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RESEARCH

Circulating tumor DNA methylation markers for the early diagnosis of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a major global health concern, being the sixth most common cancer and the third leading cause of cancer deaths worldwide. Less than 30% of HCC patients are eligible for curative treatment, primarily due to diagnosis at advanced stages. This emphasizes the importance of early detection in improving survival outcomes. In this study, we investigated the methylation levels of certain genes and miRNAs in liquid biopsy and developed a methyl predictive model (MPM-8G). The AUC for MPM-8G was found to be significantly higher than that for AFP (alpha-fetoprotein) alone. When MPM-8G and AFP were combined, the AUC increased notably, indicating that the combined use of MPM-8G and AFP offers superior diagnostic performance and enhances the accuracy of HCC detection. Furthermore, the combination of MPM-8G and AFP proved to be a powerful tool for early diagnosis of HCC. This study successfully identified differences in the methylation levels of certain genes and miRNAs in liquid biopsy from HCC patients, leading to the construction of a predictive model for early diagnosis. The impressive performance of these methylation markers underscores their potential for further clinical application in the management of HCC.

Keywords Hepatocellular carcinoma · DNA methylation · Early diagnosis · Circulating tumor DNA

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Abbreviations

ABL1	ABL proto-oncogene 1
ACC	Accuracy
AFP	Alpha-fetoprotein
APC	Adenomatous polyposis coli
AUC	Area under curve
COX2	Cyclooxygenase-2
EMT	Epithelial–mesenchymal transition
FN	False negative
FP	False positive
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
MPM-8G	Methyl predictive model
NER	Neutrophil-to-eosinophil ratio
NLR	Neutrophil-to-lymphocyte ratio
NPV	Negative predictive value
PLR	Platelet-to-lymphocyte ratio
PPV	Positive predictive value
qMSP	Quantitative methylation-specific PCR
RASSF1A	Ras association domain family member 1
RFA	Radiofrequency ablation
RGS10	Regulator of G-protein signaling proteins 10

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ROC curve	Receiver operating characteristic curve
Sen	Sensitivity
SOX4	Sry-box transcription factor 4
Spe	Specificity
ST8SIA6	ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 6
TCF/LEF	T cell factor/lymphoid enhancer factor
VIM	Vimentin

Introduction

Hepatocellular carcinoma (HCC) poses a significant global health challenge, ranking as the sixth most frequently diagnosed cancer and the third leading cause of cancer-related deaths [1]. This highly malignant tumor carries a poor prognosis and high mortality rate, with annual HCC deaths nearly equaling the number of new diagnoses [2]. HCC incidence varies geographically, likely due to regional differences in hepatitis B virus (HBV) and hepatitis C virus (HCV) exposure [1]. In addition to viral hepatitis, cirrhosis from any cause is a significant risk factor for HCC [3].

HCC exhibits relative resistance to systemic therapies, including chemotherapy, targeted therapy, and even immunotherapy [4]. Currently, no therapeutic agents have demonstrated a dramatic improvement in overall survival. The primary curative treatments for HCC are surgical interventions, specifically partial liver resection and liver transplantation. However, fewer than 30% of HCC patients are surgical candidates, primarily due to late diagnosis and the presence of multiple lesions in cirrhotic or fibrotic livers. Consequently, early HCC diagnosis is crucial for improving overall survival.

Serum alpha-fetoprotein (AFP) is the most widely used tumor marker for HCC screening and surveillance, despite its limited sensitivity and specificity [5]. Using a cutoff of 20 ng/mL, AFP sensitivity for HCC in cirrhotic patients ranges from 41 to 65% [6]. However, detection rates can be as low as one-third in early HCC, as 80% of small HCC cases do not exhibit elevated serum AFP levels [7, 8]. Furthermore, elevated AFP levels are observed in other chronic liver diseases, such as cirrhosis and hepatic inflammation, and in other cancers, including nonseminomatous germ cell tumors and gastrointestinal cancers [9]. Therefore, additional biomarkers are needed to complement AFP and improve diagnostic accuracy, particularly for early HCC.

In recent decades, DNA methylation has gained recognition as a valuable biomarker for early cancer detection and diagnosis [10]. DNA methylation plays a crucial role in numerous physiological processes. Aberrant DNA methylation contributes to various human diseases, including cancer [11]. Methylation of promoter or 5' region CpG islands can repress downstream gene expression. Mounting evidence suggests that DNA hypermethylation can downregulate tumor suppressors and DNA repair genes, while hypomethylation can upregulate oncogenes during early carcinogenesis [12, 13]. Beyond silencing tumor suppressor genes, DNA methylation also plays a crucial role in silencing tumor suppressor miRNAs in cancer cells [14]. These miRNAs, small (~22 nucleotide) noncoding RNAs, regulate gene expression by complementary binding to the 3' UTR of target mRNAs, resulting in mRNA cleavage or translational inhibition. Because a methyl group is covalently bound to genomic DNA, it exhibits greater stability than protein or RNA markers. Moreover, these methylation markers can be detected in liquid biopsies, showing potential for monitoring cancer progression [15].

In our previous study, we used a whole-genome approach to identify significant DNA methylation profiles in HCC cell lines and tissues [16–18]. From these profiles, we selected a panel of eight genes and miRNAs regulated by DNA methylation and measured their methylation levels in plasma cell-free DNA. Furthermore, we examined their predictive capability for early HCC diagnosis, both independently and in combination with AFP.

Materials and methods

Participants

Three hundred and eighteen plasma samples, including healthy donors ($n=52$), chronic hepatitis B patients ($n=61$), chronic hepatitis B patients with cirrhosis ($n=46$), and HBV-related HCC patients ($n=159$), were obtained from three medical centers in Taiwan: National Cheng Kung University (NCKU) Hospital (Tainan), Taipei Veterans General Hospital (Taipei), and An Nan Hospital, China Medical University (Tainan) (Table 1). All experimental protocols and study methods were approved by the institutional review board of the three hospitals. Written informed consent was obtained from the patients who provided the specimens.

Plasma samples were obtained from patients diagnosed with HCC who had not received any treatments, such as radiotherapy or chemotherapy, before whole blood collection. Plasma samples were stored at − 80 °C until needed. Cell-free DNA was extracted from plasma samples using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's manual.

Cell-free DNA extraction from plasma samples

Blood samples were collected in BD Vacutainer Blood Collection Tubes with K2 EDTA and were further processed to plasma in less than 6 h. The plasma fraction was separated from the blood cells by centrifugation for 15 min at room

Table 1 Clinicopathological characteristics of patients

	Healthy donor ($N=52$)	Hepatitis ($N=61$)	Cirrhosis ($N=46$)	HCC ($N=159$)
Age, median (range)	58 (29–78)	46 (24–84)	57.5 (26–92)	61 (32–94)
Gender				
Male	34	43	36	131
Female	17	18	10	28
TNM classification				
I	–	–	–	24
II	–	–	–	29
III	–	–	–	11
AFP				
median, ng/ mL	2.38	2.43	3.25	3.84
> 20, ng/ mL (%)	0	0	2.2	27.8

temperature at 2000 × g. The collected plasma was aliquoted and stored at − 80 °C until use. cfDNA was extracted from the plasma using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions.

Real-time quantitative methylation analysis

Cell-free DNA from plasma samples was bisulfite-converted using the EZ DNA methylation kit (Zymo Research) and amplified by real-time quantitative methylation-specific PCR (qMSP) using fluorescent probes. Each reaction involved 1 × Kapa Probe Fast qPCR Master Mix, 0.5 µM of each primer, and 0.25 µM of probe in a total volume of 20 µl. Amplification was performed on the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). As previously described, the methylation level was calculated as the difference in Ct value between beta-actin and candidates using the following formula: 2^[Ct (beta-actin)—Ct (candidate)] × 100.

Statistical analysis

A paired sample t-test was used to determine the difference in methylation levels between HCC patients and normal controls. Logistic regression models were used to establish the methylation prediction model of six genes and two miRNAs (MPM-8G) for diagnosis. MPM-8G was calculated as follows: 4.802 + 0.128 * methylation level of ln(APC) + 0.154 * methylation level of ln(COX2) + 0.116 * methylation level of ln(miR-203) + 0.148 * methylation level of ln(RASSF1A) + 0.257 * methylation level of ln(VIM) + 0.088 * methylation level of ln(RGS10) + 0.082 * methylation level of ln(ST8SIA6) + 0.059 * methylation level of ln(miR-129–2). To assess the diagnostic effect, receiver operating characteristic (ROC) curve analysis was used to estimate the area under the curve (AUC), cutoff value, sensitivity, and specificity. Statistical significance was accepted when $P < 0.05$ for all tests.

Results

Differential methylated genes and miRNAs of cell-free DNA in HCC patients

In our previous work, we performed a whole-genome approach to identify novel genes regulated by DNA methylation [16–18]. Based on our findings and public domain, we selected eight genes and miRNAs with significant methylation levels for further examination in liquid biopsy samples from normal and HCC patients using qMSP methods. These genes and miRNAs include APC, COX2, miR-203, RASSF1A, RGS10, miR-129–2, ST8SIA6, and VIM. Our results show that the methylation levels of these six genes and two miRNAs were significantly higher in HCC patients compared to normal controls, including healthy donors, hepatitis, and cirrhosis patients (Fig. 1). This result suggests that these candidates may potentially act as methylation markers for the diagnosis of HCC in liquid biopsy.

The detective performance of methylation prediction model for HCC diagnosis

We combined the eight methylation markers to establish a methyl predictive model (MPM-8G) using a stepwise logistic regression algorithm. The performance of MPM-8G for the diagnosis of HCC was evaluated by an ROC curve (receiver operating characteristic curve). The area under the curve (AUC) of MPM-8G was 0.875 (95% CI: 0.837–0.912, $P < 0.01$) (Fig. 2A). AFP is the most used serum marker for HCC diagnosis, but its AUC was only 0.635 (95% CI 0.559–0.710, $P < 0.01$) (Fig. 2B). When the cutoff value of AFP was set at 20 ng/mL, which is a conventional cutoff value in clinical practice, the AUC was 0.614 (95% CI 0.539–0.689, $P = 0.003$) (Fig. 2C). By combining the methylation marker and serum AFP, there was a notable increase in the AUC to 0.905 (95% CI 0.867–0.943, $P < 0.01$) (Fig. 2D), indicating an enhancement in detective capacity.

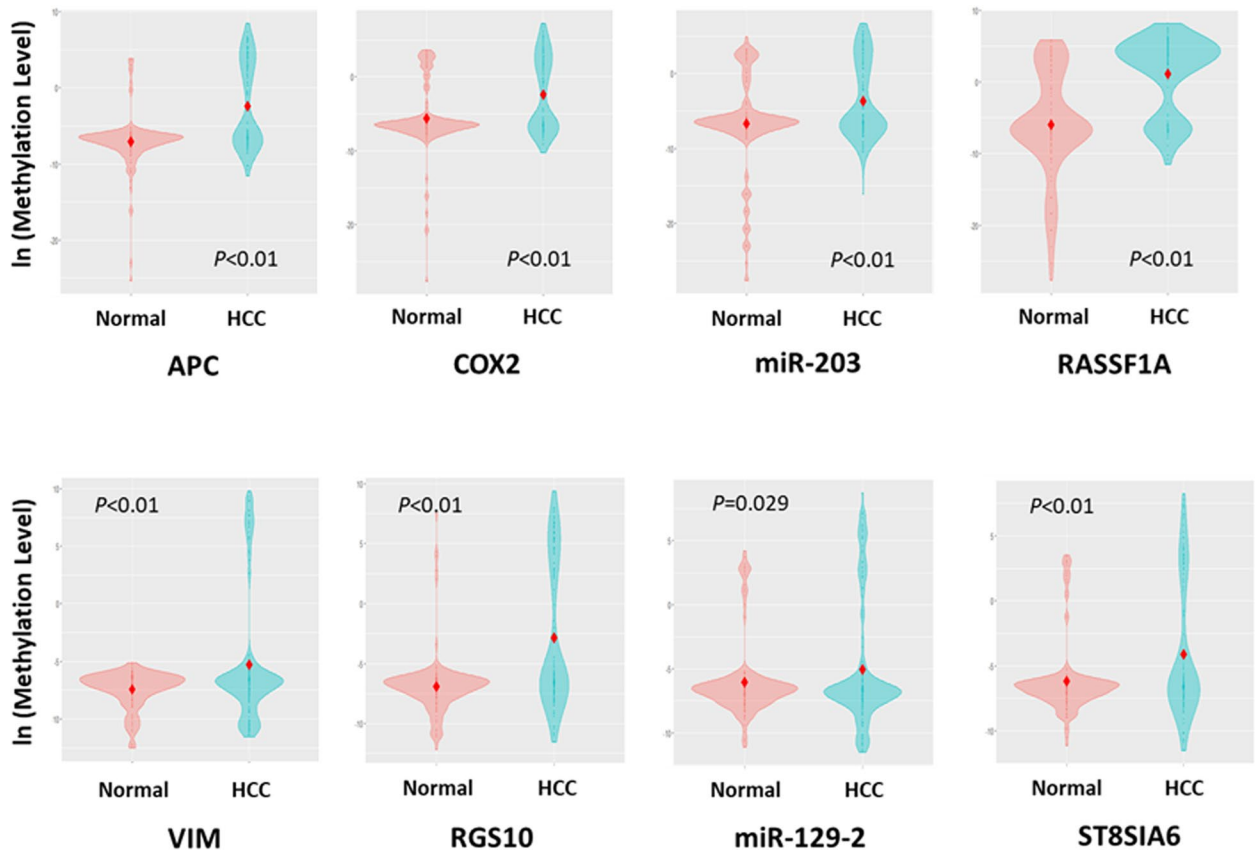

Fig. 1 Methylation levels of candidates in HCC and normal control. The methylation levels of eight candidate markers were examined in HCC and normal control, which includes healthy donors, hepatitis, and cirrhosis patients. The methylation levels were transformed by the natural logarithm. A paired t-test was used to determine statistical significance

The sensitivity (Sen), specificity (Spe), positive predictive value (PPV), negative predictive value (NPV), accuracy (ACC), false positive (FP), and false negative (FN) of AFP and MPM-8G were also measured, either individually or in combination in Table 2. AFP shows the excellent specificity and positive predictive value at the cutoff value of 20 ng/mL. However, its sensitivity is only 23.5%. The optimal cutoff value of AFP in our study is 4.5 ng/mL, elevating the sensitivity to 43.1%, which is still unacceptable for diagnosis. MPM-8G shows around 80% of Sen, Spe, PPV, NPV, ACC, and around 20% of FP and FN. By combination of AFP and MPM-8G, diagnostic ability makes a huge progress in Sen (86.3%), NPV (87.8%), and FN (12.2%). These results showed the superior performance of MPM-8G over the current serum tumor marker, and that the integration of the methylation marker and AFP provides more accurate detection ability for HCC diagnosis.

MPM-8G and AFP levels in non-tumor and tumor patients

To further evaluate the diagnostic ability of MPM-8G, we examined the MPM-8G score and AFP level in HCC and non-tumor controls, including healthy donors, hepatitis, and cirrhosis. The AFP value was transformed to a natural logarithm before analysis. The median AFP levels for healthy donors, hepatitis, cirrhosis, and HCC are 0.8961, 0.8755, 1.1663, and 1.2892, respectively. There is no significant difference in AFP levels between tumor and non-tumor controls (Fig. 3A). Regardless of the cutoff value used at 4.5 or 20 ng/mL, the median AFP of HCC patients remained below the cutoff value, indicating that over 50% of HCC patients could not be detected using any AFP cutoff values. On the other hand, the MPM-8G score is significantly higher in HCC patients (median, 0.7613)

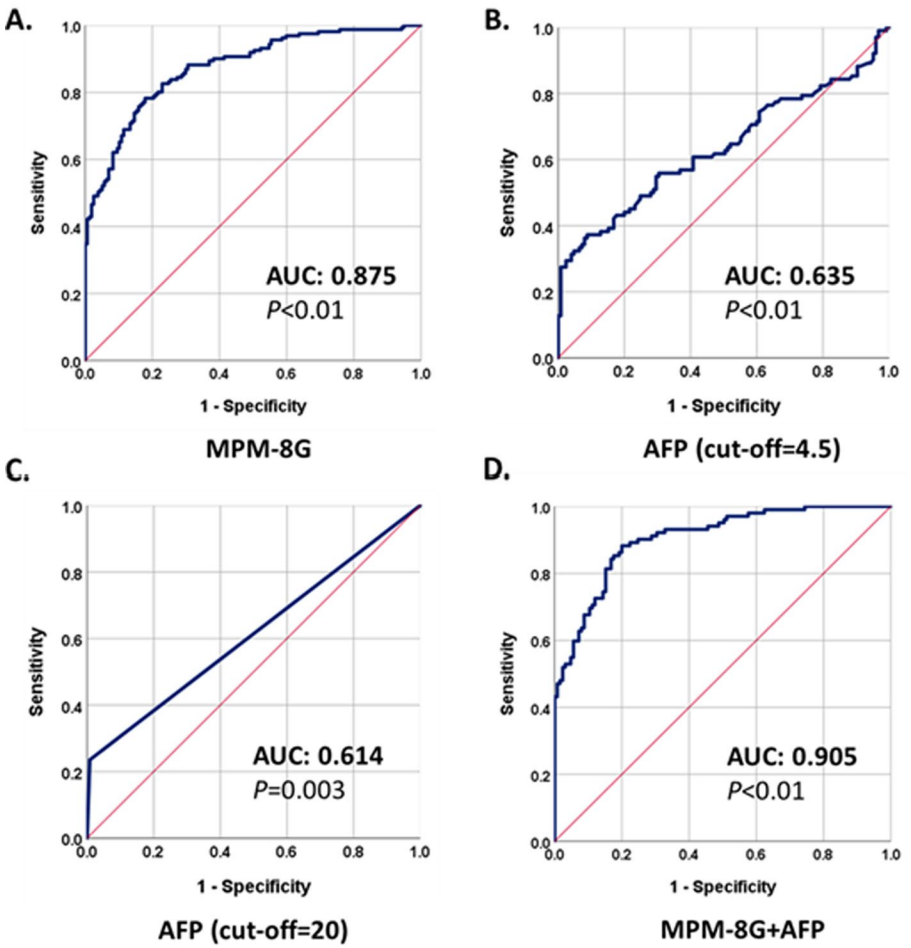

Fig. 2 The performance of MPM-8G and AFP for HCC diagnosis as measured by receiver operator characteristic (ROC) curves. **A** ROC analysis of MPM-8G, with an AUC of 0.875 ($P < 0.01$). **B** ROC analysis of AFP at the cutoff value of 4.5 ng/mL, with an AUC of 0.635 ($P < 0.01$). **C** ROC analysis of AFP at the cutoff value of 20 ng/mL, with an AUC of 0.614 ($P = 0.003$). **D** ROC analysis of the combination of MPM-8G and AFP, with an AUC of 0.905 ($P < 0.01$)

Table 2 Diagnostic parameters of AFP and MPM-8G

	AFP (%)	AFP (%)	MPM-8G (%)	MPM-8G + AFP (%)
cutoff	20	4.5	0.478	0.424
SEN	23.5	43.1	80.1	86.3
SPE	99.2	82.4	78.3	80.8
PPV	96	66.7	79.1	78.6
NPV	61.4	64.0	79.4	87.8
ACC	65.2	64.8	79.2	83.3
FP	4	33.3	22.2	21.4
FN	38.6	36.0	20.6	12.2

SEN sensitivity, *SPE* specificity, *PPV* positive predictive value, *NPV* negative predictive value, *ACC* accuracy, *FP* false positive, *FN* false negative

compared to non-tumor controls (median values: healthy donors: 0.2745, hepatitis: 0.2173, and cirrhosis: 0.2833) (Fig. 3B). When used in combination with AFP, MPM-8G demonstrated a superior ability to differentiate between non-tumor and HCC patients (Fig. 3C).

Predictive ability of MPM-8G in the early stage of HCC

To be a valuable diagnostic biomarker, we evaluated whether the early stage of HCC can be detected by MPM-8G. Based on the cutoff value of MPM-8G, the detection rates of MPM-8G in stage I, II, and III are 75.0%, 75.0%, and 81.8%, respectively (Fig. 4A). In contrast, the detection rates of AFP raise from 17.4% in stage I to 36.4% in stage III (Fig. 4B). Despite the stage-dependent manner AFP showed, the detection rate remains significantly low among patients in the first stage of HCC. In combination with MPM-8G and AFP, the detection rate can be elevated to 91.5% in stage I, 86.2% in stage II, and 90.9% in stage III (Fig. 4C). These results indicate the combination of MPM-8G and AFP may act as a very powerful tool for HCC early diagnosis.

Discussion

Early diagnosis is crucial for all cancers, especially HCC, due to its resistance to systemic therapy [4]. Curative HCC treatments, such as hepatectomy and radiofrequency ablation

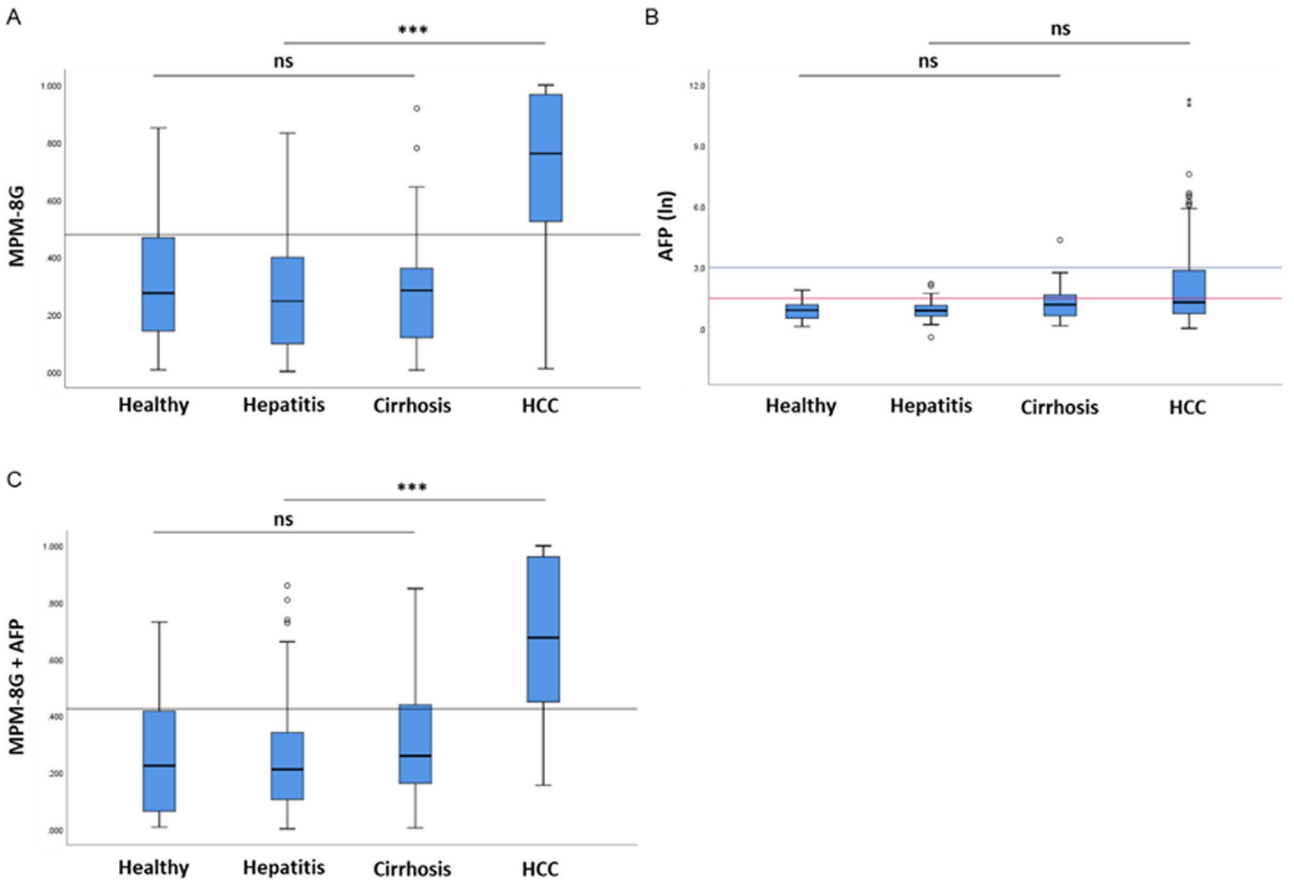

Fig. 3 Levels of MPM-8G and AFP in healthy donors, Hepatitis, cirrhosis, and HCC patients. **A** MPM-8G. **B** AFP. **C** MPM-8G + AFP. The AFP level was transformed by the natural logarithm. The cutff values for MPM-8G and MPM-8G + AFP are 0.478 and 0.424, respectively. The cutoff values for AFP at 4.5 and 20 ng/mL are indicated by red and blue lines, respectively. A paired t-test was used to determine statistical significance. ns, not significant. ***, $P < 0.01$

(RFA), are only feasible in the early stages. However, even with curative treatment, 5-year recurrence rates remain high. Early diagnostic tools are essential not only for high-risk individuals to detect HCC early but also for post-treatment surveillance in all HCC patients, regardless of treatment methods, including curative treatment, systemic therapy, targeted therapy, or immunotherapy [19–22].

Beyond conventional serum protein markers, considerable research focuses on identifying more effective recurrence predictors. Several factors have been reported, including larger tumor size, multiple tumors, microvascular invasion, absence of a tumor capsule, and poor tumor differentiation [23]. Patient-related risks include liver cirrhosis, hepatic dysfunction (Child–Pugh, MELD score, or Albumin-Bilirubin scores), high bilirubin, and systemic inflammation markers like the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and neutrophil-to-eosinophil ratio (NER) [24]. The results of our study may imply the potential of methylation markers for predicting HCC treatment outcomes.

In recent decades, the application of advanced epigenomic technologies has been adopted for the discovery of novel biomarkers for HCC diagnosis [25]. DNA methylation, a key mechanism for regulating gene expression, plays a crucial role in various physiological events, including embryonic development, X-chromosome inactivation, imprinting, and suppression of parasitic DNA sequences [26]. Reasonably, aberrant DNA methylation can lead to various human diseases, including cancers. Recent evidence suggests that DNA methylation is associated with the repression of not only tumor suppressor genes but also tumor suppressor microRNAs (miRNAs) in many cancer cells. In the present study, we identified six genes and two miRNAs, including APC, COX2, RASSF1A, RGS10, ST8SIA6, VIM, miR-129-2, and miR-203, as methylation markers for HCC early diagnosis according to our previous work [16–18].

RASSF1A, a key cell cycle regulator, is often inactivated in various cancers, including HCC. This tumor suppressor plays a crucial role in maintaining genomic stability and regulating the cell cycle [27]. Hypermethylation of

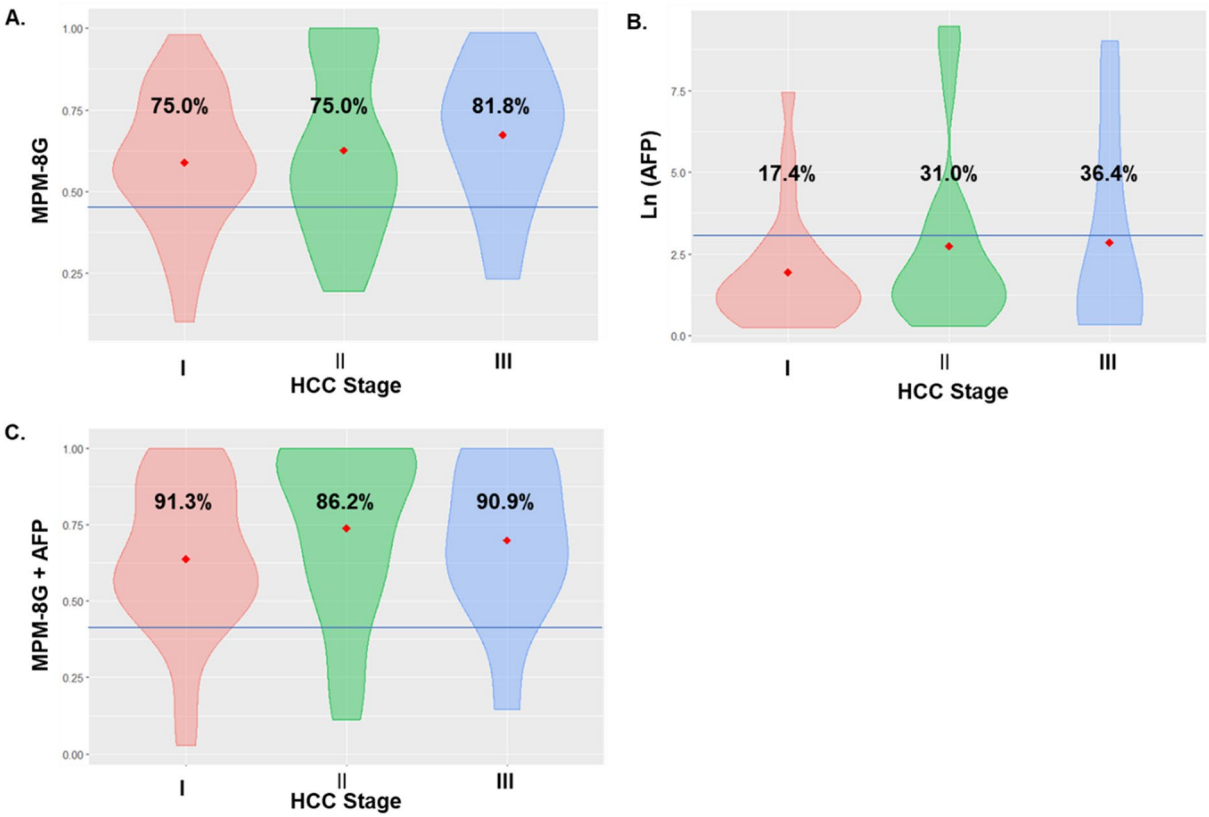

Fig. 4 Levels of MPM-8G and AFP in different stages of HCC. **A** MPM-8G. **B** AFP. **C** MPM-8G + AFP. The AFP level was transformed by natural logarithm. The cutoff values are indicated by the blue line. The X-axis represents the TNM staging classification

RASSF1A has been observed in the serum of 93% of HCC patients, 58% of HBV carriers, and 8% of healthy individuals [28]. APC also found to be hypermethylated in a significant percentage of HCC plasma samples, plays a vital role in cell cycle regulation, apoptosis, and cell migration [29]. It interacts with β-catenin [30], promoting its degradation and inhibiting the WNT signaling pathway, a crucial pathway in HCC development. Vimentin, a type III intermediate filament protein, has been associated with increased metastatic potential and poor survival rates in various cancers due to its expression in tumor tissues. It plays a significant role in managing the metastatic process, including epithelial-mesenchymal transition (EMT), invasion, and migration [31]. The methylation of VIM promoters occurs at a significantly higher rate in HCC with frequencies of 61.67%, which was considerably higher than those observed in 24.14% of liver cirrhosis, 13.64% of chronic hepatitis B patients, and 12% of healthy controls [32]. RGS10, a member of the Regulator of G-Protein Signaling (RGS) proteins, plays a significant role in cellular regulation. Changes in RGS10 expression have been linked to various diseases, including cancers [33]. ST8SIA6, a member of the six sialyltransferases family, is known to create ligands for Siglec-E, which modulates immune responses to tumors [34]. The upregulation of lncRNA ST8SIA6-AS1 has been linked to enhanced growth, movement, invasion, and apoptosis resistance in HCC [35–37]. However, the role of ST8SIA6 in the development of HCC remains unclear.

Our previous studies have shown that miR-203 and miR-129-2 have higher methylation levels in HCC compared to normal controls, suggesting their role as tumor suppressors [12, 17]. MiR-203 targets ABL1 and BCR-ABL1, an oncogenic fusion gene. When miR-203 is silenced, it activates the BCR-ABL1 gene, leading to increased tumor cell growth [38]. This silence is common in various cancers, including oral cancer and HCC [12, 39]. MiR-203 also targets Bmi-1, a component of a histone modifier complex, and its expression can trigger apoptosis and inhibit cell growth [40]. MiR-129-2 directly targets SOX4, a transcription factor involved in the Wnt pathway, which is crucial in HCC development. SOX4 can bind to TCF/LEF or β-catenin, stabilizing the β-catenin protein [41, 42]. These findings highlight the significant roles of miR-203 and miR-129-2 in cancer development and their potential as therapeutic targets.

In summary, this study investigated a novel, non-invasive liquid biopsy method using methylated genes and miRNAs in cell-free DNA for HCC diagnosis. The MPM-8G model exhibited superior diagnostic performance

compared to AFP alone, with further improvement when combined with AFP, demonstrating increased AUC, sensitivity, and specificity. Importantly, the combined MPM-8G and AFP approach shows promise for early HCC diagnosis, which is a critical factor for improved patient outcomes.

Despite these strengths, this study has several limitations. First, while the MPM-8G model shows promising results, the sample size is limited. Larger, more diverse cohorts are needed to validate these results in a multi-center study design. Second, the study focuses on methylation markers and AFP. While the combination improves diagnostic accuracy, other potential biomarkers or clinical factors were not explored, which might further enhance diagnostic performance. Finally, the study used a retrospective design, which can introduce biases due to incomplete data or patient selection. A prospective study design would be more robust.

Conclusion

In this study, we constructed a methyl predictive model (MPM-8G) for HCC early diagnosis using methylated genes and miRNAs, showing higher sensitivity, specificity, and accuracy than conventional serum AFP. Our results suggest the value and potential clinical applications of methylation markers in HCC early diagnosis.

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Author contributions Conceptualization by C.L., C.C., and C.Y.; methodology by P.P., S.H., and M.C.; validation and investigation by P.P., S.H., and M.C.; formal analysis by C.L., K.T., and C.H.; writing—original draft preparation, visualization, and funding acquisition by C.L.; writing—review and editing, supervision, and project administration by C.L., C.C., C.Y., K.T., and C.H. All authors read and approved of the final manuscript.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This study was approved by the Institutional Review Board of Taipei Veterans General Hospital (2015-06-012AC), National Cheng Kung University Hospital (B-ER-102-074), and An Nan Hospital (ANHRF112-16/TMANH112-REC016).

Consent to participate Informed consent was obtained from all individual participants included in the study.

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