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Methylation analysis of *p16*, *SLIT2*, *SCARA5*, and *Runx3* genes in hepatocellular carcinoma

Gaofeng Sun, MS[a,b], Chen Zhang, PhD[c], Min Feng, MS[d], Wensheng Liu, BS[e], Huifang Xie, PhD[a,*], Qin Qin, BS[b], E. Zhao, BS[b], Li Wan, BS[b]

Abstract

This study is to investigate the methylation status of multiple tumor suppressor 1 (*p16*), secreted glycoprotein 2 (*SLIT2*), scavenger receptor class A, member 5 putative (*SCARA5*), and human runt-related transcription factor 3 (*Runx3*) genes in the peripheral blood of hepatocellular carcinoma (HCC).

This is a case–control study. The peripheral blood samples were collected from 25 HCC patients, 25 patients with high risk of HCC (defined as "internal control group"), and 25 healthy individuals (defined as "external control group"), respectively. Then the methylation status of *p16*, *SLIT2*, *SCARA5*, and *Runx3* genes in the blood samples were analyzed by pyrosequencing. The relationship between the methylation and the clinical features of HCC patients were evaluated.

The methylation levels in the 7 CpG loci of *p16* gene in HCC patients were low and without statistically significant difference ($P > .05$) compared to the control groups. Although the methylation levels of CpG3 and CpG4 in *SLIT2* gene loci were higher than those of the control groups, there was no statistically significant difference ($P > .05$). However, the methylation rate of CpG2 locus in *SCARA5* gene in HCC patients was significantly higher ($P < .05$). And the methylation rates of CpG1, CpG2, CpG3, CpG4, CpG5, and CpG8 in *Runx3* gene in HCC patients were significantly different to that of control groups ($P < .05$). We also have analyzed the correlations between the CpG islands methylation of *Runx3* or *SCARA5* genes and the age, gender, hepatitis B, liver cirrhosis, alpha fetal protein, or hepatitis B surface antigen (HBsAg) of the HCC patients, which all showed no significant correlations ($P > .05$).

The methylation status of *SCARA5* and *Runx3* genes are abnormal in HCC patients, which may further be used as molecular markers for early auxiliary diagnosis of liver cancer.

Abbreviations: AFP = alpha fetal protein, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, p16 = multiple tumor suppressor 1, Runx3 = human runt-related transcription factor 3, SCARA5 = scavenger receptor class A, member 5 putative, SLIT2 = secreted glycoprotein 2.

Keywords: hepatocellular carcinoma, methylation, pyrosequence

1. Introduction

Primary hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms, the morbidity and mortality of which respectively ranks the 5th and the 2nd in malignant tumors worldwide.[1] HCC is particularly prevalent in the South and Southeast Asia, in which covering more than 50% of diagnosed cases all over the world.[2] Moreover, hepatitis is a great challenge in China, and there are about 1.3 million hepatitis B virus (HBV) carriers.[3] But till now, the pathogenesis of HCC is still unclear. It was though that the epigenetic changes in the DNA methylation might play an important role in the occurrence and development of HCC.[4] Studies have showed that the methylation disorders for the inactivation of tumor suppressor genes are the one common reason for the occurrence of liver cancer. The abnormal methylation of CpG-islands in the promoter region of the multiple tumor suppressor 1 (*p16*) gene has been observed in the tumorigenesis, hyperplasia, and metaplasia.[5] Park et al[6] have showed that the methylation of human runt-related transcription factor 3 (*Runx3*) gene was an important event in the early tumorigenesis of liver cancer. Besides, the abnormal methylation of scavenger receptor class A, member 5 putative (SCARA5) promoter played an important role in the development and progression of HCC.[7] The low expression of secreted glycoprotein 2 (*SLIT2*) gene in the liver cancer has also been found; however, its function remains to be further studied.[8]

The methylation detection of the 4 genes in a combination in the liver cancer genomes has not been well studied. In this study, the methylation status of *p16*, *SLIT2*, *SCARA5*, and *Runx3* gene in HCC patients were detected by pyrosequencing. And the correlation between the methylation and HCC occurrence was analyzed. Our findings may provide experimental evidence for identifying new marker for early diagnosis of HCC.

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[a] School of Public Health, Xinjiang Medical University, [b] Department of Chronic and Non-communicable Diseases Control, City Center for Disease Control and Prevention, [c] The Fifth Affiliated Hospital of Xinjiang Medical University, [d] Department of Inspection, Affiliated Tumor Hospital of Xinjiang Medical University, [e] Urumqi Health and Family Commission, Urumqi, China.

[*] Correspondence: Huifang Xie, School of Public Health, Xinjiang Medical University, No. 393 Xinyi Road, Xin shi Zone, Urumqi 830011, China (e-mail: 504900852@qq.com).

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2. Material and methods

2.1. Patients

Twenty-five patients with HCC were enrolled from June 2014 to June 2015 in Affiliated Tumor Hospital of Xinjiang Medical University. There were 13 male patients (52.0%) and 12 females (48.0%). The average age was 56.36 ± 8.85 years old. The diagnosis of HCC was determined according to the latest published diagnostic criteria of liver cancer by Anti-Cancer Association.[9]

Based on the "Harvard Cancer Risk Index"[10] and the common cancer epidemiological data of China[11] in the past 20 years, we developed a comprehensive cancer risk evaluation system[12] suitable for Chinese population using the formula recommended by the Harvard Cancer Risk Index and evaluated the risk of cancer. Based on this evaluation system, the cut-off value of the risk level was determined after analyzing and assigning values to the epidemiological risk factors, including basic information, eating habits, living environment, lifestyle and habits, psychology and emotion, past history of liver and gallbladder disease (including chronic hepatitis B, chronic hepatitis C, liver cirrhosis, fatty liver, other liver diseases, etc.), and family history of liver cancer. And, the population with cut-off value of 2.0 was defined as the high-risk population for HCC. Finally, 25 patients with high risk of HCC were also enrolled in this study as the "internal control group," all were determined by the "China Urban Cancer Early Disease Prevention and Treatment Project Anti-cancer Risk Assessment Questionnaire." And 25 healthy subjects were enrolled as "external control group." The average age of the subjects in the internal control group was 55.52 ± 8.47 years old and in the external control group 56.48 ± 7.66 years old. The ratios of male to female in both control groups were similar to that of HCC patient group.

Prior written and informed consent were obtained from every patient, and the study was approved by the ethics review board of Affiliated Tumor Hospital of Xinjiang Medical University.

2.2. Questionnaires and biochemical markers

The questionnaires of Influencing Factors for HCC were designed based on the "Cancer Risk Assessment Questionnaires for Early Diagnosis and Treatment of Cancer in Urban."[10] The investigators were trained to perform the epidemiological survey in one-to-one case. The alpha fetal protein (AFP) and hepatitis B surface antigen (HBsAg) in blood samples were detected by enzyme-linked immune sorbent assay (ELISA).[13,14]

2.3. The extraction of genomic DNA

Briefly, 1 mL blood sample was collected from the subjects with 5% EDTA for anticoagulation. The genomic DNA was extracted by Human Whole Blood Genomic DNA Extraction Kit (TIAGEN biotech, Beijing, China) according to the instructions.

2.4. The sulfitation of DNA

Genomic DNA was subjected to sulfite treatment according to the instructions of DNA Methylation-Gold kit (Tiagen, Beijing, China). After the sulfitation DNA was purified and stored at −20 °C until use.

2.5. Measurement of DNA methylation

The primers for *p16*, *SLIT2*, *SCARA5*, and *Runx3* genes were designed according to a previous report[15] using PyroMark Assay Design 2.0 (QIAGEN, Hilden, Germany) and synthesized by Life Technology Inc. (ThermoFisher, CA). The primer sequences were shown in Table 1. The PCR was performed with EPIK Amplification Kit (Qiagen, Hilden, Germany) according to the provided instructions. The 25 μL PCR system included 2×EPIK Amplification Mix (12.5 μL), upstream primer (0.6 μL), downstream primer (0.6 μL), DNA template (0.5 μL), and ddH_2O (10.8 μL). The conditions of PCR amplification were as follows: 95 °C for 2 minutes; 95 °C for 15 seconds, 56 °C for 15 seconds, and 72 °C for 30 seconds, 40 cycles; 4 °C holding. Pyrosequencing was performed on PyroMark Q96 real-time quantitative pyrophosphate sequence analyzer (Qiagen, Hilden, Germany).

Table 1

Primers for *p16*, *SLIT2*, *SCARA5*, and *Runx3* genes.

Gene	Primer	Sequences
p16	p16-F	AGAGGATTTGAGGGATAGGG
	p16-R-bio	TACCTACTCTCCCCCTCT
	p16-S	GGTTGGTTGGTTATTAGA
SLIT2	SLIT2-F	TGAATTAGTTTGGTTAGGGTTGTAAGGA
	SLIT2-R-bio	ATACCTATTAAAATCCCCTCTTCTAT
	SLIT2-S	GTTTTTTGTTTTTTAAGGATGAAT
SCARA5	SCARA5-F	AGGAGGATTAGGTTAAGATGTAATT
	SCARA5-R-bio	AAAAAAAAACCCTATTCCAAACCCCATTAC
	SCARA5-S	GTAGTTTTTTTAGGTATTGTTTGA
Runx3	Runx3-F	AAGGGGTGATTTGTAGTGAAGTTTA
	Runx3-R-bio	CCAACCCCACTTCTTCTTA
	Runx3-S	GGGTAGGTAGTGTTTTG

p16 = multiple tumor suppressor 1, Runx3 = human runt-related transcription factor 3, SCARA5 = scavenger receptor class A, member 5 putative, SLIT2 = secreted glycoprotein 2.

The average methylation rate of each group was calculated. As the methylation loci are different in each gene, the average methylation rate at different sites can be calculated. The background value of methylation was set to 0% to 5%. The methylation was determined when the index was ≥5%.[16]

2.6. Statistical analysis

SPSS17.0 (IBM) statistical package was used to analyze the data. Count data were analyzed by chi-square test. Multiple sets of individual measurement data were compared with single factor analysis of variance. The F test was used to compare the variance. The Dunnett method was used for multiple comparisons. Welch approximate F test was used for variance. And Dunnett T3 method was used for multiple comparisons. $P < .05$ was considered statistically significant. The methylation rates of CpG loci were analyzed by homogeneity of variance test.

3. Results

3.1. The characteristics of the study cohort

The epidemiological characteristics of the patients were collected and analyzed. As shown in Table 2, there were no significant differences in age, gender, ethnicity, and educational level among the 3 groups ($P > .05$). There were significant differences in occupation, occupational exposure, and per capita income among the three groups ($P < .05$). Additionally, the medical history was also analyzed and the results were shown in Table 3. There were significant differences among the 3 groups in the presence of HBV, liver cirrhosis, AFP (+), and HBsAg (+) ($P < .05$). But there was no significant difference in fatty liver, hypertension, diabetes mellitus, and BMI among the 3 groups

Table 2

Demographic characteristics of included subjects.

	HCC patients		High risk for HCC		Healthy individuals			
	N	Percentage, %	N	Percentage, %	N	Percentage, %	x^2	*P*
Gender								
Male	13	52.0	13	52.0	13	52.0	0.00	1.00
Female	12	48.0	12	48.0	12	48.0		
Age								
40–49	4	16.0	7	28.0	4	26.5	2.27	.69
50–59	12	48.0	8	32.0	12	37.3		
60–69	9	36.0	10	40.0	9	36.3		
Nationality								
Han	25	100.0	22	88.0	24	96.0	3.70	.16
Minority	0	4.0	3	12.0	1	4.0		
Education								
Primary school or below	8	32.0	3	12.0	4	16.0	5.72	.46
Junior high school	5	20.0	6	23.5	8	20.0		
Senior high school	8	20.0	7	24.0	6	23.5		
Bachelor or above	4	16.0	9	36.0	7	24.0		
Marriage								
Married	25	100.0	22	88.0	23	92.0	3.20	.53
Divorce	0	0.0	2	8.0	1	4.0		
Widowhood	0	0.0	1	4.0	1	4.0		
Career								
Worker	2	8.0	6	24.0	0	0.0	38.26	.00
Peasant	7	28.0	1	4.0	6	24.0		
Clerical work	6	24.0	11	44.0	19	76.0		
Retired	8	32.0	1	4.0	0	0.0		
Others	2	8.0	6	24.0	0	0.0		
Contact history*								
Never	21	84.0	21	84.0	20	80.0	21.23	.00
Radiation	0	0.0	0	0.0	0	0.0		
Pesticide	4	16.0	0	0.0	1	4.0		
Heavy metal	0	0.0	0	0.0	4	16.0		
Organic solvent	0	0.0	4	16.0	0	0.0		
Income								
0–1000	0	0.0	0	0.0	0	0.0	12.59	.00
1001–2000	2	8.0	8	32.0	11	44.0		
2001–3000	18	72.0	13	52.0	12	48.0		
3001–5000	5	20.0	4	16.0	2	8.0		

HCC = hepatocellular carcinoma.

* Pesticide (agriculture, forestry, and fishery industries), heavy metal (such as mining, smelting, electric welding, battery semiconductor industry workers, etc.), and organic solvent (such as shoemaking drugs, chemical glue, oil processing, paint coating workers, etc.).

($P > .05$). Thus, HBV, liver cirrhosis, AFP (+), and HBsAg (+) may be the influencing factors to HCC.

3.2. The analysis of CpG islands

The methylation of the following CpG islands was analyzed. Specifically, these CpG islands included the 17–23 locus in the 3rd methyl island (nt21974846-nt21974981) upstream of the transcription start site of *p16* gene, the 46–53 locus of the 1st methyl island (nt24931240-nt24931399) upstream of the transcription start site of *Runx3* gene, the 24–28 locus in the 1st methylated island (nt27992469-nt27992738) upstream of the *SCARA5* gene transcription start site, and the 129–132 locus in the 2nd methyl island upstream (nt20253337-nt20253571) of the *SLIT2* gene transcription.

3.3. The preparation of pyrophosphate sequencing template and pyrosequencing

Gel electrophoresis (120 V, 15 minutes) was carried out in 1.5% agarose. The pyrophosphate sequencing template for *p16*, *Runx3*, *SCARA5*, and *SLIT2* was successfully constructed. As shown in Fig. 1, the expected length of the amplified fragments was 135, 159, 269, and 234 bp, respectively. The methylation of *p16*, *Runx3*, *SCARA5*, and *SLIT2* genes was sequenced. The results of sequencing analysis were shown in Fig. 2.

3.4. The methylation of p16 gene

There was no methylation in sites of CpG2, CpG3, CpG4, CpG5, and CpG6 in *p16* gene in the 3 groups. And, the methylation rates of CpG1 and CpG7 in the 3 groups were not significantly different. Thus, there were no significant differences in the methylation of all the 7 CpG sites of *p16* gene.

3.5. The methylation of SLIT2 gene

Homogeneity of variance test was performed to analyze the methylation rates of CpG1, CpG2, CpG3, and CpG4 loci in *SLIT2* gene. The results were F1 = 1.048, P1 = 0.356, F2 = 0.420, P2 = 0.659, F3 = 0.673, P3 = 0.514, and F4 = 1.580, P4 = 0.216, respectively, indicating that the variance had homogeneity. And,

Table 3

The comparison of medical history of included subjects.

	HCC patients		High risk for HCC		Healthy individuals			
	N	Percentage, %	N	Percentage, %	N	Percentage, %	x^2	*P*
HBV								
Yes	16	64.0	6	24.0	1	4.0	21.95	.00
No	9	36.0	19	76.0	24	96.0		
Fatty liver								
Yes	13	52.0	9	36.0	11	44.0	1.30	.52
No	12	48.0	16	64.0	14	56.0		
Liver cirrhosis								
Yes	9	36.0	0	0.0	0	0.0	20.46	.00
No	16	64.0	25	100.0	25	100.0		
Hypertension								
Yes	7	28.0	7	28.0	6	24.0	0.14	.93
No	18	72.0	18	72.0	19	76.0		
Diabetes								
Yes	5	20.0	1	4.0	3	12.0	3.03	.22
No	20	80.0	24	96.0	22	88.0		
AFP (+)								
Yes	15	60.0	2	8.0	0	0.0	30.27	*.00*
No	10	40.0	23	92.0	25	100.0		
HBsAg (+)								
Yes	17	68.0	5	20.0	1	4.0	26.09	*.00*
No	8	32.0	520	80.0	24	96.0		
BMI								
Normal	15	60.0	11	44.0	10	40.0	8.18	*.09*
Over weight	10	40.0	10	40.0	8	32.0		
Obesity	0	0.0	4	16.0	7	28.0		

AFP = alpha fetal protein, BMI = body mass index, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, HCC = hepatocellular carcinoma.

there were no significant differences in the methylation of four loci in *SLIT2* gene among the 3 groups ($P > .05$) (Table 4).

3.6. The methylation of SCARA5 gene

The methylation rates of CpG1, CpG2, CpG3, CpG4, and CpG5 loci in *SCARA5* gene were analyzed by homogeneity of variance test. The results were F1 = 1.324, P1 = 0.261, F2 = 1.127, P2 = 0.330, F3 = 1.442, P3 = 0.244, F4 = 0.418, P4 = 0.660, and F5 = 0.100, P5 = 0.905, respectively. Thus, there was homogeneity of variance. As shown in Table 5, there were no significant differences in the methylation of CpG1, CpG3, CpG4, and CpG5 in *SCARA5* gene between the 3 groups ($P > .05$). However, there was significant difference in the methylation of CpG2 loci, suggesting that the CpG2 locus may be involved in the epigenetic regulation of HCC.

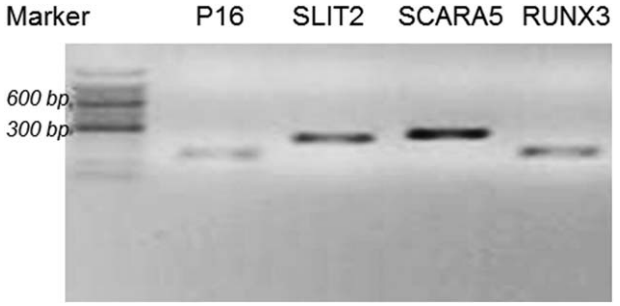

Figure 1. Electrophoresis results for pyrophosphate sequencing template. The target gene was amplified by using the primers based on the sulfite-treated pyrophosphate sequencing template. The results showed that *p16*, *Runx3*, *SCARA5*, and *SLIT2* pyrophosphate sequencing templates were successfully amplified. p16 = multiple tumor suppressor 1, Runx3 = human runt-related transcription factor 3, SCARA5 = scavenger receptor class A, member 5 putative, SLIT2 = secreted glycoprotein 2.

3.7. The methylation of Runx3 gene

The methylation rates of CpG1, CpG2, CpG3, CpG4, CpG5, CpG6, CpG7, and CpG8 loci in *Runx3* gene were analyzed by homogeneity of variance test. The results showed that there was homogeneity of variance, with F1 = 1.341, P1 = 0.268, F2 = 0.144, P2 = 0.866, F3 = 0.822, P3 = 0.443, F4 = 0.515, P4 = 0.600, F5 = 1.552, P5 = 0.219, F6 = 3.268, P6 = 0.044, F7 = 1.399, P7 = 0.253, and F8 = 1.723, P8 = 0.186, respectively. The methylation rate of CpG6 and CpG7 in *Runx3* gene was not statistically significant ($P > .05$) (Table 6). However, the methylation rate of CpG1, CpG2, CpG3, CpG4, CpG5, and CpG8 were significantly different ($P < .05$), which may be involved in the epigenetic regulation of HCC.

3.8. The relationship of CpG island methylation in Runx3 and SCARA5 genes with clinical features of HCC patients

We analyzed the relationships of the methylation status of *Runx3* and *SCARA5* genes with the clinical features of HCC patients, including the age, gender, HBV, liver cirrhosis, AFP, and HBsAg. The *Runx3* gene in all of the 25 cases showed hyper-methylation in all the 25 cases of HCC patients. The methylation rate was 100%, with no statistically significant difference. Both *Runx3* and *SCARA5* genes showed no significant relationships to the clinical features of patients with HCC ($P > .05$) (Table 7). This data indicate that the CpG island methylation in *Runx3* and

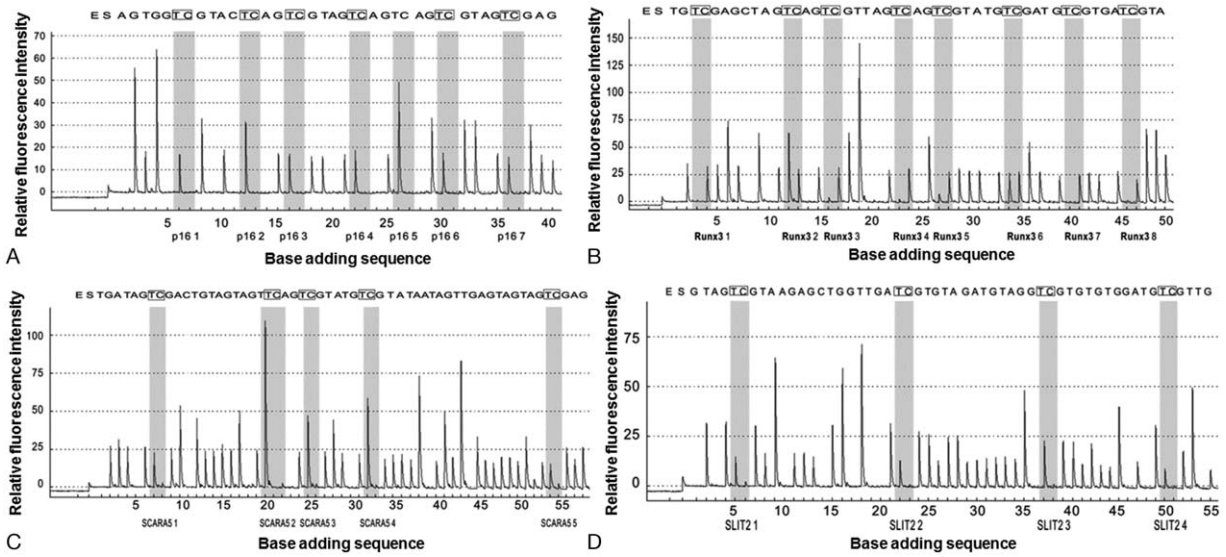

Figure 2. Sequencing analysis of the methylation regions. After pretreatment of the PCR template, the methylation regions of *p16*, *Runx3*, *SCARA5*, and *SLIT2* genes were sequenced. The results of each sample sequencing analysis were shown. (A) *p16*, (B) *Runx3*, (C) *SCARA5*, and (D) *SLIT2*. p16 = multiple tumor suppressor 1, PCR = polymerase chain reaction, Runx3 = human runt-related transcription factor 3, SCARA5 = scavenger receptor class A, member 5 putative, SLIT2 = secreted glycoprotein 2.

Table 4

The methylation rate of CpG islands in *SILT2* gene (%).

	N	HCC patients	High risk for HCC	Healthy individuals	F	*P*
CpG1	25	13.08 ± 2.40	12.28 ± 2.35	13.24 ± 3.21	0.917	.404
CpG2	25	8.68 ± 2.11	8.30 ± 2.26	9.57 ± 3.33	1.260	.291
CpG3	25	10.83 ± 2.44	10.41 ± 2.40	10.70 ± 2.38	0.175	.840
CpG4	25	10.29 ± 1.90	10.28 ± 2.32	9.42 ± 2.06	1.040	.361

HCC = hepatocellular carcinoma, SLIT2 = secreted glycoprotein 2.

Table 5

The methylation rate of CpG islands in *SCARA5* gene (%).

	N	HCC patients	High risk for HCC	Healthy individuals	F	*P*
CpG1	25	10.05 ± 3.97	8.45 ± 2.48	9.19 ± 2.06	1.558	.219
CpG2	25	14.00 ± 1.51	11.35 ± 1.92	12.13 ± 2.17	11.962	.000
CpG3	25	9.38 ± 3.98	9.41 ± 2.97	10.08 ± 3.66	0.296	.745
CpG4	25	13.76 ± 2.95	13.08 ± 3.39	13.56 ± 2.99	0.305	.738
CpG5	25	14.96 ± 4.91	14.60 ± 4.65	15.36 ± 4.58	0.162	.850

HCC = hepatocellular carcinoma, SCARA5 = scavenger receptor class A.

Table 6

The methylation rate of CpG islands in *Runx3* gene (%).

	N	HCC patients	High risk for HCC	Healthy individuals	F	*P*
CpG1	25	97.20 ± 1.58	96.00 ± 1.89	96.24 ± 1.23	3.975	.023
CpG2	25	96.96 ± 2.34	97.20 ± 2.45	94.00 ± 2.21	14.189	.000
CpG3	25	90.76 ± 1.16	89.20 ± 2.69	88.28 ± 1.54	10.733	.000
CpG4	25	91.80 ± 1.35	90.52 ± 2.55	90.02 ± 1.22	5.462	.006
CpG5	25	82.56 ± 1.53	79.12 ± 3.09	80.02 ± 1.12	17.697	.000
CpG6	25	99.60 ± 1.04	98.64 ± 1.68	99.36 ± 1.04	2.912	.064
CpG7	25	96.96 ± 2.01	96.32 ± 2.97	96.08 ± 2.38	0.838	.437
CpG8	25	90.44 ± 1.26	88.68 ± 2.84	89.04 ± 1.21	5.838	.004

HCC = hepatocellular carcinoma, Runx3 = human runt-related transcription factor 3.

Table 7

The correlation between the CpG2 methylation of SCARA5 and the clinical feature of HCC patients.

Clinical features	N	Methylation of SCARA5		x^2	P
		+	−		
Age				0.586	.444
>60	16	15	1		
<60	9	9	0		
Gender				1.128	.288
Male	13	13	0		
Female	12	11	1		
HBsAg				2.214	.137
+	17	17	0		
−	8	7	1		
AFP				0.694	.405
+	15	14	1		
−	10	10	0		
Cirrhosis				0.586	.444
Yes	9	9	0		
No	16	15	1		
HBV				0.586	.444
Yes	16	15	1		
No	9	9	0		

AFP = alpha fetal protein, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, SCARA5 = scavenger receptor class A.

SCARA5 gene was not associated with the clinical features of HCC patients.

4. Discussion

In this study, we found the potential risk factors for HCC. The occupation, occupational exposure, and per capita income distribution were different among the 3 groups, consistent with the study of Clifford et al.[17] The rates of HBV infection, liver cirrhosis, AFP (+), and HBsAg (+) were different among the 3 groups, which was consistent with the study reported by Zhang et al.[18] And the above mentioned factors may be closely related with HCC.

Pyrosequencing has become one of the most suitable techniques for the quantitative detection of DNA methylation in clinical diagnosis.[19] Studies have shown that the methylations of *p16*, *Runx3*, *SLIT2*, and *SCARA5* genes are closely related to the development of other cancers.[20–23] In this study, the methylation status of different CpG sites in *p16*, *SLIT2*, *SCARA5*, and *Runx3* genes were detected. The methylation levels of the 7 sites in *p16* gene were very low. This inconsistence may because of that the methylation level of *p16* is high in tumor, but low in blood, and further studies with larger sample sizes are warranted to clarify this issue.

We also detected the methylation in *SLIT2* gene, which showed there was no statistically significant difference among the 3 groups. It was necessary to further extend the sample size to verify the differences of the methylation in SLIT2.

Cheng et al[24] reported that SCARA5 methylation rate was 60.7% in HCC, significantly higher than the adjacent tissue 11.6%. Studies have shown that SCARA5 is closely related to the invasion and migration of cancer and overexpression of SCARA5 may inhibit the proliferation, aggregation, and metastasis of multiple tumor cell.[23,25] Consistently, we found that the methylation of CpG2 in *SCARA5* gene was statistically significant. These results suggested that the methylation of CpG2 in *SCARA5* gene might be involved in the epigenetic regulation of HCC.

Runx3 is tumor suppressor gene. Its methylation or expression is abnormal in a variety of human tumors.[26–28] Zhang and Yun[28] reported that the methylation of Runx3 promoter region was closely related to the early stage of HCC and could be used as a molecular marker for early diagnosis of HCC and a target for molecular therapy. A meta-analysis demonstrates a strong association between RUNX3 promoter methylation and HCC risk.[29] Our results were consistent with these reports. However, unlike this meta-analysis, we further analyzed the methylation of the specific gene loci of Runx3. We found that the methylation of CpG2, CpG3, CpG4, CpG5, and CpG8 locus in the *Runx3* gene were significantly different among the 3 groups, indicating that the 6 loci of *Runx3* gene might been involved in the epigenetic regulation of HCC. Thus, the abnormal methylation of Runx3 in plasma may provide a new screening marker for liver cancer patients.[30] Furthermore, we found that there was no significant correlation between the methylation of *SCARA5* and *Runx3* gene and the clinical feature of HCC patients. Whether Runx3 and SCARA5 could be used as molecular markers for the early diagnosis of liver cancer needs to be further investigated.

This study has several limitations. First, due to limited materials, only the 4 representative genes of *p16*, *Runx3*, *SLIT2*, and *SCARA5* were analyzed. Second, the methylation status of these genes is not specific for HCC. Third, due to the limitation of time and research funding, the samples of the study were slightly insufficient, and the study population needs to be further improved.

In conclusion, we analyzed that the methylation of *p16*, *SLIT2*, *SCARA5*, and *Runx3* in liver cancer and found that *Runx3*, *SCARA5* gene could be used as molecular markers for the early diagnosis of liver cancer. It is of great value in the early diagnosis and prognosis of HCC.

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