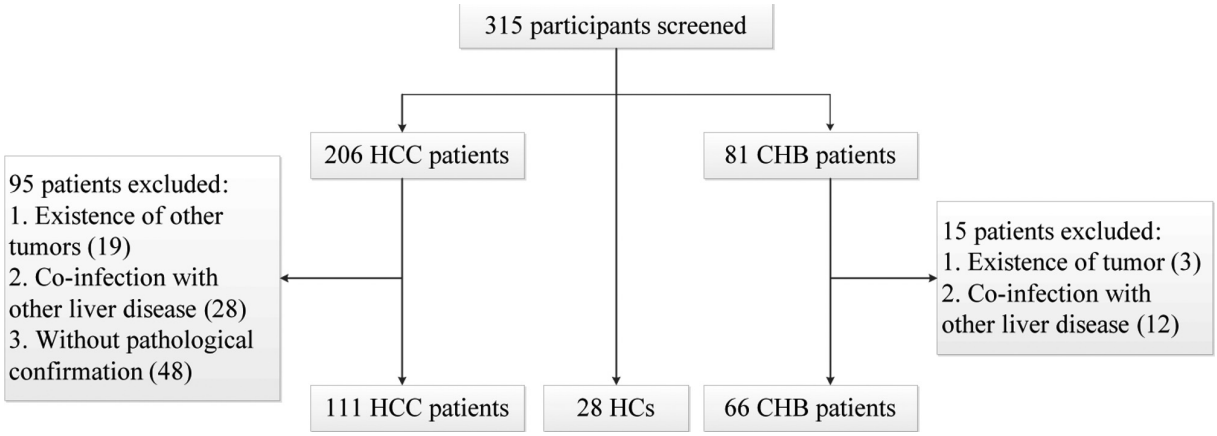

Figure 1. Flowchart depicting the selection process of the participants.

Table 1. Primer for MSP of the *SOX 11* Gene

Primer	Primer sequence(5‘ - 3‘)	Annealing temp. (°C)	Product size (bp)
U	F: GTTGGGTTATATTAAGTGGTT	52	156
	R: ACAAAATCTTCTCACTATCCT		
M	F: TCGGGTTATATTAAGCGGTC	52	156
	R: CGAAATCTTCTCGCTATCCT		

M – methylated sequence; U – unmethylated sequence; F – forward; R – reverse.

Statistical analysis. All statistical analysis was performed using the IBM SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). The χ^2 -test, and Student's t-test were used to generate P values and $P < 0.05$ was considered statistically significant. The difference of serum *SOX11* methylation status between different groups were analyzed using the χ^2-test. The association between serum *SOX11* promoter methylation status of HCC patients and their clinicopathological parameters were analyzed by χ^2-test and multivariate logistic regression. The receiver operating characteristic (ROC) curves was made by "Comparison of ROC curves" in MedCalc Statistical Software 11.5.0. The area under the receiver operating characteristic curves (AUC) was used to estimate the diagnostic value of *SOX11* gene promoter methylation and AFP level in discriminating CHB from HCC.

The diagnostic value of AFP, *SOX11* methylation and AFP combined with *SOX11* methylation was also statistically assessed by sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV). Se is the proportion of positive test in HCC patients; Sp is the proportion of negative test in CHB or HCs; PPV means the proportion of the true positive in the positive group; NPV means the proportion of the true negative in the negative group.

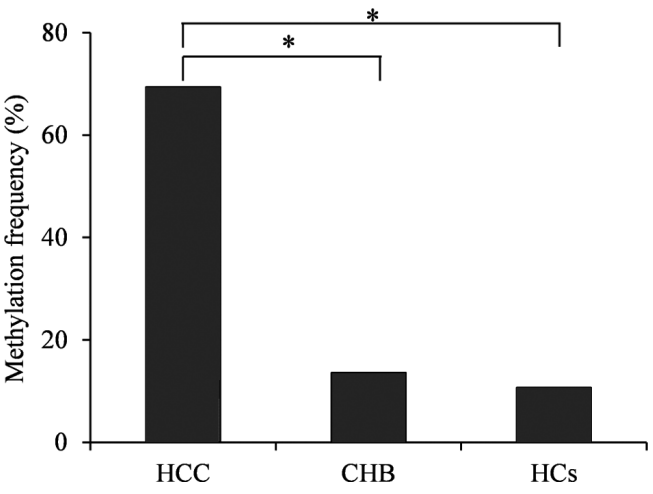

Figure 2. Methylation frequency of *SOX11* promoter in the serum from patients with HCC (69.4%), CHB (13.6%) and HCs (10.7%). * *P*<0.05.

Results

Methylation status of *SOX11* promoter in serum. The baseline characteristics of all the subjects were presented in Table 2. Figure.2 demonstrated that methylation of serum *SOX11* promoter were detected in 77 of 111 (69.4%) HCC patients, 9 of 66 (13.6%) CHB patients and 3 of 28 (10.7%) HCs. The frequency of serum *SOX11* promoter methylation in HCC patients was significantly higher than that in CHB patients ($\chi^2 = 51.467$, $P< 0.05$) and HCs ($\chi^2 = 31.489$, $P< 0.05$), respectively. However, there were no statistical differences of serum *SOX11* promoter methylation between CHB patients and HCs ($\chi^2 = 0.007$, $P > 0.05$). Representative agarose gel electrophoresis results for methylated *SOX11* were presented in Figure 3.

Correlation between *SOX11* promoter methylation and clinicopathological parameters. Table 3 revealed that the

Table 2. Baseline Characteristics of the Participants

Variable	HCC group (*n* = 111)	CHB group (*n* = 66)	HC group (*n* = 28)
Age (years)	59 (51 – 64)	47 (42 – 54)	28 (25 – 31)
Gender (M/F)	94 / 17	47 / 19	9 / 19
HBeAg (+/-)	26 / 85	28 / 38	NA
HBsAg	3553.00 (1583.00 – 5126.00)	4297.50 (849.00 – 5515.00)	NA
ALT (U/L)	43.00 (26.00 – 85.00)	25.00 (16.75 – 45.75)	NA
AST (U/L)	77.00 (45.00 – 147.00)	24.50 (19.00 – 38.25)	NA
TBIL (μmol/L)	25.30 (14.20 – 43.30)	12.45 (9.55 – 17.60)	NA
ALB (g/L)	36.10 (31.30 – 40.70)	47.40 (45.38 – 49. 63)	NA
PT-INR	1.11 (1.04 – 1.34)	1.05 (1.00- 1.10)	NA
AFP (ng/ml)	51.00 (6.13 – 430.60)	6.69 (3.09 – 32. 09)	1.00 (0.89 – 1.35)
Methylation, *N* (%)	77 (69.37%)	9 (13.63%)	3 (10.71%)

HCC – hepatocellular carcinoma; CHB – chronic hepatitis B; HC – healthy control; M – male; F – female; ALT – alanine aminotransferase; AST – aspartate aminotransferase; TBIL – total bilirubin; ALB – albumin; PT-INR – prothrombin time-international normalized ratio; AFP – alpha-fetoprotein; NA – not available.

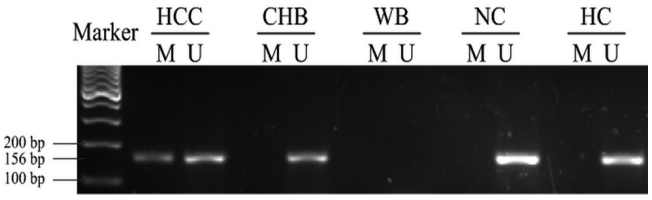

Figure 3. Representative results for methylation of *SOX11* by methylation-specific polymerasechain reaction. A 100-bp DNA ladder marker was used. Lanes M and U indicate the amplified products with primers recognizing methylated and unmethylated sequences, respectively. NC, negative control; WB, water blank

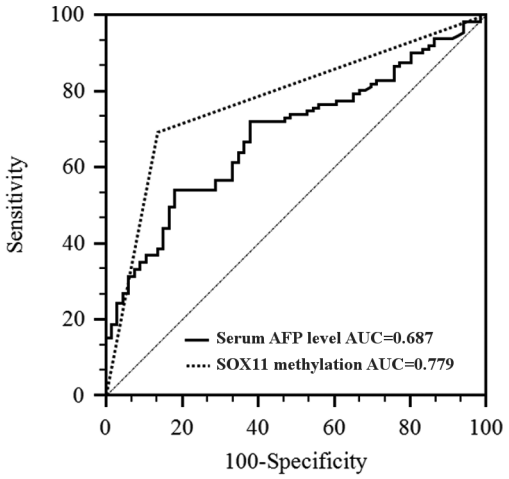

Figure 4. The receiver operating characteristic (ROC) curves of AFP and *SOX11* promoter methylation in discriminating HCC from CHB patients. Areas under ROC curves between *SOX11* promoter methylation and AFP were compared using MedCalc statistical software 11.5.0. Areas under the ROC curve, AUROC.

serum *SOX11* promoter methylation frequency in HCC patients with vascular invasion (49/58) was significantly higher than those without vascular invasion (28/53; χ^2 = 13.058, $P < 0.001$). However, *SOX11* promoter methylation status was not significantly associated with gender, age, HBeAg, serum AFP level, alcohol, smoking, tumor number, tumor size, histological grading, or Tumor node metastasis (TNM) staging (all $P > 0.05$), respectively. In Table 4, the multivariate logistic regression showed that there were no independent parameters for *SOX11* promoter methylation except vascular invasion ($P<0.05$).

The diagnostic value of *SOX11* promoter methylation and combination with AFP in HCC. When trying to discriminate HCC from CHB patients, methylation of the serum *SOX11* gene promoter showed a sensitivity of 69% and a specificity of 86%, and AFP showed a sensitivity of 57% and a specificity of 66%. Moreover, *SOX11* methylation showed a sensitivity of 69% (77/111), a specificity of 89% (25/28) in discriminating HCC from HCs (Table 5). The area under AUC (ROAUC) of serum *SOX11* promoter methylation (0.779, 95% CI: 0.710-0.837) was significantly

Table 3. Clinicopathological Parameters with *SOX11* Promotor Methylation in HCC Patients

	SOX11 methylation status			
Parameters	Methylated	Unmethylated	Statistics	*P* value
Age	58.00 (51.00 – 64.00)	59.00 (49.75 – 63.25)	T= -0.534	0.595[a]
Gender (M/F)	67/10	27/7	X^2 = 1.051	0.305[b]
HBeAg (-/+)	58/19	27/7	X^2 = 0.220	0.639[b]
Smoking (-/+)	36/41	21/13	X^2 = 2.218	0.145[b]
Alcohol (-/+)	39/38	23/11	X^2 = 2.764	0.096[b]
Number (single/multiple)	41/36	23/11	X^2 = 2.003	0.157[b]
Vascular invasion (-/+)	28/49	25/9	X^2 = 13.058	< 0.001[b]
AFP	61.00 (4.42 – 589.14)	39.12 (6.21 – 139.00)	Z = -0.358	0.720[c]
Size			X^2 = 0.001	0.970[b]
≤3 cm	32	14		
>3 cm	45	20		
Histological grading			X^2 = 2.514	0.285[b]
Poor	30	8		
Moderate	30	17		
Well	17	9		
TNM staging			X^2 = 0.226	0.635[b]
I/II	37	18		
III/IV	40	16		

M – male; F – female; TNM – tumor node metastasis.
[a]Student's*t*-test; [b]X^2 test; [c]Mann-Whitney*U*-test.

Table 4. Multivariate Logistic Regression Analysis of Clinicopathological Parameters with *SOX*11 Promoter Methylation in HCC

Variable	Coefficient	OR	95%CI	*P* value
Vascular invasion	2.102	8.181	1.578-42.407	0.012
Gender	-0.075	0.928	0.246-3.493	0.912
Age	0.007	1.007	0.956-1.060	0.794
HBeAg	0.21	1.233	0.379-4.011	0.728
Smoking	0.035	1.035	0.354-3.026	0.949
Alcohol	0.323	1.381	0.466-4.086	0.560
Number	0.242	1.274	0.254-6.398	0.768
Size	-0.127	0.881	0.320-2.421	0.805
AFP	0.000	1.000	1.000-1.000	0.796
Histological grading	-0.034	0.966	0.493-1.895	0.920
TNM staging	-1.278	0.276	0.073-1.042	0.057

OR – odds ratio; CI – confidence interval

higher than serum AFP (ROAUC = 0.687, 95% CI: 0.613-0.754; $P < 0.05$; Figure. 4).

Furthermore, we compared the diagnostic value of the combination of the AFP and serum *SOX11* methylation with AFP alone in detecting HCC from CHB (Figure 5). As the serum AFP was at the level of less than 20 ng/ml, the HCC detection rate in *SOX11* methylated group (32/47, 86%) was significantly higher than that in *SOX11* unmethylated group (16/45, 29%; $P < 0.05$). As the serum AFP was at the level of ≥ 20 ng/ml, the frequency of HCC was also significantly higher in *SOX11* methylated group (45/49, 92%) than in *SOX11* unmethylated

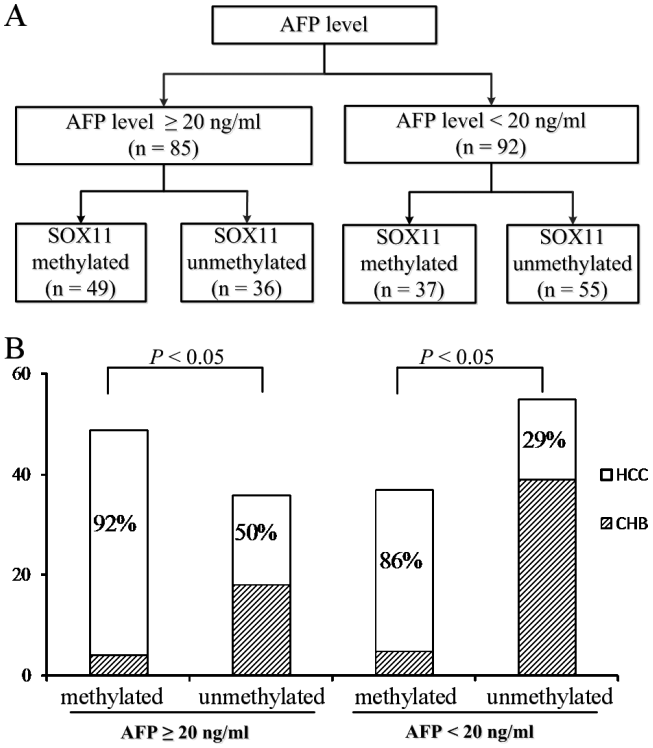

Figure 5. (A). Classification of patients according to serum AFP level and *SOX11* promoter methylation. (B). HCC detection rates in methylated and unmethylated group at the AFP cut-off points of 20 ng/ml. *P* values refer to the differences of proportion of HCC between methylated and unmethylated group ($P < 0.05$).

Table 5. Diagnostic Value of Serum *SOX11* Methylation and AFP in Predicting HCC

	SOX11 methylation	AFP	AFP + *SOX11* methylation	Statistic	*P* value
HCC from CHB					
Se (%)					
TP / (TP+FN)	77/111 (69%)	63/111 (57%)	95/111 (85%)	$X^2 = 15.321$	< 0.05
Sp (%)					
TN / (TN+FP)	57/66 (86%)	44/66 (66%)	39/66(59%)	$X^2 = 0.811$	0.368
PPV (%)					
TP / (TP+FP)	77/86 (90%)	63/85 (74%)	95/122 (78%)	$X^2 = 0.390$	0.532
NPV (%)					
TN / (TN+FN)	57/91(63%)	44/92 (48%)	39/55 (71%)	$X^2 = 7.461$	< 0.05
HCC from HCs					
Se (%)					
TP / (TP+FN)	77/111 (69%)	63/111 (57%)	95/111 (85%)	$X^2 = 15.321$	< 0.05
Sp (%)					
TN / (TN+FP)	25/28 (89%)	28/28 (100%)	25/28 (89%)	$X^2 = 3.170$	0.075
PPV (%)					
TP / (TP+FP)	77/80 (96%)	63/63 (100%)	95/98 (97%)	$X^2 = 1.965$	0.161
NPV (%)					
TN / (TN+FN)	25/59 (42%)	28/76 (37%)	25/41 (61%)	$X^2 = 6.26$	< 0.05

P values refer to the differences between AFP only and combination of AFP and *SOX11* methylation. Se (%), TP/(TP + FN); Sp (%), TN / (TN + FP); PPV(%), TP/(TP+FP) and NPV (%), TN / (TN+FN) were calculated and plotted. TP – True positive; FN – False negative; FP – False positive; TN –True negative

group (18/36, 50%; $P < 0.05$). In Table 5, we set AFP ≥ 20 ng ml or *SOX11* methylation as positive, and identify 122 patients in patients with HCC and CHB as positive including 95 HCC patients (true positive) and 27 CHB patients (false positive). Meanwhile, 55 patients with AFP < 20 ng/ml and unmethylated *SOX11* were set as negative, which containing 39 CHB patients (true negative) and 16 HCC patients (false negative). Therefore, the diagnostic value of *SOX11* methylation plus AFP has been calculated with a sensitivity of 85% (95/111), a specificity of 59% (39/66), PPV of 78% (95/122) and NPV of 71% (39/55) in discriminating HCC form CHB. Of note, the sensitivity ($\chi^2 = 15.321$, $P < 0.05$) and NPV ($\chi^2 = 7.461$, $P < 0.05$) are significantly higher than AFP alone, respectively. In addition, *SOX11* alone showed a sensitivity of 69% (77/111), a specificity of 89% (25/28), PPV of 96% (77/80) and NPV of 42% (25/59) in discriminating HCC from HCs. *SOX11* plus AFP has been calculted with a sensitivity of 85% (95/111), a specificity of 89% (25/28), PPV of 97% (95/98) and NPV of 61% (25/41) in discriminating HCC form HCs.

Discussion

To our knowledge, this present study was the first report to determine the potential value of serum *SOX11* promoter methylation as a noninvasive biomarker for the diagnosis of HCC. Our results showed that the serum *SOX11* methylation frequency was significant higher in HCC compared with CHB and HCs. We also reported that *SOX11* promoter methylation was significantly associated with vascular invasion in HCC patients. Furthermore, we demonstrated that *SOX11* promoter methylation showed higher diagnostic accuracy than APF alone in discriminating HCC from CHB. *SOX11* promoter methylation might improve the diagnostic accuracy of AFP in the diagnosis of HCC. Therefore, our results strongly suggested that serum *SOX11* promoter methylation might serve as a useful and noninvasive biomarker for the diagnosis of HCC.

The transcription factor *SOX11* plays an important role in regulating cell differentiation, proliferation and survival in the pathogenesis of tumor [25, 26]. *SOX11* gene could be epigenetically silenced through DNA methylation, which results in the down-regulation of *SOX11* protein [29, 30]. Several studies showed that *SOX11* methylation existed in gastric cancer [31], hematopoietic malignancies [29], and nasopharyngeal carcinoma [30]. In our study, we found that the *SOX11* methylation rate was obviously higher in HCC patients than in both CHB and HCs, indicating the potential role of *SOX11* in the pathogenesis of HCC. We also found a strong relationship between *SOX11* methylation and vascular invasion. The results agreed with Zhang's study that *SOX11* methylation might contribute to the growth and invasion of nasopharyngeal carcinoma [30]. In HCC patients, the possible association between methylation and invasion might be explained from the following aspects: First, *SOX11* has been demonstrated as a tumor suppressor gene and could be transcriptional inactivation mediated by promoter methylation [34]. The methylation silienced *SOX11* expression might therefore result in the growth and invasion of tumor cells [30]. Second, tumor-derived mutant DNA in situ tissue might migrate into peripheral circulation and then lead to the detectable level of serum *SOX11* promoter methylation [13, 35].

Until now, methylation abnormalities of some specific genes in the serum have been used as biomarkers in the diagnosis of HCC, such as *IGFBP7* [13], *RASSF1A* [21] *P15* and *P16* [20, 22]. In this present study, we demonstrated that *SOX11* showed potential diagnostic value with high sensitivity and specificity in the diagnosis of hepatitis B virus-related HCC. Our study also compared the combination of AFP and *SOX11* methylation with AFP alone in discriminating HCC patients from CHB patients. The sensitivity increased to 85% (combination of two markers) from 57% (AFP alone). Also, the NPV is higher in combination of two markers (39/55, 71%) than AFP alone (44/92, 48%). Notably, the specificity of *SOX11* promoter methylation alone in detecting HCC was 86% but significantly decreased to 59% when combined with AFP with a 20 ng/ml cut-off value. Therefore, our results suggest that the methylation of serum *SOX11* promoter can improve the sensitivity of AFP in diagnosis of HCC. Meanwhile, serum *SOX11* methylation may be applied to ensure a rather low false-positive rate in detecting HCC.

However, there are some limitations in our study. First, the MSP method we used is just a qualitative method to identify whether methylation occurs or not. Other methods, such as direct sequencing, might provide more exact and detailed information to determine methylation. However MSP can be performed in most clinical laboratories easily and rapidly. Although MSP is not very informative, we can still use it to select methylated cases that could be further examined. Second, the patients were from our single unit and data from a multi-center, large-scale cohort would be more helpful.

In conclusion, our study demonstrated that *SOX11* promotor methylation could be frequently detected in the serum of patients with HCC. Furthermore, S*OX11* promotor methylation might be a useful noninvasive biomarker for diagnostic of HCC. Combination of AFP and serum S*OX11* promotor methylation could enhance the sensitivity of AFP alone in the diagnostic of HCC.

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