Intensive Hypermethylation of the CpG Island of Ras Association Domain Family 1A in Hepatitis B Virus-associated Hepatocellular Carcinomas

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ABSTRACT

Purpose and Experimental Design: The human Ras association domain family 1A gene (RASSF1A) is a newly isolated tumor suppressor gene. In this study, we analyzed the methylation status of the promoter region of RASSF1A using bisulfite sequencing and PCR-RFLP in four liver cancer cell lines (Hep3B, HepG2, SK-HEP-1, and Huh-7) and a cohort of 43 hepatitis B virus-associated hepatocellular carcinoma (HCC) tissues and their corresponding nontumor tissue specimens.

Results: The methylation of the CpG islands in the RASSF1A promoter was not detected in 4 samples of normal liver tissue or 10 samples of peripheral blood mononuclear cells from normal subjects. However, the CpG islands were completely methylated, and transcription of the RASSF1A was silenced in the four cell lines. Treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine reactivated the expression of RASSF1A in the Hep3B and HepG2 cells. In 41 of 43 (95%) HCC specimens studied, the promoter region of RASSF1A was intensively methylated at its CpG sites. Although heterogeneous methylation was also detected in 16 of the 23 (70%) corresponding nontumorous tissues analyzed, the level of methylation was significantly lower than in the corresponding tumor tissues.

Conclusions: HCC has the highest incidence of promoter methylation of RASSF1A among all malignancies yet reported suggesting that hypermethylation of the CpG island promoter of RASSF1A may play an important pathological role in this tumor.

INTRODUCTION

Worldwide, HCC is the fourth most common cause of cancer-related mortality, but the molecular mechanisms leading to hepatocyte transformation are still poorly understood. In Hong Kong, HCC is a leading cause of cancer death among men, with a male:female ratio of 4:1. Although p53 mutations have been found in substantial proportion of HCCs in China (1–3), other abnormalities of proto-oncogenes and TSGs have not been reported consistently (4). Most HCC cases in our region arise in the setting of chronic HBV infection (5, 6). Dietary carcinogens, such as aflatoxin B1, also contribute to hepatic carcinogenesis (7, 8). Frequent LOH at chromosomal arms 1p, 3p11.2-p14.2, 4q, 6q, 8p, 13q, and 16p has been reported suggesting sites of putative TSGs (9–14).

The RASSF1A gene, at 3p21.3, has been suggested to be a major target tumor suppressor on the basis of its frequent epigenetic silencing and LOH in lung cancers (15). RASSF1 encodes several isoforms, including RASSF1A, RASSF1B, and RASSF1C, which are all derived from alternative mRNA splicing and promoter usage (15). It has been reported that RASSF1A is inactivated frequently in breast, ovarian, gastric, and nasopharyngeal cancer; renal cell lines; and primary tumors by de novo methylation at the CpG islands in the promoter region (16–24). In small cell lung cancers, allelic deletion at 3p21.3 is associated with RASSF1A methylation, suggesting that both genetic and epigenetic steps are crucial for RASSF1A inactivation in some tumor types. The tumor suppressor function of RASSF1A has been additionally suggested by observations that introduction of exogenous expression of RASSF1A decreases colony formation, suppresses anchorage-independent growth, and dramatically reduces tumorigenicity in vivo (15, 22). With these tumor suppression effects, the presence of a Ras association domain suggests that RASSF1 proteins may function as effector molecules in Ras or related growth inhibitory signaling pathways.

It is widely accepted that both alleles of a TSG need to be inactivated by genetic alterations such as chromosomal deletions or loss-of-function mutations in the coding region of a gene (25). As an alternative mechanism, epigenetic alterations of TSGs may occur and result in gene inactivation. Recent studies have demonstrated that the CpG islands of several genes are methylated frequently in human cancers (26–29). Because methylation plays an important role in both biological processes and cancer development, various methods have been developed...
to detect and characterize such alterations (30–34). These include bisulfite sequencing, methylation-specific PCR, and procedures based on the use of restriction endonucleases, such as PCR-RFLP. The gold standard among these methods is genomic bisulfite sequencing that provides a positive display of m5C at specific CpG sites in virtually any stretch of DNA (30). The most sensitive method is by cloning and sequencing of multiple DNA clones.

In this study, we performed a comprehensive study to investigate the methylation status using two complimentary techniques: the bisulfite sequencing and TaqI cleavage, in a series of liver cancer cell lines, and a cohort of 43 HBV-associated HCC tissues and their corresponding nontumor tissue specimens. We also studied the correlation between methylation status and the gene expression of RASSF1A using the DNA methylation inhibitor 5-aza-2′-deoxycytidine in liver cancer cell lines.

MATERIALS AND METHODS

Tissue Specimens and Human Cell Lines. Surgically resected HCC and nontumor liver tissues from patients with HCC were collected. Nonmicrodissected frozen primary tumor and nontumorous samples were used for DNA extraction and subsequent analysis of HBV-DNA by Southern blot hybridization and PCR. All of the DNA samples were tested for HBV DNA by Southern hybridization or PCR using multiple primers in different parts of the HBV genome (35). Forty-three tumor and 23 corresponding nontumor specimens, in which integrated form of HBV-DNA was detected, were chosen for this study. Four human HCC cell lines (HepG2, Hep3B, SK-HEP-1, and Huh-7) were obtained from the American Type Culture Collection (Rockville, MD). Four samples of normal liver tissue and PBMCs from 10 healthy blood donors were also included as negative controls. DNA from the tissues, cell lines, and PBMCs from 10 healthy blood donors were also included as negative controls. DNA from the tissues, cell lines, and PBMCs was prepared as described previously (35). Extraction of total cellular RNA and synthesis of cDNA were performed as described previously (15).

Sodium Bisulfite Conversion. Bisulfite modification of DNA (1 μg) was carried out by the CpGenome DNA Modification kit (Intergen, Purchase, NY) according to the manufacturer’s instructions. Modified DNA samples were precipitated with ethanol and resuspended in TE buffer [10 mM Tris and 1 mM EDTA (pH 7.5)] and used immediately or stored at −80°C until use.

Methylation Status Detection by Bisulfite Sequencing and PCR-RFLP. DNA was isolated from cells and tumors, and the methylation status of the RASSF1A promoter region was determined by a bisulfite genomic sequencing protocol (15). DNA sequences were amplified by mixing 100 ng of bisulfite-treated DNA with primers MU379 (5′GTTTGGTAGTTTATGAGTTTAGTTTTT) and ML730 (5′ACCCTCTCCTCTAACACAATAAACTAACC) in 20 μl of reaction buffer containing 200 μM of each deoxynucleotide triphosphate and Taq polymerase (AmpliTaq Gold; PE Applied Biosystems, Foster City, CA) and incubated at 95°C for 15 s, 55°C for 15 s, and 74°C for 30 s for 20 cycles. A seminested PCR was performed using 1 of 20 of the initially amplified products, and an internal primer ML561 (5′CCCCACAATCTCCTACAC-CCAAAT) and primer MU379 in 50 μl of total reaction volume with similar conditions as described for the preceding PCR amplification but for 30 cycles. The PCR products containing bisulfite-resistant cytosines were ligated into the pGEM-T vector (Promega, Shanghai, China), where four clones were sequenced. All of the described sequences were determined by cycle sequencing and run on an automated DNA sequencer to determine the methylation status.

For the restriction enzyme analysis of PCR products from bisulfite-treated DNA (24), 50 ng of the PCR products were digested with three units of TaqI (New England Biolab, Beverly, MA) according to conditions specified by the manufacturer of the enzyme and analyzed on a nondenaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination. TaqI digestion of the 204-bp PCR product generates three fragments of 92, 81, and 31 bp on complete digestion or partially digested fragments of 173 and 112 bp. The density of resultant DNA bands on a gel was estimated by a scanner (GS-700; Bio-Rad, Hercules, CA). The proportion of methylation in a sample was calculated by dividing the density of complete methylation (bands 92, 81, and 31 bp) and partial methylation (bands 173 or 112 bp) by density of all of the DNA bands on the gel image. Differences in the degree of methylation between the tumor and corresponding nontumor tissues were analyzed using the paired samples t test (SPSS for Windows, Version 10). A P < 0.05 was considered to be significant.

RT-PCR Analysis. RT-PCR was performed as described previously (15). First-strand synthesis of cDNA was performed by using an oligodeoxynucleotidic acid primer and Superscript II reverse transcriptase according to the manufacturer’s instructions (Life Technologies, Inc.). A 5-μg aliquot of total cellular RNA was used for each reverse transcription reaction, and one-tenth of this reaction was used for PCR. Lower primer at exon 4 in combination with primer exon 2 or 3 and Sγ, according to a previous report (15), were used to amplify RASSF1A and RASSF1C, respectively. For both RASSF1A and RASSF1C genes, PCR was performed at 95°C for 30 s, 60°C for 30 s, and 74°C for 1 min for 35 cycles. PCR products were separated on 2% Tris-borate EDTA agarose gels, stained with ethidium bromide, and visualized under UV illumination.

Demethylating Agent Treatment. Cell lines were cultured in MEM growth medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell lines HepG2 and Hep3B at the stage of exponential growth were seeded at a density of 3 × 10⁶/100-mm culture dish. The cells were allowed to attach overnight before the addition of freshly prepared 5-aza-deoxycytidine (Sigma) at 5 μM up to 4 days. The cells where then harvested at day 2, 3, and 4, and RNA was isolated using the Rneasy Extraction kit (Qiagen, Hilden, Germany). Before RT-PCR, the RNA was treated with 10 units of DNase (Sigma Chemical Co., St. Louis, MO) as described in the Qiagen RNA clean-up protocol.

RESULTS

Methylation Status of the RASSF1A Region. We initially used bisulfite sequencing of genomic DNA to determine the methylation status of the CpG islands that initiate RASSF1A
transcription. In this method, sodium bisulfite is used to convert all of the unmethylated cytosine nucleotides to uracil and then to thymine nucleotides during the subsequent PCR step. Because 5-methylcytosine remains nonreactive, the cytosines after sequence analysis represent only methylated cytosines. Therefore, all of the cytosines presented after sequencing are derived from methylated cytosines. The promoter region (nucleotide 139 to 3), containing three Sp1 consensus binding sites and the transcription and translation initiation sites of RASSF1A, spanning 16 CpG sites was amplified by PCR using sodium bisulfite genomic DNA as templates. We analyzed the methylation pattern of the 4 liver cancer cell lines: Hep3B, HepG2, Huh-7, and SK-HEP-1, 4 normal liver tissues, 10 samples of PBMCs, and randomly selected 6 HCCs and their corresponding nontumor tissues. All of the 16 CpG sites in these four liver cell lines were completely methylated. However, three HCC specimens showed complete methylation across the entire CpG islands in the promoter region (S1, S5, and S9). The remaining HCC samples (S2, S4, and S10) and all of the corresponding adjacent nontumor tissues showed partial methylation, including those constituting one of the TaqI recognition sites. Methylation was not detected in the normal liver tissues. Furthermore, no RASSF1A promoter methylation was found in any of the PBMCs from normal blood donors (Fig. 1).

A second method by which to analyze the PCR fragments obtained from bisulfite-modified DNA is additional digestion with a restriction enzyme that has a CpG in its consensus sequence. TaqI (5' TCGA-3') will only cut previously methylated DNA after bisulfite treatment and PCR (24). The consensus sequence will be lost in unmethylated samples. The analyzed fragment has two TaqI sites. Restriction digestion of the methylated fragment results in more than one band (complete methylation results in three bands: 92, 81, and 31 bp; partial methylation results in extra two bands: 173 and 112 bp). The inability of TaqI to cut the PCR products would indicate that the original genomic fragment is unmethylated. By using this semi-quantitative assay, bisulfite-treated DNA samples were analyzed initially using bisulfite sequencing method. As shown in Fig. 2 and Table 1, PCR fragments from the cell lines and the 3 HCCs (S1, S5, and S9) that showed complete methylation by bisulfite sequencing demonstrated complete digestion at the TaqI sites, whereas the remaining HCCs (S2, S4, and S10) and all of the corresponding nontumor tissues showed partial digestion. These results were as expected from the data obtained by bisulfite sequencing as methylation involved in TaqI recognition sites in some DNA clones (Fig. 1). This suggests that the presence of TaqI partial digestion products identified in tissue specimens is because of partial methylation. Bisulfite sequencing-negative
cases (4 normal livers and 10 PBMCs) were uniformly found to be no methylation. This concordance between results from bisulfite sequencing and PCR-RFLP assays of hypermethylation status revealed that PCR-RFLP is a reliable assay for assessing RASSF1A promoter methylation status in HCC samples. The CpG island methylation of RASSF1A gene was not detected in the 4 normal liver tissues or the 10 samples of PBMCs using both methods, indicating that RASSF1A promoter hypermethylation is tumor specific.

We next sought to determine the incidence and level of RASSF1A promoter hypermethylation in the cohort of 43 primary tumor samples and their corresponding nontumor samples available. Of the 43 HCC samples analyzed, 41 (95%) were methylated in the promoter region in various levels; 38 of the 41 (92%) showed relatively high level of methylation, indicated by the relative density of methylated bands versus all of the bands by >50%. Methylation was also detected in 16 of the 23 (70%) corresponding nontumorous tissues analyzed. However, the levels of methylation were <50% in all of the cases and significantly lower than the corresponding tumor tissues (P < 0.05).

A preliminary of clinical and pathological data examination showed that hypermethylation of RASSF1A promoter was not correlated with clinical pathological variables (cirrhosis or serum α-fetoprotein level). No association was apparent between methylation and patient age or sex.

Loss of RASSF1A Expression and Restoration of Its Expression in Liver Cancer Cell Lines. To establish the functional significance of the promoter hypermethylation observed in liver carcinogenesis, we analyzed the expression of RASSF1A and RASSF1C transcripts in our Hep3B, HepG2, and SK-HEP-1 and Huh-7 cell lines by RT-PCR using isoform-specific primers (Fig. 3a). Although the RASSF1A transcript was not detectable in any of the cell lines, the alternative transcript RASSF1C was present in all (Fig. 3b). Thus, expression of RASSF1A was lost in 100% of the liver cell lines, correlating with promoter hypermethylation status.

To test whether inactivation of the RASSF1A gene is mediated by its CpG island DNA methylation, Hep3B and HepG2 cell lines were treated with the DNA methylation inhibitor 5-aza-2'-deoxycytidine. As shown in Fig. 4, 5-aza-2'-deoxycytidine treatment reactivated expression of RASSF1A in both cell lines after 4 days of treatment. However, RASSF1C expression transcript was present in all of the samples and was not influenced by treatment with 5'-aza-2'-deoxycytidine. This confirmed that promoter hypermethylation is responsible for the absence of the RASSF1A gene expression.

DISCUSSION

HBV-associated HCC comprises the majority of HCCs (~90%) in our population. In this study, we performed a comprehensive study on RASSF1A promoter hypermethylation status, and demonstrated that frequent and extensive methylation of RASSF1A promoter occurs in such HCC tissues. Transcriptional activity of the promoter of RASSF1A was not detected in any of the liver cell lines studied, consistent with the hypermethylation status in these liver cell lines. Furthermore, application of the methylase inhibitor 5-aza-2'-deoxycytidine restored expression of RASSF1A in the HepG2 and Hep3B cell lines, demonstrating that aberrant methylation is responsible for the gene silencing in HCC. As there was no intact RNA available from the tissues specimens, we were unable to assess the correlation between RASSF1A gene promoter hypermethylation and its gene expression in the primary tumors. However, as epigenetic silencing of RASSF1A gene was found in all of the HCC cell lines studied, RASSF1A gene expression may also be affected by its promoter CpG islands hypermethylation in the primary tissue. Similarly, epigenetic silencing of the RASSF1A gene had been observed in other types of cancers (15–21). Our data represent the highest incidence of promoter methylation of RASSF1A yet reported in malignancies and suggest that hypermethylation of the CpG island promoter of RASSF1A may play an important role in the development of HBV-associated HCC.

By using both bisulfite sequencing and PCR-RFLP, we could perform a comprehensive study of the nature of RASSF1A gene methylation in HCC. We demonstrated that a heterogeneous methylation pattern was present in a substantial percentage of HCC tissues and all of the nontumor tissues. To our knowledge, this is the first report to show extensive heterogeneous methylation pattern of RASSF1A gene in both tumor tissues and their associated (cirrhotic) nontumorous tissues. However, complete methylation at CpG sites was found in all of the four liver cancer cell lines analyzed and in about one-third of the HCC tissues. A degree of methylation of >50% would suggest either
of the studied cell lines, Hep3B and HepG2 (data not shown).

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may indeed involve both alleles. In agreement with such a

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and that the other one is lost. Interestingly, we could detect an

of RASSF1A inactivation was present in 70%

that both alleles are methylated or that one allele is methylated and the other one is lost. Interestingly, we could detect an

intensive methylation of RASSF1A in all of the cell lines and the majority of primary tumor tissues, indicating that methylation may indeed involve both alleles. In agreement with such a

notion, LOH of 3p21 has not been reported in HCC (9–14), and an abnormality in the 3p21.3 region has not been found in two of the studied cell lines, Hep3B and HepG2 (data not shown).

However, both cell lines showed restoration of RASSF1A expression after 5-aza-2’-deoxycytidine treatment. In addition, low level (<50%) of RASSF1A inactivation was present in 70%

of nontumorous tissues, which were mostly classified as cirrhosis or chronic hepatitis B infection, suggesting that methylation may be a progressive process extending from one allele to the other. Thus, methylation of RASSF1A is likely to be an early event in the carcinogenesis in the majority of HBV-associated HCCs.

The reason for intensive hypermethylation of the CpG island of RASSF1A promoter region in our HBV-associated HCC is currently unknown. It has been reported that increased DNA methyltransferase mRNA expression occurs in HCC tissues and in liver tissues showing hepatitis (36, 37). In addition, recent studies have suggested that chronic inflammation may be associated with aberrant gene promoter methylation and silencing in ulcerative colitis and gastritis (38, 39). Interestingly, we have found frequent silencing of multiple genes, including P16 (data not shown) and GSTP1 (40) genes in the same cohort of HCC patients. It is possible that the abnormality in DNA methyltransferase activity during chronic hepatitis B infection may contribute to the abnormal de novo CpG island hypermethylation changes affecting RASSF1A, P16, GSTP1, and other genes (41–44), which accumulate during the pathogenesis of human HCC. Similarly, frequent RASSF1A promoter methylation was reported in other virus-related malignancies, such as EBV-associated nasopharyngeal carcinoma in this locality (21), and SV40-associated mesotheliomas (45, 46). In addition, a very low rate of methylation of RASSF1A gene was reported in

Table 1 The RASSF1A promoter methylation status measured by PCR-RFLP in the 43 HCC and corresponding nontumor tissue specimens

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gender/age</th>
<th>Cirrhosis</th>
<th>Serum AFP (µg/liter)</th>
<th>PCR-RFLP analysis of RASSF1A methylation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2</td>
<td>M/60</td>
<td>+</td>
<td>&lt;10</td>
<td>79% Tumor, 79% Nontumor</td>
</tr>
<tr>
<td>H3</td>
<td>M/39</td>
<td>+</td>
<td>&lt;10</td>
<td>90% Tumor, 90% Nontumor</td>
</tr>
<tr>
<td>H4</td>
<td>M/71</td>
<td>+</td>
<td>9</td>
<td>78% Tumor, 78% Nontumor</td>
</tr>
<tr>
<td>H5</td>
<td>F/54</td>
<td>–</td>
<td>36</td>
<td>61% Tumor, 61% Nontumor</td>
</tr>
<tr>
<td>H6</td>
<td>M/12</td>
<td>–</td>
<td>na</td>
<td>20% Tumor, 20% Nontumor</td>
</tr>
<tr>
<td>H7</td>
<td>M/53</td>
<td>–</td>
<td>1257</td>
<td>64% Tumor, 64% Nontumor</td>
</tr>
<tr>
<td>H8</td>
<td>M/58</td>
<td>–</td>
<td>5060</td>
<td>14% Tumor, 14% Nontumor</td>
</tr>
<tr>
<td>H10</td>
<td>M/41</td>
<td>–</td>
<td>115</td>
<td>68% Tumor, 68% Nontumor</td>
</tr>
<tr>
<td>H11</td>
<td>M/68</td>
<td>–</td>
<td>na</td>
<td>60% Tumor, 60% Nontumor</td>
</tr>
<tr>
<td>H12</td>
<td>F/59</td>
<td>+</td>
<td>&lt;10</td>
<td>61% Tumor, 61% Nontumor</td>
</tr>
<tr>
<td>H13</td>
<td>M/45</td>
<td>+</td>
<td>4</td>
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</tr>
<tr>
<td>H14</td>
<td>F/46</td>
<td>–</td>
<td>124</td>
<td>57% Tumor, 57% Nontumor</td>
</tr>
<tr>
<td>H15</td>
<td>M/55</td>
<td>+</td>
<td>511</td>
<td>100% Tumor, 100% Nontumor</td>
</tr>
<tr>
<td>H16</td>
<td>F/66</td>
<td>+</td>
<td>8480</td>
<td>80% Tumor, 80% Nontumor</td>
</tr>
<tr>
<td>H17</td>
<td>M/66</td>
<td>–</td>
<td>137</td>
<td>79% Tumor, 79% Nontumor</td>
</tr>
<tr>
<td>H18</td>
<td>M/64</td>
<td>+</td>
<td>35</td>
<td>52% Tumor, 52% Nontumor</td>
</tr>
<tr>
<td>H19</td>
<td>M/64</td>
<td>+</td>
<td>&lt;10</td>
<td>20% Tumor, 20% Nontumor</td>
</tr>
<tr>
<td>H20</td>
<td>F/69</td>
<td>–</td>
<td>675</td>
<td>na Tumor, na Nontumor</td>
</tr>
<tr>
<td>H21</td>
<td>M/74</td>
<td>–</td>
<td>&lt;10</td>
<td>30% Tumor, 30% Nontumor</td>
</tr>
<tr>
<td>H22</td>
<td>M/68</td>
<td>+</td>
<td>439</td>
<td>100% Tumor, 100% Nontumor</td>
</tr>
<tr>
<td>H23</td>
<td>M/41</td>
<td>–</td>
<td>3140</td>
<td>79% Tumor, 79% Nontumor</td>
</tr>
<tr>
<td>H24</td>
<td>M/48</td>
<td>+</td>
<td>309</td>
<td>78% Tumor, 78% Nontumor</td>
</tr>
<tr>
<td>H25</td>
<td>M/67</td>
<td>+</td>
<td>38</td>
<td>75% Tumor, 75% Nontumor</td>
</tr>
<tr>
<td>S1</td>
<td>M/37</td>
<td>+</td>
<td>622</td>
<td>100% Tumor, 100% Nontumor</td>
</tr>
<tr>
<td>S2</td>
<td>F/53</td>
<td>–</td>
<td>2692</td>
<td>68% Tumor, 68% Nontumor</td>
</tr>
<tr>
<td>S3</td>
<td>M/36</td>
<td>+</td>
<td>49</td>
<td>78% Tumor, 78% Nontumor</td>
</tr>
<tr>
<td>S4</td>
<td>M/63</td>
<td>–</td>
<td>9</td>
<td>55% Tumor, 55% Nontumor</td>
</tr>
<tr>
<td>S5</td>
<td>M/41</td>
<td>+</td>
<td>2387</td>
<td>100% Tumor, 100% Nontumor</td>
</tr>
<tr>
<td>S6</td>
<td>M/53</td>
<td>+</td>
<td>&lt;10</td>
<td>56% Tumor, 56% Nontumor</td>
</tr>
<tr>
<td>S7</td>
<td>F/52</td>
<td>–</td>
<td>154</td>
<td>87% Tumor, 87% Nontumor</td>
</tr>
<tr>
<td>S8</td>
<td>M/22</td>
<td>+</td>
<td>400</td>
<td>79% Tumor, 79% Nontumor</td>
</tr>
<tr>
<td>S9</td>
<td>M/45</td>
<td>+</td>
<td>na</td>
<td>100% Tumor, 100% Nontumor</td>
</tr>
<tr>
<td>S10</td>
<td>M/61</td>
<td>–</td>
<td>387</td>
<td>55% Tumor, 55% Nontumor</td>
</tr>
<tr>
<td>S11</td>
<td>M/59</td>
<td>–</td>
<td>2</td>
<td>58% Tumor, 58% Nontumor</td>
</tr>
<tr>
<td>S12</td>
<td>M/56</td>
<td>+</td>
<td>215</td>
<td>54% Tumor, 54% Nontumor</td>
</tr>
<tr>
<td>S13</td>
<td>M/38</td>
<td>–</td>
<td>1696</td>
<td>77% Tumor, 77% Nontumor</td>
</tr>
<tr>
<td>S14</td>
<td>M/34</td>
<td>+</td>
<td>1860</td>
<td>77% Tumor, 77% Nontumor</td>
</tr>
<tr>
<td>S15</td>
<td>M/45</td>
<td>+</td>
<td>20</td>
<td>86% Tumor, 86% Nontumor</td>
</tr>
<tr>
<td>S16</td>
<td>M/47</td>
<td>+</td>
<td>20</td>
<td>54% Tumor, 54% Nontumor</td>
</tr>
<tr>
<td>S17</td>
<td>M/53</td>
<td>+</td>
<td>1279</td>
<td>89% Tumor, 89% Nontumor</td>
</tr>
<tr>
<td>S18</td>
<td>M/47</td>
<td>+</td>
<td>1826</td>
<td>88% Tumor, 88% Nontumor</td>
</tr>
<tr>
<td>S19</td>
<td>M/41</td>
<td>+</td>
<td>500</td>
<td>83% Tumor, 83% Nontumor</td>
</tr>
<tr>
<td>S20</td>
<td>F/32</td>
<td>+</td>
<td>154</td>
<td>81% Tumor, 81% Nontumor</td>
</tr>
</tbody>
</table>

* na, sample not available.
hepatoblastomas, which is HBV-unrelated disease (47), also in consistent to such a notion. We have considered the possibility that aberrant methylation occurring during the regeneration process that is characteristic of chronic liver disease may involve TSGs. Such a hypothesis might be one explanation for the strong association between chronic liver disease and HCC.

The high frequency of epigenetic inactivation of the RASSF1A gene in HCC supports its role as a putative TSG. Although mutational analysis was not performed in this study, other studies have shown that somatic mutations appear to be rare in this gene (15, 17, 18, 21). Thus, epigenetic inactivation may be the major loss of function pathway for the RASSF1A gene in carcinogenesis in chronic HBV carriers. A recent study has shown that RASSF1C binds RAS in a GTP-dependent manner (48), similar to the mammalian Ras effector Nore1 (49). Overexpression of RASSF1C induces apoptosis. Because RASSF1A and RASSF1C share the identical Ras association domain, it is possible that RASSF1A binds to RAS in the same manner. Activated RAS proteins are usually associated with transformation. However, RAS can also induce growth-inhibitory effects manifested by senescence, terminal differentiation, or apoptosis (50–52). RASSF1 might be responsible for the RAS-dependent growth inhibition through its proapoptotic function (47). Loss of RASSF1 expression by methylation in HCC may shift the balance of RAS activities toward a growth-promoting effect without the necessity of RAS-activating mutations. Unlike RASSF1C, RASSF1C expression was not affected in the four liver cell lines analyzed, and, presumably, the RASSF1C promoter region was not methylated in the liver cancer cell lines. Thus, additional molecular biological studies will be required to define the precise role of the alternative expression of RASSF1 isoforms in the development of liver cancer.

The finding of frequent RASSF1A methylation in HCC was unexpected, as studies had failed to detect of 3p allele loss in HCC. However, a recent report showed that frequent deletion in this arm (3p11.2-p14.2) 3p21.3 was not involved (14). The results presented here suggest that methylation of this arm (3p11.2-p14.2) 3p21.3 was not involved (14). The results presented here suggest that methylation of the RASSF1A promoter has a critical role in tumor development, which is not activated by epigenetic silencing in regions with infrequent allele loss.

REFERENCES


Intensive Hypermethylation of the CpG Island of Ras Association Domain Family 1A in Hepatitis B Virus-associated Hepatocellular Carcinomas

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