

Q2 *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

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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, 180, 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 WBCs. It was reasoned that physiologic methylation occurring in WBCs would contaminate tumors, eliminating the ability to distinguish abnormal methylation occurring in tumor epithelial cells from normal white cell infiltration.

DNA and RNA extraction. DNAs from frozen primary tissue specimens were extracted using previously published protocols (20, 21). Cell line DNAs were purified with Proteinase K and extracted onto silica-gel membranes using DNeasy (Qiagen, Valencia, CA). Cell line RNA was isolated with phenol-chloroform and guanidine isothiocyanate according to the specifications of the manufacturer (TRIzol, Invitrogen, Carlsbad, CA; ref. 22).

qMSP. DNA methylation of *Reprimo* was determined by qMSP using the ABI 7700 Taqman system (23). MSP distinguishes methylated alleles of a given gene based on DNA sequence alterations after bisulfite treatment of DNA. Bisulfite treatment converts unmethylated but not methylated cytosines to uracils. 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 $\mu\text{mol/L}$), 0.25 μL forward and reverse primer for both *Reprimo* and *β -actin* (10 $\mu\text{mol/L}$), 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, 1×10^5 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% CO_2 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 1. Patient demographics

Tissue type	n	Age (y)	Sex	Race	Other
EAC	75	63.5 \pm 11.9	70 m (93.3%), 5 f (6.6%)	65 White (86.6%), 5 Asian (6.6%), 5 AA (6.6%)	UICC stage I: 7 (9.3%), stage II: 16 (21.3%), stage III: 35 (46.6%), stage IV: 17 (22.6%)
HGD	11	70.3 \pm 7.9	11 m	11 White	
BE	25	61.2 \pm 14.7	24 m (96%), 1 f (4%)	20 White (80%), 5 AA (20%)	9 short segment, 16 long segment
NE	19	62.6 \pm 11.5	14 m (74.7%), 5 f (26.3%)	18 White (94.7%), 1 AA (5.3%)	
ESCC	45	62.4 \pm 7.8	33 m (73.3%), 12 f (26.7%)	28 White (62.2%), 15 AA (33.3%)	UICC stage not available

Abbreviations: UICC, Unio Internationale Contra Cancrum; AA, African American.

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

Tissue type	NMV	Student's <i>t</i> test, <i>P</i>	Mann-Whitney test, <i>P</i>	Methylation status (% of <i>n</i>)
NE (<i>n</i> = 19)	0.004	NA	NA	0 (0%)
BE (<i>n</i> = 25)	0.111	0.019	0.001	9 (36%)
HGD (<i>n</i> = 11)	0.223	0.003	0.001	7 (64%)
EAC (<i>n</i> = 75)	0.249	0.02	0.00003	47 (63%)
ESCC (<i>n</i> = 45)	0.088	0.171	0.67	6 (13.3%)

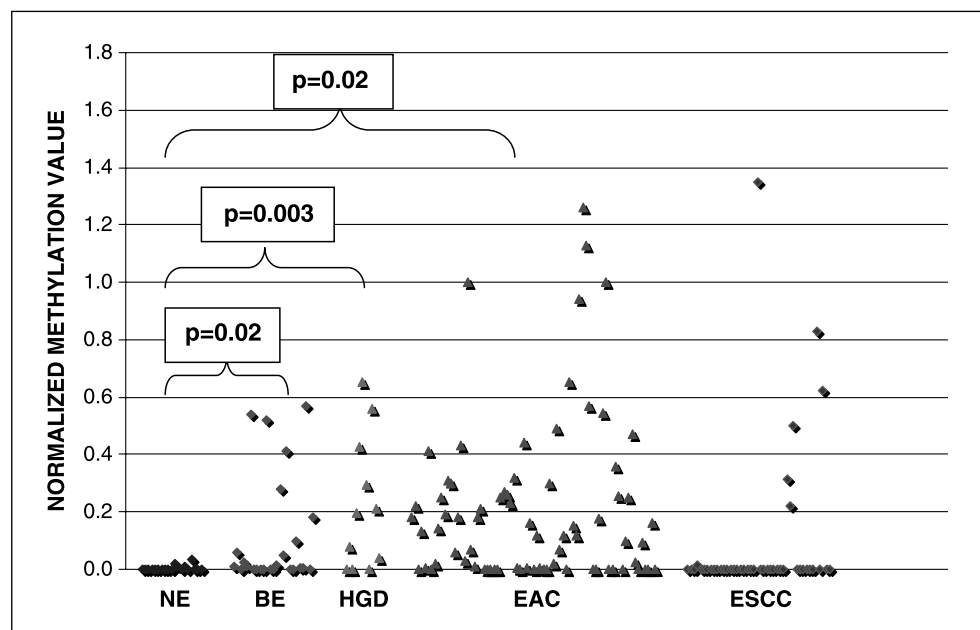
to provide further statistical rigor, and because the data were asymmetrical and did not fit a normal Gaussian distribution (e.g., many data points were equal to zero), further nonparametric testing was done using Mann-Whitney's *U* test (Statistica 6.0). To show the ability of *Reprimo* methylation 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 \pm 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).



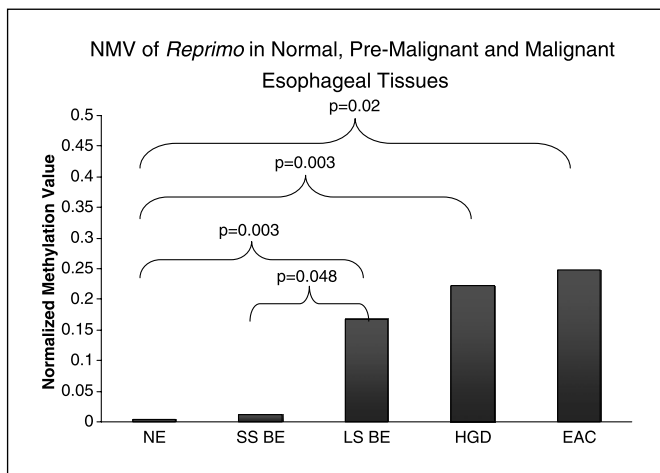


Fig. 2. The mean NMV of *Reprimo* in NE, short-segment BE (SS BE), long-segment BE (LS BE), HGD, and EAC. The differences in mean NMV between NE and SS BE, SS BE and LS BE, NE and HGD, as well as NE and EAC were significant (Student's *t* test). There were no statistically significant differences in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

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.

F3 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.

SF1 *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 may 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.

ST2 *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.

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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

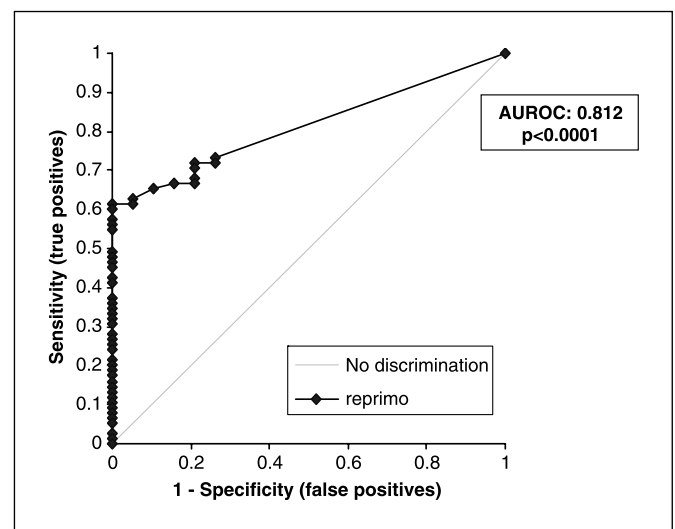


Fig. 3. ROC curve of NMVs of EAC versus NE. The area under the ROC curve (AUROC = 0.812) for *Reprimo* conveys the accuracy of this gene in distinguishing EAC from NE in terms of its sensitivity and specificity.

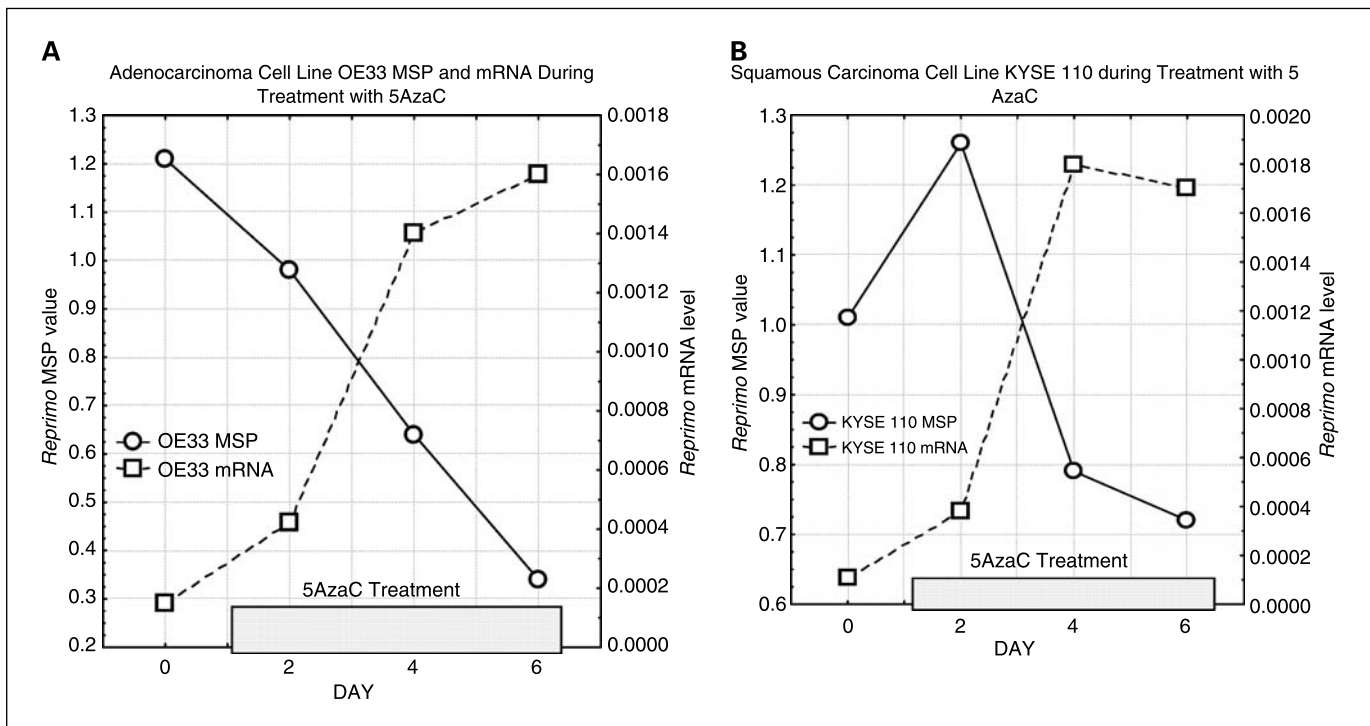


Fig. 4. A, results of EAC cell line OE33 treatment with 5AzaC begun after 24 hours of cell growth. Left Y axis, decreasing MSP value for *Reprimo*. Right Y axis, the corresponding increase in *Reprimo* mRNA level. B, results of ESCC cell line KYSE 110 treatment with 5AzaC begun after 24 hours of cell growth. Left Y axis, decreasing MSP value for *Reprimo*. Right Y axis, the corresponding increase in *Reprimo* mRNA level.

significantly more 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 HGD versus

ESCC 0.002, and 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

1. Ohki R, Nemoto J, Murasawa H, et al. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G₂ phase. *J Biol Chem* 2000; 275:22627–30.
2. Otsuka T, Kohno T, Mori M, Noguchi M, Hirohashi S, Yokota J. Deletion mapping of chromosome 2 in human lung carcinoma. *Genes Chromosomes Cancer* 1996;16:113–9.
3. Knudson AG. Two genetic hits (more or less) to cancer. *Nat Rev Cancer* 2001;1:157–62.
4. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002;16:6–21.
5. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999;21:163–7.
6. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3:415–28.
7. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042–54.
8. Kopelovich L, Crowell JA, Fay JR. The epigenome as a target for cancer chemoprevention. *J Natl Cancer Inst* 2003;95:1747–57.
9. Sato N, Fukushima N, Maitra A, et al. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. *Cancer Res* 2003;63:3735–42.
10. Wong TS, Kwong DL, Sham JS, Wei WI, Yuen AP. Methylation status of Reprimo in head and neck carcinomas. *Int J Cancer* 2005;117:697.
11. Suzuki M, Shigematsu H, Takahashi T, et al. Aberrant methylation of Reprimo in lung cancer. *Lung Cancer* 2005;47:309–14.
12. Takahashi T, Suzuki M, Shigematsu H, et al. Aberrant methylation of Reprimo in human malignancies. *Int J Cancer* 2005;115:503–10.
13. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;55:74–108.
14. Pera M, Manterola C, Vidal O, Grande L. Epidemiology of esophageal adenocarcinoma. *J Surg Oncol* 2005;92:151–9.
15. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. *CA Cancer J Clin* 2005;55:10–30.
16. Shaheen NJ. Advances in Barrett's esophagus and esophageal adenocarcinoma. *Gastroenterology* 2005; 128:1554–66.
17. Schulmann K, Sterian A, Berki A, et al. Inactivation of p16, RUNX3, and HPP1 occurs early in Barrett's-associated neoplastic progression and predicts progression risk. *Oncogene* 2005;24:4138–48.
18. Feagins LA, Souza RF. Molecular targets for treatment of Barrett's esophagus. *Dis Esophagus* 2005; 18:75–86.
19. Hamilton JP, Sato F, Greenwald BD, et al. Promoter methylation and response to chemotherapy and radiation in esophageal cancer. *Clin Gastroenterol Hepatol* 2006;4:701–8.
20. Meltzer SJ, Yin J, Manin B, et al. Microsatellite instability occurs frequently and in both diploid and aneuploid cell populations of Barrett's-associated esophageal adenocarcinomas. *Cancer Res* 1994;54: 3379–82.
21. Sato F, Shibata D, Harpaz N, et al. Aberrant methylation of the HPP1 gene in ulcerative colitis-associated colorectal carcinoma. *Cancer Res* 2002; 62:6820–2.
22. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162:156–9.
23. Eads CA, Lord RV, Wickramasinghe K, et al. Epigenetic patterns in the progression of esophageal adenocarcinoma. *Cancer Res* 2001;61:3410–8.
24. Shibata DM, Sato F, Mori Y, et al. Hypermethylation of HPP1 is associated with hMLH1 hypermethylation in gastric adenocarcinomas. *Cancer Res* 2002;62: 5637–40.
25. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 1982;143:29–36.
26. Sharma P, Morales TG, Sampliner RE. Short segment Barrett's esophagus—the need for standardization of the definition and of endoscopic criteria. *Am J Gastroenterol* 1998;93:1033–6.
27. Zhang Z, Li J, Lantry LE, et al. p53 transgenic mice are highly susceptible to 1,2-dimethylhydrazine-induced uterine sarcomas. *Cancer Res* 2002;62: 3024–9.
28. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991;253: 49–53.
29. Levine AJ, Finlay CA, Hinds PW. P53 is a tumor suppressor gene. *Cell* 2004;116:S67–9, 1p following S9.
30. Gottlieb TM, Oren M. p53 in growth control and neoplasia. *Biochim Biophys Acta* 1996;1287:77–102.
31. Sherr CJ. Cancer cell cycles. *Science* 1996;274: 1672–7.
32. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323–31.
33. el-Deiry WS. Regulation of p53 downstream genes. *Semin Cancer Biol* 1998;8:345–57.
34. Yu J, Zhang L, Hwang PM, Rago C, Kinzler KW, Vogelstein B. Identification and classification of p53-regulated genes. *Proc Natl Acad Sci U S A* 1999;96: 14517–22.
35. Taylor WR, Stark GR. Regulation of the G₂-M transition by p53. *Oncogene* 2001;20:1803–15.
36. Casson AG, Mukhopadhyay T, Cleary KR, Ro JY, Levin B, Roth JA. p53 gene mutations in Barrett's epithelium and esophageal cancer. *Cancer Res* 1991; 51:4495–9.
37. Bennett WP, Hollstein MC, Metcalf RA, et al. p53 mutation and protein accumulation during multistage human esophageal carcinogenesis. *Cancer Res* 1992; 52:6092–7.
38. Cameron AJ. Management of Barrett's esophagus. *Mayo Clin Proc* 1998;73:457–61.
39. Jankowski JA, Wright NA, Meltzer SJ, et al. Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am J Pathol* 1999;154:965–73.
40. Enzinger PC, Mayer RJ. Esophageal cancer. *N Engl J Med* 2003;349:2241–52.
41. Montgomery E, Goldblum JR, Greenson JK, et al. Dysplasia as a predictive marker for invasive carcinoma in Barrett esophagus: a follow-up study based on 138 cases from a diagnostic variability study. *Hum Pathol* 2001;32:379–88.
42. Weston AP, Sharma P, Topalovski M, Richards R, Cherian R, Dixon A. Long-term follow-up of Barrett's high-grade dysplasia. *Am J Gastroenterol* 2000;95: 1888–93.
43. Hage M, Siersema PD, van Dekken H, Steyerberg EW, Dees J, Kuipers EJ. Oesophageal cancer incidence and mortality in patients with long-segment Barrett's oesophagus after a mean follow-up of 12.7 years. *Scand J Gastroenterol* 2004;39:1175–9.
44. Wang S, Zhan M, Yin J, et al. Transcriptional profiling suggests that Barrett's metaplasia is an early intermediate stage in esophageal adenocarcinogenesis. *Oncogene* 2006;25:3346–56.
45. Momparler RL. Epigenetic therapy of cancer with 5-aza-2'-deoxycytidine (decitabine). *Semin Oncol* 2005;32:443–51.
46. Lemaire M, Momparler LF, Bernstein ML, Marquez VE, Momparler RL. Enhancement of antineoplastic action of 5-aza-2'-deoxycytidine by zebularine on L1210 leukemia. *Anticancer Drugs* 2005;16:301–8.
47. Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP. Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res* 1998;58:5489–94.
48. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat Genet* 1994;7:536–40.
49. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 1999; 96:8681–6.
50. Rashid A, Issa JP. CpG island methylation in gastroenterologic neoplasia: a maturing field. *Gastroenterology* 2004;127:1578–88.