

Plasma *SGIP1* methylation in diagnosis and prognosis prediction in hepatocellular carcinoma

Guo-Feng XIE^{1,2,#}, Yu-Xia XU^{2,#}, Fei XU^{1,2}, Li-Yue SUN^{1,2}, Zu-Lu YE^{1,2}, Jiang-Jun MA^{1,2}, Hai-Yun WANG^{1,2,3,*}, Jian-Yong SHAO^{1,2,*}

¹State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, Guangzhou, 510060, China; ²Department of Molecular Diagnostics, Sun Yat-sen University Cancer Center, Guangzhou, 510060, China; ³Guangzhou Institute of Pediatrics, Guangdong Provincial Key Laboratory of Research in Structural Birth Defect Disease, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, Guangdong, China

*Correspondence: wanghaiy@sysucc.org.cn; shaojy@sysucc.org.cn

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Aberrant methylation of some genes can serve as promising biomarkers in hepatocellular carcinoma (HCC). This study aimed to investigate the diagnostic and prognostic value of plasma *SGIP1* methylation in HCC. The study included a total of 269 subjects, of which 129 were with HCC, 45 with liver cirrhosis (LC), 45 with chronic hepatitis B (CHB), and 50 were healthy controls (HCs). The aberrant methylation was detected by quantitative methylation-specific polymerase chain reaction (qMSP). The area under the curve (AUC) was 0.872 in distinguishing HCC from HCs, with a sensitivity of 85.3% and a specificity of 88%. The AUC was 0.728, when it distinguished HCC from CHB, with a sensitivity of 43.4% and a specificity of 97.8%. The AUC was 0.728 in distinguishing HCC from LC, with a sensitivity of 43.4% and a specificity of 97.8%. Elevated levels of *SGIP1* methylation in HCC patients showed poorer overall survival (OS), progression-free survival (PFS), and metastasis-free survival (MFS) than those with low levels (Kaplan-Meier method and the log-rank test, $p < 0.05$). *SGIP1* methylation in different study groups demonstrated different sensitivities. *SGIP1* methylation detection in the plasma may serve as a non-invasive diagnostic and prognostic biomarker for HCC.

Key words: *SGIP1*, methylation, hepatocellular carcinoma, plasma, diagnosis, matrix-assisted laser desorption ionization time-of-flight mass spectrometry

At present, the incidence of malignant tumors is increasing worldwide due to environmental conditions, genetics, and smoking [1, 2]. China has a high incidence of liver cancer, with more than 50% of new cases and deaths worldwide occurring in China [3, 4]. Treatment for liver cancer includes surgical resection, radiofrequency ablation, transcatheter arterial chemoembolization, and targeted therapy [5–8]. The early symptoms for patients with liver cancer are often not obvious, and it is usually diagnosed during the advanced stages of the disease. The treatment efficacy for patients with early-stage liver cancer is much higher compared to patients with advanced-stage [9, 10]. Hence, early detection and diagnosis are vital for improving the prognosis of patients with liver cancer. Research focusing on early diagnosis has been intensively studied in recent years [11, 12].

Alpha-fetoprotein (AFP) is currently the only widely used serum biomarker for HCC [13–15]. However, several studies have demonstrated negative AFP in some HCC [16–18]. This limits its clinical utility to detect all forms of HCC. Hence, the search for a more sensitive non-invasive biomarker is

urgently needed. Current studies have demonstrated that the methylation status of tumor-related genes could be used for tumor diagnosis, efficacy observation, and prognosis [19, 20]. SH3 domain GRB2 like endophilin interacting protein 1 (*SGIP1*) is an endocytosis protein [21]. *SGIP1* is located on chromosome 1p31.3. *SGIP1* was reported that it is a neuronal protein that regulates energy balance and plays an important role in clathrin-mediated endocytosis. It has been reported to be involved in the occurrence and development of gastric and intestinal cancers [22, 23]. Through the Cancer Genome Atlas (TCGA) database (<http://ualcan.path.uab.edu/analysis.html>), we found that *SGIP1* methylation in liver cancer tissue is higher than that in normal liver tissue [24]. Can *SGIP1* methylation be used for the diagnosis of liver cancer? At present, no studies have been published with regard to the methylation status of *SGIP1* in HCC. Our study investigated the role and methylation status of *SGIP1* for liver cancer diagnosis. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to detect *SGIP1* methylation status in HCC

tissues and matched normal tissues. Quantitative methylation-specific PCR was used to detect SGIP1 methylation status from 129 liver cancer patient plasma samples prior to treatment. Forty-five patients with hepatitis B and 45 patients with liver cirrhosis were confirmed to meet the inclusion criteria. Methylation of the *SGIP1* gene in the plasma of 50 healthy subjects was also included. The sensitivity, specificity, and accuracy of the ROC curve were used to analyze the value of *SGIP1* gene methylation status for the diagnosis of liver cancer. In addition, the clinical characteristics of liver cancer patients i.e., gender, age, AFP, and TNM stage were collected. The correlation between SGIP1 methylation status and clinical characteristics of liver cancer patients were then analyzed.

Patients and methods

Tissue samples. HCC tissues and matched normal tissues (3 cm from the tumor margin) were collected from 16 HCC patients who were diagnosed and underwent the surgery at the Department of Liver Surgery in Sun Yat-sen University Cancer Center between October 2017 and November 2017. All patients provided written informed consent, which was approved by the Ethics Committee of Sun Yat-sen University Cancer Center. The patients' characteristics are shown in Table 1.

Blood samples. A total of 269 subjects who were admitted to Sun Yat-sen University Cancer Center and the Third Affiliated Hospital of Sun Yat-sen University between October 2017 and August 2018 were enrolled in this study. Of those subjects, 129 were diagnosed with hepatocellular carcinoma, 50 were healthy controls (HC), 45 were diagnosed with liver cirrhosis (LC), 45 were diagnosed with chronic hepatitis B (CHB). It is worth mentioning that patients with LC and CHB have been excluded from HCC detection by ultrasound, CT, and MRI. Blood samples were obtained from the subject above by phlebotomy using 5 ml EDTA-treated tubes, and the prepared plasma (2 ml) from blood samples within 2 h of collection by centrifugation (1600 ×g, 10 min), followed by transferring the supernatant into a 2 ml centrifugation tube and subsequent centrifugation (16000 ×g, 10 min). Plasma samples were stored at -80 °C until analysis. All participants provided written informed consent. This study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center.

Methylated primer design. The full CpG island sequence of the *SGIP1* gene was queried on the UCSC website, followed by designing of the primers at the EpiDesigner website.

Tissue DNA extraction and sodium bisulfite modification. After thawing the sample stored at -80 °C, the DNA was extracted from the tissues by phenol/chloroform and ethanol precipitation according to the manufacturer's protocol. The DNA concentration and purity of the sample were detected by NanoDrop 2000. The total amount of DNA was made up to ~3–5 µg, and its concentration was greater than 50 ng/µl.

The DNA extracted from each specimen was then subjected to bisulfite treatment using the EZ conversion kit (Zymo Research, Orange, CA, USA).

MALDI-TOF MS analysis. MALDI-TOF MS analysis technology combines a base-specific digestion reaction and MALDI-TOF detection principle to realize multiple CpG analysis and detection. The bisulfite-treated DNA was subjected to PCR with specific primer pair. The primers are shown in Table 2. The amplification of 1 ml of bisulfite-treated DNA (20 ng/ml) was carried out using HotStar Taq Polymerase in 5 ml of reaction volume, and using PCR primers at a final concentration of 200 mM. After treatment with shrimp alkaline phosphatase, 2 ml of the PCR products were used as a template for *in vitro* transcription to obtain RNA transcripts. The reverse product was subjected to base-specific cleavage, and the resulting samples were conditioned and spotted on a 384-pad SpectroCHIP. The resultant methylation calls were performed by using EpiTyper software v1.0.

RNA extraction and qRT-PCR. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from HCC tissues and normal tissues according to the manufacturer's protocol. Reverse transcription and qRT-PCR were performed with the Applied Biosystems 7500 Real-Time PCR System (ABI7500; Life Technologies, Darmstadt, Germany) using SYBR Green qRT-PCR kit from Takara Biotechnology (Takara, Dalian, China). *GAPDH* was used as an internal control for the quantification of the mRNA levels of genes. The primers used were synthesized by Ruibo (Ruibo, Guangzhou, Guangdong, China) and are shown in Table 3.

Table 1. The characteristics of tissue samples.

Characteristics	Cases	(%)
Total case number	16	
Gender		
Male	15	93.8
Female	1	6.2
Age (years)		
≤44	8	50
>44	8	50
T		
T1+T2	7	43.8
T3+ T4	9	56.2
N		
N0	14	87.5
N1	2	12.5
Clinical stage		
I+II	7	43.8
III+IV	9	56.2
AFP (µg/l)		
≥25	11	68.8
<25	5	31.2

Abbreviations: T-tumor; N-node

Table 2. MALDI-TOF MS primers of *SGIP1*.

Primer name	Primer sequence (5'-3')
<i>SGIP1</i> -1 F	TTTAGGTTTTTATTTGGGTGAATGA
<i>SGIP1</i> -1 R	CCTCCCCTCCAACCTAAACTC
<i>SGIP1</i> -2 F	TTGTTATTTTAATAGGGGATTTGG
<i>SGIP1</i> -2 R	CCACAAACACTAACATTTAAAATTCCT
<i>SGIP1</i> -3 F	AGGAATTTTAAATGTTAGTGTTTGTGG
<i>SGIP1</i> -3 R	CTACCTACTCCCCTTTCCAACCT
<i>SGIP1</i> -4 F	TTTTTTGTTAAGGTGGTAGTTTTTGA
<i>SGIP1</i> -4 R	AACCTACATCTCCTAACACAATACCC
<i>SGIP1</i> -5 F	TTGTGTTAGGAGATGTAGGTTGGTT
<i>SGIP1</i> -5 R	TCCTTAATTTCCAAAAAATCTAAAA

Abbreviations: MALDI-TOF MS; Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Table 3. PCR primers of *SGIP1* mRNA and quantitative methylation-specific *SGIP1* PCR primers

Primer name	Primer sequence (5'-3') for <i>SGIP1</i> mRNA
<i>SGIP1</i> -1 F	CAAGAACCCTGTCCCAAGA
<i>SGIP1</i> -1 R	TGTGGTGAAGCTCTGTTTCAT
<i>GAPDH</i> F	GGGAACTGTGGCGTGAT
<i>GAPDH</i> R	GAGTGGGTGTCGCTGTTGA
Methylation-specific primers (5'-3') and TaqMan probes	
<i>SGIP1</i> -1 F	GCCTTTGGAATACGGAAGAAAGA
<i>SGIP1</i> -1 R	TGCTATTGTAGGTTGGTTCGT
<i>SGIP1</i> -1 FAM	GCGCGTCGCGGAGAAACGTGTT
<i>GAPDH</i> F	TACTAGCGGTTTACGGGGC
<i>GAPDH</i> R	TCGAACAGGAGGAGCAGAGAGCGA
<i>GAPDH</i> VIC	GAGAAGTTGAGTTATGGGTAGTTGGAAAAGGA

Immunohistochemistry (IHC). IHC was performed to examine the *SGIP1* expression in human HCC specimens. Briefly, the tissue sections were fixed using 4% formaldehyde overnight and then embedded in paraffin. After deparaffinization and hydration, the sections were pretreated with sodium citrate buffer in a microwave for antigen retrieval and blocked using normal goat serum. The sections were then stained using rabbit anti-*SGIP1* antibodies (Abcam, USA) overnight at 4°C, and then incubated in biotinylated goat anti-rabbit IgG secondary antibodies for 1 h. Finally, the sections were stained with an avidin-biotin peroxidase complex (GeneTex, USA). The section scoring was performed by two independent pathologists who were blinded to patient data of the samples. The immunostaining was scored according to the German immunoreacted score [25]. Scores representing percentage of tumor cells positively stained were 0 (<5%), 1 (5–25%), 2 (25–50%), 3 (50–75%), or 4 (>75%). The score standards for the staining intensity were 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The total score of ≥ 4 was defined as the high-expression group.

Plasma DNA extraction and sodium bisulfite modification. The plasma DNA was extracted from 2 ml of plasma samples using the QIA amp DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA extracted from each specimen was

then subjected to bisulfite treatment using the EZ conversion kit (Zymo Research, Orange, CA, USA).

Quantitative methylation-specific polymerase chain reaction. Methylation of *SGIP1* was analyzed by using the quantitative methylation-specific quantitative PCR (qMSP), which is a high-throughput quantitative methylation assay that combines fluorescence-based real-time PCR (TaqMan) technology with methylation-specific quantitative PCR. The qMSP was conducted with the Applied Biosystems 7500 Real-Time PCR System (ABI7500; Life Technologies, Darmstadt, Germany). The primers were synthesized by Ruibo (Ruibo, Guangzhou, Guangdong, China). The primers and TaqMan probes are listed in Table 3. The PCR was performed under the following conditions: 94°C denaturation for 5 min, 20 cycles of 95°C for 15 s, 66°C for 30 s followed by 30 cycles of denaturation at 95°C for 10 s, annealing step at 58°C for 31 s. Each PCR was carried out in a 96-well plate composed of positive and negative controls. *GAPDH* was used as the endogenous reference to standardize plasma *SGIP1* methylation levels. Three parallel auxiliary wells were used for each quantitative PCR reaction. The *SGIP1* methylation levels for each sample was determined after normalization using the $2^{-\Delta}$ method relative to the internal reference *GAPDH*. CT calculation, $\Delta CT = \text{mean CT } SGIP1 - \text{mean CT } GAPDH$ (the CT value was the threshold cycle for each sample). Afterward, *SGIP1* methylation levels for each HCC patient (relative to healthy *SGIP1* methylation levels) were expressed as fold change: Fold change = $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (CT \text{ } SGIP1 - CT \text{ } GAPDH) - (CT \text{ normal} - \text{mean CT } GAPDH \text{ normal})$.

Statistical analysis. All statistical analyses were performed by using SPSS 16 (SPSS; IBM, Chicago, IL, USA) software. Mann-Whitney U test was used to determine differences in nonparametric variables. The optimal cut-off values were determined by simultaneously maximizing both the sensitivity, as well as the specificity, for detecting HCC using receiver-operating characteristics (ROC) curves. ROC curves were used to analyze diagnostic performance. Survival curves were adopted to analyze prognostic performance.

Overall survival (OS) was defined as the time between diagnosis and either death or the time of the last follow-up. Progression-free survival (PFS) was defined as the time from diagnosis to the time of progress or the last follow-up. Metastasis-free survival (MFS) was defined as the time from diagnosis to the time of metastasis or the last follow-up. Kaplan-Meier method and the log-rank test were adopted to analyze the survival time. A p-value <0.05 was considered statistically significant.

Results

Methylation status of *SGIP1* in liver cancer tissues. We have studied 58 CpG sites through MALDI-TOF MS analysis. In the comparison of 16 cases of liver cancer tissue and normal adjacent tissues, the methylation of liver cancer tissues at the locus of *SGIP1*_1 and *SGIP1*_3; *SGIP1*_1CPG_15.16.17.18;

20.21.22; 23.24; *SGIP1*_1CPG_25; *SGIP1*_1CPG_26; *SGIP1*_1CPG_27.28; and *SGIP1*_3CPG_4.5.6.7.8.9.10.11.12 were higher than those of normal adjacent tissues. The differences of these sites between the two groups were statistically significant, but not the other sites (Figure 1).

mRNA expression of SGIP1 and immunohistochemistry (IHC) in liver cancer tissues and normal adjacent tissues. To explore whether high methylation will affect gene expression, we have performed mRNA expression of 16 cases of liver cancer tissues and normal adjacent tissues. The

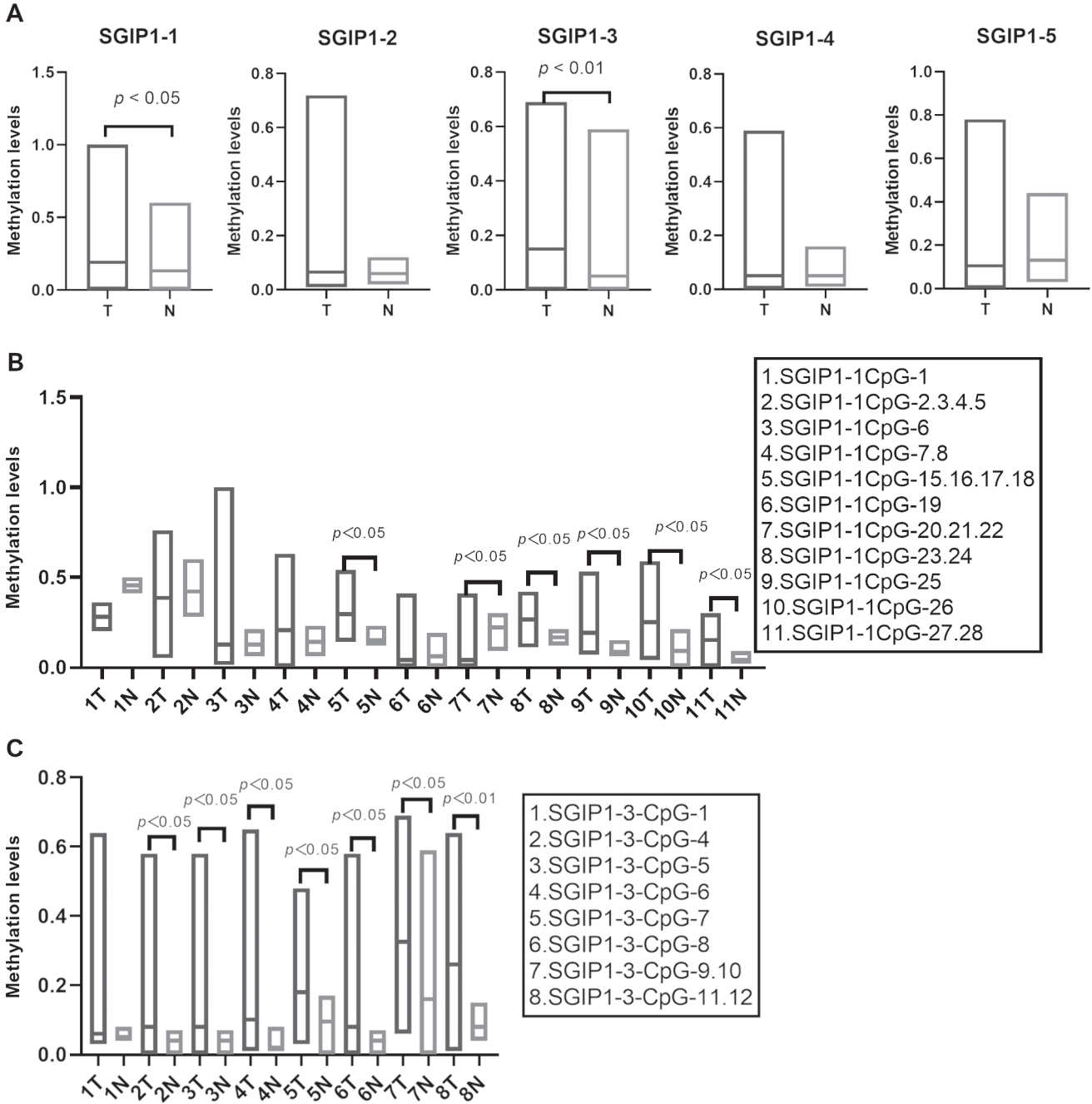


Figure 1. Methylation status of SGIP1 (A), SGIP1-1 (B), and SGIP1-3 (C) in liver cancer tissues and matched normal adjacent tissues. The methylation of liver cancer tissues at locus of *SGIP1*_1 and *SGIP1*_3 was higher than those of normal adjacent tissues ($p < 0.05$); the methylation of liver cancer tissues at locus of *SGIP1*_1CPG_15.16.17.18,20.21.22,23.24,25,26,27.28 were higher than those of normal adjacent tissues ($p < 0.01$); the methylation of liver cancer tissues at locus of *SGIP1*_3CPG_4.5.6.7.8.9.10,11.12 were higher than those of normal adjacent tissues ($p < 0.05$). Abbreviations: T-liver cancer tissues; N-normal adjacent tissues

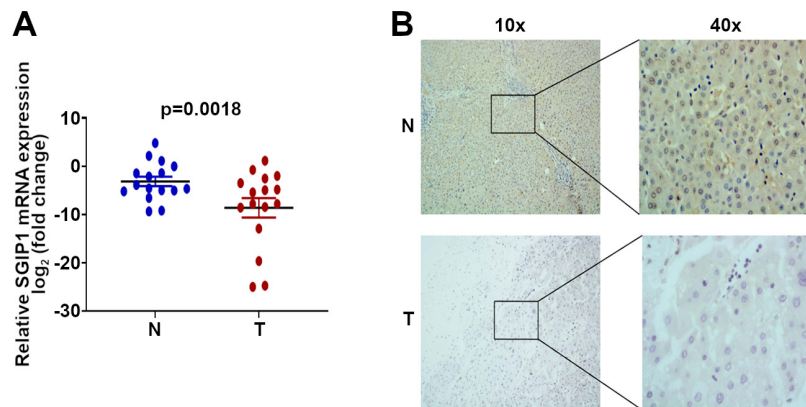


Figure 2. mRNA expression of SGIP1 and immunohistochemistry (IHC) in liver cancer tissues and normal adjacent tissues. A) The mRNA of SGIP1 in normal adjacent liver tissues was higher than liver cancer tissues ($p < 0.01$). B) Protein levels of SGIP1 in normal liver tissues were higher than liver cancer tissues ($p < 0.01$). Abbreviations: T-liver cancer tissues; N-normal adjacent tissues

results showed that the mRNA of *SGIP1* in normal adjacent liver tissues was higher than liver cancer tissues ($p < 0.01$, Figure 2A). Furthermore, protein expression was detected in liver cancer tissues and normal adjacent tissues via immunohistochemistry. The immunoreacted scores of SGIP1 in normal adjacent liver tissues (3.81 ± 1.328) ($\bar{x} \pm s$) were higher than liver cancer tissues (2.44 ± 0.964) ($p < 0.01$, Figure 2B). These data suggest that high methylation may be responsible for the downregulation of mRNA and protein expression in liver cancer.

Levels of plasma SGIP1 methylation in different samples. The median methylation levels of plasma *SGIP1* in HC, CHB, LC, and HCC samples were 4 (0.5, 20) (Q_L , Q_U), 6.55×10^4 (0.5, 1.97×10^5), 6.55×10^4 (1.64×10^4 , 2.62×10^5), and 2.62×10^5 (1.64×10^4 , 4.19×10^6), respectively. The difference between HC and CHB, HC and LC, HC and HCC, CHB or LC and HCC were statistically significant ($p < 0.01$, Figure 3).

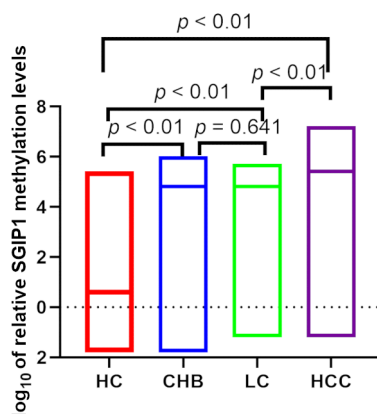


Figure 3. The levels of plasma *SGIP1* methylation in different samples. The difference between healthy controls (HC) and chronic hepatitis B (CHB), HC and liver cirrhosis (LC), HC and hepatocellular carcinoma (HCC), CHB or LC and HCC were statistically significant ($p < 0.01$).

The methylation levels of plasma *SGIP1* in LC were slightly higher compared to CHB but the difference was not statistically significant ($p = 0.641$).

Diagnostic value of plasma SGIP1 methylation and AFP in HCC. ROC curve was used to analyze the diagnostic value of *SGIP1* gene methylation in distinguishing HCC from HC, CHB, and LC. The optimal threshold was selected based on the threshold of the maximum Jordanian index. As shown in Table 4 and Figure 4, *SGIP1* gene methylation displayed a high diagnostic ability in discriminating HCC from HC with an AUC of 0.872, with 85.3% sensitivity and 88% specificity. Meanwhile, the diagnostic ability of *SGIP1* gene methylation in distinguishing HCC from CHB or LC resulted in a low sensitivity (43.4% and 43.4%, respectively) and a high specificity (97.8% and 100%, respectively), with an AUC of 0.728 and 0.675, respectively. The diagnostic ability of *SGIP1* gene methylation in distinguishing HCC from non-HCC was similar to that in distinguishing HCC from CHB. When *SGIP1* was combined with AFP25 ($\text{AFP} \geq 25$), it assisted in distinguishing between HCC and HCs by 95.3%, and distinguishing HCC from benign liver diseases such as hepatitis and cirrhosis by 86%, as shown in Supplementary Tables S1–S5.

Correlation with clinicopathologic parameters. *SGIP1* methylation levels were higher in male patients compared to female patients, higher in T3 and T4 patients compared to T1 and T2 patients, higher in patients with distant metastasis compared to patients with no distant metastasis, and higher in III and IV patients compared to I and II patients. Furthermore, *SGIP1* methylation levels in patients with tumor diameters greater than or equal to 5 cm were higher compared to patients with tumor diameters less than 5 cm and were higher in patients with abnormal AFP levels compared to patients with normal AFP levels. The differences mentioned above were statistically significant, except for the remaining clinical factors, as shown in Table 5.

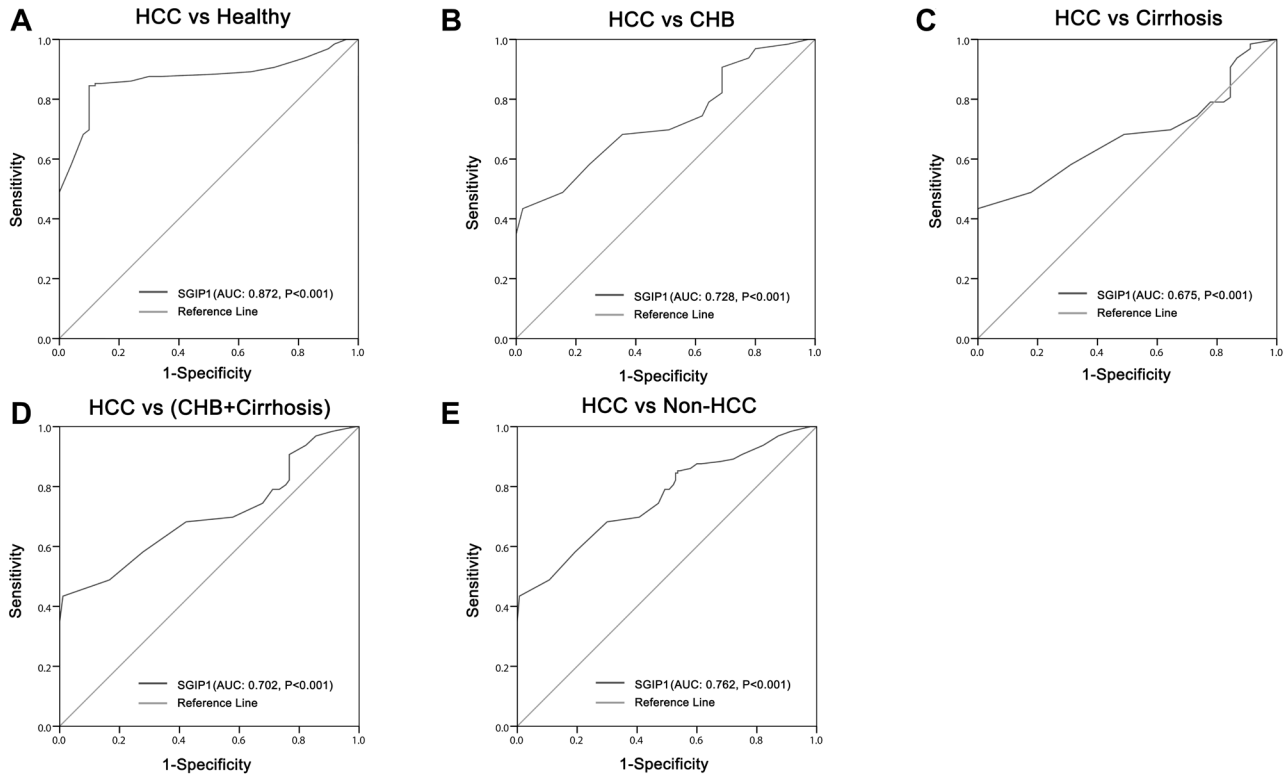


Figure 4. Diagnostic value of plasma *SGIP1* methylation in HCC. A) *SGIP1* gene methylation to discriminate patients with hepatocellular carcinoma (HCC) from healthy controls. The area under the curve (AUC) was 0.872, with 85.3% sensitivity and 88% specificity. B) *SGIP1* gene methylation to discriminate HCC from chronic hepatitis B (CHB). The AUC was 0.728, with 43.4% sensitivity and 97.8% specificity. C) *SGIP1* gene methylation to discriminate HCC from liver cirrhosis (LC). The AUC was 0.675, with 43.4% sensitivity and 100% specificity. D) *SGIP1* gene methylation to discriminate HCC from CHB and LC. The area under the curve (AUC) was 0.702, with 43.4% sensitivity and 98.9% specificity. E) *SGIP1* gene methylation to discriminate HCC from non-HCC (CHB+LC+HC). The AUC was 0.762, with 43.4% sensitivity and 99.3% specificity.

Table 4. Diagnostic value of *SGIP1* methylation in distinguishing HCC in the different populations.

Case vs. Control	Cut off	Performance			
		AUC (95%)	Sensitivity (%)	Specificity (%)	p-value
HCC vs. HC	160	0.872 (0.819–0.924)	85.3	88	<0.001
HCC vs. CHB	786432	0.728 (0.653–0.804)	43.4	97.8	<0.001
HCC vs. LC	786432	0.640 (0.525–0.755)	43.4	100	<0.001
HCC vs. (CHB+LC)	786432	0.672 (0.570–0.774)	43.4	98.9	<0.001
HCC vs. non-HCC	786432	0.749 (0.666–0.832)	43.4	99.3	<0.001

Abbreviations: HCC-hepatocellular carcinoma; CHB-chronic hepatitis B; LC-liver cirrhosis; HC-healthy controls; non-HCC-(CHB+LC+HC)

Correlation with OS, PFS, and MFS. Based on the median plasma *SGIP1* methylation levels in the 129 patients with liver cancer, patients were divided into the high-level group (> median) and low-level group (\leq median). There were 8 patients lost during follow-up, including 5 in the high-level group and 3 in the low-level group. The 1-year survival rate of patients in the high-level group was 71.5% and 85.6% in the low-level group. The difference was statistically significant ($p < 0.01$, Figure 5A). The one-year progression-free survival rate of patients in the high-level group was 37.1%, while it was 55.5% for the low-level group ($p < 0.01$, Figure 5B). The

one-year metastasis-free survival rate of patients in the high-level group was 83.1%, while it was 94.9% for the low-level group ($p < 0.01$, Figure 5C).

Discussion

SGIP1 has been reported to be involved in the occurrence and development of gastric and intestinal cancers [22, 23], through the Cancer Genome Atlas (TCGA) database, we found that *SGIP1* methylation in liver cancer tissue is higher than that in normal liver tissue. Can *SGIP1* methylation be

used for the diagnosis of liver cancer? So, we carry out this research to explore it.

Our results demonstrated that the *SGIP1* gene methylation levels were higher in CHB and LC patient samples compared to HC patient samples, while it was higher in HCC

Table 5. Correlations between plasma *SGIP1* methylation and clinicopathological characteristics of the HCC patients.

Clinicopathological factors	Cases	<i>SGIP1</i> M (Q_L , Q_U)	p-value
Gender			
Male	112	5.24×10^5 (3.28×10^4 , 4.19×10^6)	0.035
Female	17	3.28×10^4 (1.25 , 1.31×10^6)	
Age (years)			
≤ 51	61	1.05×10^6 (3.28×10^4 , 4.20×10^6)	0.196
> 51	68	2.62×10^5 (7.17×10^3 , 2.10×10^6)	
T			
T1+T2	55	3.28×10^4 (2.56×10^3 , 2.62×10^5)	< 0.001
T3+ T4	74	2.10×10^6 (2.62×10^5 , 4.19×10^6)	
N			
N0	115	2.62×10^5 (1.64×10^4 , 2.10×10^6)	0.247
N1	14	3.15×10^6 (2.29×10^5 , 8.39×10^6)	
M			
M0	119	2.62×10^5 (1.64×10^4 , 2.10×10^6)	< 0.001
M1	10	4.19×10^6 (3.41×10^6 , 8.39×10^6)	
Clinical stage			
I+II	54	3.28×10^4 (2×10^3 , 3.28×10^5)	< 0.001
III+IV	75	2.10×10^6 (2.62×10^5 , 4.19×10^6)	
Tumor size (cm)			
≥ 5	81	1.05×10^6 (1.97×10^5 , 4.19×10^6)	< 0.001
< 5	48	3.28×10^4 (20 , 2.62×10^5)	
AFP ($\mu\text{g/l}$)			
≥ 25	107	5.24×10^5 (3.28×10^4 , 4.19×10^6)	0.005
< 25	22	2.46×10^4 (13 , 3.28×10^5)	
ALT (U/l)			
≤ 40	75	2.62×10^5 (1.28×10^3 , 2.10×10^6)	0.065
> 40	54	5.24×10^5 (5.73×10^4 , 4.19×10^6)	

Abbreviations: T-tumor; N-node; M-metastasis

samples compared to CHB or LC samples. These differences were statistically significant. Plasma *SGIP1* gene methylation has a high sensitivity for distinguishing HCC from HC, while it has a low sensitivity and high specificity for distinguishing HCC from CHB or LC. A combined diagnosis with AFP could improve its sensitivity. Plasma *SGIP1* methylation in HCC is related to gender, tumor stage, tumor diameter, and AFP levels. Patients with higher *SGIP1* methylation levels have worse overall survival, progression-free survival, and metastatic-free survival.

At present, there are many plasma or serum methylation testing methods for diagnosing HCC [26–30]. Some studies have compared HCC with normal healthy individuals [26], and a few others compared HCC with hepatitis and cirrhosis patients [27–29]. Do different comparison groups make differences in sensitivity and specificity? There are few studies that compared different groups at the same time [30]. In our study, the sensitivity of *SGIP1* gene methylation in distinguishing HCC from normal individuals was 85.3%, and 43.4% in distinguishing HCC from hepatitis or cirrhotic patients. This study, as well as the above study, indicated that different comparison groups could lead to different sensitivities.

In the studies that diagnose gene methylation in HCC, the sensitivity of most of the genes was not high, such as the sensitivity of *RASSF1A* was 52–73.3% [28, 29], and *GSTP1* was 17.4–38.7% [28, 30]. To increase the sensitivity, researchers have used a combination of multiple genes or with alpha fetoprotein (AFP). For example, Xu et al. have used a combination of 10 genes, and achieved a sensitivity of 85.7% and a specificity of 94.3% in distinguishing HCC from normal individuals [26]. In our study, when *SGIP1* was combined with AFP25, it assisted in distinguishing between HCC and HCs by 95.3% and distinguishing HCC from benign liver diseases such as hepatitis and cirrhosis by 86%. More importantly, the results of our study showed that different study groups could lead to different sensitivities. Practically, the highly sensitive study groups can be used as the detection group. For example, in the study conducted by

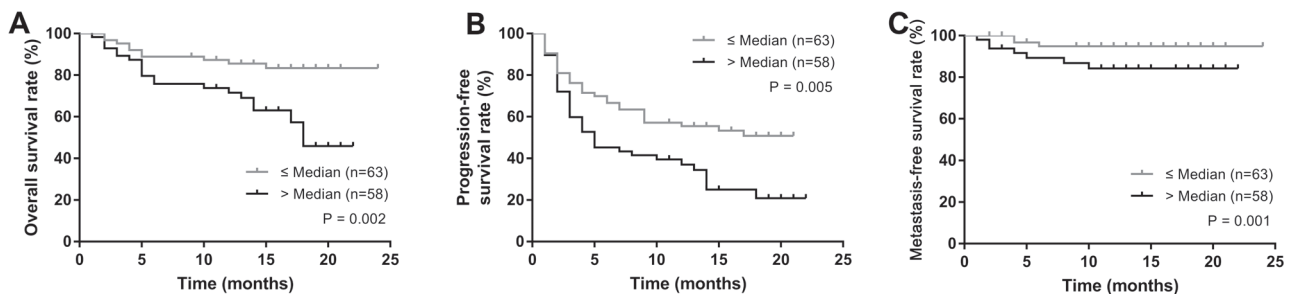


Figure 5. The relation between the levels of plasma *SGIP1* methylation and survival. A) The 1-year survival rate of patients in the high-level (> median) group was 71.5% and 85.6% in the low-level (\leq median) group ($p < 0.01$). B) The one-year progression-free survival rate of patients in the high-level (> median) group was 37.1%, while it was 55.5% for the low-level (\leq median) group ($p < 0.01$). C) The one-year metastasis-free survival rate of patients in the high-level (> median) group was 83.1%, while it was 94.9% for the low-level (\leq median) group ($p < 0.01$).

Huang et al., *APC* demonstrated high sensitivity in distinguishing HCC from benign liver disease patients [30], and so it was used to screen HCC in patients with benign liver disease. While *SGIP1* methylation was used to screen HCC in healthy people in our study.

There are several studies on the effect of gene methylation with regard to the survival of HCC. Xu et al. have demonstrated that they could effectively predict the prognosis of patients with HCC by combining the methylation of eight genes [26]. In our study, univariate analysis showed that HCC patients with high levels of *SGIP1* gene methylation had worse OS, PFS, and metastatic-free survival. In multivariate analysis, *SGIP1* gene methylation was shown to be an independent prognostic factor that affected metastatic-free survival. In addition, by analyzing the correlation between *SGIP1* gene methylation and clinicopathological factors, the results showed that the plasma *SGIP1* gene methylation was not only related to tumor stage and tumor burden but also related to distant metastasis. The possible reason for this is that the tumor locally invades the liver, and then the *SGIP1* can enter the blood circulation system through local blood vessels, or the methylation of the *SGIP1* gene promotes tumor proliferation and metastasis. *SGIP1* gene methylation acts as an independent prognostic factor that affects metastatic-free survival, suggesting that patients with HCC can take different treatment options according to different levels of *SGIP1* gene methylation. For example, patients with high methylation levels might require a more rigorous detection of distant metastases and need stronger systemic treatment.

There were several limitations to our study. First, this was a single-center study. Our results should be validated using larger multi-center patient cohorts. Second, the follow-up period in this study was short. Longer follow-up work should be performed to determine whether plasma *SGIP1* methylation levels are related to prognosis.

In summary, our study demonstrated that plasma *SGIP1* gene methylation levels had high sensitivity in distinguishing HCC from HC, while it had low sensitivity and high specificity in distinguishing HCC from CHB and LC. Diagnosis with a combination of AFP levels could improve its sensitivity. *SGIP1* plasma methylation levels could be used as a non-invasive diagnostic method for HCC and need to be further validated using larger multi-center patient cohorts.

Supplementary information is available in the online version of the paper.

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References

- [1] BRAY F, FERLAY J, SOERJOMATARAM I, SIEGEL RL, TORRE LA et al. Global cancer statistics 2018: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68: 394–424. <https://doi.org/10.3322/caac.21492>
- [2] CAI Z, LIU Q. Understanding the global cancer statistics 2018: Implications for cancer control. *Sci China Life Sci* 2019. <https://doi.org/10.1007/s11427-019-9816-1>
- [3] FENG RM, ZONG YN, CAO SM, XU RH. Current cancer situation in china: Good or bad news from the 2018 global cancer statistics? *Cancer Commun (Lond)* 2019; 39: 22. <https://doi.org/10.1186/s40880-019-0368-6>
- [4] ALLEMANI C, MATSUDA T, DI CARLO V, HAREWOOD R, MATZ M et al. Global surveillance of trends in cancer survival 2000–14 (concord-3): Analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. *Lancet* 2018; 391: 1023–1075. [https://doi.org/10.1016/S0140-6736\(17\)33326-3](https://doi.org/10.1016/S0140-6736(17)33326-3)
- [5] GUI CH, BAEY S, D'CRUZ RT, SHELAT VG. Trans-arterial chemoembolization + radiofrequency ablation versus surgical resection in hepatocellular carcinoma – a meta-analysis. *Eur J Surg Oncol* 2020; 46: 763–771. <https://doi.org/10.1016/j.ejso.2020.01.004>
- [6] TAMURA S, OKAMURA Y, SUGIURA T, ITO T, YAMAMOTO Y et al. A comparison of the outcomes between surgical resection and proton beam therapy for single primary hepatocellular carcinoma. *Surg Today* 2020; 50: 369–378. <https://doi.org/10.1007/s00595-019-01888-5>
- [7] HUO YR, CHAN MV, CHAN C. Resection plus post-operative adjuvant transcatheter arterial chemoembolization (tace) compared with resection alone for hepatocellular carcinoma: A systematic review and meta-analysis. *Cardiovasc Intervent Radiol* 2020; 43: 572–586. <https://doi.org/10.1007/s00270-019-02392-6>
- [8] KIM JJ, MCFARLANE T, TULLY S, WONG WWL. Lenvatinib versus sorafenib as first-line treatment of unresectable hepatocellular carcinoma: A cost-utility analysis. *Oncologist* 2020; 25: e512–e519. <https://doi.org/10.1634/theoncologist.2019-0501>
- [9] GOUTTE N, SOGNI P, BENDERSKY N, BARBARE JC, FALISSARD B et al. Geographical variations in incidence, management and survival of hepatocellular carcinoma in a western country. *J Hepatol* 2017; 66: 537–544. <https://doi.org/10.1016/j.jhep.2016.10.015>
- [10] MANCEBO A, VARELA M, GONZALEZ-DIEGUEZ ML, NAVASCUES CA, CADAHIA V et al. Incidence and risk factors associated with hepatocellular carcinoma surveillance failure. *J Gastroenterol Hepatol* 2018; 33: 1524–1529. <https://doi.org/10.1111/jgh.14108>
- [11] GE Y, MU W, BA Q, LI J, JIANG Y et al. Hepatocellular carcinoma-derived exosomes in organotrophic metastasis, recurrence and early diagnosis application. *Cancer Lett* 2020; 477: 41–48. <https://doi.org/10.1016/j.canlet.2020.02.003>

- [12] BAKHEET AMH, ZHAO C, CHEN JN, ZHANG JY, HUANG JT et al. Improving pathological early diagnosis and differential biomarker value for hepatocellular carcinoma via rnascope technology. *Hepatol Int* 2020; 14: 96–104. <https://doi.org/10.1007/s12072-019-10006-z>
- [13] KOMOROWSKI AL, HSU CC, JULKA KD, VASAVADA B, LIN CC et al. Afp role in predicting recurrence of hepatocellular carcinoma after living donor liver transplantation in hcv patients. *Neoplasma* 2018; 65: 455–460. https://doi.org/10.4149/neo_2018_170315N184
- [14] WU G, WU J, PAN X, LIU B, YAO Z et al. Racial disparities in alpha-fetoprotein testing and alpha-fetoprotein status associated with the diagnosis and outcome of hepatocellular carcinoma patients. *Cancer Med* 2019; 8: 6614–6623. <https://doi.org/10.1002/cam4.2549>
- [15] KIM MN, KIM BK, KIM SU, PARK JY, AHN SH et al. Longitudinal assessment of alpha-fetoprotein for early detection of hepatocellular carcinoma in patients with cirrhosis. *Scand J Gastroenterol* 2019; 54: 1283–1290. <https://doi.org/10.1080/00365521.2019.1673478>
- [16] LI L, CHEN J, XU W, DING X, WANG X et al. Clinical characteristics of hepatocellular carcinoma patients with normal serum alpha-fetoprotein level: A study of 112 consecutive cases. *Asia Pac J Clin Oncol* 2018; 14: e336–e340. <https://doi.org/10.1111/ajco.12816>
- [17] SHE WH, CHAN ACY, CHEUNG TT, LO CM, CHOK KSH. Survival outcomes of liver transplantation for hepatocellular carcinoma in patients with normal, high and very high preoperative alpha-fetoprotein levels. *World J Hepatol* 2018; 10: 308–318. <https://doi.org/10.4254/wjh.v10.i2.308>
- [18] SHIRAKI T, ISHIZUKA M, KUBOTA K, KATO M, MATSUMOTO T et al. An elevated neutrophil-to-lymphocyte ratio predicts a poor postoperative survival in primary hepatocellular carcinoma patients with a normal preoperative serum level of alpha-fetoprotein. *Surg Today* 2019; 49: 661–669. <https://doi.org/10.1007/s00595-019-01781-1>
- [19] ZHANG C, GE S, WANG J, JING XT, LI HL et al. Epigenomic profiling of DNA methylation for hepatocellular carcinoma diagnosis and prognosis prediction. *J Gastroenterol Hepatol* 2019; 34: 1869–1877. <https://doi.org/10.1111/jgh.14694>
- [20] LI GX, DING ZY, WANG YW, LIU TT, CHEN WX et al. Integrative analysis of DNA methylation and gene expression identify a six epigenetic driver signature for predicting prognosis in hepatocellular carcinoma. *J Cell Physiol* 2019; 234: 11942–11950. <https://doi.org/10.1002/jcp.27882>
- [21] ZHANG Y, FENG Y, XIN Y, LIU X. Sgip1 dimerizes via intermolecular disulfide bond in muhd domain during cellular endocytosis. *Biochem Biophys Res Commun* 2018; 505: 99–105. <https://doi.org/10.1016/j.bbrc.2018.09.075>
- [22] BAE JH, KANG MJ, YANG KM, KIM TO, YI JM. Epigenetically silenced micrnas in gastric cancer: Functional analysis and identification of their target genes. *Oncol Rep* 2015; 34: 1017–1126. <https://doi.org/10.3892/or.2015.4036>
- [23] LI JN, ZHAO L, WU J, WU B, YANG H et al. Differences in gene expression profiles and carcinogenesis pathways between colon and rectal cancer. *J Dig Dis* 2012; 13: 24–32. <https://doi.org/10.1111/j.1751-2980.2011.00551.x>
- [24] CHANDRASHEKAR DS, BASHEL B, BALASUBRAMANYA SAH, CREIGHTON CJ, PONCE-RODRIGUEZ I et al. Ualcan: A portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia* 2017; 19: 649–658. <https://doi.org/10.1016/j.neo.2017.05.002>
- [25] TANG L, TAN YX, JIANG BG, PAN YF, LI SX et al. The prognostic significance and therapeutic potential of hedgehog signaling in intrahepatic cholangiocellular carcinoma. *Clin Cancer Res* 2013; 19: 2014–2024. <https://doi.org/10.1158/1078-0432.CCR-12-0349>
- [26] XU RH, WEI W, KRAWCZYK M, WANG W, LUO H et al. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. *Nat Mater* 2017; 16: 1155–1161. <https://doi.org/10.1038/nmat4997>
- [27] TAO LP, FAN XP, FAN YC, ZHAO J, GAO S et al. Combined detection of insulin-like growth factor-binding protein 7 promoter methylation improves the diagnostic efficacy of afp in hepatitis b virus-associated hepatocellular carcinoma. *Pathol Res Pract* 2018; 214: 144–150. <https://doi.org/10.1016/j.prp.2017.10.004>
- [28] DONG X, HOU Q, CHEN Y, WANG X. Diagnostic value of the methylation of multiple gene promoters in serum in hepatitis b virus-related hepatocellular carcinoma. *Dis Markers* 2017; 2017: 2929381. <https://doi.org/10.1155/2017/2929381>
- [29] LIU XY, FAN YC, GAO S, ZHAO J, CHEN LY et al. Methylation of sox1 and vim promoters in serum as potential biomarkers for hepatocellular carcinoma. *Neoplasma* 2017; 64: 745–753. https://doi.org/10.4149/neo_2017_513
- [30] HUANG ZH, HU Y, HUA D, WU YY, SONG MX et al. Quantitative analysis of multiple methylated genes in plasma for the diagnosis and prognosis of hepatocellular carcinoma. *Exp Mol Pathol* 2011; 91: 702–707. <https://doi.org/10.1016/j.yexmp.2011.08.004>