Reprimo Methylation Is a Potential Biomarker of Barrett’s-Associated Esophageal Neoplastic Progression

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Abstract Purpose: Reprimo, a candidate tumor-suppressor gene, regulates p53-mediated cell cycle arrest at G2 phase, and tumor-suppressor gene methylation is involved in the pathogenesis and progression of esophageal cancer. Our aim was to determine whether and at what phase of neoplastic progression Reprimo methylation occurs in Barrett’s adenocarcinogenesis, as well as its columnar or squamous cell-type specificity. We also sought to determine whether Reprimo expression could be restored in vitro by the demethylating agent 5-aza-deoxycytidine (5AzaC).

Experimental Design: Quantitative methylation-specific PCR for Reprimo was done using an ABI7700 (Taqman) apparatus on 175 endoscopic biopsy specimens. In addition, reverse transcription-PCR and quantitative methylation-specific PCR were done on esophageal carcinoma cells before and after treatment with 5AzaC.

Results: In Barrett’s esophagus (BE; P = 0.001), high-grade dysplasia (HGD; P = 0.001), and esophageal adenocarcinoma (EAC; P = 0.00003), the level and frequency of Reprimo methylation were significantly higher than in normal esophagus (NE). There was no statistically significant difference between BE and EAC, HGD and EAC, or NE and esophageal squamous cell carcinoma (ESCC). Reprimo methylation occurred in 0 of 19 NE samples, 6 (13%) of 45 ESCC, 9 (36%) of 25 BE, 7 (64%) of 11 HGD, and 47 (63%) of 75 EAC. Analysis of Reprimo methylation in EAC versus NE revealed an area under the receiver-operator characteristic curve of 0.812 (P < 0.00001; 95% confidence interval, 0.73-0.90). In vitro 5AzaC treatment of OE33 EAC cells reduced Reprimo methylation and increased Reprimo expression.

Conclusions: Reprimo methylation occurs significantly more frequently in BE, HGD, and EAC than in NE or ESCC, suggesting that this epigenetic alteration is a specialized columnar, cell-specific early event with potential as a biomarker for the early detection of esophageal neoplasia.

Reprimo is a candidate tumor-suppressor gene that is involved in the regulation of the p53-mediated cell cycle arrest at the G2 phase (1). The Reprimo gene maps to chromosome 2q23, a locus that commonly shows allelic imbalance in human cancers (2). Allelic imbalance is one mechanism of gene inactivation, but other examples include point mutation, deletion, and methylation (3). It is now well established that DNA methylation correlates with silencing of gene transcription (4–6). In addition, there is a growing body of evidence showing that the abnormal methylation of DNA is an early event in carcinogenesis (6–8). In previous reports, Reprimo was frequently methylated in multiple human malignancies, including esophageal cancer (9–12). However, these studies contained too few specimens (five) to accurately describe the prevalence of aberrant methylation of Reprimo in esophageal cancer (12). Esophageal cancer ranks as the eighth most common cancer and the sixth most frequent cause of cancer death worldwide (13). Moreover, the incidence of esophageal adenocarcinoma (EAC) has increased rapidly over the past 25 years in both the United States and in several Western European countries (14). In 2005, there were an estimated 14,500 new cases in the United States alone (15). Despite therapy, 5-year survival rates remain dismal (3-25% depending on stage; ref. 16). Clearly, novel early detection biomarkers and therapeutic targets are needed, and DNA methylation represents a potential target for the early detection of cancer and novel therapeutic strategies (8, 17, 18). We have recently shown that Reprimo methylation predicts a poor response to chemotherapy and radiation in esophageal cancer (19). Accordingly, our goal was to determine the methylation status of Reprimo in esophageal cancer, premalignant lesions [henceforth called Barrett’s esophagus (BE) or BE...
with high-grade dysplasia (HGD), normal esophageal epithelium (NE), and esophageal squamous cell carcinoma (ESCC). In addition, we sought to examine the methylation of Reprimo in tissue that has been associated with neoplastic progression, i.e., BE segment length and the presence of HGD within a field of BE.

Materials and Methods

Tissue samples. One hundred seventy-five esophageal samples were serially obtained endoscopically from 25 patients with BE, 45 with ESCC, 75 with EAC, 11 with HGD, and 19 with refractory gastroesophageal reflux symptoms. This patient population was not representative of the general population as a whole due to the tertiary referral nature of the study institutions. Samples were immediately frozen on dry ice and stored at −80 °C until DNA extraction. Tissue from cancers, BE, or NE was also sent for histology to confirm the pathologic diagnosis. The NE samples showed no endoscopic or microscopic evidence of premalignant or malignant lesions. Patients with BE had neither endoscopic nor microscopic evidence of dysplasia or tumor. A separate category of patients with BE had histologically confirmed HGD. All samples were obtained from patients at the University of Maryland Medical Center or the Baltimore Veterans Affairs Medical Center, and these patients willfully consented to the research protocol. The Institutional Review Board of these respective facilities approved this protocol. The demographics of the patients can be found in Table 1.

Cell lines. Three EAC cell lines (BIC, OE33, and SEG) and 11 ESCC cell lines (KYSE 30, 70, 110, 140, 170, 220, 410, 520, 770, and 850) were obtained from collaborating investigators in Japan (Y.S.) and Michigan (D.B.). These cell lines were stored at −80 °C.

Primer and probe design. Primers and probe for quantitative methylation-specific PCR (qMSP) were designed based on the University of California Santa Cruz Human Genome Browser sequence data, and manufactured by Integrated DNA Technologies (Coralville, IA). Probe and primer sequences are listed in Supplementary Table S1.

Prescreening of Reprimo for methylation in normal WBC. Before testing in any human esophageal tissues, Reprimo was tested for methylation in normal WBC. The assumption was made that methylation of a tumor-suppressor gene should not occur in normal WBC. The assumption was made that methylation of a given gene based on DNA sequence alterations after bisulfite treatment of DNA. Bisulfite treatment converts unmethylated but not methylated cytosines to uracil. Subsequent PCR using primers and probe specific to the corresponding methylated DNA sequence is then done. β-Actin was selected as an internal control, and analyses were based on previously published primer and probe sequences (23). Briefly, 1.0 μg genomic DNA was denatured by treatment with 2 mol/L NaOH and modified by 3 mol/L sodium bisulfite. DNA samples were purified using Wizard DNA cleanup resin (Promega, Madison, WI), treated with 3 mol/L NaOH, precipitated with 100% ethanol, and resuspended in 50 μL water. The PCR mixture consisted of 12.5 μL Taqman Universal Master Mix without UNG (Applied Biosystems, Foster City, CA). 2.0 μL of probe for both Reprimo and β-actin (2.5 μmol/L), 0.25 μL forward and reverse primer for both Reprimo and β-actin (10 μmol/L), 50 ng bisulfite-treated DNA, and water (up to a total volume of 25 μL). PCR and real-time data collection were done using an ABI7700 Sequence Detection System (Taqman, Applied Biosystems) for activation of Taq polymerase at 95 °C for 10 minutes and then 50 cycles consisting of denaturation at 95 °C for 15 seconds and annealing and extension for 1 minute at 60 °C. Cpg Universal Methylated DNA (Intergen, Burlington, MA) was used to generate a standard curve for each reaction and represented totally methylated control DNA. Reaction mix without any bisulfite-treated DNA served as a negative control.

Analyses of MSP results. The normalized MSP value (NMV) was calculated by dividing the ratio of the qMSP value for Reprimo to β-actin for each sample by the ratio of the qMSP value for Reprimo to β-actin for Universal Methylated DNA (21, 24). Qualitative MSP status was determined by analyzing the NMV. A NMV of 0.05 was assigned as the cutoff point for classifying methylation status as qualitatively positive (≥0.05) or negative (<0.05). This cutoff point had been previously determined by receiver-operator characteristic (ROC) curve analysis (19).

5-Aza-2′-deoxycytidine treatment of esophageal cancer cell lines. To show the gene-silencing effect of Reprimo methylation in esophageal carcinoma, two cancer cell lines were subjected to treatment with 5-aza-2′-deoxycytidine (5AzaC; Sigma, St. Louis, MO). The squamous carcinoma (KYSE 110) and the adenocarcinoma cell line (OE33) that showed the highest quantitative values of methylation in their respective tissue types were chosen for 5AzaC treatment. This treatment protocol has been published previously (24). Briefly, 1 × 10⁵ cells/mL were seeded in 100-mm culture dishes and grown in a mixture of 47.5% RPMI 1640 (Life Technologies, Inc., Rockville, MD), 47.5% Ham’s medium (Invitrogen), and 5% fetal bovine serum (Invitrogen). Cell cultures were incubated at 5% CO₂ for 24 hours at 37 °C. Then, 1 μL of 5 mmol/L 5AzaC per milliliter of cells was added every 24 hours for 6 days. Cells were harvested at days 0, 2, 4, and 6. Harvested cells were stored at −20 °C until DNA extraction. Medium was changed every 72 hours.

Statistical analysis. The NMVs for each tissue type were compared with each other using Student’s paired t test (Statistica 6.0). In addition,

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>n</th>
<th>Age (y)</th>
<th>Sex (%)</th>
<th>Race (%)</th>
<th>Other (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>F</td>
<td>White</td>
<td>Asian</td>
</tr>
<tr>
<td>EAC</td>
<td>75</td>
<td>63.5 ± 11.9</td>
<td>5 (6.6)</td>
<td>65 (86.6)</td>
<td>5 (6.6)</td>
</tr>
<tr>
<td>HGD</td>
<td>11</td>
<td>70.3 ± 7.9</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>BE</td>
<td>25</td>
<td>61.2 ± 14.7</td>
<td>4 (4)</td>
<td>18 (94.7)</td>
<td>1 (5.3)</td>
</tr>
<tr>
<td>NE</td>
<td>19</td>
<td>62.6 ± 11.5</td>
<td>19</td>
<td>28 (62.2)</td>
<td>15 (33.3)</td>
</tr>
<tr>
<td>ESCC</td>
<td>45</td>
<td>62.4 ± 7.8</td>
<td>33 (73.3)</td>
<td>28 (62.2)</td>
<td>15 (33.3)</td>
</tr>
</tbody>
</table>

Abbreviations: UICC, Unio Internationale Contra Cancrum; AA, African American.
to provide further statistical rigor, and because the data were asymmetrical and did not fit a normal Gaussian distribution (e.g., many data points were equal to zero), further nonparametric testing was done using Mann-Whitney’s U test (Statistica 6.0). To show the ability of Reprimo methylation to distinguish between NE and EAC, ROC curve analysis (Analyze-it; ref. 25) was done using the NMV of the 75 EAC and 19 NE tissues (26). A P value of <0.05 was considered to be significant for all statistical calculations.

Results

Patient characteristics. The demographics of the patients enrolled in the current study are displayed in Table 1. The samples consisted of 75 EAC, 45 ESCC, 25 BE, 11 HGD, and 19 NE. All patients were of similar age (Student’s t test, NE versus BE, P = 0.74; NE versus HGD, P = 0.06; NE versus EAC, P = 0.76; NE versus ESCC, P = 0.96). In all histologic types, the overwhelming majority of patients were White (NE, 94.7%; BE, 80%; HGD, 100%; EAC, 86.6%; ESCC, 62.2%) and male (NE, 74.7%; BE, 96%; HGD, 100%; EAC, 93%; ESCC, 73.3%). According to generally accepted criteria, BE was defined as “long segment” if it was >3 cm and “short segment” if <3 cm (27). In this study, there were nine short-segment cases of BE and 16 long-segment cases of BE. Among the patients with EAC, there were seven cases with Unio Internationale Contra Cancrum stage I disease, 17 with stage II, 35 with stage III, and 16 with stage IV disease. Tumor stage data was not available for all of the ESCC cases.

MSP of esophageal tissues. MSP results are displayed in Fig. 1 and Table 2. NMVs and methylation status were determined as described. The mean NMV for NE was 0.004 ± 0.009 (mean ± SD); 0 of 19 patients had positive methylation status. The mean NMV for BE was 0.111 ± 0.191; 9 (36%) of 25 had positive methylation status. The mean NMV for the patients with HGD was 0.223 ± 0.229; 7 (64%) of 11 had positive methylation status. In patients with EAC, the mean NMV was 0.249 ± 0.448, and 47 (63%) of 75 had positive methylation status. Last, the mean NMV for ESCC was 0.088 ± 0.259, and 6 (13%) of 45 patients had positive methylation status. The difference in quantitative methylation of NE versus BE was significant (Student’s t test, P = 0.02; Mann-Whitney’s U test, P = 0.001), as were the differences in quantitative methylation of NE versus HGD (Student’s t test, P = 0.003; Mann-Whitney’s U test, P = 0.001) and between NE and EAC (Student’s t test, P = 0.02; Mann-Whitney’s U test, P = 0.00003). There were no statistical differences in quantitative methylation between NE and ESCC (Student’s t test, P = 0.171; Mann-Whitney’s U test, P = 0.67), BE and EAC (Student’s t test, P = 0.143; Mann-Whitney’s U test, P = 0.18), or HGD and EAC (Student’s t test, P = 0.86; Mann-Whitney’s U test, P = 0.55). Interestingly, there was a significant difference in the NMV for Reprimo between short-segment (mean NMV, 0.012) and long-segment (mean NMV, 0.168) BE (Student’s t test, P = 0.048). The mean NMVs for NE; short-segment BE; and long-segment BE, HGD, and EAC are displayed in Fig. 2.

![Fig. 1. qMSP results for human esophageal tissue. The mean NMVs for each tissue type are as follows: 0.004 for NE, 0.111 for BE, 0.222 for HGD, 0.249 for EAC, and 0.088 for ESCC. The differences between NE and BE, NE and HGD, and NE and EAC are significant (Student’s t test).](image-url)

**Table 2. Normalized quantitative methylation values (NMV) and qualitative methylation status for human esophageal tissues**

<table>
<thead>
<tr>
<th>Tissue type (n)</th>
<th>NMV</th>
<th>Student’s t test, P</th>
<th>Mann-Whitney test, P</th>
<th>Methylation status (% of n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE (19)</td>
<td>0.004</td>
<td>NA</td>
<td>NA</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>BE (25)</td>
<td>0.111</td>
<td>0.019</td>
<td>0.001</td>
<td>9 (36%)</td>
</tr>
<tr>
<td>HGD (11)</td>
<td>0.222</td>
<td>0.003</td>
<td>0.001</td>
<td>7 (64%)</td>
</tr>
<tr>
<td>EAC (75)</td>
<td>0.249</td>
<td>0.02</td>
<td>0.00003</td>
<td>47 (63%)</td>
</tr>
<tr>
<td>ESCC (45)</td>
<td>0.088</td>
<td>0.171</td>
<td>0.67</td>
<td>6 (13.3%)</td>
</tr>
</tbody>
</table>

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The differences between methylation levels of columnar tissues and ESCC were highly significant. The P value of EAC versus ESCC by Mann-Whitney testing was 0.000002, for HGD versus ESCC 0.002, and for BE versus ESCC 0.0001.

ROC curve analysis was done using the NMVs for the 75 EAC and 19 NE tissues (Fig. 3). The area under the ROC curve was 0.812 (P < 0.0001; 95% confidence interval, 0.73-0.90) and conveys Reprimo’s accuracy in distinguishing between EAC and NE, although we do not intend to replace the light microscope to make this distinction. The area under the ROC curve generated using the NMVs for the 75 EAC and 25 BE tissues was 0.59 (P = 0.08; 95% confidence interval, 0.47-0.71), suggesting that Reprimo methylation may indeed underscore a similarity between the two tissue types.

qMSP of esophageal cancer cell lines. Eleven ESCC cell lines and three esophageal EAC cell lines were tested for Reprimo methylation. Seven of the 11 ESCC cell lines were methylated and one of the three EAC cell lines were methylated. The qMSP results are displayed graphically in Supplementary Fig. S1A and S1B. The raw MSP data can be found in Supplementary Table S2. KYSE 110 was found to have the highest level (0.67) of Reprimo methylation of all the ESCC cell lines, whereas OE33 was the EAC cell line with the highest amount (0.59) of Reprimo methylation. These two cell lines were chosen for treatment with 5AzaC.

5AzaC treatment of esophageal cancer cell lines. qMSP was done on DNA from the cell lines KYSE110 and OE33 after treatment with 5 mmol/L 5AzaC. Real-time PCR for Reprimo RNA was done on samples harvested at identical time points. Quantitative results for the MSP and the reverse transcription-PCR reactions are displayed in Fig. 4A and B. An inverse relationship between Reprimo methylation and mRNA expression was observed when the cell lines were treated with the demethylating agent.

Discussion

In the current study, we found that Reprimo methylation in EAC is a cell type–specific early event showing potential as a biomarker for early neoplasia detection. DNA hypermethylation should be viewed as a global process whereby many loci are affected, but only a few are functionally significant. Reprimo is a cytoplasmic protein belonging to a family of molecules controlled by p53 that inhibits cell-cycle progression (28). p53, the tumor suppressor gene, is the most commonly mutated gene in human cancer (29, 30). In healthy cells, upon exposure to genotoxic agents or other noxious particles and stresses, the p53 protein is activated, resulting in abrogation of the cell cycle (31–33). This arrest in growth allows for coordination of cellular repair mechanisms and permits the organism to eliminate damaged cells (33). The function of p53 is mediated primarily through activation of target genes (34, 35). Indeed, previous research has shown that expression of Reprimo is dependent on p53 (36) and that overexpression of Reprimo leads to arrest at the G2 phase of the cell cycle (1). Furthermore, in a murine model of uterine sarcoma, Reprimo was significantly increased in normal uteri of p53 wild-type mice, but Reprimo expression was not increased in either normal uteri or in uterine sarcomas of p53 knockout animals (28). In addition, our group found that Reprimo methylation is significantly greater in patients who do not respond to chemoradiotherapy for esophageal cancer compared with those who do (19). Finally, reduced expression of p53 is common in patients with esophageal cancer (37–39). Thus, it seems that p53 and Reprimo are closely linked in pathways leading toward apoptosis, and that derangements in the functions of either gene are likely to constitute primary carcinogenic events as well as strong candidate markers of disease progression.

The pathogenesis of EAC requires a coordinated accumulation of genomic and epigenetic abnormalities that is initiated by the chronic reflux of acidic fluid into the esophagus (39–41). Chronic reflux leads to the gradual replacement of normal squamous epithelium with specialized columnar cells or BE (39). A small but significant portion of patients with BE will proceed to develop HGD and then EAC (42, 43). It is apparent that the length of the Barrett’s segment is an important predictor of this neoplastic progression (44). In the current study, we found that Reprimo methylation was significantly more

![Fig. 2. The mean NMV of Reprimo in NE, short-segment BE (SS BE), long-segment BE (LS BE), HGD, and EAC. The differences in mean NMV between NE and LS BE, SS BE and LS BE, NE and HGD, as well as NE and EAC were significant (Student’s t test). There were no statistically significant differences in mean NMV of Reprimo between NE and SS BE, LS BE and EAC, or HGD and EAC.](image)

![Fig. 3. ROC curve of NMVs of EAC versus NE. The area under the ROC curve (AUROC = 0.812) for Reprimo conveys the accuracy of this gene in distinguishing EAC from NE in terms of its sensitivity and specificity.](image)
common in BE, HGD, and EAC than in NE. In addition, within the set of patients with Barrett’s, those with long-segment BE had significantly more Reprimo methylation than those with short-segment disease (Student’s t test, \( P = 0.048 \)). Moreover, the levels of Reprimo methylation between BE and EAC were not statistically different, nor were they different between patients with HGD and EAC. A prior study by our group used gene expression profiles to show that BE is an early intermediate stage of EAC (45); thus, the current finding of similarity in Reprimo methylation between BE and EAC is not surprising. In fact, we found that Reprimo methylation levels in EAC (0.249) were more than double their levels in BE (0.111), implying that they actually increased during progression from BE to EAC. Reprimo methylation levels in ESCC were not statistically different from those in NE. Thus, methylation of Reprimo may represent an early event that is critical for and unique to esophageal adenocarcinogenesis.

In the cell line experiments, methylation of Reprimo in EAC and ESCC cell lines was associated with reduced expression of Reprimo mRNA. However, treatment with a demethylating agent lead to increased Reprimo mRNA expression and concomitant reduced Reprimo methylation. These data suggest that hypermethylation constitutes a mechanism by which Reprimo expression is silenced. In addition, 5AzaC or its derivatives have shown potential as therapeutic anticancer drugs (46, 47), and Reprimo thus represents a novel potential target for molecular-based therapies involving demethylation. Finally, although methylation was apparently more frequent in ESCC than in EAC lines, discrepancies between in vitro and primary tissue in vivo results such as these are often found in tumor genetics studies. To reemphasize, the \( P \) value of EAC versus ESCC by Mann-Whitney testing was 0.000002, for BE versus ESCC 0.0001, suggesting a highly significant tendency for Reprimo methylation to target specialized columnar rather than squamous human esophageal cells in vivo.

Prior studies have shown that normal physiologic methylation occurs with aging (48, 49). This discovery has led to the proposal that there are two types of methylated genes, type A and type C (50). Methylation of type A genes is age related, whereas methylation of type C genes is cancer specific. The ages of patients enrolled in this study were similar for all histologic types, yet methylation of Reprimo was nonexistent in NE (0.004) but significantly greater in BE (0.0111), HGD (0.223), and EAC (0.249). This contrast suggests that methylation of Reprimo is disease specific, rather than related to aging.

In conclusion, based on the current findings, it seems that Reprimo methylation occurs commonly in premalignant BE, in particular, long-segment BE, as well as in HGD and EAC. The level and frequency of Reprimo methylation increase in a stepwise fashion along the progression cascade toward EAC. Methylation of Reprimo was not commonly detected in ESCC or in NE, suggesting this represents a cell type–specific biomarker for EAC more so than ESCC. Further large-scale prospective longitudinal validation studies of this biomarker in progression from BE to HGD or EAC are supported by these data. It also remains to be determined whether arresting epigenetic silencing via demethylation represents a viable strategy to prevent or treat this deadly neoplasm.

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References


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