RESEARCH ARTICLE

Application of multiplex methylated-specific PCR with capillary electrophoresis to explore prognostic value of TSGs hypermethylation for hepatocellular carcinoma

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Background: Hepatocellular carcinoma (HCC) is a malignant tumor that severely threatens human health. To date, early detection for HCC patients is particularly significant due to their poor survival rates even after liver resection.

Methods: Therefore, an efficient and sensitive detection method for monitoring liver cancer, multiplex methylation-specific PCR (MSP) coupled with capillary electrophoresis, is developed.

Results: Simulations demonstrated that the methylation status of *RASSF1A*, *p16*, *SFRP1*, and *ELF* could be detected even when DNA equaled or exceeded 12.5 ng simultaneously. Also, its accuracy for methylation detection outweighed polyacrylamide gel electrophoresis (87.5%) and agarose electrophoresis (84.3%), reaching 92.1%. Subsequently, we implemented multiplex MSP with capillary electrophoresis to investigate methylation status of the four tumor suppressor genes in tissue specimens and explore the prognostic value for HCC patients. As the data suggested, multivariate cox regression analysis revealed that the recurrence-free survival of 46 patients was greatly associated with portal vein tumor thrombus (PVTT) and *p16* methylation and receiver operating characteristic (ROC) curves demonstrated that the predictive range of portal vein tumor thrombus (PVTT) combined with *p16* hypermethylation was more sensitive than that of either PVTT or *p16* hypermethylation alone with regard to disease recurrence in patients with HCC, which could be testified as a valuable biomarker in Clinical application.

Conclusion: Multiplex MSP coupled with capillary electrophoresis has an excellent prospect of clinical application for monitoring early liver cancer and screening valuable biomarkers for prognosis of HCC patients.

KEYWORDS

biomarkers, capillary electrophoresis, Hepatocellular carcinoma, methylation, Multiple MS-PCR

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is commonly developed in cirrhotic livers with mostly chronic hepatitis B virus (HBV), hepatitis C virus

or co-infected with HBV and hepatitis C virus.¹⁻³ Currently, surgery provides the best cure for HCC.⁴ However, even after liver resection of HCC patients, overall prognosis is still poor,⁵⁻⁷ which is attributed to frequent intrahepatic spread and extrahepatic metastasis, with

a dismal five-year survival rate less than 15%.¹ Additionally, DNA methylation is an epigenetic modification and cancer-specific DNA methylation alterations in tissues and blood of cancer patients can serve as markers for risk assessment, monitoring progression, early detection, and prognosis prediction.⁸ Hence, to investigate the prognostic value for HCC patients, tumor suppressor genes (TSGs; *RASSF1A*, *p16*, *SFRP1*, and *ELF*) were served for research. The RAS association domain family 1A gene (*RASSF1A*) is a key member of cell adhesion molecules,⁹ playing a vital role in tumor invasion and metastasis.^{10–16} *p16* is critical in cell cycle regulation and therefore acts as a tumor suppressor.^{17–19} Embryonic liver fodrin (*ELF*) has a pivotal effect on the control of Smad access to activate receptors involved in regulation of TGF- β -signaling,²⁰ which is a key suppressor of tumorigenesis.^{21,22} Secreted frizzled-related protein 1 (*SFRP1*) can inhibit the development of epithelial-mesenchymal transition (EMT), which is associated with tumor infiltration.²³

Multiplex methylation-specific PCR (MSP), combined with multiplex PCR (Multiplex polymerase chain reaction) and MSP, is applied to detect the methylation status of *RASSF1A*, *p16*, *SFRP1*, and *ELF* in the research. Compared with conventional MSP, the improved approach can amplify multiple regions of the template in one reaction tube with several pairs of primers, thus saving a large amount of time, labor, and template for the experiment. Furthermore, multiplex PCR products detection is coupled with capillary electrophoresis (CE), which has become an attractive separation method because of its excellent advantages including fast-separation, high-resolution, and less sample consumption,²⁴ thus giving rise to notable savings of labor and reagent costs, and errors from sample pretreatment steps can also be avoided.²⁵ Thus, this study aimed to provide a more

sensitive and efficient detection method for screening and making prognosis prediction for liver cancer.

2 | MATERIALS AND METHODS

2.1 | Materials

Acrylamide, Bis, TEMED, ammonium persulfate, Tris base, EB, EDTA, sodium thiosulfate, formaldehyde, sodium hydroxide, ethidium Bromide, and silver nitrate were obtained from Biotopped (Beijing, China). GeneScan™ LIZ-500 was purchased from ABI company in USA. SssI methylase was purchased from New England Biolabs (Beverly, MA). TIANamp Genomic DNA kit and AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit were purchased from TIANGEN and AxyPrep, respectively, in China. EZ DNA Methylation-Gold Kit was obtained from ZYMO in USA. Fluorescent primers were synthesized by Invitrogen (USA) and general primers by BGI (China). All other chemicals used for the preparation of buffers and solutions were of analytical grade and obtained from commercial sources.

2.2 | Samples and DNA extraction

With informed consent, normal liver tissue from healthy individuals who died of sudden accidents, patients' plasma, and tissue samples were obtained from the West China Hospital in Sichuan University, including liver tissue of one healthy individual and 46 HCC patients, plasma of 10 HCC patients, and 14 normal people. We also collected the placenta from West China Second University Hospital

TABLE 1 Primer sequences and polymerase chain reaction conditions for multiplex MS-PCR analysis

Primer name	Primer sequence	Annealing temp.(°C)	Product size (bp)	Ref.
RASSF1A-UF	5'-GGGGTTTTGTGAGAGTGTGTTTAG	63	175	[36]
RASSF1A-UR	5'-TAAACACTAACAAACACAAACCAAAC			
RASSF1A-MF	5'-GGGTTTTGCGAGAGCGCG	63	169	
RASSF1A-MR	5'-GCTAACAAACGCGAACCG			
p16-UF	5'-TTATTAGAGGGTGGGGTGGATTGT	63	151	[37]
p16-UR	5'-CAACCCCAAACCAACCATAA			
p16-MF	5'-TTATTAGAGGGTGGGGCGGATCGC	63	150	
p16-MR	5'-GACCCCGAACCGCGACCGTAA			
SFRP1-UF	5'-GTTTTGTAGTTTTTGGAGTTAGTGTGTGT	63	135	[38]
SFRP1-UR	5'-CTCAACCTACAATCAAAAACAACAAACA			
SFRP1-MF	5'-TGAGTTTTTCGGAGTTAGTGTGCGCG	63	126	
SFRP1-MR	5'-CCTACGATCGAAAACGACGCGAACG			
ELF-UF	5'-GGTGAGTGGTTTTTGATAAAATTATAAATT	63	107	[37]
ELF-UR	5'-ACATACCACAATTACCAAAACCCAAA			
ELF-MF	5'-GGCGAGTGGTTTTTGATAAAATTATAAAT	63	107	
ELF-MR	5'-ACATACCACGATTACCGAACCCGAA			

MF, methylated forward primer; MR, unmethylated reverse primer; UF, unmethylated forward primer; UR, unmethylated reverse primer.

The 5' position of the sense unmethylated or sense methylated primer sequences is numbered relative to the transcription start site of the gene concern.

with informed consent. These samples were immediately stored at -30°C until DNA extraction. According to the manufacturer's protocol, genomic DNA was extracted from plasma samples with a commercial DNA extraction kit (AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit; AxyPrep, China). Likewise, DNA was extracted from tissue samples by TIANamp Genomic DNA Kit (TIANGEN, China).

2.3 | Bisulfite modification and multiplex methylation-specific PCR

DNA (200 ng) was subjected to sodium bisulfite modification using the EZ DNA Methylation kit (Zymo Research) according to the protocol described in the user manual. Multiplex MS-PCR was performed in a volume of about 25 μL that contained 6.25–100 ng of the sodium bisulfite-treated DNA with Multiplex PCR kit (QIAGEN, Germany).

The multiplex MS-PCR primer sequences for *RASSF1A*, *p16*, *SFRP1*, and *ELF* were listed in Table 1. After heating at 95°C for 15 minutes, PCR was performed in a thermal cycler for 30 cycles, each of which consisted of denaturation at 94°C for 30 seconds, annealing at 63°C for 90 seconds, and extension at 72°C for 90 seconds, followed by a final 10-minute extension at 72°C . The PCR products were analyzed by agarose electrophoresis, polyacrylamide gel electrophoresis, and capillary electrophoresis.

3 | RESULTS

3.1 | Sensitivity properties of CE detection

The placenta had been used as a reference tissue, which allowed researchers to utilize an important residual tissue whose DNA

FIGURE 1 The sensitivity of capillary electrophoresis to detect multiplex MSP products. The concentration of DNA template reaches 100 ng/ μL (A), 50 ng/ μL (B), 12.5 ng/ μL (C), 6.25 ng/ μL (D), respectively. The horizontal axis and vertical axis represents product size and peak height, respectively. MSP, methylation-specific PCR

FIGURE 2 The comparison between capillary electrophoresis, polyacrylamide gel electrophoresis, and agarose electrophoresis method in terms of detection accuracy. A, Representative example of plasma samples from two HCC patients with capillary electrophoresis, polyacrylamide gel electrophoresis, and agarose electrophoresis detection. B, AUCs, sensitivity, and specificity with regard to capillary electrophoresis, polyacrylamide gel electrophoresis and agarose electrophoresis detection. AUC, area under curve

methylation patterns may prove to be powerful biomarkers for a number of diseases or disease progression.²⁶⁻²⁸ So genomic DNA from placenta was methylated in vitro with *SssI* methylase and exploited as a positive control to be dissolved in plasma of normal people and then DNA was re-extracted with AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit as multiplex MSP template. After *RASSF1A*, *p16*, *SFRP1*, and *ELF* were amplified with multiplex MSP, capillary electrophoresis was adopted to detect the methylation status of four genes. The detection results are listed as follows in Figure 1: when concentration of the template DNA equaled or exceeded 100 ng, 50 ng or 12.5 ng,

CE could detect four genes' methylation status (*RASSF1A*, *p16*, *SFRP1*, and *ELF*; Figure 1A-C); nevertheless, if the concentration of template DNA was equal to or greater than 6.25 ng, the methylation status of three genes would be tested (*RASSF1A*, *p16* and *ELF*; Figure 1D).

3.2 | CE could enhance detection accuracy compared with other two electrophoresis methods

To validate whether capillary electrophoresis outweighed polyacrylamide gel electrophoresis and agarose electrophoresis in

TABLE 2 The comparison of detection accuracy between capillary electrophoresis, polyacrylamide gel electrophoresis, and agarose electrophoresis

Detection technology	AUC	Sensitivity	Specificity	Cut off value	P value
Capillary electrophoresis	92.1%	90.0%	42.9%	0.1040254	.001
Polyacrylamide gel electrophoresis	87.5%	90.0%	42.9%	0.1138108	.002
Agarose electrophoresis	84.3%	90.0%	35.7%	0.3061776	.005

AUC, area under curve.

FIGURE 3 The specificity of capillary electrophoresis to detect multiplex MSP products. A, Placenta genomic DNA treated with *SssI* methylase and genomic DNA from normal liver tissue were used as positive control and negative control for *RASSF1A*, *p16*, *SFRP1*, and *ELF*, respectively. B, Bisulfite sequencing of *RASSF1A*, *p16*, *SFRP1*, and *ELF*. The targeted sequences were coincident with purpose fragments and CpGs in MSP product were replaced by TpGs in USP product. M: PCR product with methylation-specific primers; U: PCR product with unmethylation-specific primers

terms of detection accuracy, the three detection methods were utilized to test the TSGs methylation status among an identical cohort of plasma specimens, involving 10 HCC patients and

14 healthy people. Fifty nanogram template DNA was applied into multiplex MSP, and the products were tested with the three methods above. The representative result of two HCC plasma is

shown in Figure 2A, which validates that capillary electrophoresis could detect more hypermethylated genes compared with the other two detection methods. From the data suggested, the detection accuracy could reach 92.1%, 87.5%, and 84.3% with capillary electrophoresis, polyacrylamide gel electrophoresis and agarose electrophoresis detection, respectively (Figure 2B; Table 2), which demonstrated that capillary electrophoresis had an edge over the other two detection methods in respect of detection accuracy, sensitivity, and specificity.

3.3 | Application of multiplex MSP with CE detection to explore prognostic value for HCC patients

Placenta genomic DNA treated with *Sss*I methylase was used as a positive control, and normal liver tissue was substituted for genomic DNA as a negative control (Figure 3A). The methylation status of tumor suppressor genes with M set primers could be detected in methylated placenta while they could not be detected

FIGURE 4 Multiplex MSP analysis of *RASSF1A*, *p16*, *SFRP1*, and *ELF* in representative samples of hepatocellular carcinoma (HCC) with capillary electrophoresis detection. M, methylation, U, unmethylation. The horizontal axis and vertical axis represents product size and peak height, respectively

FIGURE 5 Summary of multiplex MS-PCR analysis of *RASSF1A*, *p16*, *SFRP1*, and *ELF* in tissue samples from 46 HCC patients. Filled boxes reveal the presence of hypermethylation, and open boxes indicate the absence of hypermethylation

DX	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Hepatocellular carcinoma	<i>RASSF1A</i>																
	<i>p16</i>																
	<i>SFRP1</i>																
	<i>ELF</i>																
	No.	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
	<i>RASSF1A</i>																
	<i>p16</i>																
	<i>SFRP1</i>																
	<i>ELF</i>																
	No.	33	34	35	36	37	38	39	40	41	42	43	44	45	46		
	<i>RASSF1A</i>																
	<i>p16</i>																
	<i>SFRP1</i>																
	<i>ELF</i>																

TABLE 3 Methylation frequency of *RASSF1A*, *p16*, *SFRP1*, and *ELF* in 46 HCC cases

Gene	n = 46 (%)
<i>RASSF1A</i>	41 (89.1%)
<i>p16</i>	34 (73.9%)
<i>SFRP1</i>	32 (69.6%)
<i>ELF</i>	31 (67.4%)

in normal liver tissue. However, they could be tested in normal liver tissue with U set primers. Also, the detection specificity was confirmed by the sequence of MSP and USP product (Figure 3B). The CpGs were frequently methylated in genomic DNA of treated placenta with *SssI* methylase, while they were replaced by TpGs in USP product. DNA was isolated from tumor tissues of 46 stage I-IV HCC patients, and dealt with sodium bisulfite. Fifty nanogram bisulfite-modified DNA was amplified by multiplex MSP. Representative samples of the multiplex MSP reactions are shown in Figure 4. As shown in Figure 5, methylation for *RASSF1A*, *p16*, *SFRP1*, and *ELF* was observed in 89.1% (41/46), 73.9% (34/46), 69.6% (32/46), and 67.4% (31/46), respectively (Table 3). Afterward, we analyzed the relationship between the recurrence-free survival after surgery in 46 patients and clinical characteristics (age, gender, differentiation, tumor size, PVTT-portal vein tumor thrombus, fibrillation grade, HBsAg, Anti-HCV, AFP level, TNM stage) plus *RASSF1A*, *p16*, *SFRP1*, and *ELF* hypermethylation. The baseline characteristics of 46 patients are summarized in Table 4. Multivariate cox regression analysis revealed that the recurrence-free survival of 46 patients was greatly associated with PVTT and *p16* hypermethylation (Table 5), which could confirmed that PVTT and *p16* hypermethylation were significant predictors for HCC. In addition, to analyze the prognostic value of combining PVTT and *p16* hypermethylation for HCC, ROC curve analysis was applied for prediction accuracy of prognosis. The area under curve (AUC) for HCC prognosis prediction determined from PVTT and *p16* hypermethylation was 57.0% ($P = .422$) and 67.7% ($P = .042$), respectively. Whereas a prognostic panel

TABLE 4 Patients' characteristics (n = 46)

Characteristics	Total
Age	
≥50, n (%)	28 (60.9)
<50, n (%)	18 (39.1)
Mean follow-up years	1.5
Gender	
Male, n (%)	35 (76.1)
Female, n (%)	11 (23.9)
Degree of cell differentiation	
Moderately, n (%)	15 (32.6)
Poorly, n (%)	31 (67.4)
Tumor size (cm)	
≥5	20 (43.5)
<5	26 (56.5)
PVTT, n (%)	18 (39.1)
Fibrillation grade	
3-4, n (%)	15 (32.6)
5-6, n (%)	31 (67.4)
HBsAg, n (%)	43 (93.5)
Anti-HCV, n (%)	5 (10.9)
AFP level (μg/L)	
≥400	17 (37.0)
<400	29 (63.0)
Clinical TNM stage	
I-II, n (%)	38 (82.6)
III-IV, n (%)	8 (17.4)
<i>RASSF1A</i> methylation, n (%)	41 (89.1)
<i>p16</i> methylation, n (%)	34 (73.9)
<i>SFRP1</i> methylation, n (%)	32 (69.6)
<i>ELF</i> methylation, n (%)	31 (67.4)

with PVTT and *p16* hypermethylation combination was effective for prognosis prediction with AUC reaching 75.4% ($P = .004$) (Figure 6; Table 6).

4 | DISCUSSION

Hepatocellular carcinoma (HCC), like multistep carcinogenesis of other cancers, has undergone a progression from hepatitis B virus (HBV) hepatitis, followed by liver cirrhosis (LC), and finally to carcinoma. In the present research, except alpha-fetoprotein (AFP) exploited extensively in clinical practice, the alterations in DNA methylation pattern have been also recognized as one of the most frequent molecular phenomenon in liver cancer.²⁹ Consequently, the detection of altered DNA methylation patterns in promoter region of cancer-related genes may provide useful tool for tumor behavior monitoring or recognition of recurrence.³⁰⁻³² Thus, the study laid stress on seeking higher-sensitivity method to detect

aberrant methylation as biomarkers for prognostic prediction of HCC patients.

At the moment, conventional MSP is time-consuming and labor intensive and also requires relatively large amounts of genomic DNA, compared with multiplex MSP that offers a more rapid alternative and requires a much lower amount of genomic DNA because of the simultaneous treatment of studied genes. Moreover, capillary electrophoresis, which can detect the PCR products that cannot be observed in agarose gel electrophoresis, are more advantageous than EB staining in terms of sensitivity. In this study, multiplex MSP with capillary electrophoresis certifies more effective and sensitive for screening and monitoring biomarkers of methylation genes in liver cancer. And three key elements should

TABLE 5 Recurrence-free survival of HCC patients in relation to clinical characteristics and *RASSF1A*, *p16*, *SFRP1*, *ELF* methylation status

Variable (n = 46)	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Age	1.133 (0.456-2.818)	.788	2.233 (0.615-8.104)	.222
Gender	0.886 (0.294-2.670)	.830	1.112 (0.197-6.274)	.904
Degree of cell differentiation	1.961 (0.651-5.913)	.232	4.164 (0.450-38.504)	.209
Tumor size	2.276 (0.915-5.664)	.077	0.854 (0.157-4.655)	.855
PVTT	0.641 (0.260-1.580)	.334	0.143 (0.029-0.701)	.016*
Fibrillation grade	2.199 (0.729-6.631)	.162	0.146 (0.017-1.245)	.079
HBsAg	1.157 (0.154-8.675)	.887	36.987 (0.488-2801.846)	.102
Anti-HCV	1.046 (0.242-4.530)	.952	14.027 (0.975-201.832)	.052
AFP level	2.545 (0.844-7.672)	.097	5.449 (0.682-43.557)	.110
Clinical TNM stage	1.887 (0.436-8.171)	.396	0.375 (0.059-2.365)	.296
<i>RASSF1A</i> methylation	0.040 (0.000-12.077)	.269	0.000 (0.000-6.233E+246)	.976
<i>p16</i> methylation	0.117 (0.016-0.880)	.037	0.026 (0.001-0.709)	.030*
<i>SFRP1</i> methylation	0.092 (0.012-0.689)	.020	0.157 (0.013-1.923)	.147
<i>ELF</i> methylation	0.020 (0.000-0.912)	.045	0.000 (0.000-2.870E+121)	.923

*Statistically significant values are highlighted in bold.

FIGURE 6 Receiver operating characteristic (ROC) curves of sensitivity vs 1-specificity. A, Receiver operating characteristic curve for the model that includes PVTT. B, Receiver operating characteristic curve for the model that includes *p16* hypermethylation. C, Receiver operating characteristic curve for the model that includes PVTT and *p16* hypermethylation

TABLE 6 The comparison of prognostic accuracy between PVTT, *p16* hypermethylation, and the two combined elements for recurrence-free survival of HCC patients

Variable	AUC	Sensitivity	Specificity	Cutoff value	P value
PVTT	57.0%	47.4%	33.3%	0.50	.422
<i>p16</i> hypermethylation	67.7%	94.7%	59.3%	0.50	.042
PVTT plus <i>p16</i> hypermethylation	75.4%	94.7%	59.3%	0.2731179	.004

AUC, area under curve.

be put emphasis on specificity of primers, appropriate annealing temperature, and primer proportion. Firstly, as base pair mismatches in the primer-binding regions can reduce affinity of the primers for the template and are intended to cause reduced or no amplification of non-targets, the location of the base pair mismatches within the primer sets is extremely important to assay specificity.³³ Then, the choices of identical annealing temperature are essential for several pairs of primers that would be put into a reaction tube to amplify with multiple MSP. Yet, several pairs of primers of genes should be chosen to have close *T_m* value so that identical and appropriate annealing temperature could be developed. Relatively high annealing temperature should be used in PCR system as the annealing temperature is elevated, and the stringency of primer annealing is also increased, leading to more specific and reproducible amplification.

In the study, the establishment of multiplex MSP system with capillary electrophoresis was applied to detect methylation status of TSGs to analyze the prognostic value for HCC patients. Cheng et al³⁴ reported that PVTT would worsen prognosis of HCC patients, which was consistent with our results. Subsequently, our data revealed that patients with *p16* hypermethylation had a high probability of recurrence and poor prognosis, matching with Eunkyung ko et al' results that *p16* hypermethylation may be associated with a poor prognosis in recurrent hepatocellular carcinoma.³⁵ However, ROC analysis delineated that combined PVTT and *p16* hypermethylation could yield the advantages with more precise than the individual indicator. This result indicated that combined biomarkers would be a more valuable marker for prognosis prediction of HCC patients.

In conclusion, multiplex MSP with capillary electrophoresis has an excellent prospect of clinical application for monitoring early liver cancer and screening valuable biomarkers for prognosis of HCC patients. And the most interesting finding was that the predictive range of PVTT combined with *p16* hypermethylation was more sensitive than that of either PVTT or *p16* hypermethylation alone with regard to disease recurrence in patients with HCC.

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