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Differential methylation of the promoter and first exon of the *RASSF1A* gene in hepatocarcinogenesis

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Abstract

Aim—Aberrant methylation of the promoter, P2, and the first exon, E1, regions of the tumor suppressor gene *RASSF1A*, have been associated with hepatocellular carcinoma (HCC), albeit with poor specificity. This study analyzed the methylation profiles of P1, P2 and E1 regions of the gene to identify the region of which methylation most specifically corresponds to HCC and to evaluate the potential of this methylated region as a biomarker in urine for HCC screening.

Methods—Bisulfite DNA sequencing and quantitative methylation-specific polymerase chain reaction assays were performed to compare methylation of the 56 CpG sites in regions P1, P2 and E1 in DNA isolated from normal, hepatic, cirrhotic, adjacent non-HCC, and HCC liver tissue and urine samples for the characterization of hypermethylation of the *RASSF1A* gene as a biomarker for HCC screening.

Results—In tissue, comparing HCC ($n = 120$) with cirrhosis and hepatitis together ($n = 70$), methylation of P1 had an area under the receiver operating characteristics curve (AUROC) of

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SUPPORTING INFORMATION

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0.90, whereas methylation of E1 and P2 had AUROC of 0.84 and 0.72, respectively. At 90% sensitivity, specificity for P1 methylation was 72.9% versus 38.6% for E1 and 27.1% for P2. Methylated P1 DNA was detected in urine in association with cirrhosis and HCC. It had a sensitivity of 81.8% for α -fetoprotein negative HCC.

Conclusion—Among the three regions analyzed, methylation of P1 is the most specific for HCC and holds great promise as a DNA marker in urine for screening of cirrhosis and HCC.

Keywords

biomarkers; cirrhosis; hepatocellular carcinoma; methylation; *RASSF1A*; urine

INTRODUCTION

Methylation of multiple tumor suppressor genes is implicated in hepatocarcinogenesis.^{1–4} These hypermethylation events also offer promise as tools to detect cancer in body fluids.^{5–9} Among these, aberrant hypermethylation of the *RASSF1A* gene (*mRASSF1A*) is found in 90% of liver cancer tissues.^{10–16} The *RASSF1A* gene is a member of the *Ras* association domain family that can regulate the cell cycle and trigger apoptosis.^{17–20} Many studies have demonstrated that the *mRASSF1A* has resulted in downregulation of gene expression. The inverse association between *mRASSF1A* and its RNA expression has been shown both *in vitro* in hepatocellular carcinoma (HCC) cell lines (HepG2 and Hep3B) and *in vivo* in patient liver samples from hepatitis, cirrhosis and HCC.^{21–24} Moreover, the increase of *RASSF1A* expression resulted in suppressed cancer properties such as proliferation, colony formation and apoptosis resistance in many cancerous cell lines, including HCC cell lines.^{25–30} Thus, the *mRASSF1A* has been suggested for its important role in hepatocarcinogenesis and its potential as a biomarker for HCC, but often with poor measures of specificity.^{10,11,31,32}

Our previous studies suggested that the locations of the CpG sites analyzed affect the sensitivity and specificity of biomarkers for HCC.^{33,34} Yan *et al.*³⁵ demonstrated by examining regions P1, P2 and E1 of the promoter and first exon of *RASSF1A*, that methylation of the P1 region was most specific for breast cancer detection. Although there are over 30 publications studying the association between *mRASSF1A* and HCC, to our knowledge, the methylation of the P1 region has not been investigated for HCC. This study compares methylation profiles of P1, P2 and E1 of the *RASSF1A* gene in HCC and non-HCC liver tissues and shows that methylation of the P1 region is most specific to liver carcinogenesis or HCC.

We and others have shown that urine contains DNA from the circulation and that this DNA is mostly derived from apoptotic cells.^{36–41} This circulation-derived urine DNA is filtered through the kidney barrier resulting in DNA fragmentation to sequences less than 300 base pairs (bp) (low molecular weight [LMW] DNA)^{38,42} which can then be used to detect cancer-derived genetic modifications.^{42–47} Encouragingly, we have also shown that the methylated P1 region detected in urine was associated with HCC development, thus suggesting that *mRASSF1A* can serve as a potential marker for hepatocarcinogenesis.

METHODS

Study subjects

Human samples were obtained with written informed consent from patients and were acquired under institutional review board approvals from the National Cheng-Kung University Medical Center, Taiwan, the Buddhist Tzu Chi Medical Center in Hualien, Taiwan, and Johns Hopkins University School of Medicine (Baltimore, MD, USA). DNA samples from normal tissues were purchased from Capital Biosciences (Rockville, MD, USA). Detailed sample information is provided in Tables 1–4.

DNA isolation, urine collection, LMW urine DNA fractionation and bisulfite (BS) treatment

Tissue DNA was isolated using the Qiagen (Valencia, CA, USA) DNeasy Tissue kit according to the manufacturer's instructions. Freshly collected urine was immediately mixed with 0.5 mol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0, to a final concentration of 10 mmol/L EDTA, and stored at -70°C . Total urine DNA was isolated by adding an equal volume of 6 mol/L guanidine thiocyanate (Sigma, St Louis, MO, USA) to thawed urine as described previously.³⁸ The LMW urine DNA, DNA less than 1 kb, was obtained from total urine DNA using carboxylated magnetic beads (Agentcourt Bioscience, Beverly, MA, USA), as previously developed by us.⁴² BS treatment was performed using Qiagen EpiTect Bisulfite conversion kits following the manufacturer's guidelines.

Preparation of reconstituted standards of methylated and unmethylated DNA for BS polymerase chain reaction (PCR) sequencing

A reconstituted standard consisted of a known amount of methylated DNA (M), Bisulfite-converted Universal Methylated Human DNA Standard (Zymo Research, Irvine, CA, USA), in a background of unmethylated DNA (UM), HeLa DNA, as shown by Yeo *et al.*¹² On the basis of quantification by BS-actin PCR,³³ reconstituted sample sets were prepared in the following ratios: (i) 0% M, 100% UM; (ii) 10% M, 90% UM; (iii) 25% M, 75% UM; (iv) 50% M, 50% UM; and (v) 100% M, 0% UM.

BS-PCR and sequencing

Bisulfite-treated DNA was amplified by PCR for P1, P2 and E1 regions. The primer sequences and annealing temperatures for each PCR reaction are listed in Table 5 and locations are indicated in Figure 1(a). Sequencing was performed at the NAPCore Facility at the Children's Hospital of Philadelphia (Philadelphia, PA, USA). Sequencing results were analyzed using ClustalW software (available at <http://www.ch.embnet.org/>) and Finch TV version 1.4.0 (Geospiza, Seattle, WA, USA).

Reference index for data analysis of BS-PCR sequencing

To normalize the primer or sequencing software bias, we established, on the basis of the BS-PCR sequencing data of the reconstituted standards from two reproducible experiments, a reference index for data analysis of each primer set (Fig. S1). Sequencing results were analyzed using chromatograms and comparisons of thymine versus cytosine peaks at each CpG site of the PCR products of reconstituted DNA standards. As shown in Figure S1, the

P1 and E1 primer pairs were not able to detect the DNA sample with 10% of methylated DNA, however, they could detect the reconstituted sample with 25% of methylated DNA. The P2 primer set, however, was the least sensitive of the three as it did not detect 25% methylated DNA. Thus, for all three primer sets, the fact that the height of the cytosine peak exceeded that of the thymine peak was an indication of greater than 50% methylation reproducibly. Therefore, we categorized the methylation of each CpG site into two groups: less than 50% methylation (T only and C > T); and 50% or more methylation (C > T and C only).

Quantitative methylation-specific PCR (qMSP) assays

Two qMSP assays, P1 and P2, were conducted in a Roche LightCycler 480 (LC480) system (Roche Applied Science, Mannheim, Germany). PCR conditions for both the P1 and the P2 assays were 95°C for 10 min (95°C for 10 s, 56°C for 15 s, 72°C for 10 s), 45 cycles with 1X LightCycler 480 Probes Master (Roche), 1.0 µM primers and 0.2 µM TaqMan probe. For the E1 assay, PCR conditions were 95°C for 5 min (95°C for 10 s, 56°C for 15 s, 72°C for 10 s) and 45 cycles.

For the short amplicon two-step qMSP targeting the P1 region, the step one reactions were set up at 10 µL with 1× Qiagen PCR buffer, 250 µM deoxyribonucleo-tide triphosphate mix, 1.0 µM primers, Hotstart Taq Polymerase Plus (Qiagen), at 95°C for 5 min (95°C for 30 s for 57°C for 30 s, 72°C for 30 s), and 25 cycles, 72°C for 4 min. The step one PCR product was diluted 1:10 and 1 µL of diluted PCR product was added as the template for the second PCR, which was set up in LC480 with 1× LightCycler 480 SYBR Green I Master (Roche) and 1.0 µM step two primers. The PCR conditions were 95°C for 5 min (95°C for 10 s, 58°C for 30 s, 72°C for 10 s) and 40 cycles. All experiments were performed in duplicate to ensure reproducibility. All MSP primer information is tabulated in Table 5.

Statistical analysis

Distribution of age and sex across the HCC and non-HCC groups was evaluated using Student's *t*-test and Fisher's exact test, respectively. For analysis of the results, appropriate statistical tests, as mentioned in the text, were performed using Graph Pad software (La Jolla, CA, USA). Receiver–operator curves (ROC) for all assays, parallel charts and distribution graphs were constructed using SPSS Statistics 20 (IBM, Armonk, NY, USA). ROC were compared using the StAR Tool⁴⁸ available online.

RESULTS

Comparison of the extent of DNA methylation on P1, P2 and E1 regions of the *RASSF1A* gene in hepatocarcinogenesis by BS–PCR sequencing

To test our hypothesis that the extent of DNA methylation of a particular promoter loci of the *RASSF1A* gene varies during the process of hepatocarcinogenesis, our strategy was first to comprehensively examine every CpG site in the region of interest by BS–PCR sequencing in a small cohort of tissue DNA and then validate the findings in a larger tissue DNA cohort using MSP, which is a higher throughput assay.

Figure 1(a) shows CpG sites (vertical bars) in the promoter and first exon regions of the *RASSF1A* gene, known as the P1, P2 and E1 regions,³⁵ along with locations of the BS-PCR sequencing primers used in this study. CpG sites within the 626-bp region studied were numbered 1–59 in the 5′-to-3′ direction.

We first established a reference index for sensitivity of each primer set for detecting methylation as detailed in Methods and in Figure S1. We then used this index to estimate the extent of DNA methylation of the three regions, P1, P2 and E1, on DNA isolated from tissues of various liver diseases. The data is summarized in Figure 1(b). To compare the methylation status of these three regions across the various tissue types, we pooled total CpG sites from all samples and assigned them to one of two categories: “low methylation” (<50% methylation) and “high methylation” (350% methylation). We then calculated the percent of CpG sites in the high-methylation category (Fig. 1c).

Hepatocellular carcinoma tissue samples had the highest level of methylation in all three regions. Although more than 85% of the CpG sites studied in each region were significantly methylated (350%) in the HCC samples, the methylation of the P1 region was significantly less in non-cancerous, diseased liver tissue (25.5% for hepatitis and 23.6% for cirrhosis), compared with E1 and P2 (57.9% and 38.6% for E1 and 77.5% and 67.5% for P2, for hepatitis and cirrhosis, respectively) as compared by Fisher's exact test ($P < 0.0001$) (Fig. 1c), suggesting that P1 is the region most specifically methylated in HCC.

DNA methylation of the *RASSF1A* gene varies among different tissue types

Next, we compared the DNA methylation pattern of normal liver tissue to that of other normal non-liver tissues. Although the methylation profile of the *RASSF1A* gene does not seem to exert a liver-specific pattern because a similar degree of DNA methylation was found in kidney and pancreatic tissue, most of the other normal tissues examined did not contain detectable levels of *mRASSF1A* when analyzed by BS-PCR sequencing. Interestingly, CpG sites 30 in the P2 region and 59 in the E1 region were consistently methylated across all tissue types examined (Fig. 1b).

Methylation of the P1 region is most specific for distinguishing HCC from hepatitis and cirrhosis of the three regions studied

In order to determine whether methylation of the P1 region is most specific for distinguishing HCC from hepatitis and cirrhosis in a larger sample size, higher throughput, qMSP assays for P1, P2 and E1, were developed, as shown in Figure 2(a). The performance of the MSP assays were first compared with BS-PCR sequencing data, using a panel of four cell lines: two hepatoma cell lines (Huh7 and HepG2) with both undetectable *RASSF1A* expression and methylated promoters^{22,24} and two non-hepatoma carcinoma cell lines (HeLa, human cervical adenocarcinoma, and SW480, colorectal adeno-carcinoma) with both high *RASSF1A* expression and unmethylated promoters.^{11,49} The data is summarized in Figure S2(a) for BS-PCR sequencing and in Figure S2(b) for the MSP assays. The methylation analysis obtained by these two different assay platforms is comparable, although only the MSP assays are quantitative (Fig. S2). Of interest, the overall methylation of the *RASSF1A* promoter region, quantified by the qMSP assays, was significantly greater

in the “RASSF1A-low” cell lines Huh7 and HepG2 than in the “RASSF1A-high” cell lines HeLa and SW480 ($P < 0.01$ by Student's t-test). Subsequently P1, P2 and E1 qMSP were performed to evaluate the finding from the BS-PCR sequencing in a larger sample size (120 HCC, 35 cirrhosis and 35 hepatitis tissues), as described in Table 3.

A ROC was constructed, and the area under the ROC (AUROC) was calculated for each region to evaluate the performance of methylation of these three regions as a biomarker to distinguish HCC from other liver diseases (cirrhosis and hepatitis) (Fig. 2b). The AUROC of the P1 (0.90) is statistically better than both E1 (0.84) and P2 (0.72) (P1 vs E1, $P = 0.0256$; P1 vs P2, $P < 0.0001$). Note, a sample size of 120 from the positive group (HCC) and 70 from the negative group (cirrhosis and hepatitis) achieves 85% power to detect a difference of 0.10 between the two diagnostic tests, with an AUROC of 0.90, and another diagnostic test with an AUROC of 0.80, using a two-sided z-test at a significance level of 0.05.^{50,51} Thus, this sample size is statistically powered to make a conclusion for the comparison of AUROC analysis. To compare the specificity, the sensitivity was fixed at 90%, 75%, 50% and 25%, as listed in the insert table in Figure 2(c); at 90% sensitivity, the P1 assay had a specificity of 72.9%, almost twice as specific as the E1 assay, which was only 38.6% specific, and far better than the P2 assay, which had a specificity of a mere 27.1% ($P < 0.0001$ by Fisher's exact two-tailed test when comparing the specificity of methylation for P1 vs P2 and P1 vs E1). A similar trend was observed at all other fixed values of sensitivity.

Methylation of the P1 region of the *RASSF1A* promoter during hepatocarcinogenesis

Having identified the P1 region as the most specifically methylated location in the *RASSF1A* gene in HCC, we studied its methylation distribution during hepatocarcinogenesis by analyzing the quantitative P1 qMSP data. We compared the methylation of hepatitis, cirrhosis, adjacent non-HCC and HCC tissues to each other by generating a 2-D dot plot (Fig. 3). Each open circle represents one case and the horizontal dark line indicates the median value.

As indicated in Figure 3, *mRASSF1A* increases gradually during the process of hepatocarcinogenesis. The amount of *mRASSF1A* is significantly greater in: (i) cirrhosis as compared with hepatitis; and (ii) HCC tissues as compared with hepatitis, cirrhosis and adjacent non-HCC tissues. There is no significant difference between the *mRASSF1A* between cirrhosis and the adjacent non-HCC tissues. To evaluate the performance of *mRASSF1A* in tissue as a marker for hepatocarcinogenesis, we constructed ROC curves (Fig. S3) and obtained AUROC values for distinguishing: (i) cirrhosis from hepatitis (AUROC = 0.697, 95% confidence interval [CI] = 0.563–0.831); and (ii) HCC and cirrhosis from hepatitis (AUROC = 0.881, 95% CI = 0.827–0.935).

Correlation of the age and methylation of the P1, P2 and E1 region of the *RASSF1A* gene

Previous studies have reported an age-dependent methylation of the *RASSF1A* promoter regions P2 and E1.^{11,31,52} To dissect the impact of age from that of hepatocarcinogenesis on the *mRASSF1A*, two analyses were performed. Spearman's rank correlation coefficient test between age and the level of methylation revealed that the age-dependent effect was only statistically significant in the hepatitis and cirrhosis group for the E1 and P1 regions ($P <$

0.05), and insignificant in adjacent non-HCC and HCC tissue ($P > 0.05$) (Table 6). To eliminate any influence of age-related methylation, we performed the Wilcoxon rank sum test between the HCC and the matched adjacent non-HCC tissue for each individual. All three regions had significantly higher methylation in the HCC tissue as compared with the matched adjacent non-HCC tissue ($P < 0.001$). Thus, the elevated *mRASSF1A* gene detected in HCC tissue resulted mainly from liver carcinogenesis.

Detection of *mRASSF1A* in the cell-free urine DNA of patients with hepatitis, cirrhosis and HCC by qMSP assay

We, and others, have shown that urine contains DNA from the circulation^{36,41,53,54} and that circulation-derived DNA in urine is fragmented into segments of length fewer than 300 bp that can be used to detect cancer-derived genetic modifications if the tumor is present. A short-amplicon, two-step qMSP assay targeting a 49-bp P1 region (including CpG sites 3–9, as referred to in the numbering in Fig. 1a) was developed for detecting cell-free circulating *mRASSF1A* in the urine to explore whether *mRASSF1A* (P1 region) can be a urine-based biomarker for HCC development.

Urine samples from 45 hepatitis, 50 cirrhosis and 78 HCC subjects were tested for the presence of *mRASSF1A* DNA using the P1 short-amplicon qMSP assays. Total urine DNA was isolated and the DNA less than 1 kb, designated as LMW urine DNA, was obtained with the purpose of enriching circulation-derived DNA. BS-converted LMW urine DNA derived from 0.2 mL urine was subjected to the P1 short-amplicon qMSP assays.

As indicated in Figure 4(a), the amount of *mRASSF1A* DNA detected was significantly higher in the urine samples from patients with cirrhosis and HCC as compared with the urine samples from patients with hepatitis (Fig. 4b, Mann–Whitney *U*-test, $P < 0.0001$, for hepatitis vs cirrhosis and hepatitis vs HCC). Also, *mRASSF1A* DNA was significantly higher in the HCC group as compared with the non-HCC group (hepatitis + cirrhosis) ($P < 0.0001$). However, there was no significant difference between the cirrhosis group versus the HCC group ($P = 0.069$), suggesting that *mRASSF1A* appears to be an early event in the hepatocarcinogenesis.

To evaluate the performance of *mRASSF1A* in urine as a biomarker for severe liver diseases or hepatocarcinogenesis, we generated ROC curves for distinguishing: (i) HCC from hepatitis; (ii) HCC from cirrhosis; (iii) HCC from cirrhosis and hepatitis; and (iv) HCC and cirrhosis from hepatitis. The AUROC was calculated for each comparison and listed in Figure 4(b). As expected, the detection of *mRASSF1A* in urine as a biomarker for severe liver diseases (cirrhosis and HCC) has an AUROC of 0.846 but an AUROC of 0.595 to distinguish HCC from cirrhosis. It was of interest to determine whether *mRASSF1A* would have the potential as a biomarker to detect α -fetoprotein (AFP) negative HCC, because this is the type of HCC that would be undetected by the most widely used biomarker, serum AFP level. We thus analyzed the incidence of *mRASSF1A* in the urine of HCC patients who were negative for serum AFP (<20 ng/mL). Encouragingly, the *mRASSF1A* DNA was detected in 36 of 44 (81.8%) urine samples (data not shown).

DISCUSSION

This study demonstrated that location of CpG dinucleotide methylation affects the specificity of *mRASSF1A* to distinguish HCC tissue from other liver diseases, such as cirrhosis and hepatitis. In addition, this study identified that among the three regions studied (P1, P2 and E1), methylation of the P1 region exerts the most specificity to HCC. To our knowledge, this is the first report examining the methylation of the P1 region in association with liver diseases, although there have been more than 30 publications studying the association of *RASSF1A* methylation and HCC carcinogenesis. We further showed that the aberrantly methylated *RASSF1A* gene could be detected in urine samples and is significantly higher in patients with cirrhosis and HCC, which suggests that *mRASSF1A* in urine can be utilized as a potential biomarker for liver carcinogenesis.

By comparing the methylation status of the three regions (P1, P2 and E1) by qMSP assays, we found that the promoter P1 region was far more specific than those examined in previous studies (P2 and E1).^{4,11,14,22,31,55–57} We also found that methylation of the *RASSF1A* promoter is an early event and increases progressively during the process of HCC pathogenesis. Wild-type Ras proteins have been described as tumor suppressors, namely, negative regulators of mitosis and the cell cycle, as well as activators of cell death; thus, it is likely that their malfunction (e.g. via aberrant hypermethylation) has great potential to trigger carcinogenesis.¹⁸

The impact on methylation by only liver carcinogenesis was demonstrated by comparing the level of methylation between adjacent non-HCC and HCC for each individual and by showing a significant elevation of methylation in all three regions. To our knowledge, this is the first report comparing the level of methylation between adjacent non-HCC and HCC by individual subjects to eliminate possible effects of age or other individual variations. It is an important comparison to distinguish HCC-related methylation from age-related methylation, particularly in this study, as the average age in the HCC group is significantly higher than that of the hepatitis and cirrhosis groups ($P < 0.05$, Table 3). Although age-related methylation was revealed in hepatitis and cirrhosis, it was significantly lower than HCC-related methylation.

Our data suggest that the P1 region outperforms the E1 region in liver tissues as a biomarker for distinguishing HCC from both cirrhosis and hepatitis. The methylated E1 region of the *RASSF1A* gene was previously detected in the serum of patients with HCC.^{10,57–60} Thus, it was of interest to explore the potential of the methylated P1 region of the *RASSF1A* gene as a screening biomarker by developing a short-amplicon qMSP assay for detection in urine. Encouragingly, similar to our tissue study (Fig. 3), *mRASSF1A* performed robustly as a biomarker with an AUROC of 0.831 for HCC when compared with hepatitis in urine (Fig. 4b). However, unlike the results obtained in the tissue study (Fig. 3), *mRASSF1A* was unable to discriminate between HCC and cirrhosis urine (AUROC = 0.595, Fig. 4). This inconsistency of the hypermethylation frequency of the *RASSF1A* gene between the tissue and periphery (in this case urine) has been previously reported in plasma and serum samples^{10,58,60} and has been attributed to *mRASSF1A* being an early common event in chronic liver diseases and the process of hepatocarcinogenesis. In spite of our efforts to map

the HCC-specific methylated P1 region in the *RASSF1A* promoter to boost its specificity as a biomarker for HCC, its performance for distinguishing HCC from cirrhosis was worse in urine than in liver tissues. One possibility is that there were undetectable cancerous cells in the cirrhotic liver that contributed to the *mRASSF1A* DNA in urine. Another possibility is that, similar to the observation in circulating DNA studies, methylation of the *RASSF1A* gene is an early event during liver carcinogenesis, which occurs either in cirrhosis or in the transition between cirrhosis to HCC.

We, and others, have suggested that circulation-derived urine DNA is mostly from DNA released by apoptotic cells. Malignant and preneoplastic cells often proliferate at abnormal rates, which is accompanied by an increase in apoptotic cell death,^{61,62} and this DNA may accumulate in the urine, which is essentially a collection of the body fluid. The statistically insignificant distinction of *mRASSF1A* observed in urine could also be the consequence of the dilution of the target DNA proportions between HCC and cirrhosis with the DNA coming from the rest of the body. Nevertheless, the amount of *mRASSF1A* DNA in the urine from patients with hepatitis was significantly less than that from patients with cirrhosis and HCC ($P < 0.001$, Fig. 4a), suggesting that *mRASSF1A* can serve as a screening marker for distinguishing HCC or cirrhosis from hepatitis (Fig. 4b) to bring patients more sophisticated imaging tests for early detection of HCC.

Unexpectedly, we observed hypermethylation of the P1 region in normal kidney tissue (Fig. 1b), and it is known that chronic hepatitis B virus/hepatitis C virus are both associated with glomerular disease.^{63,64} So, it is possible that the methylated P1 DNA detected in urine could be partially coming from the kidney, even though we only used LMW urine DNA as the substrate. In this study, the status of the existing glomerular disease for each patient was not clear but will be important to consider in the further development of this marker.

Overall, these results establish a locus-dependent CpG-site methylation pattern in the *RASSF1A* gene for the first time in liver cancer and demonstrate a progressive augmentation of *mRASSF1A* during hepatocarcinogenesis in the liver tissue. In addition, this study also demonstrated for the first time that methylation of the P1 region is associated with hepatocarcinogenesis and is the most specific region of methylation for HCC, as compared with methylation of the P2 and E1 regions. Most importantly, we also demonstrate the feasibility of using circulation-derived urine DNA as a viable approach for non-invasive early indication of hepatocarcinogenesis. We are currently exploring the prospect of combining *mRASSF1A* with other HCC-specific urine DNA markers, as suggested previously,^{8,52,53,55} to develop a sensitive and specific urine test for the identification of patients with a “high risk” of developing HCC, thereby greatly improving the prognosis of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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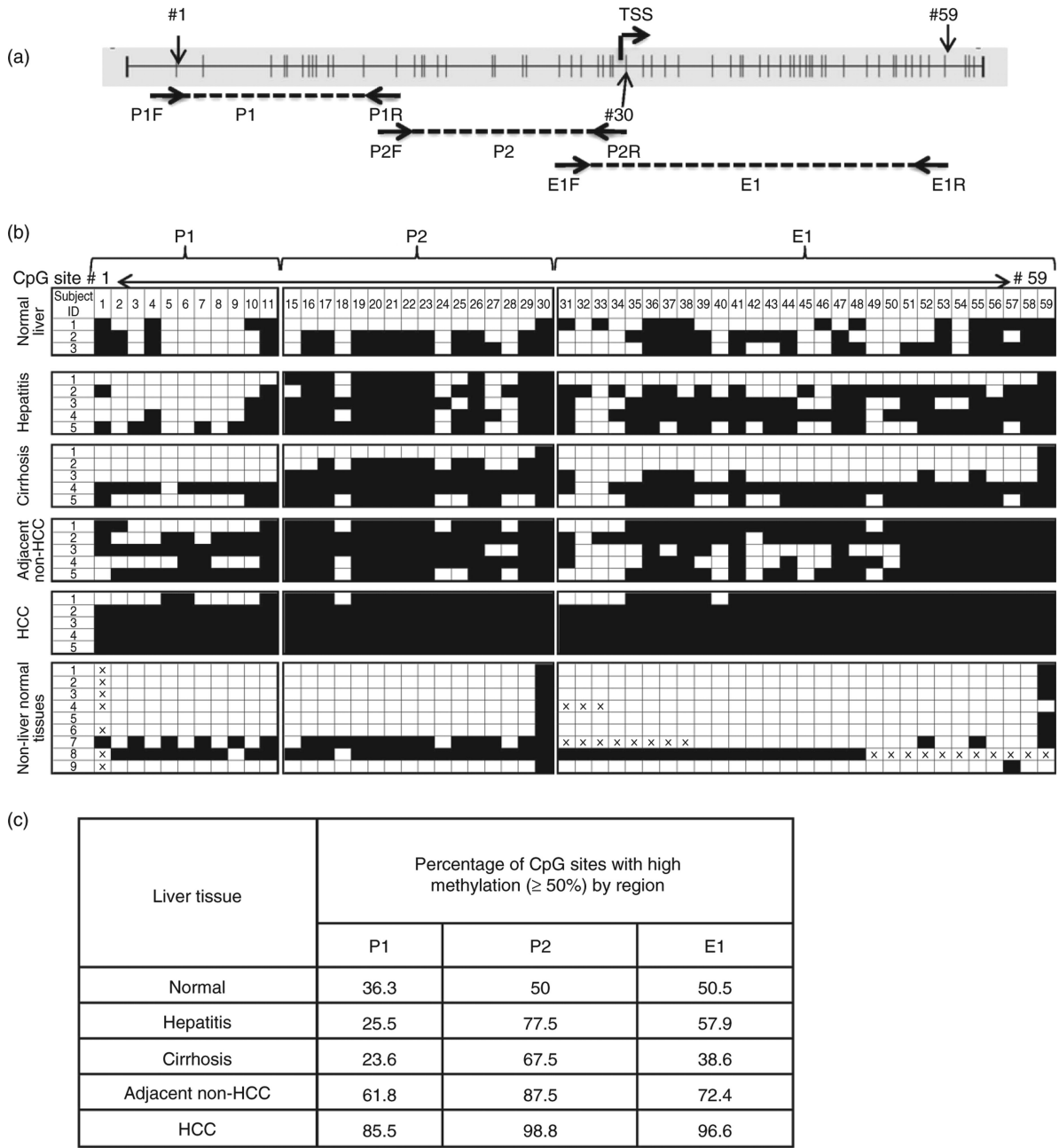


Figure 1. Bisulfite (BS) sequencing analysis of the *RASSF1A* promoter and first exon region in normal liver, diseased liver and non-liver normal tissues. (a) *RASSF1A* promoter region (GenBank accession no. DQ444319.1) and position of BS sequencing primers for three regions: P1 (nucleotides 357–548), P2 (nucleotides 530–736) and E1 (nucleotides 680–981). Vertical lines represent CpG sites; the transcription start site (TSS) is also indicated. CpG sites designated 1–59 are indicated with arrows. Note, the BS sequencing data for CpG sites 12–14 were not available due to sequencing-related technical issues; thus, they are not listed. (b) The extent of DNA methylation in three regions, P1, P2 and E1. Qualitative index of each CpG site (open boxes, $<50\%$ methylation detected; filled boxes, $\geq 50\%$ methylation detected)

is based on the relative heights of cytosine and thymine peaks within the chromatogram generated by a reconstituted standard as shown in the Figure S1. The non-liver normal tissues are: 1, spleen; 2, lung; 3, breast; 4, stomach; 5, colon; 6, trigeminal ganglion; 7, pancreas; 8, kidney; and 9, fetal liver. (c) The percentage of CpG sites with high (350%) methylation per total number of CpG sites examined in each region, P1, P2 and E1, in each liver tissue type as listed in the table. HCC, hepatocellular carcinoma.

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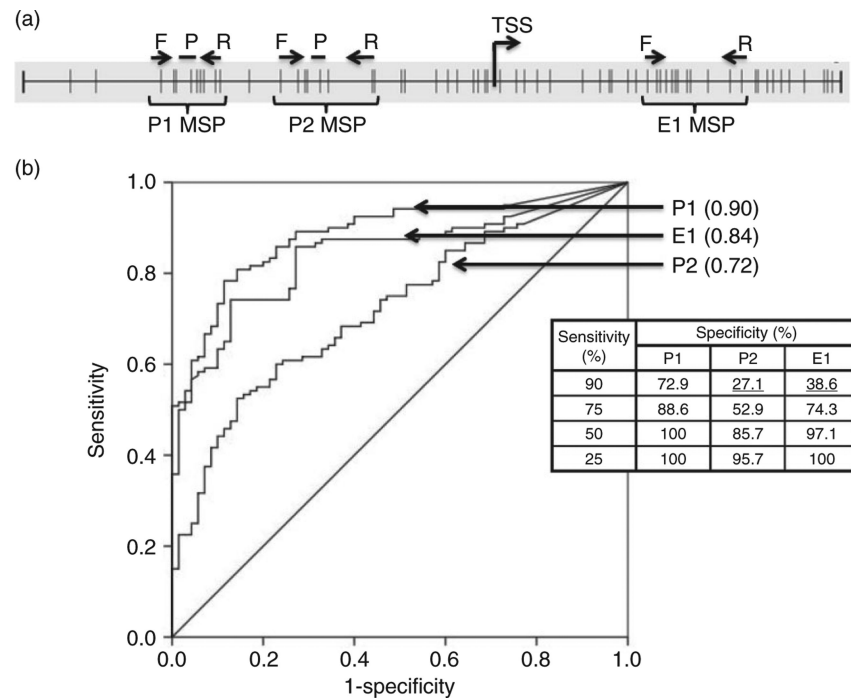


Figure 2.

Performance of the methylated P1, P2 and E1 regions of the *RASSF1A* gene as a biomarker for distinguishing hepato-cellular carcinoma (HCC) tissue from other liver disease tissues by quantitative methylation-specific polymerase chain reaction (qMSP) assays. (a) *RASSF1A* promoter. Vertical lines represent CpG sites. Forward (F) and reverse (R) primers and probes (P) are indicated for the three qMSP assays in their respective regions. The transcription start site (TSS) is also indicated. (b) Receiver–operator curves (ROC) for the *RASSF1A* gene as a marker to discriminate HCC ($n = 120$) from non-HCC liver tissues including hepatitis ($n = 35$) and cirrhosis ($n = 35$), generated by the qMSP assays of the P1, P2 and E1 regions, respectively. The quantity of methylated DNA was the average of two duplicate qMSP assays as detailed in Methods. Each area under the ROC and the specificity and sensitivity determined by the cut-off of 10 copies per input of 300 copies of DNA is shown in the inserted table.

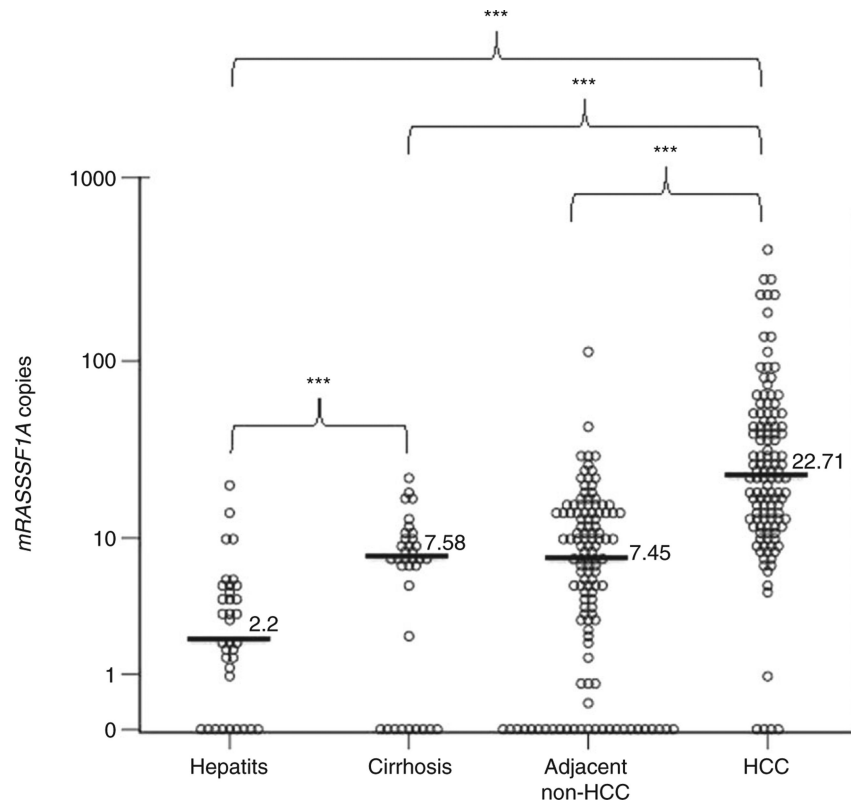


Figure 3.

Analysis of the methylated *RASSF1A* in the liver tissues during hepatocarcinogenesis. 2-D dot plot illustrating the distribution of *mRASSF1A* in the hepatitis ($n = 35$), cirrhosis ($n = 35$), adjacent nonhepatocellular carcinoma (HCC) ($n = 120$) and HCC ($n = 120$) liver tissues. The Y-axis indicates the number of *mRASSF1A* copies detected by the P1 quantitative methylation-specific polymerase chain reaction (qMSP) assay per 300 copies of bisulfite (BS)-actin input. *** $P < 0.001$ by Kruskal–Wallis test.

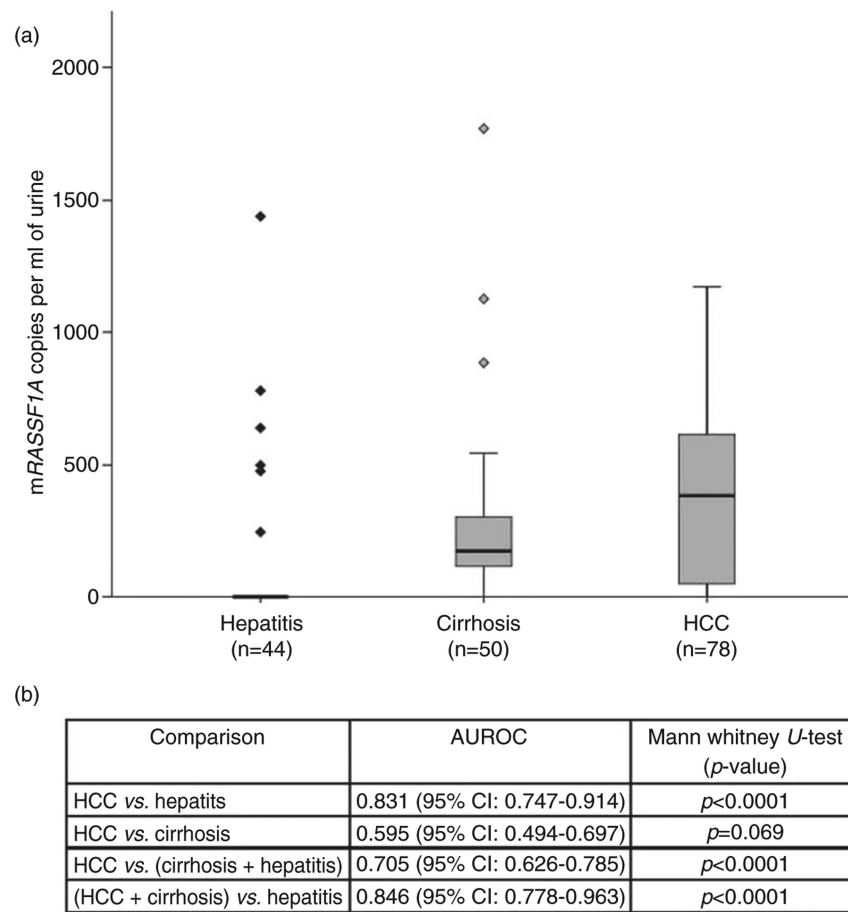


Figure 4. Analysis of methylated *RASSF1A* in the urine of patients with hepatitis, cirrhosis and hepatocellular carcinoma (HCC). (a) Box plot showing the distribution of *mRASSF1A* copies in the urine of patients with hepatitis, cirrhosis and HCC. The median value is indicated by a line within each box and the diamond symbols (\diamond , \blacklozenge) indicate outliers in the respective group. (b) Performance of *mRASSF1A* in urine as a biomarker for severe liver diseases. Area under the receiver–operator curve values for *mRASSF1A* in urine as a marker to discriminate severe liver diseases, HCC and cirrhosis from hepatitis and *P*-values of each comparison by Mann–Whitney *U*-test are listed in the inserted table.

Table 1

Clinicopathological characterization of the liver tissues analyzed by BS-PCR DNA sequencing

Characteristic	Normal (<i>n</i> = 3)	Hepatitis (<i>n</i> = 5)	Cirrhosis (<i>n</i> = 5)	HCC and adjacent non-HCC (<i>n</i> = 5)
Mean age (years) ± SD	35.67 ± 19.14 [†]	60.6 ± 14.9	60 ± 10.4	63 ± 10.7
Male/female (<i>n</i>)	3/0	1/4	3/2	4/1
HBV/HCV/both/non-viral or unknown (<i>n</i>)	–	1/2/1/1	2/2/0/1	1/2/0/2
Stage 1/2/3/4/unknown (<i>n</i>)	–	–	–	3/2/0/0/0
Grade 1/2/3/unknown (<i>n</i>)	–	–	–	0/2/3/0
Mean size of tumor (cm) ± SD	–	–	–	7.1 ± 2.2
AFP levels <20/>20 ng/mL/unknown (<i>n</i>)	–	–	–	1/4/0

AFP, α-fetoprotein; BS-PCR, bisulfite polymerase chain reaction; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; SD, standard deviation.

[†]The individual age for the three normal liver subjects is 57, 20 and 30 years.

Table 2

Subject information for non-liver tissues and fetal liver

Tissue	Sex	Age (years)	Pathological profile
Spleen	F	83	Normal
Lung	F	50	Normal
Breast	F	78	Normal
Stomach	M	27	Normal
Colon	F	85	Normal
Trigeminal ganglion	M	75	Normal
Pancreas	M	27	Normal
Kidney	F	63	Normal
Fetal liver	M	29 weeks	Normal

F, female; M, male.

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Table 3

Clinicopathological characterization of the liver tissues analyzed by MSP assays

Characteristic	Hepatitis (<i>n</i> = 35)	Cirrhosis (<i>n</i> = 35)	HCC and adjacent non-HCC (<i>n</i> = 120)	<i>P</i>
Mean age ± SD	55 ± 11.62	56 ± 13.8	60 ± 11.3	0.03 [†]
Male/female	17/18	23/12	81/39	0.16 [†]
HBV/HCV/non-viral or unknown (n)	3/22/9/1	6/16/0/13	59/29/4/28	–
Stage 1/2/3/4/unknown (<i>n</i>)	–	–	48/48/16/4/4	–
Grade 1/2/3/unknown (n)	–	–	18/74/23/5	–
Mean size of tumor ± SD	–	–	5.31 ± 3.69 cm	–
AFP levels <20 ng/mL/>20 ng/mL/unknown	–	–	62/53/5	–

AFP, α-fetoprotein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MSP, methylation-specific polymerase chain reaction; SD, standard deviation.

[†] Across all subjects (*n* = 190), age was analyzed by Student's *t*-test and sex by Fisher's exact test.

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Table 4

Clinicopathological characteristics of the urine analyzed in this study

Characteristic	Hepatitis (<i>n</i> = 44)	Cirrhosis (<i>n</i> = 50)	HCC (<i>n</i> = 78)
Mean age ± SD, years	51.4 ± 9.8	58.7 ± 10.7	58.8 ± 12.2
Male/female	25/18/1	33/17/0	58/20
HBV/HCV/both/none/unknown	2/1/21/20/0/0	11/24/4/10/0	39/19/2/9/9
Stage 1/2/3/4/unknown	–	–	21/32/18/2/5
Grade 1/2/3/unknown	–	–	9/47/18/4
Mean size of tumor ± SD, cm	–	–	5.2 ± 3.2
AFP levels, ng/mL, <20/>20/unknown	–	–	44/30/4

AFP, α-fetoprotein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; SD, standard deviation.

Table 5

Primer and probe sequences used for bisulfite DNA sequencing and MSP for detecting the P1, P2 and E1 regions of the *RASSF1A* gene (GenBank accession no. DQ444319.1)

Assay	Sequence	Annealing (°C)	CpG (#) [†]	Ref.
P1 BSP	F: 5'-gtaggtaagtgtgtttt-3'	54	1–12	26
	R: 5'-ttaccttcttccctcctt-3'			
P2 BSP	F: 5'-aggagggaaggaggtaag-3'	53	13–30	26
	R: 5'-taacttaaacgtaacaaa-3'			
E1 BSP	F: 5'-aagtcgggttcgtttgtgtt-3'	53	24–59	26
	R: 5'-ccccaaataaaatgccacaaaa-3'			
P1 MSP	F: 5'-agaaatcgggtatttcgc-3'	56	3–11	–
	R: 5'-caccccgaacgaccacaa-3'			
	Probe: 6FAM-accacaacgacgacgcgc-BHQ1			
P2 MSP	F: 5'-gggtttgcgagagcgcg-3'	56	14–20	–
	R: 5'-aaaccgcgaataaaaacc-3'			
	Probe: 6FAM-cgcgaaccgaacgaa-BHQ1			
E1 MSP	F: 5'-gtgtaacgcttgcgtatc-3'	60	42–54	17
	R: 5'-aaccgcgaactaaaacga-3'			
P1 short-amplicon MSP	Step 1	57	3–9	–
	F: 5'-aaatcgggtatttcgc-3'			
	R: 5'-gctcttcgtgtgtggaccacacgacgac-3'	58		
	Step 2			
	F: 5'-acgggtatttcgctg-3'			
R: 5'-ttcgtgtgtgtggac-3'				

BSP, bisulfite-specific polymerase chain reaction; F, forward; MSP, methylation-specific polymerase chain reaction; R, reverse.

[†]CpG site numbers refer to the numbering in Fig. 1(a). The artificial sequences for the first step PCR of the P1 short-amplicon MSP are underlined.

Table 6

Correlation between age and methylation levels of the P1, P2 and E1 regions of the *RASSF1A* gene in hepatitis and cirrhosis, adjacent non-HCC tissue and HCC tissues

Spearman's rank correlation coefficient	Age correlation with methylation, Spearman's rho (<i>P</i> -values)		
	P1	P2	E1
Hepatitis and cirrhosis	0.20 (0.045 [†])	0.09 (0.221)	0.23 (0.025 [†])
Adjacent non-HCC	-0.006 (0.474)	-0.08 (0.455)	0.01 (0.455)
HCC	-0.09 (0.171)	-0.14 (0.064)	-0.01 (0.453)

HCC, hepatocellular carcinoma.

[†]Correlation is significant at the 0.05 level (one-tailed).

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