Advances in Brief

Prognostic Importance of Promoter Hypermethylation of Multiple Genes in Esophageal Adenocarcinoma[1]

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Abstract

Purpose: **We investigated aberrant methylation patterns in esophageal adenocarcinoma and correlated the findings to patient survival and tumor recurrence.**

Experimental Design: **Gene promoter methylation was performed in 82 samples from 41 esophagectomy patients consisting of 41 adenocarcinoma samples, each with its adjacent nonmalignant tissue, which included one sample with Barretts metaplasia. The methylation status of seven genes was determined. Epigenetic silencing was confirmed using immunohistochemical staining. Kaplan-Meier plots were constructed using disease-specific survival as the primary end point and the interval from surgery to tumor recurrence as the secondary end point. The association of clinicopathological and biomolecular risk factors to survival and recurrence was performed using the Log-rank test and Cox proportional hazards model for multivariate analysis.**

Results: **Methylation frequencies of the genes analyzed were *APC*, 68%; *E-cadherin*, 66%; *O^6-methylguanine DNA methyltransferase*, 56%; *ER*, 51%; *p16*, 39%; *DAP-kinase*, 19%; and *TIMP3*, 19%. DNA methylation of some genes individually showed only trends toward diminished survival, whereas patients whose tumors had >50% of their gene profile methylated had both significantly poorer survival ($P = 0.04$) and earlier tumor recurrence ($P = 0.05$) than those without positive methylation. By multivariate analysis, the hazard ratios (HRs) with positive methylation status were more powerful predictors of survival [HR 2.7 (1.14–6.45; 95% confidence interval)] and tumor recurrence [HR 2.5 (1.11–5.6)] than age (HR 2.03 and 1.96, respectively) or stage (HR 1.48 and 1.67, respectively).**

Conclusions: **Our data suggest that positive methylation status for multiple genes in esophageal adenocarcinoma is a predictor of poor prognosis.**

Introduction

Since the 1960s, esophageal adenocarcinoma has earned the dubious distinction as the most rapidly increasing solid organ tumor in the western world. More specifically, when age-standardized incidence rates are considered, it is the rise in incidence rates for adenocarcinomas of the lower third of the esophagus that are unmatched by any other tumor (1). Demographically, Caucasian males are disproportionately affected, and most patients present at a late diagnostic stage with an associated poor prognosis. Despite this increased incidence of esophageal adenocarcoma, squamous cancer of the esophagus remains the predominant cell type worldwide. Environmental and dietary factors, such as alcohol, tobacco, as well as high levels of nitrates in the soil and drinking water, have all been associated with the etiology of esophageal squamous cancer, whereas the presence of Barretts esophagus and GE[3] reflux disease are important risk factors of esophageal adenocarcinoma.

Epigenetic changes in DNA without concomitant changes in the underlying genetic code are now known to be common events in human cancer (2). The CpG islands of genes in normal tissues are generally protected from aberrant hypermethylation, but this protection may be lost early in tumorigenesis. This has led to the use of hypermethylation of tumor suppressor genes, in particular, as biomarkers for the early diagnosis of cancer (3). Promoter hypermethylation and transcriptional repression of functionally important cancer-related genes may also affect tumor behavior, impacting clinical outcomes. Epigenetic silencing of genes that determine tumor invasiveness, growth patterns, neovascularization, and metastatic behavior, in particular, may dictate tumor recurrence after treatment and impact overall patient survival. Because each tumor may harbor multiple genes susceptible to promoter hypermethylation, individual tumors would exhibit different frequencies of hypermethylation within a particular profile of genes, a methylation profile potentially predictive of a patient's clinical outcome.

In patients with diffuse large B-cell lymphoma and gliomas, we have demonstrated that hypermethylation of the DNA

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[3] The abbreviations used are: GE, gastroesophageal; MGMT, O^6-methylguanine DNA methyltransferase; ECAD, E-cadherin; IHC, immunohistochemistry; MSP, methylation-specific PCR.

repair gene *MGMT* was a useful predictor of responsiveness to treatment with alkylating agents (4, 5). We have also reported a predictive value in the methylation status of a single gene, *p16*, if it is combined with an analysis of K-*ras* mutations in colorectal cancer patients (6). In lung cancer, patients with methylation of the pro-apoptotic gene, *DAP-kinase*, were shown to have statistically significant poorer 5-year survivals than those without hypermethylation (7). All of these studies correlated clinical outcomes with single gene promoter hypermethylation. In this study, we hypothesize that a methylation profile of multiple cancer-related genes predicts cancer recurrence and decreased disease-specific survival in patients with esophageal adenocarcinoma.

Over the past decade at the Johns Hopkins Hospital, we have amassed a tissue bank of snap-frozen resected distal esophageal adenocarcinomas, along with comprehensive outcome data on all patients with this disease. We selected a subset of patients whose tumors were treated by surgery alone and examined these tumors for promoter hypermethylation changes in well-characterized tumor suppressor genes (*p16* and *APC*), DNA repair genes (*MGMT*), those related to metastasis and invasion (ECAD and TIMP3), and genes associated with apoptosis (*DAP-kinase*) or transcription regulation (*ER*). Each of these genes, known to be methylated in other gastrointestinal malignancies, possesses a CpG island in their promoter that is unmethylated in the CpG island of nonmalignant tissue (8). We aimed to determine whether promoter hypermethylation of a profile of genes in primary esophageal adenocarcinoma is predictive of clinical outcome after surgery.

Materials and Methods

Study Design. We conducted a retrospective cohort study of 41 patients who had esophageal resections from 1991 to 1998 with curative intent for distal esophageal adenocarcinomas at the Johns Hopkins Hospital. Patient samples were drawn from the institution's tumor bank of snap-frozen esophageal tissue and selected because most patients received no neoadjuvant therapy (39 of 41). Informed consent was obtained from all patients for harvesting of their tissue fresh for subsequent molecular marker studies. Disease-specific survival was the end point of the study, and recurrence of esophageal cancer was used as a surrogate end point. Outcome data were derived from a comprehensive database that is maintained using the institution's cancer registry, patient's charts, and telephone calls to local primary physicians. Approval to perform this study was given by the institution's review board.

Tissue Samples. All esophagectomy specimens were procured at time of resection by a single pathologist (T. T. W.), frozen in liquid nitrogen, and stored at −70°C. All malignancies were examined, and tumor staging codified according to the Tumor-Node-Metastasis classification of the American Board of Cancer. For each patient, histologically paired malignant and nonmalignant sections were available for H&E staining, IHC analysis, and hypermethylation studies. An H&E stain confirmed the presence of cancer or the absence of malignancy in normal tissues. All malignant specimens had their tissue differentiation classified according to the highest graded lesion present in the sample. Corresponding paired nonmalignant tissue sections were procured at the most distant site from the distal negative margin of the tumor. In two cases, normal squamous esophageal epithelium was not present in the esophagectomy specimen. Normal gastric epithelium and esophageal epithelium with Barretts metaplasia were present and used as the corresponding paired nonmalignant tissue. One pathologist (T. T. W.) determined the site of tumor involvement in the esophagus as distal esophagus or GE junction based on the lesion's epicenter.

DNA Extraction. Genomic DNA from esophageal adenocarcinoma and adjacent nonmalignant sections was isolated from frozen section tissue slides by standard methods using a simplified proteinase K digestion method.

Methylation Specific PCR. DNA methylation patterns in the CpG islands of all seven genes were determined by MSP (9). A multiplex-nested MSP approach was used for samples with poor quality DNA or if only small amounts of DNA were available. In samples where nested and non-nested MSP methodologies were used together, there was good correlation. MSP distinguishes methylated from unmethylated alleles in a given gene by relying on chemical modification of the unmethylated, but not methylated, cytosines to uracil with subsequent PCR amplication using primers specific for either the methylated or modified unmethylated DNA. Primer sequences of p16 (10), MGMT (11), DAP-kinase (12), ECAD (13), APC (14), and ER (15) have been described previously. Cell lines used as positive controls have also been described previously (RKO methylated for p16, MDA231 for MGMT, RKO for DAP-kinase, and MDA231 for ECAD and ER). Placental DNA treated *in vitro* with *Sss*I methyltransferase (New England Biolabs, Beverly, MA) was used as the positive control for methylated alleles for APC (14). DNA from normal lymphocytes was used as a negative control for methylated genes. The annealing temperature for all reactions was 60°C, except for APC, in which reactions with an annealing temperature of 61°C were used. PCR products were analyzed as described previously (9). Investigators performing all assays were blinded to patient identifiers and outcomes.

Immunohistochemistry for ECAD and MGMT. DNA methylation has been associated with transcriptional inactivation and lack of protein expression. IHC staining was performed to demonstrate the proof of principle that aberrant promotion hypermethylation observed in this study leads to reduced gene expression of ECAD and MGMT. Fourteen neoplasms with known methylation status were blindly evaluated for ECAD and MGMT expression by a hematopathologist. We chose to examine MGMT and ECAD based on the commercial availability of well-tested, specific antibodies. Corresponding nonmalignant adjacent tissue was only examined in one sample as a representative case.

Immunoperioxidase staining was performed using diaminobenzidine as a chromogen on parallel histopathological sections from Silane-coated, paraffin-embedded tissue using the Envision System (DAKO Corp., Carpinteria, CA). MGMT gene product (mouse anti-MGMT monoclonal antibody clone MT3.1; Chemicon International, Inc., Temecula, CA) and ECAD gene product (Transduction Laboratories, Lexington, KY) were stained after antigen retrieval. IHC staining and

Table 1 The clinicopathological demographics of patients with esophageal adenocarcinoma at the Johns Hopkins Hospital

No. of patients	41
Median age at surgery (range in years)	65.4 (41.9–81.9)
Males, *n* (%)	35 (85%)
M/F ratio	7:1
Preoperative GERD,[a] *n* (%)	24 (59%)
Smoking history, *n* (%)	30 (73%)
Alcohol history, *n* (%)	22 (54%)
Site of malignancy	
Esophageal, *n* (%)	21 (51.2%)
G-E junction, *n* (%)	20 (48.8%)
Histology-adenocarcinoma, *n* (%)	41 (100%)
Associated Barretts esophagus, *n* (%)	26 (63%)
Associated Barretts high-grade dysplasia, *n* (%)	14 (34%)
Stage of tumor[b]	
I *n* (%)	1 (2%)
IIA *n* (%)	14 (34%)
IIB *n* (%)	8 (20%)
III *n* (%)	12 (29%)
IV *n* (%)	6 (15%)
Tumor differentiation	
Moderate, *n* (%)	21 (51%)
Moderate to poor, *n* (%)	6 (15%)
Poor, *n* (%)	14 (34%)
Deaths, *n* (%)	29 (71%)
Follow-up in months, median (range)	24.2 (1–96.3)

[a] GERD, gastroesophageal reflux disease.
[b] Tumor-Node-Metastasis classification by American Joint Committee on Cancer staging.

interpretation were done by an investigator blinded to MSP results (Y. A.).

Statistical Analysis. Disease-specific survival was the primary end point of the study, and the interval from surgery to tumor recurrence was the secondary end point. All other deaths were treated as censored observations. Survival was calculated from the date of surgery to the time of death or censor and was modeled using the Kaplan-Meier plot of estimated probability of survival. The association of risk factors to time-to-event or time-to-censor end points was analyzed using the Log-rank test for univariate analysis and Cox proportional hazards model for multivariate analysis. Results from this model are reported as relative risks with 95% confidence intervals (Stata Statistical Software, College Station, TX). Correlation between variables was estimated using Fisher's exact test or the Student *t* test when appropriate, all reported *P*s are two sided, and all significant associations were considered when $P \leq 0.05$.

Results

Clinical and Pathological Features. The demographics of the 41 patients in the cohort are shown in Table 1. The mean age is 65.1 ± 9.7 years (median age 65.4 years), and the male:female ratio is 7:1. The women are statistically older than the men (mean age 76 *versus* 63.2 years; $P < 0.002$), and in fact, all six women in this cohort are older than 70 years. Because all patients were selected preoperatively to undergo resections with curative intent, the pathological staging of disease in the cohort consists predominantly of stage II and III disease. Six patients who were upstaged to stage IV disease after metastases were found incidentally at surgery; four patients had celiac-positive lymph nodes, one patient had a positive subcarinal lymph node, and another had a small hepatic focus of esophageal adenocarcinoma. All six patients underwent complete esophageal resection.

Patient Outcome. Median survival for all patients in this series was only 2 years, with ~71% ($n = 29$ patients) succumbing to disease. Only one patient was lost to follow-up, and length of follow-up ranged from 1 day to 8 years. In this cohort, treated largely with surgery alone, the 5-year, disease-specific survival was 22 ± 7.4%. Recurrences occurred in 59% of patients with a median time to recurrence of 15 months. By multivariate analysis, when patient demographics are considered, only tumor stage and patient age were strong predictors of tumor recurrence and overall survival. The hazard ratios, or relative hazard (risk) of dying with regards to age and stage, are similar for overall survival, overall relapse-free survival, 2-year survival, and 2-year relapse-free survival.

Methylation Analysis. Using seven cancer-related genes, 95% (39 of 41) malignancies demonstrated methylation in at least one locus (Fig. 1). Far from being a tumor-specific or malignancy-restricted event, it is becoming evident that promoter hypermethylation may also represent preneoplastic/preinvasive genetic changes, especially in histologically normal smoking-damaged lung epithelium and Barretts esophagus (16, 17). Methylation of the CpG islands of histologically normal tissue suggests a large field of early methylation changes in tissue adjacent to malignancies, perhaps representing a clonal expansion of cells with abnormal hypermethylation. Indeed, our data demonstrate that, in most cases, the adjacent nonmalignant tissues (74% or 14 of 19) had at least one methylated locus that corresponded to the malignancy itself. We also observed that the number of adjacent nonmalignant samples with at least one positively methylated locus was slightly higher if there were associated histological Barretts changes in the resected esophagus ($n = 13$ of 26 or 50%) *versus* if there was no associated Barretts metaplasia ($n = 6$ of 15 or 40%). Furthermore, nonmalignant tissues were harvested at the most distant site away from the primary tumor to avoid cross-contamination. These results suggest promoter hypermethylation as an early preneoplastic alteration in esophageal adenocarcinoma.

We also analyzed for DNA hypermethylation using the same profile of seven genes, normal esophageal squamous mucosa from 17 patients without malignancy who underwent endoscopic biopsy procedures, and esophagectomy specimens from 4 patients with end-stage achalasia (data not shown). In the 17 patients with normal esophageal tissue, p16, APC, DAP-K, ER, and TIMP3 all had no methylation of their promoters. MGMT was methylated in 1 of 17 (6%) samples, whereas ECAD had methylated alleles in 3 of 17 (18%) biopsies. In the achalasia specimens, of the 28 loci examined, only 4 loci were methylated, 3 for the ER gene and 1 locus for the ECAD gene. Although we have no clinical correlation data for our achalasia specimens, achalasia is a known precursor of squamous esophageal cancer (18), and squamous mucosal alterations, including an increased frequency of p53 immunoreactivity, have been shown in esophagectomy specimens from patients with end-stage achalasia (19). The overall infrequent methylation events in the normal specimens without associated malignancy and achalasia specimens support the proof of principle that histo-

PATIENT #	TUMOR SAMPLES							NORMAL TISSUE						
	p16	MGMT	DAP-K	TIMP-3	E-CAD	ER	APC	p16	MGMT	DAP-K	TIMP-3	E-CAD	ER	APC
1	U	U	M	M	M	M	M	U	M	U	U	U	M	U
2	M	U	M	U	M	M	M	U	M	U	U	U	M	U
3	M	M	U	U	M	M	M	M	M	U	U	U	M	M
4	U	U	U	U	U	U	M	M	U	U	U	U	U	U
5	U	M	U	U	U	U	U	U	U	U	U	U	U	U
6	M	U	U	M	M	M	M	U	U	U	U	U	U	U
7	U	M	U	U	M	U	M	U	U	U	U	U	U	U
8	U	U	M	U	M	U	M	U	U	U	U	U	U	U
9	M	M	U	U	M	M	M	M	M	U	U	M	U	U
10	U	U	U	U	U	U	U	U	U	U	U	U	U	U
11	M	M	M	U	M	M	M	M	M	U	U	U	U	U
12	U	U	M	M	M	M	U	M	U	U	U	M	M	U
13	M	M	U	U	M	M	M	U	M	U	U	M	U	U
14	U	M	U	U	M	U	U	U	M	U	U	U	U	U
15	U	M	U	U	M	U	M	U	U	U	U	U	U	U
16	U	M	M	U	M	U	U	M	U	M	U	M	U	U
17	U	U	U	U	M	M	M	M	U	U	U	U	U	U
18	U	U	U	U	M	M	M	M	U	U	U	U	U	U
19	M	M	U	M	U	U	M	U	U	U	U	U	U	U
20	M	M	U	U	M	M	M	M	U	U	U	U	U	U
21	U	U	U	U	U	U	M	U	U	U	U	U	U	U
22	U	M	U	M	U	U	M	U	U	U	U	U	U	M
23	U	U	U	U	M	U	U	U	U	U	U	U	U	U
24	M	M	U	M	U	M	M	U	M	U	U	M	M	U
25	M	M	U	U	M	M	M	U	U	U	U	U	U	U
26	U	U	U	U	M	U	U	U	U	U	U	U	U	U
27	U	U	U	U	U	U	M	U	U	U	U	U	U	U
28	U	M	M	U	U	U	U	U	U	U	U	U	U	U
29	U	M	M	U	M	M	M	U	U	U	U	U	U	U
30	M	M	U	U	M	U	U	U	U	U	U	U	U	U
31	M	M	U	M	M	M	M	M	M	U	U	U	U	U
32	U	U	U	M	U	M	M	U	M	U	U	U	U	U
33	U	M	U	U	U	M	M	U	U	U	U	U	U	U
34	M	M	U	U	M	M	M	U	U	U	U	U	U	U
35	M	M	U	U	U	M	M	U	U	U	U	U	U	M
36	U	M	U	U	U	U	U	U	U	U	U	U	U	U
37	U	U	U	U	M	M	M	U	U	U	U	U	U	U
38	M	M	U	U	M	U	U	U	U	U	U	U	U	U
39	U	U	U	U	M	U	U	U	U	U	U	U	U	U
40	M	M	U	U	M	M	M	U	U	U	U	U	U	U
41	U	U	U	U	U	U	U	U	U	M	U	U	U	U

Fig. 1 Methylation results by MSP analysis for 41 patients with M = methylation (*in gray*) and U = unmethylation (*in white*). Each patient had an esophageal malignancy accompanied by a paired, adjacent histologically non-malignant esophageal specimen.

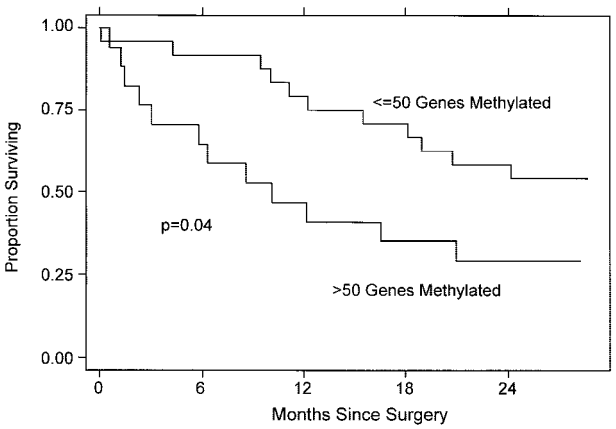

Fig. 2 Probability of 2-year survival for patients with resected esophageal adenocarcinoma in relation to the positive and negative methylation status of the primary tumor. A statistically significant difference in survival rate was observed between the methylation-positive and methylation-negative groups (p = 0.04).

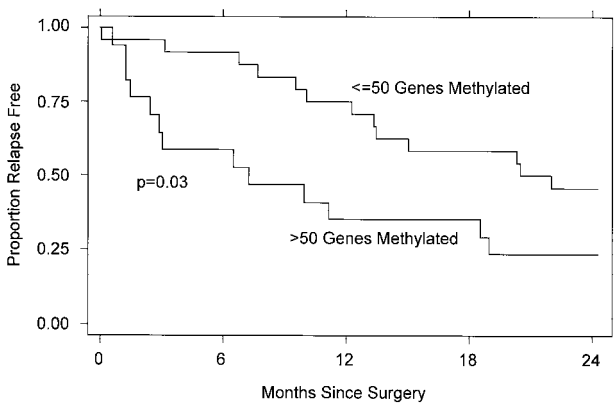

Fig. 3 Probability of having no recurrence for patients with resected esophageal adenocarcinoma in relation to the methylation status of the primary tumor. A statistically significant difference in recurrence rate was observed between the methylation-positive and methylation-negative groups (p = 0.03).

logically normal tissue, tissue at risk for preneoplastic histological changes, and frank carcinoma can all manifest different DNA methylation alterations.

There was a strong trend toward shorter survival for patients whose primary tumors were methylated for p16, MGMT, ER, and ECAD (Table 2). Median survival for patients with these methylated genes was only 1 *versus* 3 years for those patients whose primary tumors were without methylation. For each individual gene analyzed, however, this difference in median survival was not statistically significant.

When the methylation status of the seven genes in each patient was considered as a profile, however, both 2-year survival (Fig. 2) and 2-year relapse-free survival (Fig. 3) were significantly different for esophagectomy patients who had >50% of their genes methylated *versus* those that had <50% methylated.

The use of a methylation profile to correlate with clinico-

Table 2 Correlation between overall survival and the methylation status of p16, MGMT, ER, and ECAD (Kaplan-Meier Analysis of estimated probability of survival analysis)

Methylation status	*n*	Deaths	Median overall survival (months)	*P* (Log-rank)
Unmethylated p16	25	17	29	*P* = N/S
Methylated p16	16	12	11	*P* = N/S
Unmethylated MGMT	17	10	31	*P* = N/S
Methylated MGMT	24	19	14	*P* = N/S
Unmethylated ER	11	9	33	*P* = N/S
Methylated ER	30	20	18	*P* = N/S
Unmethylated ECAD	14	10	37	*P* = N/S
Methylated ECAD	27	19	12	*P* = N/S

Table 3 Multivariate Cox's regression analysis of overall and relapse-free survival

Factor	Risk ratio	95% Confidence interval	*P*
Overall survival			
Methylation status	1.95	0.89–4.26	0.095
Age of patient	1.94	1.26–2.98	0.003
Stage of tumor	1.78	1.14–2.78	0.012
2-year survival			
Methylation status	2.71	1.14–6.45	0.024
Age of patient	2.03	1.21–3.39	0.007
Stage of tumor	1.48	0.92–2.93	0.11
Overall relapse-free survival			
Methylation status	2.08	0.89–4.87	0.09
Age of patient	1.91	1.19–3.07	0.007
Stage of tumor	2.19	1.27–3.77	0.005
2-year, relapse-free survival			
Methylation status	2.5	1.11–5.6	0.027
Age of patient	1.96	1.23–3.12	0.004
Stage of tumor	1.67	1.05–2.64	0.029

pathological features has also been reported by Maruyama *et al.* (20), although they failed to appreciate any significant association between methylation status and survival in their cohort of prostate cancer patients. We also observed using multivariate Cox proportional hazards modeling (Table 3) that aberrant promoter hypermethylation in malignant esophagectomy tissue was a stronger predictor of 2-year survival and recurrence than both the age of the patient or stage of the resected tumor. Methylation status was also a strong predictor of overall and overall relapse-free survival (Table 2). Methylation events did not correlate with other clinicopathologic variables, such as gender, age, stage of malignancy, history of gastroesphogeal reflux, alcohol, smoking history, degree of tumor differentiation, number of lymph nodes with metastatic disease, tumor location (lower esophagus *versus* GE junction), or the presence of associated Barretts metaplasia in the resected specimen.

Immunostaining. Fig. 4, *A* and *B* demonstrate the relationship of promoter hypermethylation changes to the expression of the gene. There was complete concordance of the expression data and methylation status of the genes. In all malignancies with positive methylation by MSP, there was loss of protein expression immunohistochemically. Similarly, all unmethylated neoplasms had evidence of protein expression. Fig. 4*C* demonstrates a representative case of the nonmalignant adjoining esophageal squamous tissue that was methylation negative showing positive gene expression for ECAD on immunostaining. Fig. 4*D* demonstrates considerable heterogeneity of expression for ECAD, suggesting tumor evolution from a methylated clonal precursor, as well as alternative mechanisms of tumor growth advantage.

Discussion

The pathogenesis of esophageal adenocarcinoma involves a multistep progression from injury of normal esophageal mucosa through Barretts metaplasia to low- and high-grade Barretts dysplasia and, finally, malignancy. This process involves genetic and epigenetic changes. In this report, we describe epigenetic changes of individual genes that have accumulated in esophageal adenocarcinoma and correlate them to patient outcome. We found that methylation of multiple genes in esophageal adenocarcinoma was an independent and strong predictor for disease-specific recurrence and survival of patients. Furthermore, multivariate analysis confirmed that epigenetic tumor profiling by methylation status was a more powerful biomarker of risk prediction in esophageal adenocarcinoma than the classic clinicopathological features of stage and age. This may be attributable to the silencing in these tumors of critical genes affecting growth control, proliferation, apoptosis, and cell differentiation that can lead during aberrant methylation to selective growth advantage, tumor progression, recurrence, and decreased patient survival.

The potential utility of DNA methylation markers in our panel as a means to characterize malignancies has been supported by recent studies. In gastric cancer, MGMT methylation has been significantly associated with lymph node invasion ($P < 0.01$), tumor stage ($P < 0.03$), and 5-year, disease-free survival ($P < 0.02$), but it was not an independent predictor of survival in a multivariate analysis (21). In lung cancer, MGMT methylation was associated with a poorer prognosis among smokers (22), and in early staged lung adenocarcinomas, p16 methylation was an independent risk factor for predicting a short survival ($P = 0.03$). Reduced levels of ECAD protein expression have not only been observed in Barretts dysplasia and esophageal adenocarcinoma (23–25) but also have been correlated to poor prognosis (26). We have attributed previously some of this reduced ECAD expression to promoter hypermethylation (27). Elevated APC methylation levels in the serum of esophageal adenocarcinoma patients were shown to be predictive of poor prognosis (28), and although ER methylation has been documented in other gastrointestinal malignancies (3), only in adult acute myeloid leukemia has its absence been shown to improve survival (29).

APC, *DAP-kinase*, and *TIMP3* were genes that did not manifest trends favoring prolonged survival for methylation in our study. Kawakami *et al.* (28) also observed a lack of correlation between survival and APC methylation in primary esophageal adenocarcinoma specimens but demonstrated a significant association between high plasma DNA levels of hypermethylated APC and reduced survival. APC promoter hypermethylation in primary esophageal adenocarcinomas

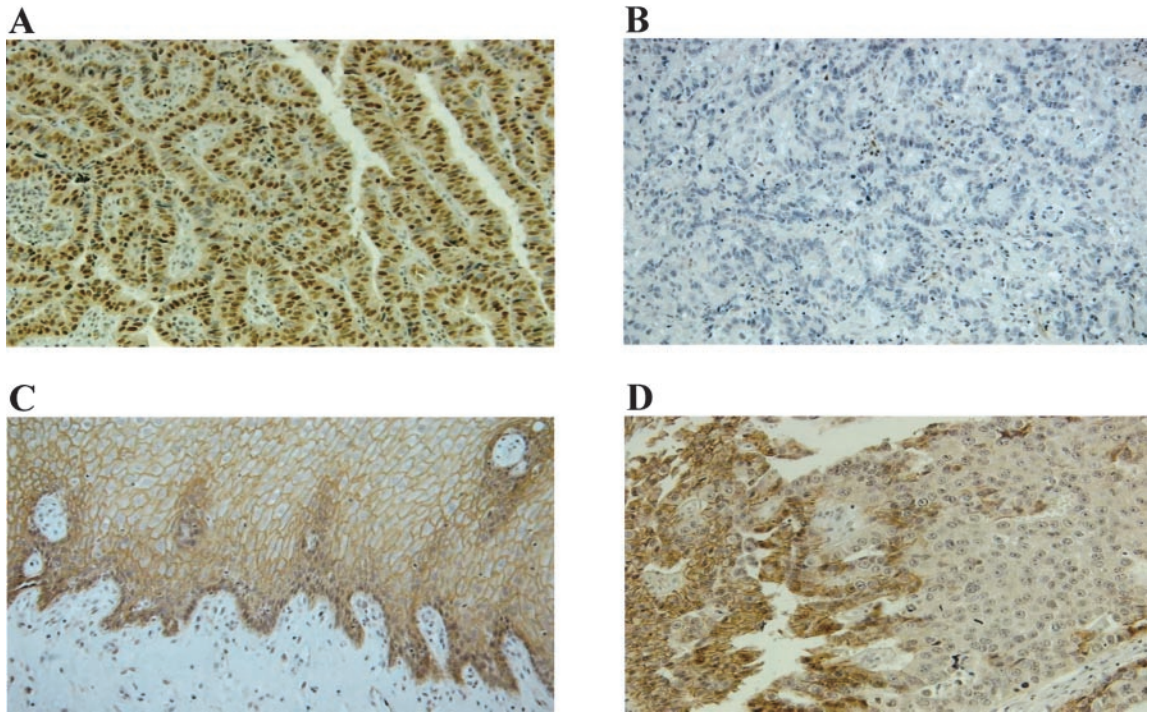

Fig. 4 Light microscope images (×40) of immunohistochemistry of esophageal adenocarcinoma specimens: *A,* Specimen with an unmethylated DNA status typically expressing MGMT. *B,* Specimen with a methylated DNA status that typically is not expressing MGMT. *C,* Normal esophageal mucosa expressing E-cadherin. *D,* Adenocarcinoma specimen with a methylated DNA status demonstrating heterogeneity of expression of E-cadherin.

occurs at such a high frequency that correlation with survival of any subgroup would be difficult to discern. The low prevalence of DAP-kinase methylation in primary esophageal adenocarcinoma tissues in this study is a likely explanation for its lack of correlation with survival. Although TIMP3 methylation in gastric cancer cell lines has been associated with a more malignant and invasive phenotype (30), we did not observe any correlation of positive methylation and shortened survival.

Because of the small number of patients analyzed and relatively short median survival of 2 years, attributable to the virulence of this cancer, DNA methylation of individual genes showed only a trend toward diminished survival and did not individually provide statistically significant correlations. When the methylation patterns of all seven genes were combined, however, a statistically significant correlation between methylation status, survival, and recurrence became apparent. The predictive value of these methylation changes is based solely on the biology of the primary tumor and is independent of tumor stage and age. This suggests that the frequency of DNA methylation events reflects disease progression and that DNA methylation events accumulate as the molecular clock of a cancer advances with time. It is encouraging that DNA methylation changes can serve as a marker of tumor virulence even with a malignancy as aggressive as esophageal adenocarcinoma and can differentiate those patients at risk for early death and tumor recurrence. It is interesting to speculate that unique epigenetic fingerprints of tumors based on multiple genes may reflect the proliferative impulses of a tumor and provide prognostic information for individual patients. Additional clinical advantages of this approach could be the ability to determine prognosis based on endoscopic biopsy samples and the avoidance of the morbidity associated with extended surgical lymphadenectomies for clinical staging of regional lymph nodes.

Using MSP as a diagnostic tool, we identified 39 of 41 patients with esophageal adenocarcinoma, a sensitivity of 95%. This sensitivity is higher than observed using MSP with about four to five genes in many other tumor types, including colon (79%), lung (68%), kidney (88%), leukemia (81%), breast (73%), and bladder (78%; Refs. 3 and 31). There were 19 of 46 patients with hypermethylation of the adjacent nonmalignant esophageal epithelia. Hypermethylation of multiple genes in nonmalignant tissues associated with malignancies has been reported (16, 17, 28, 32). Nonmalignant adjacent esophageal mucosa is perhaps "at risk" for preneoplastic changes, and they can be observed as DNA methylation alterations. In esophageal adenocarcinoma, this field cancerization phenomenon has been described mainly in relation to the premalignant changes of Barretts mucosa. Eads *et al.* (17) showed that when hypermethylation occurs in Barretts mucosa, it does so in a large contiguous field and suggested a clonal expansion of cells with abnormal hypermethylation. It is interesting to extend this concept to histological "normal" adjacent nonmalignant esophageal tissue and speculate that even Barretts changes are preceded by epigenetic alterations in a clonal field-like fashion.

We found considerable heterogeneity of ECAD methyla-

tion in esophageal adenocarcinoma even in the same tumor sample in a single individual (Fig. 4*C*). This heterogeneous loss of ECAD expression attributable to promoter hypermethylation has been described in breast cancers (33). Despite the observed heterogeneity in our study, we found that the methylation of ECAD was associated with a trend toward shortened survival. This concurs with Krishnadath *et al.* (26), who showed that reduced expression of ECAD in esophageal adenocarcinoma correlates with poor prognosis.

In summary, using a profile of genes, we determined that positive methylation status was a powerful predictor of poor prognosis for primary esophageal adenocarcinoma tissues. In addition, the methylation high frequency of esophageal adenocarcinomas may enable the use of DNA methylation also as a biomarker for the detection of this tumor. Additional validation studies are needed. The clinical utility of methylation status as a means to predict responsiveness to neoadjuvant therapy in modern esophageal adenocarcinoma protocols remains to be studied.

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