Hypermethylation of the p14ARF Gene in Ulcerative Colitis-associated Colorectal Carcinogenesis

Fumiaki Sato, Noam Harpaz, David Shibata, Yan Xu, Jing Yin, Yuriko Mori, Tong-Tong Zou, Suna Wang, Kena Desai, Anatoly Leytin, Florin M. Selaru, John M. Abraham, and Stephen J. Meltzer

Gastroenterology Division, Department of Medicine, University of Maryland School of Medicine and Gastroenterology Service, Department of Medicine, Baltimore Veterans Administration Hospital Baltimore, Maryland 21201 [F. S., Y. Y., J. Y., Y. M., T.-F. Z., S. W., K. D., F. M. S., J. M. A., S. J. M.]; Department of Pathology, The Mount Sinai School of Medicine, New York, New York 10029 [N. H., A. L.]; and Division of Surgical Oncology, Department of Surgery, University of Maryland School of Medicine, Baltimore, Maryland 21201 [D. S.]

ABSTRACT

The p14ARF protein directly inhibits the MDM-2 oncoprotein, which mediates degradation of the p53 protein. It has been shown that p14ARF expression is frequently down-regulated by p14ARF gene hypermethylation in colorectal cancer. To determine whether p14ARF inactivation was involved in ulcerative colitis (UC)-associated carcinogenesis, the frequency and timing of p14ARF methylation were investigated in four different histological stages of UC-associated carcinogenesis. Methylation-specific PCR and bisulfite sequencing were used to determine the prevalence of p14ARF gene methylation. p14ARF methylation was observed in 19 of 38 (50%) adenocarcinomas, 4 of 12 (33%) dysplasias, and 3 of the 5 (60%) nonneoplastic UC mucosa. In contrast, 3 of 40 (3.7%) normal tissues showed p14ARF methylation (χ² test; P = 0.0005). Bisulfite sequencing was used to analyze 28 CpGs of p14ARF gene in 20 samples. The number of methylated CpGs ranged from 0 to 4, 0 to 20, and 0 to 28 in the normal, dysplastic, and carcinomatous samples, respectively (Kruskall-Wallis test; P = 0.0005). Densely methylated alleles were detected only in carcinomas by bisulfite sequencing. In conclusion, our data suggest that methylation of p14ARF is a relatively common early event in UC-associated carcinogenesis. p14ARF offers potential as a biomarker for the early detection of cancer or dysplasia in UC. Finally, analyses of p14ARF methylation in other organs should explore not only frank cancers but other premalignant lesions.

INTRODUCTION

UC is a chronic disease characterized by inflammation of the mucosa and submucosa of the large intestine. The duration and extent to which a patient suffers from UC correlate directly with an increased propensity to develop colorectal carcinoma (1, 2). For patients who have had UC for >20 years, the incidence of colorectal cancer is 10–20-fold greater than that of the general population, and the average age of onset is 20 years earlier (3). UC-associated colorectal carcinoma is different from sporadic carcinoma; unlike sporadic colorectal carcinoma, which arises from adenomatous polyps, UC-associated colorectal carcinoma progresses from areas of dysplastic mucosa. Although the molecular events that facilitate the progression of adenoma to carcinoma in sporadic colorectal cancer have been well investigated (4), much remains to be learned regarding molecular events underlying the progression of UC mucosa to dysplasia and carcinoma. The p53 tumor suppressor gene is frequently inactivated in both sporadic and UC-associated colorectal carcinoma (4–6). Although point mutation and loss of heterozygosity are the most commonly reported mechanisms resulting in p53 inactivation, other genetic and epigenetic factors have been shown to modify p53 activity as well. Amplification of the MDM-2 gene (the protein product of which tags p53 for degradation) and expression of viral oncoproteins (which sequester p53) are two such examples (7).

Recently, p14ARF has been ascribed a role in modulating the cellular amounts of p53 through direct interaction with MDM-2. MDM-2, which tags p53 for degradation through the ubiquitin/proteosome pathway, is inhibited by p14ARF (8). Homozygous deletion of the p14ARF locus has been reported in a variety of cancers (9–13), and gene knockout of p14ARF correlates with tumorigenesis (14). In addition to mutation, the p14ARF gene can also be epigenetically inactivated through hypermethylation of its normally unmethylated CpG island. Esteller et al. (15) demonstrated that p14ARF hypermethylation occurs frequently in sporadic colorectal cancer. To determine whether p14ARF hypermethylation occurs during the progression of UC mucosa to carcinoma, the frequency and timing of p14ARF methylation were investigated in clinical samples ranging from nonneoplastic UC mucosa to colorectal carcinoma.

MATERIALS AND METHODS

Tissue Samples. Matching normal and tumor tissues were obtained at the time of surgical resection from 40 patients with one or more UC-associated colorectal neoplasms. The UC-associated neoplasms consisted of 38 adenocarcinomas and 12 dysplasias. Normal control samples consisted of 30 ileal mucosae, 2 smooth muscle tissues, and 3 nonmetastatic lymph nodes. All tissues were grossly dissected free of normal surrounding tissue, and parallel sections were used for histological characterization. Microdissection was not performed; however, the tissues were selected to include only those tumors containing 70% or more tumor cells by H&E staining.

DNA Extraction. Genomic normal and tumor DNAs were extracted using published protocols (16, 17). MSP. DNA methylation of p14ARF was determined by MSP (18). MSP distinguishes unmethylated alleles of a given gene based on DNA sequence alterations after bisulfite treatment of DNA, which converts unmethylated but not methylated cytosines to uracils. Subsequent PCR using primers specific to sequences corresponding to either methylated or unmethylated DNA sequences is then performed. We slightly shifted the MSP primer location used by Esteller et al. (Ref. 15; Fig. 1). Primers for MSP were designed using the GenBank L41934 sequence for p14ARF. Primer sequences of p14ARF for the unmethylated reaction were forward (5′-GTTTTTGTGATTGTTTTTGATTGGT-3′) and reverse (5′-TACCCACTCCCCCCATAAAACCACA-3′), which amplify a 94-bp product. Primer sequences for the methylated reaction were forward (5′-GTTTTTGATGTTTTTGATTGGT-3′) and reverse (5′-CAGCCCCCTGAGACCGG-3′), which amplify an 84-bp product. Briefly, 2 μg of genomic DNA were denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega Corp., Madison, WI), treated with NaOH, precipitated with ethanol, and resuspended in 20 μl of water. Two μl of modified DNAs were PCR amplified in a total volume of 50 μl. The annealing temperature for both unmethylated and methylated reactions was 58°C. PCR was performed in a thermal cycler (Biometra T-Gradient Thermoblock, Goettingen, Ger-
RESULTS

MSP of p14ARF. DNA obtained from 40 patients were analyzed by MSP. p14ARF hypermethylation was present in 19 of 38 (50%) adenocarcinomas, 4 of 12 (33%) dysplasias, and 3 of 5 (60%) nonneoplastic UC mucosa. Conversely, 3 of 40 (7.5%) normal tissues showed p14ARF hypermethylation (χ² test: P = 0.0003). No other correlations were found between hypermethylation status of p14ARF and clinicopathological features such as age, gender, histology, anatomical location of sample (right versus left colon), or Duke’s stage. All three methylated bands obtained from normal tissue were faint. Examples are shown in Fig. 2.

Bisulfite Sequencing of p14ARF. Two hundred thirty-two clones from 20 samples (>10 clones from each sample) were investigated using bisulfite sequencing. Bisulfite sequencing showed 3 negative, 2 partial, and 3 positive results among 8 MSP-positive samples, whereas all MSP-negative samples were sequence negative (Table 1). As shown in Table 2, no densely methylated clones were found in normal samples. An example is shown in Fig. 3. Partially methylated clones were observed in dysplastic samples, and densely methylated clones were detected only in carcinoma samples (Kruskall-Wallis test: P = 0.0005). In 4 samples, we detected 12 clones with methylation at all 6 MSP primer-recognized CpGs.

Table 1 Comparison between MSP and bisulfite sequencing

<table>
<thead>
<tr>
<th>Sample</th>
<th>Patient</th>
<th>Histology</th>
<th>Bisulfite sequencing</th>
<th>MSP¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H24</td>
<td>Normal tissue</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Carcinoma</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>H81</td>
<td>Normal tissue</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Carcinoma</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>H82</td>
<td>Normal tissue</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Carcinoma</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>H92</td>
<td>Normal tissue</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Carcinoma</td>
<td>Partial</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>H89</td>
<td>Normal tissue</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>Dysplasia</td>
<td>Partial</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>11</td>
<td>H73</td>
<td>Normal tissue</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>Dysplasia</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>Carcinoma</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>14</td>
<td>H69</td>
<td>Normal tissue</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>15</td>
<td>Dysplasia</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>16</td>
<td>Carcinoma 1</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>17</td>
<td>Carcinoma 2</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>18</td>
<td>H26</td>
<td>Normal tissue</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>19</td>
<td>Dysplasia</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>20</td>
<td>Carcinoma</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

¹ Negative, very low density of methylation; partial, presence of clones, more than half of which CpGs were methylated; positive, presence of clones with all six MSP-recognition CpGs positive.

² Negative and positive, absent and present of methylated band, respectively.

Fig. 2. MSP analysis of the p14ARF gene. A visible PCR product in Lane U indicates the presence of unmethylated p14ARF, a visible product in Lane M indicates the presence of methylated p14ARF. CpGenome Universal Methylated DNA (Integen, NY) was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as a negative control for methylated genes. Water controls for the PCR reaction are also shown. Lesions T1 and T2 of H69, D of H89, and T of H82 were methylation positive, whereas all others were methylation negative. N, normal tissue; D, dysplasia; T, tumor; NL, normal lymph node; TM, totally methylated control DNA.
The number of methylated CpGs in these 12 clones ranged from 20 to 28 (mean, 25.3) of 28 CpGs. No particular pattern could be observed in the distribution of methylated CpGs in different samples. We confirmed that the methylated and unmethylated control DNAs used in MSP were totally methylated and unmethylated, respectively, by bisulfite sequencing.

**DISCUSSION**

The purpose of this study was to determine the frequency and timing of p14ARF hypermethylation in UC-associated colorectal carcinogenesis. In this study, hypermethylation of p14ARF was present in 19 of 38 (50%) carcinomas and in 4 of 12 (33%) dysplasias. Esteller et al. (15) and Burri et al. (19) reported that the frequency of p14ARF hypermethylation is 28%–33% in sporadic colorectal cancers and 32% in colon adenomas. In comparison with these data, our hypermethylation frequencies are somewhat higher.

Although several groups have reported genetic abnormalities in nonneoplastic UC mucosa, such as microsatellite instability (20, 21) and p53 mutation (22–25), hypermethylation of cancer-related genes has not been reported in this setting. Hypermethylation of tumor suppressor genes has been demonstrated in other precancerous lesions associated with chronic inflammation. Hypermethylation of E-cadherin has been reported in chronic gastritis (26), and hypermethylation of APC, p16INK2a, and E-cadherin has been demonstrated in the esophagitis-associated lesion, Barrett’s esophagus (27). Previously, Esteller et al. (15) found no evidence of p14ARF hypermethylation in normal uninflamed colonic mucosa; however, we have noted p14ARF hypermethylation in 3 (60%) of 5 (60%) nonneoplastic inflamed UC mucosae. Therefore, it is possible that longstanding chronic inflammation induces aberrant methylation of the p14ARF gene in colonic mucosa. It is also possible that p14ARF methylation varies according to location within the intestinal tract (e.g., absent in the ileum but present in the colon). However, our study suggests that p14ARF methylation is a potential marker of early carcinogenesis in the setting of UC. In this regard, p14ARF may resemble the p53 tumor suppressor gene, in that alterations of p53 are regarded as relatively early events in UC-associated carcinogenesis (5, 22–25).

In this study, 3 of 20 samples studied by bisulfite sequencing showed results different from MSP. Because our samples had varying degrees of contamination by normal and stromal cells, our observed ratios of methylated CpGs could underestimate actual methylation ratios in tumor cells. However, according to our bisulfite sequencing data, the density of methylated CpGs increased during the progression from dysplasia to carcinoma. These findings suggest that aberrant methylation of the p14ARF gene may be progressive during the dysplasia-carcinoma sequence of UC.

Regarding the distribution pattern of methylated CpGs, no significant pattern was found in this study. Zheng et al. (28) described that the 3’ region of exon 1β was more densely methylated than the promoter and 5’ region of exon 1β. However, their primers for bisulfite sequencing were situated on several CpGs, and the forward primer sequence appeared to be based on the unmethylated sequence, whereas the reverse primer was based on the methylated sequence. Primers for bisulfite sequencing are generally placed in regions lacking the sequence CpG.

In conclusion, our data suggest that hypermethylation of p14ARF is a relatively common and early event in UC-associated carcinogenesis. Thus, p14ARF is worthy of study to explore its potential as a biomarker for the early detection of cancer or dysplasia in UC. Furthermore, future analyses of p14ARF methylation in other or-
gans should focus not only on frank cancers but on other prema-

lignant lesions as well.

REFERENCES

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