DNA Methylation and Environmental Exposures in Human Hepatocellular Carcinoma

Lanlan Shen, Nita Ahuja, Yu Shen, Nagy A. Habib, Minoru Toyota, Asif Rashid, Jean-Pierre J. Issa

Background: Hypermethylation of a CpG-rich promoter (CpG island) blocks expression of the corresponding gene. The CpG island methylator phenotype (CIMP), defined as a variable pattern of hypermethylation of CpG islands in tumor suppressor genes, may be associated with carcinogenesis. To determine whether CIMP is associated with the development of hepatocellular carcinoma (HCC) and with exposure to environmental agents, we examined the methylation status of CpG islands in HCCs from countries with various HCC risks. Methods: We examined the methylation status of 12 CpG islands (eight for known genes) in 85 HCC tumors from various geographic locations by use of bisulfite–polymerase chain reaction methylation assays and analyzed results with univariate and multivariable methods. All statistical tests were two-sided. Results: Eight CpG islands were hypermethylated. The frequency of hypermethylation in the 85 tumors was 62% for the estrogen receptor (ER), 42% for p16, 18% for cyclooxygenase-2, 21% for the T-type calcium channel gene, 38% for MINT31, 28% for MINT1, 15% for MINT27, and 11% for MINT2 (the latter four CpG islands are not yet associated with genes). Methylation levels of the eight frequently methylated CpG islands were positively correlated (from $R = .2$ [P = .05] to $R = .6$ [P <.001]), supporting the presence of CIMP. p16 methylation had statistically significant geographic variation (34.4% in tumors from China and Egypt versus 12.2% in tumors from the United States and Europe, difference = 22.2%; 95% confidence interval [CI] = 11.2% to 33.2%; P <.001). Similar geographic variations were observed for ER methylation and CIMP. This observation was partly related to higher methylation in tumors from patients with cirrhosis (33.6% for patients with cirrhosis versus 11.7% for those without it; difference = 21.9%; 95% CI = 10.9% to 32.8%; P <.001) or hepatitis (34.2% for patients with hepatitis versus 6.2% for those without it; difference = 28%; 95% CI = 18.3% to 37.6%; P <.001). Conclusion: Geographic variations in the methylation status of various CpG islands indicate that environmental factors may influence the frequent and concordant degree of hypermethylation in multiple genes in HCC tumors. (J Natl Cancer Inst 2002;94:755–61)

Evidence is accumulating that genetic changes (such as mutations and translocations) and epigenetic changes (such as hypermethylation) play a role in carcinogenesis. DNA methylation is one form of epigenetic inheritance in mammalian cells (1). aberrant hypermethylation in the CpG-rich promoter regions (CpG islands) of many tumor suppressor genes is associated with the lack of gene transcription and, thus, can contribute to the formation and progression of cancer by providing an alternative mechanism for the loss of tumor suppressor gene function (2,3).

Nonrandom patterns of aberrant CpG island methylation have been described in cancer (4), and the CpG island methylator phenotype (CIMP), identified by a variable incidence of hypermethylation in tumors, is responsible for the aberrant methylation of many tumor suppressor genes in the development of cancer (5). CIMP was described initially in colorectal cancer (6) and then in gastric cancer (7), pancreatic cancer (8), ovarian cancer (9), and acute myelogenous leukemia (10). Genome scanning for changes in DNA methylation in various human cancers has confirmed the presence of a hypermethylator phenotype in selected tumors (4). Thus, CIMP appears to be involved in an important new tumorigenesis pathway that leads to cancer progression by simultaneously inactivating multiple genes. The causes of CIMP, however, remain unknown.

As with other cancers, the development of hepatocellular carcinoma (HCC) is a complex, multistep process (11). HCC is the eighth most common cancer in the world, with rates that show considerable geographic variation. The Far East and sub-Saharan Africa have very high rates, and the United States and Europe have relatively low rates (12–14). Epidemiologic data indicate that hepatitis B virus and hepatitis C virus account for 50%–90% of the HCC in high-risk populations and for 1%–10% in low-risk countries. In high-incidence areas, aflatoxin exposure is also a risk factor. Chronic alcoholism probably plays a major role in the United States and Europe and may be gaining importance in developing countries of Africa and Asia. Thus, HCC provides a good model to determine whether exposure to environmental carcinogens is associated with molecular changes in a variety of genes, as observed for the p53 gene (15). Many individual genes have been reported to be abnormally methylated in HCC (11). However, most of the previous studies were limited to single target genes (16–18). In this study, we used bisulfite–polymerase chain reaction (PCR) methylation assays to examine the methylation status of 12 CpG islands in 85 HCC tumors from patients in various countries to determine whether CIMP is associated with the development of HCC and whether exposures to various environmental agents affect the likelihood of finding hypermethylation in a given tumor.

PATIENTS, MATERIALS, AND METHODS

Patient Selection

Patients with HCC in this study consisted of three cohorts selected solely on the availability of tissue for molecular analy-
ses. The first cohort of 25 patients was from Qidong and Shanghai, China, and has been studied previously (19). The second cohort of 27 patients was from London, U.K., and included patients from European and non-European countries at high risk for HCC development. Molecular analyses of these patients have also been reported previously (20). The third cohort of 33 patients was from Baltimore, MD.

Tissue Preparation and Examination

Eighty-five HCC tissue specimens were obtained from four surgical centers in Qidong and Shanghai, China; London, U.K.; and Baltimore, MD. Slides of specimens from China and Baltimore (68% of the total) were reviewed by one of us (A. Rashid) to verify the pathologic diagnoses of cirrhosis and neoplasia. All other specimens were obtained from a single surgical center (Hammersmith Hospital, London, U.K.) and were reviewed locally. DNA was extracted from neoplastic liver tissue and, when available, corresponding non-neoplastic liver tissues. Tissue collection was approved by all patients according to institutional guidelines.

Bisulfite–PCR Methylation Analysis

Bisulfite treatment was performed as reported previously (6,21). Briefly, 2 μg of genomic DNA was denatured in 0.2 N NaOH at 37 °C for 10 minutes and incubated with 3 M sodium bisulfite at 50 °C for 16 hours. After this incubation, DNA was purified with the Wizard DNA Clean-Up System (Promega, Madison, WI) and desulfonated in 0.3 N NaOH at 25 °C for 5 minutes. Desulfonated DNA was then precipitated with ammonium acetate and ethanol, washed with 70% ethanol, and resuspended in 20 μL of H2O; 2 μL of this solution was used as the template for PCRs. The methylation status of CpG islands for eight genes and four additional CpG islands was examined. The genes included p16, cyclooxygenase-2, T-type calcium channel (CACNA1G), estrogen receptor (ER), retinoic acid receptor-β2 (RARβ2), mut-L homolog-1 (MLH1), thrombospondin-1, and E-cadherin. The four additional CpG islands were MINT1, an island associated with a cDNA transcript of unknown function; MINT31, an island 2 kilobases upstream of CACNA1G; and MINT27 and MINT2, two CpG islands that are not yet associated with genes but are consistently hypermethylated in the presence of CIMP. Primer sequences and enzymes used for methylation determination are shown in Table 1. All PCRs included a denaturation step at 95°C for 30 seconds, followed by an annealing step at various temperatures for 30 seconds. For most of the assays, the annealing temperature was reduced progressively after a defined number of cycles. PCR conditions are presented in Table 1. This assay relies on bisulfite-induced restriction fragment length polymorphisms—i.e., methylated bands are digested by restriction enzymes but unmethylated bands are not. Digested PCR products were separated by electrophoresis on 6% polyacrylamide gels. Gels were stained with ethidium bromide, imaged, and quantitated in a Bio-Rad Geldoc 2000 imager (Bio-Rad, Hercules, CA). The methylation density for each sample was computed as a ratio of the density of the digested band to the density of all bands in a given lane (22). Most of the assays used have previously been published (6,23–27), including validation of methylation for multiple sites within each island, correlation with other techniques (methylation-specific PCR, Southern blotting, and sequencing), lack of PCR bias, and association with lack of expression when appropriate.

p53 Mutation Analysis

p53 mutation data were available for 44 samples from previous studies (19,20). Thirty-two additional samples for which enough DNA was available were studied for p53 mutations with methods similar to those in our previous report (19) by PCR amplification of exons 4–9 and direct sequencing of the PCR products.

Statistical Analysis

All data were generated without knowledge of the clinical status of the samples analyzed. Univariate analyses of the interaction between methylation and clinical parameters were performed with the χ2 test and Fisher’s exact test when testing small samples. The Wilcoxon rank-sum test was used to compare two groups of independent but continuous samples. Multivariable ordinal logistic regression analyses were performed to assess whether each of the risk factors—cirrhosis, hepatitis, or geographic location (with or without p53 mutations)—was associated with methylation. The generalized linear regression model was used to predict the value of p16 (and CIMP) by other risk factors such as geographic location, hepatitis, cirrhosis, and p53 mutations. All P values presented are two-sided, and a P value of less than .05 was considered statistically significant. Statistical analyses were carried out with SAS and SPLUS4.4 software packages (MathSoft, Seattle, WA).

Table 1. Summary of primer sequences, polymerase chain reaction (PCR) conditions, and restriction enzymes used for bisulfite-PCR

<table>
<thead>
<tr>
<th>Gene or CpG island</th>
<th>Primer set (forward/reverse)</th>
<th>Sequential annealing temperatures, °C (No. of cycles at a temperature)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>GGTGTGGAGTGGTTTGGTATGTATGGTTTACCTATCTCAATCTCTCTCTA</td>
<td>58(3), 56(4), 54(5), 52(23)</td>
<td>TaqI</td>
</tr>
<tr>
<td>COX2</td>
<td>GATTTGTAGTGAGGAGTTGAGGTGCACTATGTTTACCTATCTCTCTCTC</td>
<td>53(3), 51(4), 49(5), 47(26)</td>
<td>TaqI</td>
</tr>
<tr>
<td>CACNA1G</td>
<td>GGGGGTGTTTGTGSTGTTTGGTATGTATGGTTTACCTATCTCTCTCTA</td>
<td>55(3), 53(5), 51(5), 49(26)</td>
<td>EcoRI</td>
</tr>
<tr>
<td>ER</td>
<td>GGTGTGGAGTGGTTTGGTATGTATGGTTTACCTATCTCTCTCTC</td>
<td>60(3), 57(4), 54(5), 51(25)</td>
<td>BstUI</td>
</tr>
<tr>
<td>MINT1</td>
<td>GGGGTGTTTGTGSTGTTTGGTATGTATGGTTTACCTATCTCTCTCTA</td>
<td>55(35)</td>
<td>TaqI</td>
</tr>
<tr>
<td>MINT2</td>
<td>GGTGTGGAGTGGTTTGGTATGTATGGTTTACCTATCTCTCTCTC</td>
<td>60(3), 58(4), 56(5), 54(26)</td>
<td>MINT2</td>
</tr>
<tr>
<td>MINT27</td>
<td>GGGGTGTTTGTGSTGTTTGGTATGTATGGTTTACCTATCTCTCTCTA</td>
<td>55(35)</td>
<td>TaqI</td>
</tr>
<tr>
<td>MINT31</td>
<td>GAGGATGAACTGTTTTTGATGTATGTATGGTTTACCTATCTCTCTCTC</td>
<td>58(3), 56(4), 54(5), 52(26)</td>
<td>BstUI</td>
</tr>
</tbody>
</table>

*COX2 = cyclooxygenase-2; ER = estrogen receptor.
RESULTS

Patient Characteristics

Among the 85 patients with liver cancer in the study cohort, 40 were from high-risk countries (28 from China, nine from Egypt, and three from East Asia) and 45 were from low-risk countries (15 from the U.K./Europe and 30 from the United States). Clinicopathologic characteristics of the 85 patients with HCC are shown in Table 2. The presence or absence of hepatitis or cirrhosis could be assessed for 82 patients. According to all of the available clinical information, 44 of the 82 patients with HCC had underlying cirrhosis. Of these 44 patients, eight had alcoholic cirrhosis, 33 had serologic or molecular evidence of viral hepatitis (two were hepatitis B- and C-positive, 24 were hepatitis B-positive, and seven were hepatitis C-positive), and three had no evidence of alcoholic disease or viral infection. In the remaining 38 noncirrhotic HCC tumors, eight were positive for viral hepatitis and 30 were negative. p53 mutational status [reported in previous studies for 44 patients (19,20) and PCR amplification and direct sequencing of exons 4–9 in 32 additional patients] was available for 76 patients and is also shown in Table 2. Striking differences were observed between HCC specimens from low-risk and high-risk countries; the latter specimens had much higher rates of viral hepatitis (34 of 38 specimens versus 15 of 44 specimens from low-risk countries), cirrhosis (27 of 38 specimens versus 17 of 44 specimens from low-risk countries), and p53 mutations (18 of 38 specimens versus 2 of 38 specimens from low-risk countries). As expected, the presence of cirrhosis was statistically significantly associated with hepatitis ($P<.001$, Fisher’s exact test) and geographic location ($P = .007$).

Bisulfite–PCR Analysis of Methylation in Multiple CpG Islands

Methylation was examined with a semiquantitative methylation bisulfite–PCR assay (22,29). The methylation level was determined by measuring the percentage of methylated bands relative to the total density of all bands in each lane. We have previously determined, with this assay, that the level of methylation correlated with gene expression (6,10,23,24,26,27). Most of the statistical analyses were performed with methylation as a continuous variable. For CIMP classification (7,10), a specimen was considered positive if the methylation density was 15% or greater, as described (6). We examined a total of 12 CpG islands in this study. Preliminary experiments detected no substantial (>5%) CpG island methylation of four genes (E-cadherin [0 of 10 specimens], thrombospondin-1 [0 of 11 specimens], MLH1 [0 of 14 specimens], and RARβ2 [0 of 10 specimens]), and these genes were not examined further (data not shown). The other eight CpG islands were methylated at frequencies ranging from 11% to 62% in neoplastic samples (examples in Fig.1 and summary in Table 3). As shown in Table 3, methylation levels of p16, CACNA1G, cyclooxygenase-2, MINT1, MINT2, MINT27, and MINT31 CpG islands were statistically significantly higher in neoplastic samples than in the corresponding non-neoplastic samples. No statistically significant difference in the methylation of the ER CpG island, however, was found between neoplastic and non-neoplastic tissues (methylation density = 26.3 [95% confidence interval {CI} = 22.0 to 30.6] and 24.5 [95% CI =

![Fig. 1. Examples of methylation analyses. For each gene (indicated on the left), bisulfite-treated DNA was amplified with gene-specific primers, and the PCR products were digested with restriction endonucleases specific for methylated alleles (listed in Table 1). Arrows = methylated alleles; N = non-neoplastic normal tissues; T = tumor tissues; RKO and SW48 = colon cancer cell lines used as positive controls.](image-url)
22.0 to 27.0], respectively; \( P = .24 \) because, compared with the paired non-neoplastic tissues, the ER methylation level was higher in 42 neoplastic specimens, lower in 31 neoplastic specimens, and similar in 12 neoplastic specimens. No association was observed between the number of genes positive for methylation (i.e., with a methylation density of greater than 15%) and the methylation density of the positive genes.

### CIMP in HCC Tissues

We found positive correlations between the methylation densities for all eight CpG islands studied in detail (from \( R = .2 \) [\( P = .05 \) to \( R = .6 \) [\( P < .001 \)]). Thus, the methylation status of CACNA1G correlated with that of seven other islands (\( R = .3 \) to .5 and \( P < .01 \) for all). The methylation status of ER, cyclooxygenase-2, MINT1, and MINT27 correlated with that of six others (\( R = .2 \) to .6 and \( P < .01 \) for all). The methylation status of MINT2 correlated with that of five others (\( R = .4 \) to .5 and \( P < .01 \) for all). The methylation status of MINT31 correlated with that of four others (\( R = .2 \) to .4 and \( P < .05 \) for all), and the methylation status of p16 correlated with that of two others (\( R = .3 \) and \( P < .01 \) for both). This concordant methylation suggested the presence of a hypermethylator phenotype. Consistent with this result, if a 15% methylation density were the threshold for a methylation-positive specimen, then the methylation was not evenly distributed, with some specimens having no methylation (no loci) and others being highly methylated (more than two loci) (Fig. 2). Results were essentially unchanged when a 10% cutoff was used instead of the 15% cutoff. To classify HCCs by their methylation status, we arbitrarily divided the specimens into CIMP-negative (no methylated gene; 17 specimens), CIMP-intermediate (one to two methylated genes; 36 specimens), and CIMP-positive (more than two methylated genes; 32 specimens) groups.

### Methylation and Clinicopathologic Features

We next investigated the relationship between methylation and known clinicopathologic factors in HCC. We initially studied associations between the methylation of each gene separately and available clinical information including age, gender, differentiation of the tumor, presence of hepatitis, presence of cirrhosis, geographic location, and presence of p53 mutations in the tumor. The same analyses were then performed on CIMP status. The most statistically significant factors were then examined in a multivariable analysis. Fig. 2 presents all data pertaining to individual gene methylation, CIMP status, and clinicopathologic correlates in this study.

No association was found between methylation of any gene and age or tumor differentiation. Methylation of p16, ER, and CACNA1G was higher in males than in females; however, information on sex was missing from 32% of the samples. Sex was strongly associated with the presence of hepatitis and cirrhosis,

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**Table 3.** CpG island methylation (mean density and frequency) in normal and neoplastic liver samples*

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of samples studied</th>
<th>Non-neoplastic samples</th>
<th>Neoplastic samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (95% CI)</td>
<td>No. of positive samples (%)</td>
</tr>
<tr>
<td>p16</td>
<td>85</td>
<td>4.9 (2.9 to 6.9)</td>
<td>6 (7)</td>
</tr>
<tr>
<td>CACNA1G</td>
<td>85</td>
<td>0.9 (0 to 2.0)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>COX2†</td>
<td>85</td>
<td>0.8 (0.4 to 1.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>MINT1</td>
<td>85</td>
<td>2.4 (1.7 to 3.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>MINT2</td>
<td>85</td>
<td>0 (0 to 0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>MINT27</td>
<td>85</td>
<td>2.4 (1.3 to 3.5)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>MINT31</td>
<td>85</td>
<td>1.0 (0.4 to 1.6)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>ER</td>
<td>85</td>
<td>24.5 (22.0 to 27.0)</td>
<td>69 (81)</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean methylation density for each gene as well as the 95% confidence interval (CI). No. of positive samples = number of samples where methylation density is more than 15%; number in parenthesis = percentage based on the total number of samples studied. The \( P \) value was computed from two-sided Wilcoxon tests comparing the mean methylation density in non-neoplastic samples with that in neoplastic samples.

†COX2 = cyclooxygenase-2; ER = estrogen receptor.
with males more highly represented in both conditions. Thus, sex was not included in the multivariable analyses.

We next investigated whether methylation of each gene (measured as a continuous variable) was correlated with clinicopathologic features that indicate specific carcinogenic exposures, such as hepatitis, cirrhosis, geographic location (high versus low risk), and the presence of a p53 mutation. In univariate analyses, p16 methylation had a statistically significant geographic variation (34.4% in tumors from China and Egypt versus 12.2% in tumors from the United States and Europe, difference = 22.2%; 95% CI for the difference = 11.2% to 33.2%; P < .001). This observation was partly related to higher methylation in tumors from patients with cirrhosis (33.6% for patients with cirrhosis versus 11.7% for those without it; difference = 21.9%; 95% CI = 10.9% to 32.8%; P < .001) or hepatitis (34.2% for patients with hepatitis versus 6.2% for those without it; difference = 28%; 95% CI = 18.3% to 37.6%; P < .001). These findings were confirmed by Wilcoxon tests that gave statistically significant P values (Table 4). Similar, statistically significant results were obtained for the methylation of ER (results not shown). Results for CACNA1G and MINT1 followed similar trends but were not statistically significant, and no association was found between these variables and methylation of the other genes (results not shown).

Linear regression analyses assessed which of these clinicopathologic features were predictive of p16 methylation. Initially, a model not including p53 showed that hepatitis and geographic location status were independently associated with p16 methylation (for hepatitis, regression parameter = 20.4, 95% CI = 7.7 to 33.1, P < .003; for geographic location, regression parameter = −6.64, 95% CI = −9.37 to −2.9, P = .04). In addition, hepatitis and geographic location were correlated with each other (R = .56, P < .001). When p53 mutations were factored into the model, both cirrhosis and p53 mutations were independent predictors of p16 methylation (for cirrhosis, regression parameter = 7.8, 95% CI = 2.5 to 13.1, P = .005; for p53, regression parameter = 31.3, 95% CI = 19.5 to 43.1, P < .001), and p53 mutations were correlated with both hepatitis (R = .31, P = .008) and geographic location (R = .48, P < .001). No differences in methylation status were observed between specimens that had p53 mutations in codon 249 and specimens that had other mutations (Fig. 2). Linear regression analyses identified hepatitis alone as a statistically significant predictor of ER methylation (regression parameter = 17.4, 95% CI = 9.2 to 25.6, P < .001).

Similar results were observed when we considered CIMP status, which reflects global CpG island methylation. Univariate analysis showed that CIMP was associated with cirrhosis (P = .038 by the χ² test), hepatitis (P = .010 by the χ² test), country of origin (P = .021 by Fisher’s exact test), and p53 mutation status (P = .017 by Fisher’s exact test) (Table 5). For multivariable analyses, we grouped the specimens into two CIMP groups, negative (zero or one gene methylated) or positive (more than one gene methylated). Logistic regression analysis showed that, without including p53 in the model, hepatitis was a statistically significant predictor of CIMP status, with an odds ratio for a CIMP-positive tumor in patients with hepatitis of 3.5 (95% CI = 1.4 to 9.0, P = .01) compared with those who do not have hepatitis. When p53 was included in the model, the presence of p53 mutations was the best predictor of CIMP. Specifically, the odds ratio for a CIMP-positive tumor in patients with p53 mutations was 5.8 (95% CI = 1.2 to 27.5, P = .03) compared with patients without p53 mutations. Consistent with the analysis using CIMP, p53 mutation alone is a statistically significant predictor of the number of loci methylated (zero to eight), whereas the other covariates are not independent predictors of this response variable. One likely explanation for this result is that, as discussed above, cirrhosis, hepatitis, and geographic location are all correlated with p53 mutation.

### Discussion

Hypermethylation events appear to be nonrandomly distributed in human tumors (5). A distinct methylator pathway, CIMP, was recently described in colorectal tumorigenesis (6). CIMP has also been observed in gastric (7), pancreatic (8), ovarian (9), and hematopoietic tumors (10). The molecular mechanisms that result in the CIMP pathway are still not fully understood. In this study, we found that there is frequent and concordant methylation of multiple genes in HCC, indicating the presence of CIMP in a subset of tumors. By studying liver cancers from several geographic areas and with several etiologies, we were able to test the hypothesis that the CIMP phenotype was associated with

#### Table 4. Methylation of p16 by hepatocellular carcinoma risk factor

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Negative</th>
<th>Positive</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cirrhosis</td>
<td>38</td>
<td>44</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>32</td>
<td>49</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Country</td>
<td>45</td>
<td>40</td>
<td>.001</td>
</tr>
<tr>
<td>p53 mutation</td>
<td>56</td>
<td>20</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>No</th>
<th>Yes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cirrhosis</td>
<td>12</td>
<td>16</td>
<td>.038†</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>5</td>
<td>18</td>
<td>.21</td>
</tr>
<tr>
<td>Country</td>
<td>12</td>
<td>8</td>
<td>.010‡</td>
</tr>
<tr>
<td>p53 mutation</td>
<td>0</td>
<td>8</td>
<td>.017§</td>
</tr>
</tbody>
</table>

*Data are expressed as the mean methylation density as well as the 95% confidence interval (CI) for each gene in the group of patients who were either negative or positive for the listed risk factors.

†P values were calculated from two-sided Wilcoxon tests.

‡Negative = country with low risk; positive = country with high risk.

§P value calculated by Fisher’s exact test.

### Table 5. Clinicopathologic correlates of CpG island methylator phenotype (CIMP) status

<table>
<thead>
<tr>
<th>CIMP status</th>
<th>Negative</th>
<th>Intermediate</th>
<th>Positive</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cirrhosis</td>
<td>12</td>
<td>16</td>
<td>10</td>
<td>.038†</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>5</td>
<td>18</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Country</td>
<td>12</td>
<td>8</td>
<td>14</td>
<td>.021§</td>
</tr>
<tr>
<td>High risk</td>
<td>3</td>
<td>19</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>p53 mutation</td>
<td>0</td>
<td>8</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

*CIMP status was defined by the number of CpG islands methylated in each sample as follows: CIMP negative (no methylated gene), CIMP intermediate (one to two methylated genes), and CIMP positive (more than two methylated genes). Numbers represent the actual number of patients that fit the respective categories. The number of patients is not identical in all of the columns because of missing clinical or p53 data.

†P value calculated from two-sided Wilcoxon tests.

‡Negative = country with low risk; positive = country with high risk.

§P value calculated by Fisher’s exact test.
environmental exposures. We found that HCC tumors from patients with hepatitis (caused by hepatitis B virus, hepatitis C virus, or alcohol), from patients with cirrhosis, or from patients living in high-risk geographic areas have much higher levels of methylation than do tumors arising spontaneously. These differences strongly suggest a major role for environmental factors in the pathogenesis of hypermethylation in cancer. A previous study in an animal model had shown associations between specific carcinogen exposures and hypermethylation of the ER gene (29). In addition, a recent study has demonstrated an association between cigarette smoking and p16 methylation in human lung cancers, although that association was not found for another gene (30). Nevertheless, these studies support the hypothesis that environmental exposures influence the degree of methylation in cancer.

The mechanism of CIMP and environmental interactions remains to be clarified. A strong possibility is that CIMP may be caused or facilitated by inflammatory proliferative stimuli related to environmental exposures. In fact, cirrhosis reflects chronic proliferative stress caused by ongoing damage to the liver and is strongly associated with CIMP. Similar results were also found in ulcerative colitis, where chronic inflammation was associated with increased methylation of multiple CpG islands (31). Finally, Barrett’s esophagus, a condition also associated with chronic inflammation, has a high incidence of p16 methylation (32,33). Thus, abnormal methylation could be caused or accelerated by inflammatory stimuli and associated proliferative changes. Alternatively, oxidative damage related to chronic inflammation could directly affect the methylation status of DNA.

Viral exposure is another possible explanation for our findings. For example, in adenovirus-induced tumors, both virus and adjacent host DNA are frequently hypermethylated (34), and in Epstein-Barr virus-infected cells, de novo methylation of DNA has been demonstrated (35). A plausible hypothesis, then, is that hepatitis viruses activate a methylation pathway that methylates endogenous genes de novo.

Another interesting finding in this study is the association between p53 mutations and methylation status. Unlike the inverse correlation found between CIMP (or p16 methylation) and p53 mutations in colon cancer (25), we found a strong direct correlation between p53 mutations and CIMP in liver cancer. Mutations frequently occur in regions high in methylated cytosines (hot spots), and deamination of 5-methylcytosine has been considered a source of genetic mutation in tumors, as shown for the p53 gene (3). However, the p53 gene is normally methylated at mutational hotspots, whereas CIMP affects areas not previously methylated (CpG islands). The association between methylation and p53 mutations in liver cancers, then, is likely related to environmental causes, such as the association between aflatoxin B exposure and mutations in the p53 gene (15).

The current study suggests that there are two major molecular pathways to HCC: the p53/CIMP pathway, which predominates in cirrhosis-related HCC, and a separate pathway in HCC without cirrhosis. The molecular changes that characterize the latter group are unclear but may well involve a higher incidence of loss of heterozygosity at multiple loci (36). It has recently been suggested that liver tumors can be divided into two distinct groups: tumors with β-catenin mutations and tumors with chromosomal instability (37). The group with β-catenin mutations had a lower rate of p53 mutations, and it might be useful to determine whether the group with β-catenin mutations corresponds to the CIMP-negative group described herein.

One drawback of our study is the somewhat imprecise CIMP classification in which a substantial number of patients was assigned to the intermediate group. This problem is most likely related to the limited number of liver cancer-specific genes examined. We predict that large-scale methylation profiling would improve the current CIMP classification. In fact, our CIMP-intermediate group could be (retrospectively) subdivided into two groups: those with simultaneous ER/p16 methylation that are very similar to the CIMP-positive group (including the clinicopathologic correlates) and those lacking ER and p16 methylation that are similar to the CIMP-negative group. Differentially methylated CpG islands from liver cancer cells could then be cloned to refine the CIMP classification. This refined classification may then identify a subset of patients with distinct clinical and genetic characteristics, distinct cancer epidemiology, and distinct prognosis.

Genetic changes can be used as markers of carcinogen exposures. Our current work demonstrates that methylation changes ("methylation profiling") could also be used to clarify the exposures that contributed to the development of HCC. We propose that such studies of environment–epigenetic interactions (epigenetic epidemiology) may be necessary for a full understanding of neoplasia and the mechanism of action of carcinogenic exposures and could be used to identify novel opportunities for cancer prevention and treatment.

REFERENCES

REFERENCES


NOTES

Editor’s note: Dr. Issa is a consultant for and holds stock options in the biotechnology company Epigenomics, which is developing methylation-based diagnostic assays.

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