

Promoter Methylation and Response to Chemotherapy and Radiation in Esophageal Cancer

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Background & Aims: Multiple studies have shown that promoter methylation of tumor suppressor genes underlies esophageal carcinogenesis. Hypothetically, methylation resulting in tumor suppressor gene inactivation might result in tumors that are unresponsive to chemotherapy and radiation. Accordingly, our aim was to find methylation markers that could be used to predict response to chemoradiation. **Methods:** Tumor specimens were obtained before treatment from 35 patients enrolled in a uniform chemoradiation treatment protocol. Methylation-specific quantitative polymerase chain reaction was performed on all samples. Pathology reports from esophagectomy specimens were used to define response to treatment. **Results:** Thirteen (37%) of 35 patients were responders, and 22 (63%) of 35 patients were nonresponders. The number of methylated genes per patient was significantly lower in responders than in nonresponders (1.4 vs 2.4 genes per patient; Student *t* test, *P* = .026). The combined mean level of promoter methylation of *p16*, *Reprimo*, *p57*, *p73*, *RUNX-3*, *CHFR*, *MGMT*, *TIMP-3*, and *HPP1* was also lower in responders than in nonresponders (Student *t* test, *P* = .003; Mann-Whitney test, *P* = .001). The frequency (15% of responders vs 64% of nonresponders; Fisher exact test, *P* = .01) and level (0.078 in responders vs 0.313 in nonresponders; Mann-Whitney test, *P* = .037) of *Reprimo* methylation was significantly lower in responders than in nonresponders. **Conclusions:** *Reprimo* methylation occurred at significantly lower levels and less frequently in chemoradioresponsive than in nonresponsive esophageal cancer patients, suggesting potential clinical application of this single-gene biomarker in defining prognosis and management. In addition, increased methylation of a 9-gene panel correlated significantly with poor responsiveness to chemoradiation.

might lead to many complications, including mucositis, pancytopenia, infection, and rarely, death.^{1,2} Moreover, despite recent advances in treatment, 5-year survival rates are dismal (20%).³ Esophageal cancer is the eighth most common malignancy and sixth most common cause of cancer death in the world.⁴ The incidence of esophageal cancer in the United States is rising, and it is estimated that 14,500 new cancers will be diagnosed in 2005.⁵ It is clear that new techniques, markers, and medicines are needed to diagnose, stratify, and treat patients with esophageal cancer.

The molecular pathogenesis of esophageal cancer has been studied widely for a number of years. Of relevance to these studies, there is a growing body of evidence showing that the abnormal methylation of DNA is an early event in carcinogenesis. Specifically, methylation of the promoter regions of tumor suppressor genes is commonly found in many human malignancies, including esophageal carcinoma.⁶ This methylation leads to the reduced expression of tumor suppressor genes, resulting in unchecked cellular growth, tissue invasion, angiogenesis, and metastases.^{7,8} Multiple studies have shown that promoter methylation of tumor suppressor genes underlies carcinogenesis⁹⁻¹¹; in addition, aberrant methylation of multiple genes correlates with prognosis of many cancers.¹²⁻¹⁶ Although promoter methylation has been shown to predict response to combined modality treatment in some cancers,^{17,18} to our

Abbreviations used in this paper: CHFR, checkpoint with forkhead associated and ring finger; COX-2, cyclooxygenase-2; HPPI, hyperplastic polyposis; MGMT, O⁶-methylguanine-DNA methyltransferase; MSP, methylation-specific polymerase chain reaction; PCR, polymerase chain reaction; *RUNX-3*, runt-related transcription factor 3; *TIMP-3*, tissue inhibitor of metalloproteinase-3; XAF-1, X-linked inhibitor of apoptosis-1

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Although patients with localized esophageal cancer might benefit from concomitant radiation and chemotherapy,¹ these regimens are extremely grueling and

knowledge, this relationship has not been examined in esophageal cancer. Accordingly, the goal of the current study was to determine whether promoter methylation correlates with the response to chemotherapy and radiation in esophageal cancer.

Eleven candidate genes were selected on the basis of their known ability to predict responsiveness to chemoradiation and prognosis in esophageal cancer or on the basis of their role in governing the cell cycle. The G2/M phase of the cell cycle, in particular, was targeted because that is when cells are most sensitive to x-ray–induced apoptosis.^{19–21} In addition, these genes were selected on the basis of their known involvement in human carcinogenesis. For example, specifically, *Reprimo* (the Greek word for “repress”) is a mediator of p53-mediated cell cycle arrest at the G2/M phase.²² *Reprimo* is frequently methylated in a variety of human malignancies and is also induced by X-irradiation.¹¹ *O*⁶-methylguanine-DNA methyltransferase (*MGMT*), a DNA excision repair gene, is commonly methylated in esophageal cancer,⁹ and promoter hypermethylation of *MGMT* has been shown to correlate with a response to alkylating agents in brain tumors.²³ Similarly, checkpoint with fork-head associated and ring finger (*CHFR*) exists as part of an early G2/M checkpoint,²⁴ and lack of expression of *CHFR* in esophageal cancer has been linked to promoter hypermethylation.²⁵ Tissue inhibitor of metalloproteinase-3 (*TIMP-3*) encodes a potent inhibitor of angiogenesis, and silencing of this gene by promoter methylation is associated with a poor prognosis in esophageal cancer.¹³ *p16* and *p57* belong to a family of cyclin-dependent kinase inhibitors that cause cell cycle arrest at the G1 phase. Methylation and subsequent lack of expression of *p16* in esophageal cancer are associated with a poor prognosis.¹² Methylation of *p57* has been reported in multiple human malignancies.²⁶ Methylation of runt-related transcription factor 3 (*RUNX-3*) is observed in esophageal cancer, and this methylation also correlates with progression from Barrett’s esophagus with low-grade dysplasia to Barrett’s adenocarcinoma.⁶ Methylation of hyperplastic polyposis (*HPP1*) has also been shown to correlate with Barrett’s esophagus–associated neoplastic progression.⁶ Methylation of *HPP1* is found in esophageal,⁶ gastric,²⁷ and colon cancers.^{28,29} The exact function of *HPP1* has not been determined, but it encodes an epidermal growth factor domain and is therefore thought to play a role in cell growth, maturation, and adhesion.^{27,28} The role of *p73* in esophageal cancer is unclear,^{30,31} but it is a homologue of *p53*, and this family of genes functions as transcription factors that play a major role in regulating the response of mammalian cells

Table 1. Demographics and Disease Characteristics of the Patients

| Variable | n = 35 |
|-------------------------------|------------|
| Age, y, mean (range) | 61 (37–81) |
| Race (white/African American) | 32/3 |
| Sex (m/f) | 28/7 |
| UICC stage IIa | 1 |
| UICC stage IIb | 2 |
| UICC stage III | 32 |
| Adenocarcinoma | 23 |
| Squamous cell carcinoma | 12 |

UICC, Union Internationale Contre Cancer.

to stressors and damage, in part through the transcriptional activation of genes involved in cell cycle control, DNA repair, senescence, angiogenesis, and apoptosis.^{32–36} Finally, X-linked inhibitor of apoptosis-1 (*XAF-1*) and cyclooxygenase-2 (*COX-2*) were selected because of their respective roles in regulating apoptosis, the cell cycle, and inflammatory responses.^{37,38} The expression of *XAF-1* has been linked to resistance to cisplatin in vitro,³⁹ and *COX-2* expression has been reported to correlate with responsiveness to radiation and chemotherapy in gynecologic squamous cell malignancies.⁴⁰

Materials and Methods

Patient Selection and Treatment Protocol

Before any treatment and after informed consent, endoscopic biopsies were obtained from the esophageal tumors of 35 patients who were consecutively enrolled in a uniform treatment protocol at the University of Maryland, Baltimore. The clinical characteristics of the patients can be found in Table 1. The tumor samples were immediately frozen on dry ice and then stored at -80°C until DNA extraction. Tissue from the tumors was also sent for histology to confirm the diagnosis. After staging, the patients received 2 cycles of induction chemotherapy with cisplatin (75 mg/m²/day) and 5-fluorouracil (1000 mg/m²/day.) This treatment was coupled with concurrent x-ray radiation (56.4 Gy) to the tumor. At 1 month after induction, patients were re-staged with esophagogastroduodenoscopy, computed tomography scans of the chest and abdomen, and positron emission tomography scans. After restaging, patients underwent esophagectomy. In most cases, response to chemotherapy and radiation was defined after surgery. The surgical resection was examined for the presence or absence of tumor. If no tumor was present in the esophagectomy specimen, then the patient was defined as a responder. If tumor was present in the specimen, then the patient was defined as a nonresponder. In addition, during the restaging process, if metastases were found or if disease progression occurred, then that patient was also defined as a nonresponder. The Institutional Review Board and the Office of Research on Human Subjects at the University of Maryland, Baltimore,

approved the Marlene and Stewart Greenebaum Cancer Center treatment protocol # 9967.

Primer and Probe Design

Probe and primers for quantitative methylation-specific polymerase chain reaction (MSP) for the 11 candidate genes were designed on the UCSC Human Genome Browser sequence data.

Prescreening of Candidate Genes for Methylation in Normal White Blood Cells

First, before testing in any tumor tissues, candidate genes were tested for methylation in normal white blood cells. The assumption was made that methylation of esophageal tumor suppressor genes should not occur in white blood cells. It was reasoned that physiologic methylation occurring in white blood cells would contaminate tumors, eliminating the ability to distinguish abnormal methylation in tumor epithelial cells from white cell infiltration.

DNA Extraction and Quantitative Methylation-Specific Polymerase Chain Reaction

DNA from the frozen tumor specimens was extracted by using previously published protocols.^{10,41} DNA methylation of *Reprimo*, *p16*, *CHFR*, *MGMT*, *TIMP-3*, *RUNX-3*, *p57*, *p73*, *COX-2*, *HPP1*, and *XAF-1* was determined by quantitative MSP⁹ by using the Taqman (Applied Biosystems, Foster City, CA) system. MSP distinguishes methylated alleles of a given gene on the basis of DNA sequence alterations after bisulfite treatment of DNA. Bisulfite treatment converts unmethylated but not methylated cytosines to uracils. Subsequent polymerase chain reaction (PCR) by using primers and probe specific to the corresponding methylated DNA sequence is then performed. *β -Actin* was selected as an internal control, and analysis was based on previously published primer and probe sequences.^{9,10} Bisulfite-treated DNA extracted from the white blood cells of normal patients was used as an additional negative control. Briefly, 1.0 μ g of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were purified by using Wizard DNA clean-up resin (Promega Corp, Madison, WI), treated with NaOH, precipitated with ethanol, and re-suspended in 50 μ L of water. The PCR mixture consisted of 12.5 μ L of Taqman Universal Master Mix without uracil deoxyribonucleic acid glycosylase (Applied Biosystems), 2.0 μ L of probe for both the gene of interest and *β -actin* (2.5 μ mol/L), 0.25 μ L of forward and reverse primer for both the gene of interest and *β -actin* (10 μ mol/L), 50 ng of bisulfite-treated DNA, and water up to a total volume of 25 μ L. PCR and real-time data collection were performed with an ABI7700 Sequence Detection System (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, Pur-

chase, NY) was used to generate a standard curve for each reaction. Reaction mix without any bisulfite-treated DNA served as a negative control.⁹

Analysis of Results of Methylation-Specific Polymerase Chain Reaction

The normalized MSP value was calculated by dividing the ratio of the quantitative MSP value for the gene of interest to *β -actin* for each sample by the ratio of the quantitative MSP value for the gene of interest to *β -actin* for Universal Methylated DNA.^{10,27} The qualitative MSP status was determined by analyzing the normalized MSP value. A normalized MSP value of 0.05 was assigned as the cutoff point for classifying methylation status as positive (≥ 0.05) or negative (< 0.05). This cutoff point was previously determined by receiver operating characteristic curve analysis.⁶ To combine all the data for all the genes, standardization is necessary. The formula for this process is $(X - \text{mean})/\text{standard deviation}$, where X is a normalized MSP value for a particular patient for a specific gene. This calculation converts the mean MSP value for each gene to zero. Data points below the mean are assigned negative values, and data points above the mean are assigned positive values.

Statistical Analysis

The normalized methylation value of the genes was compared in responders versus nonresponders by using the Student paired *t* test (Statistica 6.0; StatSoft Inc, Tulsa, OK). In addition, because many of the data points were equal to zero, further nonparametric analysis was performed on the genes by using the Mann-Whitney *U* test (Statistica 6.0). After a qualitative methylation status was assigned, the individual genes were tested for significance with regards to response to therapy by using Fisher exact test.

Results

Response to Combined Modality Treatment

Thirteen (37%) of the 35 patients were responders. Twenty-two (63%) of the 35 patients were nonresponders. Thirty-two of the patients had their response defined at surgery. Two of the 22 nonresponders had evidence of metastasis after therapy and were no longer candidates for surgery. Seven (58%) of the 12 patients with squamous cell carcinoma responded to radiation and chemotherapy. Seven (30%) of the 23 patients with adenocarcinoma of the esophagus responded to this treatment. The difference in treatment response between the 2 tissue types was not significant (Fisher exact test, $P = .15$).

Methylation-Specific Polymerase Chain Reaction

Promoter methylation of *XAF-1* and *COX-2* was detected in the white blood cell DNA from normal

Table 2. Combined MSP Levels of 9 Genes in Cancer Cell Types, Cancer Stages, Responders, and Nonresponders

| Variable | Combined mean methylation value | Student <i>t</i> test |
|-------------------------|---------------------------------|-----------------------|
| Adenocarcinoma | 0.185 | |
| Squamous cell carcinoma | -0.312 | $P = .00001^a$ |
| Stage II disease | 0.198 | |
| Stage III disease | -0.019 | $P = .277^a$ |
| Responders | -0.215 | |
| Nonresponders | 0.127 | $P = .003^a$ |

NOTE. For combination of data from 9 genes to be statistically valid, standardization is required. Mean MSP value for each gene is set at zero. Values above zero are greater than the mean, and values below zero are less than the mean.

^aAdenocarcinoma compared with squamous cell carcinoma, stage II disease compared with stage III disease, and responders compared with nonresponders.

patients. These 2 genes were excluded from further study. In the remaining genes, the white blood cell control DNA did not show methylation.

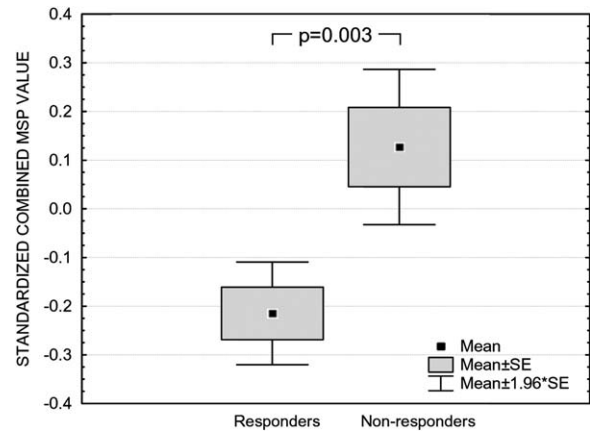
Methylation was found more often in cases of adenocarcinoma than in cases of squamous cell cancer (Student *t* test, $P = .0001$). There was no significant difference in methylation values between stage II and stage III disease (Student *t* test, $P = .27$). The overall prevalence of methylation in the remaining 9 genes was 34% in nonresponders and 14% in responders (χ^2 test, $P < .0001$). The average number of methylated genes was 2.4 per patient in nonresponders versus 1.4 genes per patient in responders (Student *t* test, $P = .026$). The normalized methylation values for all 9 genes combined were greater in nonresponders than in responders (Student *t* test, $P = .003$; Mann-Whitney test, $P = .001$). These results are found in Tables 2 and 3 and Figure 1.

Promoter methylation of *p57* and *p73* was uncommon in both responders and nonresponders. Methylation of *p57* was found in none (0%) of the 13 responders and in 1 (5%) of the 22 nonresponders. Methylation of *p73* was detected in 2 (14%) of the nonresponders and none (0%) of the responders.

Table 3. Number of Methylated Genes in Responders and Nonresponders

| Number of genes methylated | Responders (n = 13) | Nonresponders (n = 22) |
|----------------------------|---------------------|------------------------|
| 0 | 5 | 3 |
| 1 | 3 | 5 |
| 2 | 3 | 4 |
| ≥3 | 2 | 10 |
| Average per patient | 1.4 | 2.4 ^a |

^aStudent *t* test, $P = .026$.

**Figure 1.** For combination of data from 9 genes to be statistically valid, standardization is required. In this figure, 0.0 represents the mean MSP value for each gene. Values above zero are greater than the mean, and values below zero are less than the mean. This is a box-plot graph of the combined standardized values of all 9 genes in all 35 patients, demonstrating a significant difference between the means of these combined values in responders vs nonresponders (Student *t* test, $P = .003$).

RUNX-3 was methylated in 5 (38%) of the 13 responders and in 7 (32%) of the 22 nonresponders. Promoter hypermethylation of *p16*, *CHFR*, *MGMT*, *TIMP-3*, *HPP1*, and *Reprimo* was common, and it was found more frequently in nonresponders than in responders. The qualitative MSP results for the individual genes are found in Table 4. The frequency of *Reprimo* methylation was significantly greater in the nonresponders versus the responders (Fisher exact test, $P = .01$). The normalized MSP values in the nonresponders and the responders were also significantly different for *Reprimo* (Mann-Whitney test, $P = .037$; Figure 2).

On the basis of methylation frequencies, 6 genes appeared to constitute the best markers of response to chemoradiation in esophageal cancer. The percentage of patients with methylation of *p16*, *Reprimo*, *MGMT*, *CHFR*, *TIMP-3*, and *HPP1* in responders and nonresponders is shown in Figure 3. The mean normalized MSP value for this panel of genes in responders was significantly less than in nonresponders (Student *t* test, $P = .007$; Mann-Whitney test, $P = .0004$).

Discussion

In the current study, we identified epigenetic alterations that distinguish between esophageal cancer patients who respond versus those who do not respond to chemotherapy and radiation. The patient demographics showed a homogeneous cohort with little variation in age, sex, and race. The tumor stages of the patients were all nearly identical. The protocol for chemotherapy and radiation was absolutely uniform in all patients.

Table 4. Frequency and Mean Level^a of MSP for Individual Genes

| Gene name | Responders | Mean MSP (R) | Nonresponders | Mean MSP (NR) |
|----------------|--------------|--------------|--------------------------|---------------|
| <i>p57</i> | 0/13 (0%) | 0.0004 | 1/22 (5%) | 0.019 |
| <i>RUNX-3</i> | 5/13 (38%) | 0.067 | 7/22 (32%) | 0.090 |
| <i>MGMT</i> | 3/13 (23%) | 0.059 | 9/22 (41%) | 0.062 |
| <i>p73</i> | 0/13 (0%) | 0.0 | 3/22 (14%) | 0.113 |
| <i>p16</i> | 1/13 (8%) | 0.031 | 6/22 (27%) | 0.120 |
| <i>CHFR</i> | 1/13 (8%) | 0.040 | 7/22 (32%) | 0.068 |
| <i>TIMP3</i> | 2/13 (15%) | 0.022 | 10/22 (45%) | 0.107 |
| <i>HPP1</i> | 2/13 (15%) | 0.085 | 11/22 (50%) | 0.274 |
| <i>Reprimo</i> | 2/13 (15%) | 0.078 | 14/22 (64%) ^b | 0.313 |
| Total | 16/117 (14%) | | 67/198 (34%) | |

R, responder; NR, nonresponder.

^aMean MSP values for each gene include data from all 35 patients.

^bFisher exact test, $P = .01$.

The current data demonstrate that differential methylation of several genes significantly correlates with responsiveness to simultaneous chemotherapy and irradiation in patients with esophageal carcinoma. We started with 11 candidate genes but reduced that number to 9 after detecting methylation of 2 (*XAF-1* and *COX-2*) genes in white blood cells. We found significant differences between responders and nonresponders when all of these remaining 9 genes were analyzed. The 9 genes in this final panel, *p16*, *Reprimo*, *p57*, *p73*, *RUNX-3*, *CHFR*, *MGMT*, *TIMP-3*, and *HPP1*, have been shown to play pivotal roles in the molecular pathogenesis and progression of numerous human malignancies, including esophageal cancer.^{6,11,12} In addition,

one gene, *Reprimo*, was found to have significantly higher methylation levels and frequency in patients who were unresponsive to chemoradiation than in patients who were responsive to these treatments.

We were able to further reduce the number of genes in our panel to 6 (*p16*, *Reprimo*, *MGMT*, *CHFR*, *TIMP-3*, and *HPP1*). The rationale for removing *p57* and *p73* from the panel was that they had relatively low (ie, <15%) overall frequencies of methylation and therefore were not likely to be informative methylation markers of chemoradiation response. In our study, methylation of *RUNX-3* was detected more often in responders than in nonresponders, suggesting that methylation of this gene was not a useful marker of poor responsiveness to chemoradiation in esophageal cancer. The difference in methylation of the panel of 6 remaining genes between nonresponders and responders was also significant. We suspect that the methylation profile of this 6-gene panel (*p16*, *Reprimo*, *MGMT*, *CHFR*, *TIMP-3*, and *HPP1*) might have more clinical translatability than the

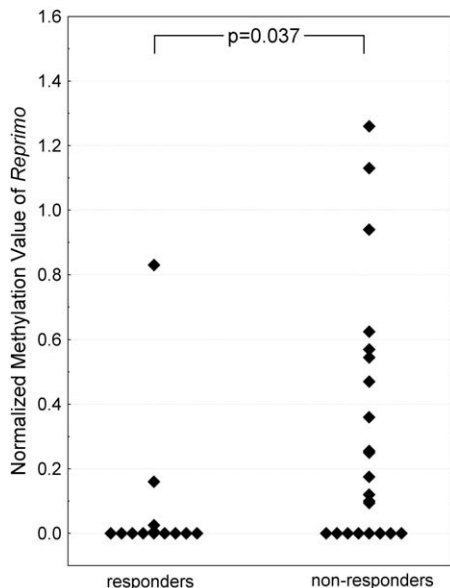


Figure 2. Normalized methylation values of *Reprimo* in responders vs nonresponders. Each black diamond represents an individual patient. The mean normalized MSP value for responders was 0.078, and the mean normalized MSP value for nonresponders was 0.313. The difference between the means of these 2 sets of patients is significant (Mann-Whitney U test, $P = .037$).

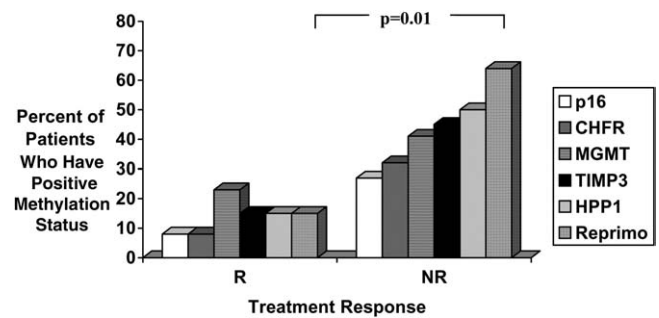


Figure 3. Treatment response vs promoter methylation status of individual genes. The percentage of patients methylated in responders (R) vs nonresponders (NR) for the 6 genes showing the most marked differences in methylation between the 2 groups. *Reprimo* showed the only significant difference (Fisher exact test, $P = .01$). A normalized MSP value of 0.05 was assigned as the cutoff point for classifying methylation status as positive (≥ 0.05) or negative (< 0.05).

panel of all 9 genes, because it will be simpler to apply and to perform this assay in the hospital clinical pathology laboratory.

The tumor genetics of *p16* have been evaluated extensively; gene silencing might occur by mutation, loss of heterozygosity, homozygous deletion, or promoter hypermethylation.^{32,42–44} Methylation of *p16* is common in esophageal adenocarcinoma, and the resultant reduced expression of *p16* is associated with a poor prognosis.¹² In this study, *p16* was methylated in 8% of patients who did respond versus 27% of patients who did not respond to treatment. This gene, a member of the cyclin-dependent kinase inhibitor (CDKI) family of genes, causes cell cycle arrest at the G1/S phase.⁴³ Its inactivation results in uncontrolled cell growth.^{44–46} Therefore, it is not surprising that *p16* methylation is associated with increased chemotherapy resistance, because methylated cells have a decreased ability to arrest at the G2/M checkpoint, where cisplatin and radiation are active.^{47,48}

The product of the *CHFR* gene is responsible for a delay in chromosomal condensation during prophase in response to microtubule injury.⁴⁹ In previous reports, *CHFR* was frequently inactivated by promoter methylation in esophageal, lung, brain, and colon cancers.^{25,50,51} In our study, *CHFR* was methylated in 32% of patients who did not respond but in only 8% of patients who did respond to the treatment of esophageal cancer. This difference in methylation could have been explained by the notion that tumor cells with inactivated *CHFR* were more likely to have unregulated cell growth, becoming less likely to be killed by the toxic effects of chemotherapy and radiation.

MGMT is a mismatch repair gene that is a primary defense against alkylation-induced mutagenesis, carcinogenesis, and apoptosis.^{23,52} The inactivation of *MGMT* by promoter methylation has been reported in esophageal cancer.^{9,52} Our results suggest that promoter methylation of *MGMT* predicts a poor response to therapy in esophageal cancer. Methylation of *MGMT* was found in 41% of patients who did not respond but in only 23% of patients who did respond to chemotherapy and radiation. Our findings are supported by prior studies demonstrating that *MGMT* methylation correlated with esophageal and lung cancer prognosis, irrespective of treatment.^{12,53} Although reduced expression of *MGMT* via promoter methylation is associated with a good response to alkylating agents in brain tumors,²³ we did not find a similar result in esophageal cancer. This discrepancy might have been due to the fact that the chemotherapy used in this protocol (cisplatin) did not include a classic alkylating agent. Our conclusion is that tumors with promoter methylation and consequent reduced expression of *MGMT* are unable to repair damage caused by

environmental alkylating agents such as nitrosamines. This deficiency might lead to more aggressive tumor biology and thus unresponsiveness to chemotherapy and radiation.

TIMP-3 inhibits the activity of matrix metalloproteinase, which is thought to play an important role in carcinoma invasion and metastasis.⁵⁴ There is an association between methylation of *TIMP-3* and esophageal cancer. Furthermore, reduced expression of the *TIMP-3* protein in esophageal cancer is associated with increased tumor invasiveness and reduced patient survival.¹³ Our results correspond accordingly; 45% of patients who did not respond to chemotherapy and radiation had methylated *TIMP-3*, whereas only 15% of patients who responded were methylated at *TIMP-3*.

HPP1 is a transmembrane protein-encoding gene that is commonly methylated in colon polyps and cancers.²⁸ Moreover, *HPP-1* has been validated as a biomarker for Barrett's esophagus-associated neoplastic progression.⁶ The molecular function of *HPP1* is unknown, but it has a follistatin domain that is thought to be a ligand for transforming growth factor- β , and its epidermal growth factor domain is speculated to bind c-erbB-4.²⁸ Given these observations and the evidence that *HPP1* is inactivated in multiple malignancies, it is believed that this gene plays an important role in controlling cell growth.^{27,28} We found that *HPP1* is more frequently methylated in nonresponders (50%) than in the responders (15%). These results are consistent with the theory that *HPP1* is potential tumor suppressor gene, and when *HPP1* is inactivated by methylation, the tumor cells grow without restraint and do not respond to chemotherapy and radiation.

Reprimo is a regulator of the *p53*-mediated cell cycle arrest point at G2/M. This abrogation of the cell cycle occurs when tumors are the most sensitive to radiation-induced damage and death.⁴⁸ In fact, certain chemotherapeutic agents (the taxanes) have been designed to arrest tumor cells at G2/M to potentiate the tumor response to irradiation.⁵⁵ Our results indicate that methylation of *Reprimo* correlates with a poor response to chemoradiation in esophageal cancer. In the current study, nearly two thirds (64%) of patients who did not respond versus only 15% of patients who did respond to chemotherapy and radiation had *Reprimo* promoter methylation. This difference was statistically significant (Fisher exact test, $P = .01$). The comparison of the normalized methylation values of the responders and nonresponders for *Reprimo* also reveals a significant difference (Mann-Whitney test, $P = .037$). We hypothesize that tumor cells with promoter methylation of *Reprimo* are likely to skip the G2/M checkpoint, resulting in reduced sensitivity to irradiation.

In the current study, both adenocarcinoma and squamous cancer were included because the treatment of both cancers is identical. In addition, multiple studies have revealed similarities between these 2 tissue types.^{14,34,41,56} Furthermore, in our study, the response to treatment did not differ, irrespective of tissue type.

Prior studies have validated the use of methylation of a panel of genes to predict prognosis in esophageal and rectal cancer.^{12,57} When the combined MSP results of a panel of genes consisting of *p16*, *Reprimo*, *p57*, *p73*, *RUNX-3*, *CHFR*, *MGMT*, *TIMP-3*, and *HPP1* were analyzed by well-validated parametric and nonparametric statistics, there was an obvious difference between esophageal cancer patients who did and did not respond to chemotherapy and radiation (Student *t* test, *P* = .003; Mann-Whitney test, *P* = .001). This study correlates methylation with responsiveness to chemotherapy and radiation in esophageal cancer.

The clinicobiologic differences between responders and nonresponders might be explained by the inactivation of these genes by promoter methylation. Four of these genes (*p16*, *CHFR*, *MGMT*, and *Reprimo*) are important in arresting the cell cycle to repair damage to DNA or induce apoptosis. Inactivation of these genes results in uncontrolled tumor growth and poor response to therapy. *TIMP-3* inhibits vascular invasion of tumors and metastasis. Inactivation of this gene results in aggressive tumors that do not respond well to therapy. *HPP1* is a candidate tumor suppressor gene, which, when methylated, also leads to uncontrolled tumor growth.²⁹ *Reprimo* showed markedly lower methylation rates in esophageal cancer patients responsive to chemoradiation, suggesting that this gene in particular might function as a biomarker in helping to define the prognosis and management of this disease. Moreover, a combined gene panel consisting of *p16*, *Reprimo*, *p57*, *p73*, *RUNX-3*, *CHFR*, *MGMT*, *TIMP-3*, and *HPP1* predicted a poor response to adjuvant chemotherapy when methylation was found.

Although the patient cohort in this study was relatively moderate in size, it consisted of a uniform group of subjects with nearly the same disease stages who received identical doses of radiation and chemotherapy. The current data might lead to development of biomarkers to stratify patients into treatment subgroups. Furthermore, the current findings might identify potential targets for molecular treatments such as demethylation therapies to sensitize tumors to chemotherapy and radiation. Finally, the current research protocol is still ongoing, and further information might be gleaned from long-term survival data.

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