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Methylation of *SOX1* and *VIM* promoters in serum as potential biomarkers for hepatocellular carcinoma

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Aberrant methylation of tumor-related genes has been identified as a promising biomarker for hepatocellular carcinoma (HCC). This study aimed to investigate the diagnostic value of SRY (sex determining region Y)-box 1 (*SOX1*) and Vimentin (*VIM*) promoter methylation for HCC. The study included 360 subjects, 240 patients with HCC, 29 with liver cirrhosis (LC), 66 with chronic hepatitis B (CHB) and 25 healthy controls (HCs). The methylation status of *SOX1* and *VIM* promoters in the serum was detected by methylation-specific polymerase chain reaction (MSP). The methylation frequencies of *SOX1* and *VIM* promoters in HCC patients were significantly higher than those in LC ($p<0.001$ and $p<0.001$), CHB ($p<0.001$ and $p<0.001$) and HC ($p<0.001$ and $p<0.001$) subjects. Furthermore, hypermethylation of *SOX1* and *VIM* promoters were found in patients with advanced TNM stage (III-IV) and larger tumor size (≥5 cm) compared with early stage (I-II) ($p<0.001$ and $p=0.004$) patients with smaller tumor size (<3 cm) ($p=0.018$ and $p=0.001$). Moreover, the *VIM* promoter methylation frequency was higher in patients with portal vein tumor thrombosis (PVTT) ($p=0.006$) and vascular invasion ($p=0.003$). In addition, the combination of α-fetoprotein (≥20 ng/ml) with *SOX1* and *VIM* promoter methylation significantly improved their diagnostic value. In conclusions, aberrant methylation of *SOX1* and *VIM* promoters may be potential biomarkers for noninvasive detection of HCC and HCC metastasis.

Key words: methylation, SOX1, VIM, hepatocellular carcinoma

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer in men and the seventh in women worldwide. It is the third most frequent cause of cancer-related mortality. In Asia and Africa, the incidence of HCC is higher than that in Southern Europe, and incidence is the lowest in most high-income countries [1]. Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are risk factors for HCC [2]. Early diagnosis of HCC is a prerequisite for any curative therapy [3]. Recently, diagnostic procedures have been greatly improved. Clinically, detection of HCC is based on serum α-fetoprotein (AFP), biopsy of the lesion and various imaging technologies, including ultrasonography, multi-detector computed tomography and diffusion-weighted magnetic resonance imaging. However, certain limitations remain in these techniques for HCC surveillance and diagnosis [4]. AFP, a specific tumor marker, has been used for clinical diagnosis of HCC for more than 30 years. However, AFP has also been found in some benign cases of liver disease, and more than 30% of HCC patients are AFP negative. Therefore, evaluation of AFP alone can lead to misdiagnosis and missed diagnoses [5]. Imaging technologies depend on examiner expertise, patient data, the presence of liver cirrhosis and tumor size, and smaller tumors are difficult to detect. Biopsy of the lesion is an invasive procedure. Thus, the development of new biomarkers is of great importance for improving the diagnosis of early-stage HCC.

Biomarkers are biological characteristics that are widely used to forecast risk, diagnose diseases and predict prognosis. They are easily and objectively measured at any stage of a disease [6-8]. Circulating cell-free tumor DNA (ctDNA) in the blood of patients with cancer can be detected as a biological biomarker [9]. It offers a simple and noninvasive method to diagnose and prognose carcinoma [10]. Epigenetic alterations play an important role in the initiation and progression of human cancer. The most common epigenetic change in tumors is aberrant DNA methylation in the promoter regions of genes, which lead to silencing of tumor-suppressor genes. Numerous

studies have confirmed that using DNA methylation changes as biomarkers for carcinoma is useful for early detection of cancer, including HCC [11]. For example, aberrant methylation of serum *ELF, RASSF1A, GSTP1, APC, SFRP1, LINE-1, GRA* has been shown to be useful as biomarkers for assessing the risk of HCC [12-16]. Abnormal hypermethylation of a CpG island in different stages of tumorigenesis is a dysfunction of tumor suppressor genes (TSGs) [17-19]. Hypermethylation of gene promoters could be markers for HCC in early or late events [20, 21].

SRY (sex determining region Y)-box 1 (*SOX1*) is a member of the SRY-box (*SOX*) family of proteins and plays an important role during embryonic and postnatal development [22]. It encodes a transcription factor implicated in the regulation of embryonic development and the determination of cell fate [23]. *SOX1* is frequently downregulated through promoter hypermethylation in HCC cell lines and tumor tissues. Ectopic expression of *SOX1* leads to significant repression of HCC growth by interfering with Wnt/β-catenin signaling. Therefore, *SOX1* is an important tumor suppressor of HCC [24]. Vimentin (*VIM*) belongs to the family of intermediate filaments, which are specifically found in connective tissues [25]. The *VIM* gene encodes Vimentin and plays an important role in various biological processes, such as maintaining cell shape and stabilizing cytoskeletal interactions. *VIM* is also related to cell migration, inflammation and signal transduction [26]. Previous studies have found that aberrant methylation of *SOX1* is correlated with cervical and non-small cell lung cancer [27, 28], and aberrant methylation of the *VIM* gene is associated with cervical and pancreatic carcinoma [26, 29]. Serum *SOX1* and *VIM* methylation could be potential predictive biomarkers for ovarian and colorectal cancer [30, 31]. Aberrant methylation of *SOX1* and *VIM* has been detected in HCC tissues [32, 33]. However, the role of *SOX1* and *VIM* expression in the serum of HCC patients is unclear. Therefore, we chose these two genes and aimed to evaluate the potential value of serum *SOX1* and *VIM* promoter methylation as noninvasive biomarkers for the diagnosis of HCC.

In this study, we used methylation-specific polymerase chain reaction (MSP) to determine the promoter methylation of *SOX1* and *VIM* genes in patients with HCC, CHB, LC and in healthy blood donors. Then, we evaluated the methylation status among them and assessed the diagnostic value for HCC.

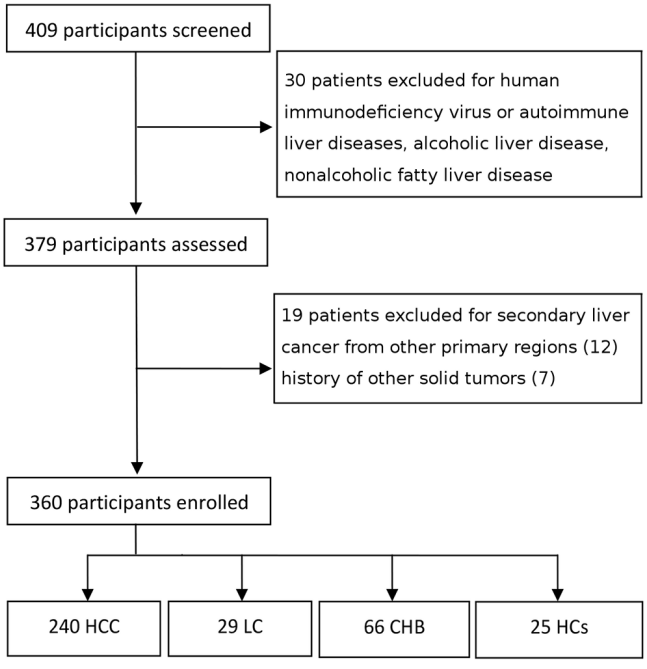

Figure 1. Flowchart depicting the selection process of the participants. HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; CHB: Chronic hepatitis B; HCs: Healthy controls.

Patients and methods

Study populations. The study enrolled 360 subjects, including 240 patients with HCC, 29 cases with LC, 66 cases with CHB and 25 HCs, at the Department of Hepatology, Qilu Hospital of Shandong University from July 2014 to July 2015. All subjects with HCC were diagnosed according to the criteria of the American Association for The Study of Liver Diseases (AASLD), which was updated in 2010 [34]. The 2009 Edition of AASLD Practice Guidelines were used to diagnose CHB patients in this study [35]. A history of other tumors, human immunodeficiency virus or autoimmune liver diseases, alcoholic liver disease, nonalcoholic fatty liver disease and other causes of chronic liver disease were the exclusion criteria (Figure 1). All patients who agreed to participate in this study signed a written informed consent, and the study protocol was approved by the Ethics Committee of Qilu Hospital. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (ALB) and prothrombin time-international normalized ratio (PT-INR) were measured using a Roche automated biochemical analyzer cobas c311 (Roche Diagnostic Ltd, Germany). Hepatitis B e antigen (HBeAg) and serum AFP levels were detected with an electrochemiluminescence immunoassay using an automatic analyzer (COBAS e 601, Roche Diagnostics, Mannheim, Germany). AFP concentrations higher than 20 ng/ml were considered abnormal. Tumor size was determined by computed tomography and is presented as the longest diameter.

Serum DNA extraction and sodium bisulfite modification. DNA was extracted from 400 µl of serum with a QIAamp DNA Blood Mini Kit (Qiagen, Mainz, Germany) according to the protocol for DNA purification from blood as recommended by the manufacturer. Then, 200 µl of serum DNA was eluted and stored at -20 °C until use. An EZ DNA Methylation-Gold Kit™ (Zymo Research, USA) was used to treat 20 µl of DNA according to the manufacturer's instructions. Finally, 20 µl of modified DNA was obtained as a template for MSP or stored at -20 °C.

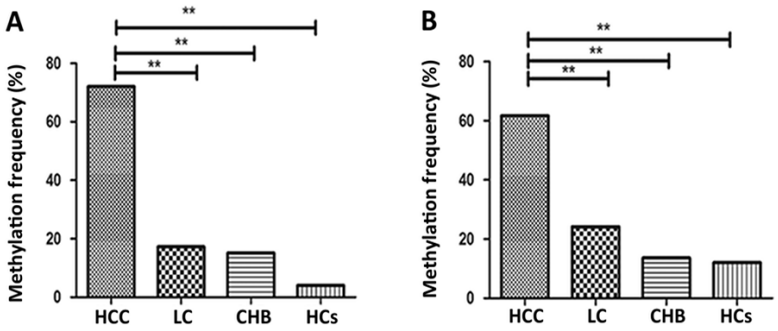

Figure 2. The methylation frequency of the serum *SOX1* and *VIM* promoters in hepatocellular carcinoma (HCC), liver cirrhosis (LC), chronic hepatitis B (CHB), and healthy control (HC) group. A: Percentage methylation of *SOX1*; B: Percentage methylation of *VIM*. **$p < 0.001$.

Methylation-specific polymerase chain reaction (MSP). Methylated and unmethylated primers of *SOX1* and *VIM* for MSP were used to amplify the bisulfite-modified DNA. The MSP primer sequences were obtained from previous studies [27, 36]. The expected size of the PCR product of *SOX1* was 135 bp and that of *VIM* was 151 bp (Table 1). Briefly, 12.5 μl of Premix Taq (Zymo Research, USA), 10.5 μl of nuclease-free water, 0.5 μl of each primer and 1 μl of bisulfite-treated DNA were combined to a volume of 25 μl in the MSP reaction system. The PCR protocol was composed of an initial denaturation step at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 40 s, and primer extension at 72 °C for 40 s; finally, an extension step was set at 72 °C for 10 min. The negative control was water without DNA. A total of 7 μl of PCR products were then electrophoresed on a 2% agarose gel, stained with Gel Red and visualized under UV illumination. According to the guidelines of the MSP procedure, methylation was identified if only the M band or both the M and U bands were positive, while unmethylation was identified if the U band was positive [37, 38].

Statistical analysis. All data were analyzed using SPSS v.20.0 software. The methylation percentage was calculated by the ratio of the methylated number/total number in each group. Chi-square test was used to compare the differences in serum *SOX1* and *VIM* methylation status between different groups. Then, we evaluated the correlation between serum *SOX1* and *VIM* methylation status in HCC patients and their clinicopathological parameters via a chi-square test. Univariate logistic regression was also used to investigate the relationship between clinicopathological parameters and *SOX1* and *VIM* promoter methylation. The *p* value for entry into the regression model was 0.05 and that for removal was 0.1. Diagnostic values of *SOX1* and *VIM* methylation and serum AFP level were assessed by the area under the receiver operating characteristic curve (AUC). As to the combination of *SOX1* and *VIM* methylation and AFP, patients with AFP ≥ 20 ng/ml or methylated *SOX1* and *VIM* were regarded as positive. Patients with AFP < 20 ng/ml and unmethylated *SOX1* and *VIM* were regarded as negative. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. The statistical significance was defined as $p < 0.05$.

Results

Methylation status of *SOX1* and *VIM* in serum. First, we examined the methylation status of the serum *SOX1* and *VIM* promoter in 240 HCC patients, 29 LC patients, 66 CHB patients and 25 HCs. Baseline characteristics of the participants are shown in Table 2. The chi-square test analysis indicated that the percentage of *SOX1* promoter methylation was significantly higher in the HCC group (72.08%) than in the LC group (17.24%, $p<0.001$), the CHB group (15.15%, $p<0.001$) and the HCs (4.00%, $p<0.001$). However, there were no significant differences for *SOX1* between the LC group and the CHB group ($p=0.061$), the CHB group and the healthy control group ($p=0.057$), or the LC group and the healthy control group ($p=0.059$; Figure 2A). The methylation frequency of the *VIM* promoter was also higher in the HCC group (61.67%) than in the LC group (24.14%, $p<0.001$), the CHB group (13.64%, $p<0.001$) and the HCs (12.00%,

Table 1. Primers of the *SOX1* and *VIM* Gene for MSP

Primer	Primer sequence (5'-3')	Product size (bp)	Annealing temp (°C)
SOX1			
M	F:CGTTTTTTTTTTTTCGTTATTGGC R:CCTACGCTCGATCCTCAACG	135	58
U	F:TGTTTTTTTTTTTTTTGTTATTGGTG R:CCTACACTCAATCCTCAACAAC	135	56
VIM			
M	F:GGATTTTTTTGGTTTAGTTTTAGGC R:AACATAATCCCGTTACTTCAACG	151	58
U	F:ATTTTTTTGGTTTAGTTTTAGGTGG R:ACATAATCCCATTACTTCAACACT	151	58

MSP, methylation-specific polymerase chain reaction M, methylated sequence; U, unmethylated sequence; F, forward; R, reverse.

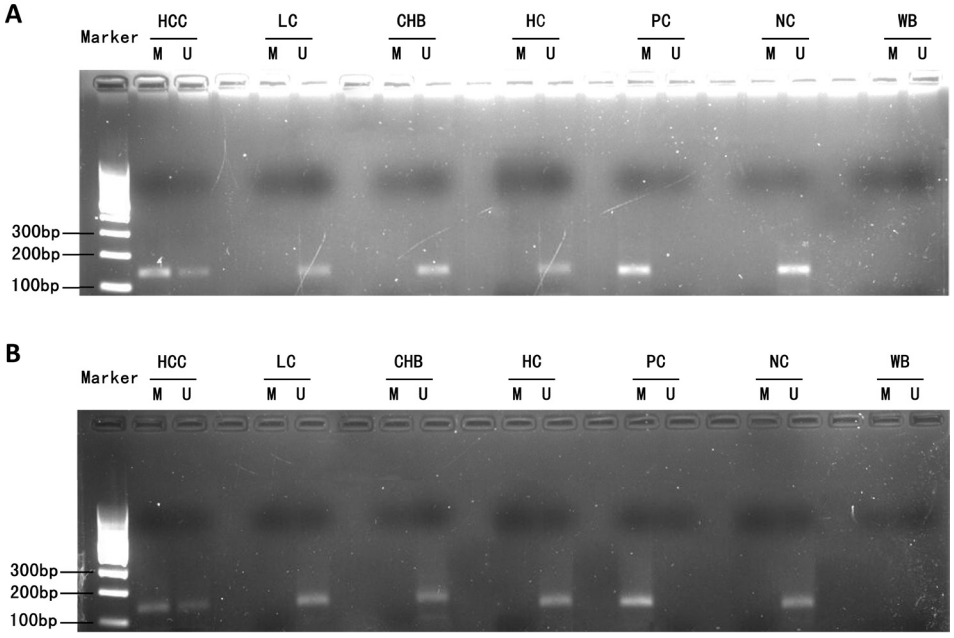

Figure 3. Representative results showing the *SOX1* and *VIM* promoter methylation status identified by MSP in serum samples from hepatocellular carcinoma (HCC) patients, liver cirrhosis (LC) patients, chronic hepatitis B (CHB) patients, and healthy controls (HCs). A: The methylated and unmethylated sequences of *SOX1*; B: The methylated and unmethylated sequences of *VIM*. A 100-bp DNA ladder marker was used. M and U indicate the amplified products with primers recognizing methylated and unmethylated sequences, respectively. PC, positive control; NC, negative control; WB, water blank.

$p<0.001$). Moreover, no significant differences in *VIM* were found between the LC group and the CHB group ($p=0.083$), the CHB group and the healthy control group ($p=0.074$), or the LC group and the healthy control group ($p=0.052$; Figure 2B). Typical results of MSP for the *SOX1* and *VIM* promoters are presented in Figure 3.

Correlation between *SOX1* and *VIM* promoter methylation and clinicopathological parameters. The association between the methylation status of the *SOX1* and *VIM* promoters and clinicopathological characteristics of HCC patients is shown in Table 3. There was no significant correlation between the methylation status of *SOX1* and *VIM*

Table 2. Baseline Characteristics of the Participants

Variable	HCC group (n=240)	LC group (n=29)	CHB group (n=66)	HCs group (n=25)
Age (years)	57.0 (21.0-83.0)	47.0 (38.0-50.0)	43.0 (18.0-75.0)	28.0 (23.0-42.0)
Gender (M/F)	178/62	15/14	39/27	10/15
HBeAg (+/-)	202/38	16/13	42/24	NA
ALT (U/I)	80.72 (11.00-1762.00)	188.72 (85.00-1061.00)	129.44 (24-256.00)	NA
AST (U/I)	114.22 (20.00-1008.00)	154.00 (28.00-793.00)	80.86 (35.00-200.00)	NA
TBIL (μmol/l)	22.83 (4.30-47.80)	24.36 (17.90-52.20)	24.43 (9.90-36.20)	NA
ALB (g/l)	37.45 (22.30-204.00)	36.18 (28.00-46.00)	35.47 (31.00-42.00)	NA
PT-INR	1.15 (0.93-1.93)	1.35 (1.01-1.93)	1.17 (1.09-1.25)	NA
AFP (ng/ml)	621.41 (1.44-24200.00)	7.61 (2.67-41.80)	15.96 (2.50-39.9)	NA
Methylation (%)				
SOX1	72.08	17.24	15.15	4.00
VIM	61.67	24.14	13.64	12.00

HCC, hepatocellular carcinoma; CHB,chronic hepatitis B ; LC, liver cirrhosis; HCs, healthy controls ; 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

and patient gender, age, viral infection, smoking status, alcohol use, Child-Pugh stage or Milan standards. We also found that *SOX1* promoter methylation was related to the tumor number ($\chi2$=6.107, p=0.013), tumor size ($\chi2$=7.986, p=0.018) and TNM stage ($\chi2$=12.458, p=0.001). The *VIM* promoter had a higher methylation frequency in patients with portal vein tumor thrombosis ($\chi2$=7.528, p=0.006), tumor number ($\chi2$=9.997, p=0.002), tumor size ($\chi2$=19.451, p=0.001), TNM stage ($\chi2$=8.291, p=0.004) and vascular invasion ($\chi2$=8.832, p=0.003). Furthermore, univariate logistic regression was used to analyze the correlation between *SOX1* and *VIM* promoter methylation and the characteristics of HCC (Tables 4 and 5). The serum *SOX1* promoter methylation was more frequent in HCC patients with multiple tumors (OR=0.208, p<0.001) and TNM stage (III–IV) (OR=4.987, p<0.001). *VIM* promoter methylation was highly correlated with PVTT (OR=0.006, p<0.001), TNM stage (III–IV) (OR=3.978, p<0.001), tumor size>5 cm (OR=2.340, p=0.037) and vascular invasion (OR=33.681, p<0.001).

Diagnostic value of serum concentrations of AFP and promoter-methylated *SOX1* and *VIM* in HCC. To discriminate HCC from LC and CHB, the sensitivity is 72.08% for *SOX1* and 61.67% for *VIM*, higher than AFP alone (56.67%, $\chi2$=12.436, p< 0.001; $\chi2$=1.242, p=0.265). When we combined *SOX1* and *VIM* promoter methylation, higher sensitivity (82.50%) but lower specificity (78.95%) for discriminating HCC from CHB and LC was found, as shown in Table 6. Then, we compared the diagnostic value of AFP combined with *SOX1* and *VIM* methylation, and the two methods are presented in

Table 3. Correlations Between *SOX1* and *VIM* Promoter Methylation Status and Clinicopathological Characteristics of the HCC Patients

Parameters	*SOX1*		$\chi2$	*p* value	*VIM*		$\chi2$	*p* value
	Methylated	Unmethylated			Methylated	Unmethylated		
Age								
<55	82	28	0.612	0.434	71	39	0.712	0.399
≥55	91	39			77	53		
Gender (M/F)	127/46	51/16	0.185	0.667	112/36	66/26	0.459	0.498
Viral infection								
HBV	157	62			131	88		
HCV	9	3	1.199	0.753	10	2	4.225	0.238
HBV and HCV	1	1			2	0		
Non HBV,non HCB	6	1			5	2		
Smoking(+/-)	107/66	39/28	0.269	0.604	95/53	51/41	1.825	0.177
Alcohol (+/-)	119/54	45/22	0.059	0.809	107/41	57/35	2.804	0.094
Child – Pugh								
A	39	20			41	18		
B	71	31	3.665	0.160	60	42	3.035	0.362
C	63	16			47	32		
AFP (ng/ml)								
<20	79	25	1.372	0.242	65	39	0.003	0.956
≥20	94	42			83	53		
Size (cm)								
<3	35	19			24	30		
3 – 5	49	27	7.986	0.018	40	36	19.451	0.001
≥ 5	89	21			84	26		
PVTT (+/-)	17/156	7/60	0.021	0.886	21/127	3/89	7.528	0.006
TNM stage								
I/II	53	37	12.458	0.001	45	45	8.291	0.004
III/IV	120	30			103	47		
Milan standard								
Yes	79	38	2.361	0.124	78	39	2.414	0.120
No	94	29			70	53		
Tumor multiplicity								
single	117	56	6.107	0.013	96	77	9.997	0.002
multiple	56	11			52	15		
Vascular invasion or metastasis(+/-)	52/121	19/48	0.067	0.796	54/94	17/75	8.832	0.003

M, male; F, female; TNM, tumor node metastasis; PVTT, portal vein tumor thrombus

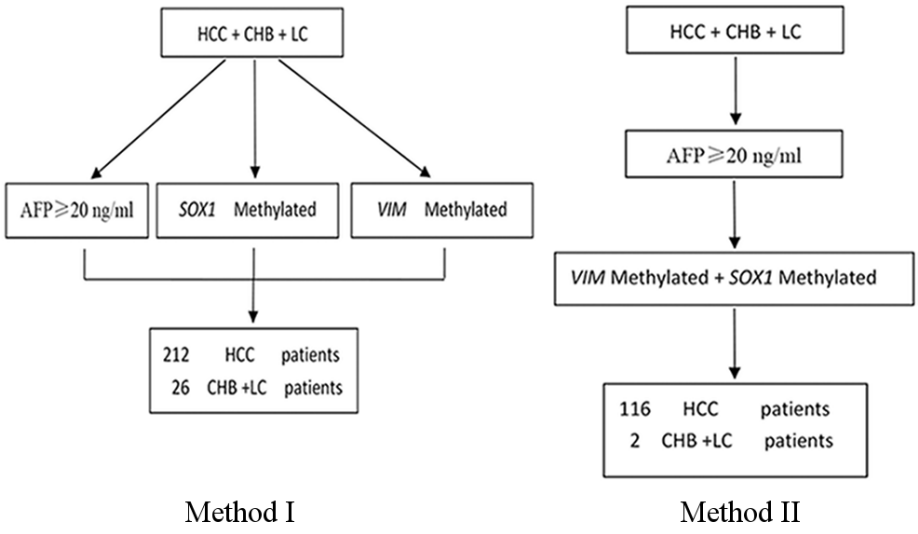

Figure 4. Diagnostic algorithms of Method I and Method II to discriminate HCC patients from CHB and LC patients.

Figure 4. When AFP was more than 20 ng/ml or at least one gene of *SOX1* and *VIM* was methylated, the sensitivity, specificity, PPV and NPV were 88.33%, 72.63%, 89.08% and 71.13%, respectively. When AFP was more than 20 ng/ml and *SOX1* and *VIM* were both methylated, the sensitivity, specificity, PPV, and NPV were 48.33%, 97.89%, 98.31% and 42.86%, respectively. From Table 7, we can conclude that Method I provides higher sensitivity than Method II ($\chi 2=68.912$, $p<0.001$) and AFP alone ($\chi 2=25.960$, $p<0.001$). The specificity of Method II for discriminating HCC from LC and CHB patients is higher. The correlation coefficient between AFP and *VIM* was 0.271, $p<0.001$; and AFP and *SOX1* was 0.186, $p=0.004$. Moreover, the AUC of *SOX1* and *VIM* methylation was significantly higher than that of AFP in discriminating HCC from CHB and LC patients (0.805 versus 0.742; $p=0.012$) (Figure 5).

Table 4. Univariate Logistic Regression Analysis of *SOX 1* Promoter Methylation with Clinicopathological Characteristics in HCC

SOX1	Tumor size >5 cm	Tumor multiplicity	TNM stage III-IV
P	0.177	0.001	0.001
OR	1.542	0.208	4.987
95% CI	0.822-2.892	0.095-0.456	2.113-11.768

OR, odds ratio; CI, confidence interval; TNM, tumor node metastasis

Table 5. Univariate Logistic Regression Analysis of *VIM* Promoter Methylation with Clinicopathological Characteristics in HCC

VIM	Tumor size >5 cm	PVTT	invasion	TNM stage III-IV
p	0.037	0.001	0.001	0.001
OR	2.340	0.006	33.681	3.978
95% CI	1.053-5.198	0.001-0.040	9.410-120.561	1.789-8.847

PVTT, portal vein tumor thrombus; OR, odds ratio; CI, confidence interval.

Discussion

In this study, we first demonstrated that *SOX1* and *VIM* promoter methylation could be detected in the serum of patients with HCC, LC, CHB and in HCs. The frequency of *SOX1* and

Table 6. Sensitivity, Specificity, PPV and NPV of gene promoter methylation and AFP to discriminate HCC from LC and CHB

Marker	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
SOX1	72.08	84.21	92.02	54.42
VIM	61.67	83.16	90.24	46.20
SOX1/VIM	82.50	78.95	90.83	64.10
AFP	56.67	83.16	89.47	43.17

AFP, alpha-fetoprotein; PPV, positive predictive value; NPV, negative predictive value.

Table 7. Diagnostic Value of Two Methods for Discriminating HCC from LC and CHB

Marker	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
AFP/*SOX1*/*VIM* Methylation(Method I)	88.33	72.63	89.08	71.13
AFP+*SOX1*+*VIM* Methylation(Method II)	48.33	97.89	98.31	42.86

AFP, alpha-fetoprotein; PPV, positive predictive value; NPV, negative predictive value.

VIM promoter methylation in HCC was 72.08% and 61.67%, respectively, which was significantly higher than that in LC (17.24%, 24.14%) and CHB (15.15%, 13.64%) patients and in HCs (4%, 12%). The results were consistent with previous studies that showed that the *SOX1* and *VIM* promoters are methylated in HCC tissues [24, 33]. HBV is a major public health problem worldwide, with approximately 2 billion individuals infected and 400 million people with chronic HBV infection [39]. Previous studies have indicated that chronic hepatitis – cirrhosis – HCC is a continuous progress [40]. The results of our study showed that the frequency of *SOX1* and *VIM* promoter methylation gradually increased from the HCs to patients with, CHB, LC and HCC. This trend may provide a simple and practical method to predict disease progression earlier.

For diagnosis, the status of serum *SOX1* and *VIM* promoter methylation showed a higher sensitivity (72.08%; 61.67%) and specificity (84.21%; 83.16%) than that of AFP (56.67%, 83.16%) in discriminating HCC from CHB and LC. However, the combination of *SOX1* and *VIM* promoter methylation elevated the sensitivity to 82.50% in diagnosing HCC. Furthermore, we combined measurement of AFP level (≥ 20) with the methylation of *SOX1* and *VIM* promoters, and if there is at least one positive index, then the diagnostic ability increases to 88.33%. If all the indexes are positive, then the specificity significantly improves to 97.89%. The false negative rate of AFP is high, but epigenetic changes occur early and are stable, and thus, can compensate for the deficiency of AFP and increase the detection rate of HCC.

SOX1 is a member of the *SOX* transcript factor superfamily. Recently, the function of *SOX1* promoter methylation has received more attention. It has been demonstrated that *SOX1* is a tumor suppressor that interferes with Wnt/β-catenin signaling in the development of HCC. Moreover, researchers have found that *SOX1* is frequently downregulated in HCC cells and tissues. Furthermore, they have observed that constitutive overexpression of *SOX1* can suppress cell proliferation, colony formation and invasion ability in HCC cell lines, and knockdown of *SOX1* can partially restore these functions [24]. In addition, it has also been reported that there is a significant correlation between downregulation of *SOX1* expression and promoter methylation in HCC tissues. The percentage of methylation is 57.3%, which is significantly higher than that of chronic hepatitis and cirrhosis tissues [32]. In addition to its function in HCC, methylation of *SOX1* is more frequently associated with non-small cell lung cancer [41]. A recent study on cervical cancer found that *SOX1* was frequently methylated in squamous cervical cell carcinomas, and the aberrant methylation rate is 81.5% according to a quantitative methylation-specific PCR assay [42]. Vimentin is a marker of mesenchymal cells that is associated with cell adhesion, cytoplasmic microtubule assembly, and cytoskeleton remodeling [29]. Furthermore, previous studies have indicated that vimentin participates in epithelial-mesenchymal transition (EMT). EMT is a process in which tumor cells

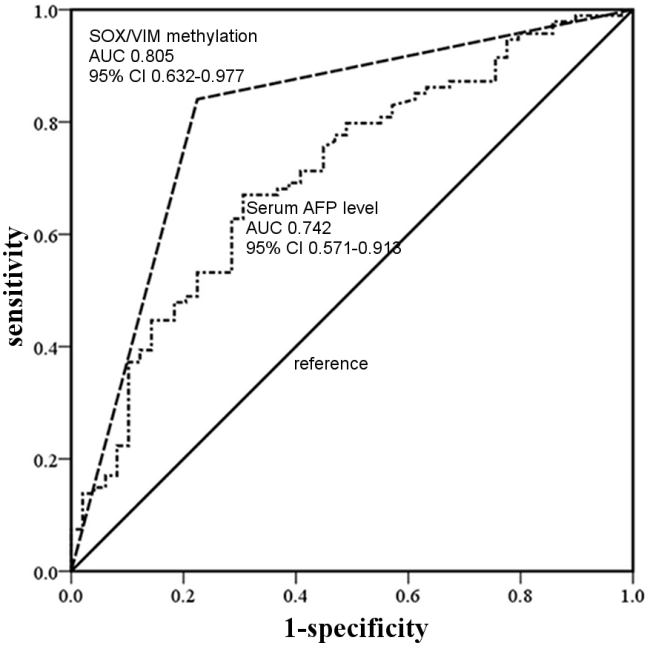

Figure 5. Receiver operating characteristic curves (ROCs) of serum AFP level ≥20 ng/ml and the methylation of *SOX1* and *VIM* promoters in discriminating HCC from CHB and LC.

lose the characteristic polarity of epithelial cells and acquire the characteristics of stroma cells. EMT is a critical event in invasion and metastasis of malignant cells [43, 44]. From our study, we evaluated whether promoter methylation of serum *SOX1* and *VIM* was associated with clinicopathological parameters in HCC patients. We found that *SOX1* promoter methylation was associated with multiple tumors and TNM stage (III–IV). *VIM* promoter methylation was highly associated with PVTT, TNM stage (III–IV), tumor size (>5 cm) and vascular invasion. Promoter methylation of *SOX1* and *VIM* may affect the expression of other related genes, which may lead to clinicopathological changes in HCC. Patients with portal vein tumor thrombus and metastasis showed an elevated percentage of serum *VIM* promoter methylation (p=0.006, p=0.003). This indicated that *VIM* promoter methylation was correlated with HCC progression, migration and proliferation, although the molecular mechanisms were unclear. Univariate logistic regression analysis indicated that risk factors of *VIM* promoter methylation were PVTT (OR=0.006) and metastasis (OR=33.681). Based on the results, we propose that the combination of serum *SOX1* and *VIM* promoter methylation might be a useful predictor of HCC progression.

The present study has some limitations. First, the observations of *SOX1* and *VIM* promoter methylation were from a small number of patients within a single site. More studies with a larger number of HCC patients from multiple locations are expected in the future. Second, we need long-term follow-

up observations to reveal the prognostic value of *SOX1* and *VIM* methylation in HCC patients. Third, we did not quantify the mRNA and protein levels of *SOX1* and *VIM* in the serum and tissue of HCC patients. We will perform further studies to detect the relationship between *SOX1* and *VIM* expression and promoter methylation in HCC patients. However, the main aim of the present study was to confirm whether *SOX1* and *VIM* promoter methylation were present in the serum of HCC patients and whether they could function as biomarkers for HCC diagnosis.

In summary, we primarily determined the status of *SOX1* and *VIM* promoter methylation in serum and evaluated the potential value of serum *SOX1* and *VIM* promoter methylation in the diagnosis of HCC. We report that serum *SOX1* and *VIM* methylation showed a higher sensitivity than AFP alone for the diagnosis of HCC, suggesting that serum *SOX1* and *VIM* promoter methylation might be potential noninvasive biomarkers for HCC. Further studies are needed to validate the mechanism and assess the clinical value.

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