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Evaluation of *INK4A* promoter methylation using pyrosequencing and circulating cell-free DNA from patients with hepatocellular carcinoma

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Abstract

Background—Hyper-methylation of CpG dinucleotides in the promoter region of inhibitor of cyclin-dependent kinase 4A (*INK4A*) has been reported in 60%–80% of hepatocellular carcinoma (HCC). As *INK4A* promoter hypermethylation event occurs early in HCC progression, the quantification of *INK4A* promoter methylation in blood sample may represent a useful biomarker for non-invasive diagnosis and prediction of response to therapy.

Methods—We examined *INK4A* promoter methylation using circulating cell-free DNA (ccfDNA) in a total of 109 serum specimens, including 66 HCC and 43 benign chronic liver diseases. Methylation of the individual seven CpG sites was examined using pyrosequencing.

Results—Our results showed that there were significantly higher levels of methylated *INK4A* in HCC specimens than controls and that the seven CpG sites had different levels of methylation and might exist in different PCR amplicons. The area under receiver operating characteristic (ROC) curve was 0.82, with 65.3% sensitivity and 87.2% specificity at 5% (LOD), 39.0% sensitivity and 96.5% specificity at 7% LOD, and 20.3% sensitivity and 98.8% specificity at 10% LOD, respectively.

Conclusions—Our results support additional studies incorporating *INK4A* methylation testing of ccfDNA to further validate the diagnostic, predictive, and prognostic characteristics of this biomarker in HCC patients. The knowledge of the existence of epi-alleles should help improve assay design to maximize detection.

Keywords

circulating cell-free DNA; DNA methylation; hepatocellular carcinoma; *INK4A*; pyrosequencing

Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death worldwide. The HCC incidence in the USA has tripled since the early 1980s [1, 2]. Although various

therapeutic strategies are available to treat HCC including surgical resection, ablation, liver transplantation, transcatheter arterial chemoembolization (TACE), and the multikinase inhibitor sorafenib, the 5-year survival of HCC is still low, especially when the disease is diagnosed at advanced stages [2]. Therefore, there is an urgent need to develop more effective diagnostic and treatment strategies for HCC. Several genetic/epigenetics changes have been identified that play key roles at the tumor initiation, promotion, or progression stages of HCC [3, 4]. Inhibitor of cyclin-dependent kinase (CDK) 4 (*INK4A*) gene encodes the p16 protein, a critical cell cycle regulator [5, 6]. p16 interacts with CDK4 and CDK6 (CDK4/6) and blocks their ability to phosphorylate and inactivate retinoblastoma (RB) protein; this suppresses cell cycle progression and cell proliferation [5, 6]. Promoter hypermethylation of *INK4A*, resulting in the block of *INK4A* RNA expression [7] and reduced *INK4A* RNA in HCC tissue specimens [8, 9], has been attributed to the loss of p16 expression in 60%–80% of HCC specimens [10–14].

Recently biomarkers targeting tumor DNA in blood samples have shown clinical utility as a non-invasive “liquid biopsy” for solid tumors. For example, the plasma-based septin 9 (*SEPT9*) hypermethylation assay has been used clinically in colorectal cancer [15, 16]. Studies have also supported the use of a blood-based *INK4A* methylation assay as a biomarker for HCC. For example, it has been reported that as HCC progresses an increasing amounts of cell-free (cf) tumor DNA is released into the circulation [17]. *INK4A* DNA hypermethylation is detected not only at high frequencies in HCC liver tissues, but also in cf DNA in blood samples of HCC patients [18–20]. Molecular predictive biomarkers have been studied to stratify HCC patients for the most effective treatment strategies [21, 22] and analysis of *INK4A* methylation may be critical in the development of agents targeting DNA methylation or p16-CDK4/6-RB pathway.

Much of the studies have evaluated *INK4A* promoter methylation using a variation of an allele-specific PCR assay known as methylation-specific PCR (MSP) which relies on the conversion of cytosine to uracil by sodium bisulfite [23]. However, this method cannot identify which CpG dinucleotides are methylated or determine the levels of methylation at nucleotide resolution. MSP is also subject to PCR allele dropout due to the existence of heterozygous epi-alleles after bisulfite conversion of CpG sequences [24]. Pyrosequencing is a valuable tool for methylation analysis for several reasons. It is a highly quantitative method that measures the percentage of methylation at a given CpG site by calculating the ratio of C/T (methylated to unmethylated C) in a sample. Quantitative measurement of methylation is important because low levels of methylation (e.g., below the threshold of transcriptional silencing) may not be biologically important. In the case of *INK4A*, where a certain degree of methylation may occur in benign liver disease, the quantitative nature of the pyrosequencing method allows one to establish a limit of quantification value to provide a more accurate assessment of tumor-specific hyper-methylation. Also, several CpG dinucleotides can be analyzed simultaneously, which allows specific sites to be studied separately. Additionally, forward and reverse polymerase chain reaction (PCR) primers can be designed to anneal to sequences that do not have CpG sites; therefore, the PCR amplification is not subject to the status of CpG methylation. Subsequent pyrosequencing

can detect and quantify various sequences in the PCR amplicons and may, therefore, increase coverage of CpG sites and sensitivity of the methylation assay [25].

In this study, we examined *INK4A* methylation using circulating cell-free DNA from patients with HCC and benign liver conditions by pyrosequencing. The purpose of the study was to evaluate the clinical sensitivity and specificity of pyrosequencing *INK4A* methylation assay using serum DNA of HCC patients.

Materials and methods

Blood specimens

This study included 109 blood specimens collected during the period of March 2012 to June 2013 from 66 patients with HCC and 43 with benign liver diseases (chronic hepatitis and liver cirrhosis). Whole blood samples were collected without preservatives and stored at 4°C, serum was then separated within 72 h and stored at –70°C until DNA extraction [26]. HCC was diagnosed based on imaging characteristics, α fetal protein (AFP), pathology evaluation, and past medical history following established practice guidelines [2, 27, 28]. The study was approved by the UTMB's Institutional Review Board (IRB).

Control cell lines

Human melanoma cell lines Mel888, A375, and Mel624 were used as methylation controls. *INK4A* has been reported to be highly methylated in Mel888 but not in A375 cells [29]. A375 and 624Mel cells harbor *INK4A* nonsense mutation and an 18 bp deletion, respectively [30]. Mel888, A375, and Mel624 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum in plastic tissue culture plates in a humidified atmosphere containing 5% CO₂ at 37°C. These cell lines were kindly provided by Dr. Ruth Halaban (Yale University School of Medicine, New Haven, USA) and Dr. Stuart Aaronson (Mount Sinai School of Medicine in New York City, USA), respectively.

DNA extraction

For the *INK4A* methylation assay, genomic DNA was extracted and purified from 1 to 2 mL of serum using QIAvac 24 Plus and QIAamp Circulating Nucleic Acid Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Purified DNA was eluted with 60 μ L of elution buffer and stored at –20°C. The typical amount of DNA purified from 1 to 2 mL of serum was approximately 1–2 μ g/60 μ L. Genomic DNA was extracted from cultured melanoma cells using Blood & Cell Culture DNA Mini Kit (Qiagen Inc.) according to the manufacturer's instructions.

Sodium bisulfite treatment

Conversion of unmethylated cytosine to uracil in the genomic DNA was performed with EpiTect Bisulfite Kit (Qiagen Inc.) according to the manufacturer's instructions. Briefly, 500 ng of extracted circulating DNA in a volume of 20 μ L of each sample was incubated with sodium bisulfite (pH 5.0) for 16 h at 50°C. For genomic DNA extracted from cell lines and EpiTect PCR Control DNA, 200 ng of DNA was used in the conversion reaction. Bisulfite

conversion was performed in 85 μ L of Bisulfite Mix Solution, and 35 μ L of DNA Protect Buffer. The total reaction volume was 140 μ L. The procedure was performed according to the manufacturer's instructions. After the conversion reaction and a cleanup step, bisulfite-converted DNA was eluted in 30 μ L elution buffer and used for methylation analysis or stored at -20°C .

PCR and pyrosequencing

Bisulfite-treated genomic DNA was PCR amplified using P16 Pyro-Mark PCR Kit (Qiagen Inc.) with primers designed to bind only to CpG-free sequences in the *INK4A* promoter [25]; therefore, amplification was not affected by methylation status of the templates. Briefly, *INK4A* sequence was amplified from 5 μ L of bisulfite-treated DNA in a 26 μ L PCR reaction using biotin-labeled PCR primers. PCR reaction was performed using Applied Biosystems GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA, USA). The biotinylated PCR amplicons from 10 μ L of PCR reaction mix was captured on streptavidin-coated beads and underwent pyrosequencing using PyroMark Q24 CpG p16 Kit (Qiagen Inc.). The methylated fraction (%) of each of the seven CpG sites (Figure 1A) was measured by pyrosequencing as shown in representative pyrograms (Figure 1B, C).

Each PCR and pyrosequencing assay included a PCR contamination control without DNA template, unmethylated DNA to control background levels of methylation, a low positive control (10%) of assay quantification from mixing of fully methylated and un-methylated control DNA using EpiTect PCR Control DNA Set (Qiagen Inc.). Genomic DNA extracted from Mel888 cells that show methylation of all the seven CpG sites was used to control bisulfite conversion as well as to serve as a high level methylation positive control.

Methylation-specific PCR

Methylation-specific PCR (MSP) was performed using a protocol used for several HCC-related studies [11, 18, 23]. The forward PCR primer ends at the C of the third CpG of the region covered by PyroMark pyrosequencing (Figure 1A). The unmethylated primer sequences were 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'-CAACCCCAAACCACAACCATAA-3'. The methylated primer sequences were 5'-TTATTAGAGGGTGGGGCGGATCGC-3' and 5'-GACCCCGAACCGCGACCGTAA-3'. Five microliters bisulfite-treated genomic DNA, as described above, was subjected to methylation-specific PCR using GoTaq polymerase (Promega, Madison, WI, USA) in a 25 μ L PCR reaction. The PCRs were carried out by denaturation at 95°C for 5 min, followed by 55 cycles including denaturation, annealing and elongation at 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s, respectively, and a final extension of 72°C for 4 min. The unmethylated PCRs were run as above except by using 65°C annealing temperature for 30 s [11, 18, 23]. PCR amplicons were separated by electrophoresis on 3% agarose gel.

Subcloning of PCR amplicons and sequencing analysis of CpG epi-alleles

Five microliters of bisulfite-converted circulating DNA was used for PCR amplification in a volume of 25 μ L reaction containing 1X Green GoTaq Reaction Buffer, 0.2 mM dNTP, 15 pmol of forward and reverse primers, 1.5 units of GoTaq DNA Polymerase (Promega). Primers consisted of the following: forward primer 5'-

GGAGGGGTTGGTTGGTTATTAG-3', reverse primer 5'-CTACCTACTCTCCCCCTCTC-3' that generated a 77 bp PCR amplicon (NM_000077.4; Figure 1A). The primers were placed to only cover unique sequences lacking CpG sites as designed for pyrosequencing assays [25]. Amplification was in an Applied Biosystems GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA, USA) using 35 cycles. Cycling parameters were: denaturation at 95°C for 45 s, annealing at 55°C for 40 s and elongation at 72°C for 40 s. Hot start was at 95°C for 5 min and 72°C for 5 min at the end allowed complete elongation of all DNA products. PCR products were purified using QIAquick Gel Extraction Kit (Qiagen Inc.) according to the manufacturer's instructions. The purified PCR product was cloned into TA cloning vector using TOPO TA Cloning Kit for Sequencing (Life Technologies, Grand Island, NY, USA) following the company's instruction manual. About 3 µL of the cloning reaction was transformed into Top10 *Escherichia coli* competent cells; the cells were plated onto LB plates containing kanamycin (50 µg/mL). Plasmid DNA was prepared using QIAprep Spin Miniprep Kit (Qiagen Inc.) from overnight culture of a single colony. Sequencing of plasmid DNA with PCR product was performed by Applied Biosystem's 3130xL Genetic Analyzer (Life Technologies, Grand Island, NY, USA).

Statistical analysis

Statistical analyses were performed using the computing environment R (R Development Core Team, 2005. <http://cran.r-project.org>) and SAS version 9.2 (SAS Institute, Cary, NC, USA). Descriptive statistics were calculated, and the Wilcoxon rank-sum test was used to determine whether *INK4A* methylation differed significantly between non-HCC controls and patients with primary HCC. Non-parametric Spearman's rank order correlation coefficients (ρ) were calculated to determine whether associations existed between *INK4A* methylation and AFP. Logistic regression models were compared for their ability to discriminate HCC from control patients using the area under the curve (AUC) of receiver operating characteristic (ROC) plots from software Sigmaplot (Systat Software, San Jose, CA, USA). Data were further analyzed by Student's t-test and analysis of variance (ANOVA) model using GraphPad statistical software (GraphPad Software Inc., San Diego, CA, USA) and by Mann-Whitney test using the unpaired Wilcoxon rank sum test from R. The statistics of the pyrosequencing data were calculated by natural logarithm and unpaired t-test with Welch's correction using GraphPad statistical software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant at the α level of 0.05 for two-sided tests.

Results

Quantification of *INK4A* promoter methylation using pyrosequencing

Pyrosequencing was used to measure DNA methylation levels of seven CpG sites (underlined) within the 36 bp sequence 5'-gggtggggcggaccgcgcgcctcggcgcgcgcga-3' in exon 1 of the *INK4A* gene (Ensembl: ENSG00000147889) [25, 31], corresponding to nucleotide positions 235–270 of *INK4A* mRNA (NM_000077.4; Figure 1A).

The sensitivity, accuracy, and precision of the pyrosequencing assay was assessed in a series of dilutions, in which fully methylated DNA (Qiagen Inc.) was diluted stepwise (100%–2%)

with unmethylated DNA (Qiagen Inc.) and analyzed in triplicates. The methylation levels of the seven sites for the unmethylated DNA (0%), 2%, 5%, 10%, 20%, 50% diluted DNA, and fully methylated DNA (100%) were 1.8, 3.5, 1.6, 1.8, 3.3, 1.5, 1.5 for 0%; 2.2, 3.5, 2.0, 2.2, 3.5, 1.7, 1.5 for 2%; 5.2, 7.1, 5.0, 5.2, 6.5, 4.7, 4.6 for 5%; 9.9, 12.5, 9.9, 9.9, 11.7, 8.9, 8.6 for 10%; 18.2, 21.2, 17.8, 17.8, 20.3, 16.8, 15.8 for 20%; 47.7, 50.8, 47.0, 45.5, 50.0, 43.0, 40.3 for 50%; and 96.1, 99.9, 96.2, 94.6, 99.7, 90.5, 85.3 for 100%, respectively. We also examined *INK4A* methylation of genomic DNA samples extracted from cell pellets of human melanoma cell lines Mel888, A375, and Mel624. Our PyroMark Q24 CpG p16 results showed that all the seven CpG sites were methylated at levels between 80% and 100% in Mel888 cells, but not methylated in A375 and Mel624 cells based on a limit of detection (LOD) of 5% (data not shown). We further confirmed the methylated/unmethylated status of unmethylated and methylated EpiTect control DNAs and DNAs extracted from melanoma cell lines using a published *INK4A* MSP assay that shares *INK4A* sequence coverage with the PyroMark Q24 CpG p16 test [11] (data not shown).

***INK4A* methylation in circulating cell-free DNA from patients with HCC and benign liver diseases**

INK4A methylation levels of the individual seven CpG sites were assessed using serum samples from patients diagnosed with HCC (66 samples from 66 patients) and benign chronic liver diseases (43 samples from 43 patients). Methylation of the individual seven specific CpG sites was quantified using pyrosequencing. Supplemental Tables 1 and 2 (which accompanies the article at <http://www.degruyter.com/view/j/cclm.2014.52.issue-6/issue-files/cclm.2014.52.issue-6.xml>) listed methylation assay results in HCC and non-HCC cases, respectively, along with patient demographic information (ethnicity, age, and gender), hepatitis C virus (HCV)/hepatitis B virus (HBV) positivity, and AFP results. Also included are tumor stages measured around the same time period of blood collection for *INK4A* assay (Supplemental Table 1), and clinical diagnoses of benign liver diseases (Supplemental Table 2). As shown in Figure 2A, when the highest value of methylation of the seven CpG sites was used for each of the samples, there were significantly higher levels of *INK4A* methylation in HCC samples than in the benign liver disease samples (odds ratio=1.38 with 95% confidence interval of 1.05–1.79; $p<0.05$, Wald test). Further analysis showed that there was significant difference between levels of *INK4A* methylation in early stages of HCC (stage I and II) and non-HCC cases (t-test, $p=0.00$).

For distinguishing HCC from non-HCC cases using methylation levels, we used ROC analysis to set the optimal cut-off to achieve the best sensitivity and specificity of the test. When the highest value of methylation of the seven CpG sites was used for each of the samples, the AUC was 0.82 (Figure 2B), with 65.3% sensitivity and 87.2% specificity at 5% limit of detection (LOD), 39.0% sensitivity and 96.5% specificity at 7% LOD, and 20.3% sensitivity and 98.8% specificity at 10% LOD, respectively (Table 1). The average methylation levels of the seven sites were 6.1%, 8.7%, 6.0%, 6.3%, 7.7%, 5.6%, and 5.6% for HCC cases, and 1.6%, 2.9%, 1.5%, 2.1%, 2.5%, 1.3% and 2.1% for non-HCC cases. There was no statistical difference between levels of CpG methylation of the individual sites in HCC and non-HCC cases. HCC samples were significantly more methylated than non-HCC samples at each of the CpG sites and on average across all sites (non-HCC: 2.0% vs.

HCC: 6.6%) ($p < 0.05$ for individual sites as well as the average of the seven CpG sites, t-test).

Using the highest value of methylation of the seven CpG sites in each of the samples, the positive rates were 74.2% (49/66) at 5% LOD, 50.0% (33/66) at 7% LOD, and 27.3% (18/66) at 10% LOD for HCC cases, and 18.6% (8/43) at 5% LOD, 4.7% (2/43) at 7% LOD, and 3.3% (1/43) at 10% LOD for non-HCC cases. The Wald test in ANOVA model showed that there was significant difference among the mean values of *INK4A* methylation across the HCC stages. As a multiple comparison, post hoc test (LSD) procedures showed that stages I and IV, stages II and IV, and stages III and IV were statistically significant ($p = 0.02$).

Since *INK4A* methylation and AFP results were skewed distributions beyond the normal distribution, we calculated the Spearman's correlation coefficients to check the association between *INK4A* methylation and AFP. In the HCC cases, the correlation coefficient was statistically significant at the 0.05 level (two-tailed), but the strength was not strong (Spearman's correlation coefficient = 0.30). No significant correlation was detected in non-HCC cases. In combined HCC and benign cases, the correlation was significant at the 0.05 level (two-tailed, Spearman's correlation coefficient = 0.21). Using LOD cut-offs of 5% for *INK4A* methylation [31] and 200 $\mu\text{g/L}$ for AFP [28], 49 and 30 positives, respectively, were detected for the 66 HCC patients; 23 of the 49 *INK4A* positive HCC cases were only positive for *INK4A*, five of the 30 AFP positive HCC cases were only positive for AFP; 26 cases were positive for both *INK4A* and AFP (Supplemental Table 1); eight and one positives, respectively, were detected for the 29 non-HCC cases that had both values, and none of the *INK4A* and AFP positive cases co-existed in non-HCC samples (Supplemental Table 2). Adding *INK4A* methylation (LOD at 5%) to AFP (LOD at 200 $\mu\text{g/L}$) increased positive rates of the 66 HCC cases from AFP's 45.5% (30/66) and *INK4A*'s 74.2% (49/66) to the combined 80.3% (53/66) (Figure 2C).

CpG epi-alleles

In methylation positive specimens, the seven CpG sites may exist as fully methylated and unmethylated alleles, or as mixtures of heterogeneously methylated epi-alleles [24]. Although capable of quantifying methylation at individual CpG sites, the bisulfite-pyrosequencing method we used cannot phase the seven CpG epi-alleles that may exist in a sample and therefore, cannot determine whether co-existing methylated CpG dinucleotides occur in the same (cis) or different (trans) strand of *INK4A* DNA sequence. To examine heterogeneous methylation of the seven CpG sites, we performed PCR and TA cloning of PCR amplicons from three bisulfite-treated genomic DNA samples (cases 46, 58, and 110, listed in Table 2). After bacterial transformation, subcloning, and mini-plasmid DNA preparation, *INK4A* DNA inserts were analyzed by Sanger sequencing of 40 single clones of each case for a total of 120 plasmid DNA preparations that carried *INK4A* PCR amplicons. As summarized in Figure 3, we identified 13 epi-alleles including eight in cases 46 and 110, and seven in case 58. Sequences with methylation between one and six CpG sites were 32.5% of the 120 clones. Therefore, the seven CpG sites in the *INK4A* promoter could have different levels of methylation, and might exist in different DNA strands (i.e., in trans).

Discussion

In this study, we used pyrosequencing to retrospectively examine methylation status of seven CpG sites in the promoter region of *INK4A* using circulating cell-free DNA from patients with HCC and benign liver conditions. We found that pyrosequencing assay provided comprehensive coverage and quantification of multiple CpG sites, consistent with previous reports [25, 31]. The assay identified significantly higher levels of *INK4A* methylation in the HCC group than in benign liver disease groups and in advanced than in early stage HCC. The AUC curve was 0.82 with 65.3% sensitivity and 87.2% specificity at 5% (LOD), 39.0% sensitivity and 96.5% specificity at 7% LOD, and 20.3% sensitivity and 98.8% specificity at 10% LOD, respectively. Further analysis showed that there was significant difference between levels of *INK4A* methylation in early stages of HCC (stage I and II) and non-HCC cases (t-test, $p=0.00$).

It is well known that localized liver cancers can have curable outcomes, but most patients are not diagnosed until the cancer has advanced. Currently, many small liver nodules cannot be accurately diagnosed using available diagnostic tools, such as imaging scans and serum AFP levels. Rather, such nodules are allowed to increase in size for several months in order to diagnose HCC [2, 28]. This may result in decreased treatment efficacy of HCC cases. Therefore, there is an urgent need to diagnose HCC when the tumor nodules are small. As summarized in Table 2, several studies have evaluated the use of circulating *INK4A* methylation to improve the diagnosis of HCC. The positive rates of methylated *INK4A* in these studies vary, which may be due to differences in assay sensitivity and patients enrolled in the studies. Of note, when 5% LOD is used, our study has a positive rate of 74.2%, the highest of the reported using ccfDNA as listed in Table 2. Although an increase in assay sensitivity may lead to more false positives, *INK4A* methylation is only part of the several markers used for HCC diagnosis [28]. For example, *INK4A* positivity in non-HCC cases listed in Supplemental Table 2 does not provide a diagnosis of HCC without imaging, AFP and other clinical parameters. However, given the knowledge of *INK4A* in HCC, such positive result may indicate closer follow-up of these cases, as detection of circulating methylated *INK4A* is linked to subsequent diagnosis of HCC [20].

The increased positive rate may be attributed to the design of the PyroMark CpG p16 assay to cover more CpG sites to capture additional epi-alleles. MSP was used in several HCC studies (Table 2). The C residue of the third CpG of the region covered by PyroMark pyrosequencing (Figure 1A) is the last nucleotide at the 3' terminus of the forward primer sequence of a commonly used *INK4A* MSP assay [8, 10, 13, 18, 23], which is an extremely important site for the accurate, successful amplification of the template [37]. Due to the existence of epi-alleles by heterogeneous methylation of neighboring CpG sites, a fraction of methylation CpGs that do not fit the specific MSP primer design may fail to be detected. In general, real-time PCR can reach higher assay sensitivity than pyrosequencing. For example, real-time PCR can detect somatic *KRAS* mutation as low as 1% of total DNA [38], whereas to our knowledge the most sensitive *KRAS* mutation assay based on pyrosequencing is 2.3% of total DNA [39]. The knowledge of *INK4A* CpG epi-alleles (Figure 3) may be used to facilitate the design of a multiplex allele-specific real-time PCR assay to cover several CpG sites to increase assay sensitivity.

Further analyses showed that levels of circulating methylated *INK4A* weakly correlate with levels of AFP, consistent with a previous report [18]. However, such correlation has not been identified in two other studies [20, 33]. The reasons for the discrepancy are not clear but may relate to different sensitivity of *INK4A* methylation assays, various cut-off values of AFP, and differences in the patient population included in the studies. Importantly, our data suggest that circulating cell-free methylated *INK4A* may complement AFP to improve the positivity in HCC; the positive rates are 45.5%, 74.2%, and 80.3% in the 66 HCC cases for AFP, *INK4A* methylation, and in combination, respectively, using LOD cut-offs of 200 µg/L for AFP [28] and 5% for *INK4A* methylation [31]. Supporting this notion, it has been reported that adding *INK4A* methylation status to AFP can increase the diagnostic sensitivity of HCC from 60% to 80% [18]. Additional investigations including prospective studies are needed to examine the clinical performance of the *INK4A* assay for HCC diagnosis when combined with imaging scans, pathology findings, and AFP measurement.

We detected increased levels of circulating methylated *INK4A* in patients with late stages of HCC. The origin of circulating cf tumor DNA may include shedding from necrotic/apoptotic tumor cells in tumor nodules, tumor-derived exosomes, cellular fragments or lysis of circulating tumor cells (CTCs) in the bloodstream [15, 40]. Increased circulating tumor DNA should correlate with invasion, dissemination and active metabolism of tumor cells, hallmarks of cancers [41]. It has been reported that *INK4A* promoter methylation was associated with HCC metastasis [42, 43]. The higher levels of methylated *INK4A* in blood samples of later stage HCC should promote assay specificity, which may allow the use of circulating *INK4A* methylation as a standalone predictive marker for HCC treatment. Two DNA methyltransferase inhibitors, azacitidine (Vidaza; Celgene) and decitabine (Dacogen; Eisai), have gained approval by the Food and Drug Administration (FDA) for myelodysplastic syndromes (MDS). Studies are continuing to refine the use of these drugs for leukemia as well as solid tumors, including HCC [44]. *INK4A* hypermethylation leads to loss of p16 expression; p16 loss can functionally mimic CDK4 activation in tumor cells that have constitutively activated mitogens [45, 46]. Circulating *INK4A* hypermethylation may serve as a companion biomarker for treatment with CDK4 inhibitors that are in active clinical development (<http://clinicaltrials.gov/>) and may be effective in p16-negative HCC. Therefore, a sensitive, specific, non-invasive, and quantitative *INK4A* methylation assay may prove valuable in the future treatment of HCC.

The biological significance of various patterns and cis versus trans configurations of the seven CpG sites in the *INK4A* promoter regions is unknown, but may reflect epi-genetic heterogeneity of HCC tumor DNA. We performed virtual laboratory search to analyze potential transcription factors that can bind to the *INK4A* promoter sequence covered by the PyroMark Q24 CpG p16 assay. Importantly, the seven CpG sites are within the consensus binding sequences of different transcription factors that can regulate gene transcription in response to extra- and intra-cellular environments. Methylation of different CpG sites may affect the binding of different transcription factors that mediate specific molecular pathways in the pathogenesis of HCC, and may also be influenced by these transcription factors. Recent studies have shown that *INK4A* DNA hyper-methylation is associated with environmental carcinogens [47–50]. Levels and patterns of CpG methylation of *INK4A*

DNA may therefore serve as a marker of both exposure and effect of HCC carcinogens. Most patients with HCC have an underlying liver disease caused by chronic HCV or HBV infection and non-viral etiologic risk factors such as alcohol consumption, smoking, dietary aflatoxin exposure, and fatty liver disease. Whether *INK4A* methylation can be used to measure exposure and the effect of specific risk factors will be tested in follow-up studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Quantification of seven CpG sites in the *INK4A* promoter region

(A) *INK4A* DNA sequence including the seven CpG sites analyzed by PyroMark p16 methylation assay. The cytosines in red are predicted to be methylated (<http://bio.dfci.harvard.edu/Methylator/>). The PyroMark p16 methylation assay sequenced the 36 bp in bold that contains the seven CpG sites. The underlined show where the primers were placed for subcloning PCR amplicons, which were comparable with the Qiagen PyroMark Q24 CpG p16 test (Qiagen Inc., Valencia, CA, USA) based on sequencing analyses. (B, C) Representative pyrograms. Shown are pyrograms of 1%–2% (B) and 34%–41% (C) methylation of the seven *INK4A* CpG sites. The seven sites are highlighted in light blue. Peak heights of the T (unmethylation) and C (methylation) sequence within each CpG sites are used to calculate the ratio of methylation, which are the numbers shown in blue on top of each of the CpG sites. The second last cytosine residue of the above sequence (in red) is non-CpG and was used as built-in control to verify bisulfite conversion. 10 ng bisulfite converted DNA was used in the PyroSeq assay.

Figure 2. Performance of pyrosequencing *INK4A* promoter methylation in HCC and benign liver diseases. The highest value of methylation of the seven CpG sites was used for each of the samples

(A) HCC patients had significantly higher levels of *INK4A* methylation than non-HCC cases (odds ratio=1.38, 95% confidence interval 1.05–1.79; $p<0.05$, Wald test). Samples with outlier values were marked above the bars. (B) Receiver operating characteristic curves of sensitivity versus 1-specificity. When the highest value of methylation of the seven CpG sites was used for each of the samples, the area under ROC curve (AUC) was 0.82. (C) Using LOD cut-offs of 5% for *INK4A* methylation [31] and 200 µg/L for AFP [28], 49 and 30 positives, respectively, were detected for the 66 HCC patients; 23 of the 49 *INK4A* positive HCC cases were only positive for *INK4A*, four of the 30 AFP positive HCC cases were only positive for AFP; 26 cases were positive for both *INK4A* and AFP. Adding *INK4A* methylation to AFP (LOD at 200 µg/L) increased positive rates of the 66 HCC cases from AFP's 45.5% (30/66) and *INK4A*'s 74.2% (49/66) to the combined 80.3% (53/66). Numbers shown at the bottom left are numbers of cases in each of the four quadrants.

Case 46	CpG site:	1	2	3	4	5	6	7		
	% methylation	40	41	38	38	38	35	34		
CpG site:	1	2	3	4	5	6	7	# Clones	Epi-allele	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	16	A	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	C	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	D	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	2	E	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	F	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	J	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	2	L	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	16	M	
Methylated at least one site: 24/40=60.00%										
Methylated all 7 sites of the methylated clones: 16/24=66.67%										
Not-all sites methylated of the methylated clones: 8/24=33.33%										
Case 58	CpG site:	1	2	3	4	5	6	7		
	% methylation	40	51	41	40	52	35	30		
CpG site:	1	2	3	4	5	6	7	# Clones	Epi-allele	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	17	A	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	2	C	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	G	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	2	I	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	3	K	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	L	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	14	M	
Methylated at least one site: 26/40=65.00%										
Methylated all 7 sites of the methylated clones: 17/26=65.39%										
Not-all sites methylated of the methylated clones: 9/26=34.62%										
Case 110	CpG site:	1	2	3	4	5	6	7		
	% methylation	47	54	50	51	50	45	42		
CpG site:	1	2	3	4	5	6	7	# Clones	Epi-allele	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	19	A	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	B	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	D	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	3	E	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	H	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	I	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	J	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	13	M	
Methylated at least one site: 27/40=67.5%										
Methylated all 7 sites of the methylated clones: 19/27=70.37%										
Not-all sites methylated of the methylated clones: 8/27=29.63%										
Total										
CpG site:	1	2	3	4	5	6	7	# Clones	Epi-allele	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	52	A	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	B	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	3	C	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	2	D	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	5	E	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	F	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	G	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	1	H	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	3	I	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	2	J	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	3	K	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	3	L	
gggtgggg	Ggac	G	Gtg	Gct	Gg	Ggctg	Gga	43	M	
Methylated at least one site: 77/120=64.175%										
Methylated all 7 sites of the methylated clones: 52/77=67.53%										
Not-all sites methylated of the methylated clones: 25/77=32.47%										

Figure 3. Heterogeneous methylation alleles of the seven CpG sites

Shown are summary of epi-alleles including fully methylated and fully unmethylated sequences identified in samples 46, 58, and 110 (Supplemental Table 1). PCR amplicons were analyzed using TA cloning, transformation, plasmid DNA preparation, and Sanger sequencing of DNA inserts of single clones. Analyses of 120 single clones (40 in each case) that carried *INK4A* PCR amplicons identified a total of 13 epi-alleles (A-M), including eight in cases 46 and 110, and seven in case 58, that have different configurations of methylated

CpG dinucleotides. CG, methylated; TG, unmethylated. Sequences with methylation between one and six CpG sites were 32.5% of the 120 clones.

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Table 1

Sensitivity and specificity at different CpG cut-offs.

Cut-off	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PV+%	PV-%
5	65.3 (51.8–77.1)	87.2 (73.8–95.1)	84.4	71.7
7	39.0 (26.6–52.3)	96.5 (86.0–99.7)	92.0	61.3
10	20.3 (11.0–32.8)	98.8 (89.8–100)	95.5	55.4

CI, confidence interval; PV+%, percent positive-predictive value; PV-%, percent negative-predictive value.

Table 2Summary of published circulating *INK4A* methylation results in HCC and controls.

References	Serum/plasma samples of HCC cases	Serum/plasma samples of chronic hepatitis/cirrhosis cases	Method	Association with serum AFP	Tumor samples
Wong et al. [14]	59.1% (13/22) including 81% (13/16) tumors with methylation and 0% (0/6) tumors without methylation	0% (0/38)	MSP	NA	72.7% (16/22)
Wong et al. [18]	53% (24/45) including 80% (20/30) tumors with methylation and 0% (0/15) tumors without methylation	0% (0/35)	MSP	Significant association in cases with tumoral <i>INK4A</i> methylation	66.7% (30/45)
Wong et al. [32]	31.1% (14/45) including 46.7% (14/30) tumors with methylation and 0% (0/15) tumors without methylation	0% (0/50)	RTQ-MSP	NA	66.7% (30/45)
Chu et al. [33]	47.8% (22/46) of HCC	17.4% (4/23) of liver cirrhosis (LC)	MSP	No association	NA
Zhang et al. [34]	30.8% (12/39) of HCC	NA	MSP	NA	62.5% (25/40)
Zhang et al. [20]	44.0% (22/50) and 64.3% (9/14), respectively in samples collected 0–9 years and 1–3 years prior to diagnosis of HCC	4.0% (2/50) non-HCC controls	MSP	No association	
Chang et al. [35]	19.2% (5/26)	0% (0/16)	MSP	NA	78.9% (15/19)
Iyer et al. [36]	46.4% (13/28) HCC	NA	MSP	NA	71.4% (13/28)
This study	74.2% (49/66), 50.0% (33/66), and 27.3% (18/66) at 5%, 7% and 10% LOD, respectively	18.6% (8/43), 4.7% (2/43), 3.3% (1/43) at 5%, 7% and 10% LOD, respectively	Pyrosequencing	Weak association	NA

NA, not available. RTQ-MSP, real-time quantitative methylation-specific PCR.