Background: The adenomatous polyposis coli (APC) locus on chromosome 5q21–22 shows frequent loss of heterozygosity (LOH) in esophageal carcinomas. However, the prevalence of truncating mutations in the APC gene in esophageal carcinomas is low. Because hypermethylation of promoter regions is known to affect several other tumor suppressor genes, we investigated whether the APC promoter region is hypermethylated in esophageal cancer patients and whether this abnormality could serve as a prognostic plasma biomarker. Methods: We assayed DNA from tumor tissue and matched plasma from esophageal cancer patients for hypermethylation of the promoter region of the APC gene. We used the maximal chi-square statistic to identify a discriminatory cutoff value for hypermethylated APC DNA levels in plasma and used bootstrap-like simulations to determine the P value to test for the strength of this association. This cutoff value was used to generate Kaplan–Meier survival curves. All P values were based on two-sided tests. Results: Hypermethylation of the promoter region of the APC gene occurred in abnormal esophageal tissue in 48 (92%) of 52 patients with esophageal adenocarcinoma, in 16 (50%) of 32 patients with esophageal squamous cell carcinoma, and in 17 (39.5%) of 43 patients with Barrett’s metaplasia but not in matching normal esophageal tissues. Hypermethylated APC DNA was observed in the plasma of 13 (25%) of 52 adenocarcinoma patients and in two (6.3%) of 32 squamous carcinoma patients. High plasma levels of methylated APC DNA were statistically significantly associated with reduced patient survival (P = .016). Conclusion: The APC promoter region was hypermethylated in tumors of the majority of patients with primary esophageal adenocarcinomas. Levels of hypermethylated APC gene DNA in the plasma may be a useful biomarker of biologically aggressive disease in esophageal adenocarcinoma patients and should be evaluated as a potential biomarker in additional tumor types. [J Natl Cancer Inst 2000;92:1805–11]
APC by this mechanism is a fundamental process involved in the development of many cancers, including esophageal cancers, in which hypermethylation of the cyclin-dependent kinase inhibitor CDKN2/\(p16\) (also known as INK4a) has been observed frequently (22,23). This latter finding was an important one because, although the \(p16\) locus on chromosome 9p21 undergoes frequent LOH in esophageal tumors (13,14,24–27), inactivating point mutations affecting the \(p16\) gene are relatively rare in most cancer types, including esophageal adenocarcinomas (14,26,28).

Other critical genes targeted by hypermethylation in human cancers include the DNA mismatch repair gene hMLH1 (human mut L homologue 1), the estrogen receptor and progesterone receptor genes, and the E-cadherin gene (19,20,29,30).

The purpose of this study was to determine whether CpG island hypermethylation in the promoter region of the APC gene occurs in primary esophageal carcinomas and premalignant lesions, whether freely circulating hypermethylated APC DNA is detectable in the plasma of these patients, and whether the presence and quantity of hypermethylated APC in the plasma have any relationship with outcome.

### Materials and Methods

#### Patients and Specimens

Fifty-two patients with esophageal adenocarcinoma, 32 with esophageal squamous cell carcinoma, 43 with nondysplastic Barrett’s esophagus, 20 with normal esophageal mucosa only, and 23 with gastritis were studied. Of the 52 patients with adenocarcinoma, all had their primary tissue and plasma assayed for hypermethylated APC DNA levels; follow-up data were available for all 52 patients with adenocarcinoma, which were used in survival calculations. All of the 32 patients with squamous cell carcinoma had tissue and plasma available that was assayed for hypermethylated APC DNA. All 43 Barrett’s esophagus patients were studied for tissue hypermethylated APC DNA levels; only 11 had plasma available, which was also evaluated for hypermethylated APC DNA levels. Matching normal stomach tissue was obtained from 37 of the Barrett’s esophagus patients. Twenty normal esophageal epithelial samples were obtained from patients without Barrett’s esophagus or esophageal cancer. Biopsies were performed at the time of upper gastrointestinal endoscopy, before any treatment, and the specimens were stored in liquid nitrogen until use. The normal gastric biopsies were always performed before the biopsies on the Barrett’s esophageal tissue or tumor epithelium to avoid contamination of normal mucosa by these preneoplastic or neoplastic tissues. Plasma specimens were obtained before endoscopy. All participating institutions had approval to conduct the study from their respective Institutional Review Boards. Written informed study consent was obtained from all patients at all participating institutions.

#### DNA Isolation

Tissue samples ranged from 0.05 to 2.0 g each. Genomic DNA was obtained from tissue by standard methods (31). Briefly, large pieces of tissue (>0.2 g) were ground into a powder under liquid nitrogen; smaller pieces were not ground up. The tissue was digested with proteinase K (500 \(\mu\)g/mL), extracted with an equal volume of phenol–chloroform, and precipitated with 2\(\frac{1}{2}\) volumes of 100% ethanol. The pellet was then washed with 70% ethanol, dried, and resuspended in water to yield a final concentration of greater than 100 \(\mu\)g/mL.

For the isolation of freely circulating DNA from blood, 300 \(\mu\)L of plasma was digested overnight at 48 °C with an equal volume of 1% sodium dodecyl sulfate (SDS) containing a 50-\(\mu\)L aliquot of proteinase K (20 mg/mL stock solution; Life Technologies, Inc. [GIBCO BRL], Rockville, MD). The proteinase K stock solution was prepared by dissolving the entire contents of the lyophilized bottle, Life Technologies, Inc. [GIBCO BRL], Rockville, MD) for equilibrating 120 mL of phenol and 160 mL of chloroform with 80 mL of TE 8 buffer (i.e., 250 mL of 1 M Tris [pH 8.0], 50 mL of 0.2 M EDTA, 1.25 mL of 4 M NaCl, and 199 mL H\(_2\)O). The 4-mL upper layer from the second extraction was precipitated overnight at ~20 °C by adding 12 mL of 100% ethanol, 1.3 mL of 7.5 M ammonium acetate, and 7 \(\mu\)L of 1% sodium dodecyl sulfate (1% SDS) solution; Ambion, Austin, TX). The resulting pellet was washed with 10 mL of 70% ethanol and then dissolved in 40 \(\mu\)L of H\(_2\)O.

### Bisulfite Treatment

Bisulfite treatment

Deamination of DNA with bisulfite was done by a modification of a previously published method (32,33). DNA in a volume of 18 \(\mu\)L was denatured by being heated for 10 minutes at 97 °C and then placed on ice for 5 minutes. We added 3 \(M\) NaOH (2 \(\mu\)L) and heated the solution for 20 minutes at 48 °C. Freshly made bisulfite solution (500 \(\mu\)L) was added to each sample, and incubation was continued for 12 hours at 45 °C in the dark. We prepared the bisulfite solution (2.5 \(M\) sodium metabisulfite and 125 \(mM\) hydroquinone) by dissolving 1.9 \(g\) of sodium metabisulfite in 2.5 mL water at 48 °C and adding 0.5 \(mL\) of 1 M hydroquinone solution (i.e., 2 \(M\) hydroquinone solution and 0.7 \(mL\) of 2 \(M\) NaOH). The 1 \(M\) hydroquinone solution was freshly made by dissolving 0.11 \(g\) of hydroquinone in 1 \(mL\) of water at 48 °C. Modified DNA was purified by use of the Wizard DNA purification resin according to the manufacturer (Promega Corp., Madison, WI) and eluted in 45 \(\mu\)L of water. The modified DNA was treated with 5 \(\mu\)L of 3 \(M\) NaOH and incubated at 37 °C for 10 minutes. We then added 75 \(\mu\)L of 5 \(M\) ammonium acetate, and incubated the reaction mixture for 5 minutes at room temperature. Finally, the modified DNA was precipitated by adding 2.5 volumes of 100% ethanol and 7 \(\mu\)L of 1% sodium dodecyl sulfate (5 mg/mL; Ambion). The pellet was washed with 70% ethanol, dried, and dissolved in 20 \(\mu\)L 5 \(mM\) Tris (pH 8.0).

#### Methylation-Specific, Real-Time Polymerase Chain Reaction

To determine whether and how frequently hypermethylation of the APC gene promoter region (hypermethylated APC DNA) occurred in esophageal carcinoma patients, as well as to assess the clinical significance of hypermethylated APC DNA, we investigated bisulfite-treated DNA from the primary tissues and plasma of patients with esophageal cancerous or precancerous lesions by use of a quantitative real-time (TaqMan®) methylation-specific polymerase chain reaction (PCR) assay (ABI 7700; PE Biosystems, Foster City, CA), as described previously (34). Real-time PCR plots the PCR product on a curve as it accumulates at each cycle of the reaction, in contrast to conventional PCR, which only displays PCR product at the final cycle. Briefly, primers and probes were designed to specifically amplify bisulfite-converted DNA in the promoter region of the methylated version of the gene of interest, APC, as well as an internal reference gene, unmethylated myoD. The ratio of methylated APC promoter DNA to the control gene myoD DNA represented relative APC methylation level. Fifty cycles of PCR were run for all assays, since it is standard to run TaqMan analyses to the end of the plateau phase and to ensure complete absence of product in hypermethylated APC DNA-negative cases. However, the precise quantitative hypermethylated APC DNA value was read at the midpoint of the linear portion of the S-shaped real-time PCR curve (i.e., the Ct point, or threshold cycle, meaning the threshold at which we read the PCR product quantity). In more than 90% of assays yielding myoD or hypermethylated APC DNA PCR products, this threshold level occurred at less than 36 cycles (range, 28–35 cycles). In no case did PCR products first appear beyond 39 cycles.

The primers and probe sequences used were as follows: 1) methylated APC, primers: TTATATGTCCGTTATCCTGCTATAT and GAACAAAAC-GCTCCCCCAT; probe, 6FAM (carboxyfluorescein) 5′-CCCCGTGAAAGACCC-GCGGATT-3′ TAMRA (N,N′,N′-tetramethyl-6-carboxyrhodamine); and 2) myoD, primers: CCAACTCCAAATCCCCCTCCTT and TGATTTATTAG-ATGGGTTTAGAGAAGGA; probe, 6FAM5′-TCCCTTCATTCC-TAAATCCAACTTAAATCCTCC-3′ TAMRA.

#### Statistical Analyses

Continuous data were summarized with scatter plots and medians; groups were compared by use of the Wilcoxon signed rank test. Percentages were calculated to summarize categorical data. APC gene methylation was analyzed in two ways: as a continuous variable and as a dichotomous variable (i.e., positive or negative). As a continuous variable, the association of APC methylation with type of tumor (adenocarcinoma versus squamous cell carcinoma), with type of tissue (normal esophagus versus Barrett’s esophagus versus normal stomach, or normal esophagus versus gastritis versus Barrett’s esophagus versus adenocarcinoma) or with stage was summarized with scatter plots and medians (2). Groups were compared by use of the Wilcoxon signed rank test. To summarize
the association between APC methylation (coded as positive or negative) with type of tumor, we calculated type of tissue, or stage, percentages; we compared groups with the use of 2 × 2 tables and Fisher’s exact test.

Hazard ratios were used to calculate the relative risks of death. These calculations were based on the Pike estimate, with the use of the observed and expected number of events as calculated in the log-rank test statistic (35). The maximal chi-square method of Miller and Siegmund (36) and Halpern (37) was adapted to determine which hypermethylated APC DNA value best segregated patients into poor- and good-prognosis subgroups (in terms of likelihood of surviving), with the stratified log-rank test as the statistic used to measure the strength of the grouping. To determine a P value that would be interpreted as a measure of the strength of the association based on the maximal chi-square analysis, 1000 bootstrap-like simulations were used to estimate the distribution of the maximal chi-square statistic under the hypothesis of no association (37). All P values reported were based on two-sided tests.

RESULTS

Hypermethylation of the APC Gene Promoter Region in Primary Esophageal Cancer Tissue, Barrett’s Esophageal Tissue, and Normal Gastric Epithelium

Fig. 1 shows TaqMan real-time PCR analyses of hypermethylated APC DNA in the tissues and plasma of patients with esophageal adenocarcinoma. In tissues that were positive for hypermethylated APC DNA, myoD and hypermethylated APC DNA tracings crossed the threshold line (see the “Materials and Methods” section) approximately 2.5 cycles apart. In contrast, in hypermethylated APC DNA-negative tissues, myoD crossed the threshold at cycle 31, but no hypermethylated APC DNA signal was apparent, even at 50 cycles. In the plasma shown, myoD and hypermethylated APC DNA signals in the positive sample were separated by 3.5 cycles, but in the hypermethylated APC DNA-negative sample, the hypermethylated APC DNA signal failed to appear, even at 50 cycles.

Fig. 2 plots the ratios of hypermethylated APC DNA to unmethylated myoD levels in normal esophagus, Barrett’s esophagus, normal stomach, squamous cell carcinoma, and adenocarcinoma tissues. Sixteen (50%) of 32 esophageal squamous cell carcinoma tissues showed detectable methylated APC DNA, whereas 48 (92%) of 52 esophageal adenocarcinoma tissues had detectable hypermethylated APC DNA levels (P = .0001 for adenocarcinoma versus squamous cell carcinoma; Fisher’s exact test; two-tailed). Moreover, the two histologic subtypes of esophageal carcinoma manifested considerably different distributions of hypermethylated APC/myoD DNA ratios. Although the ranges of ratios were similar in the two subtypes (0–2.85 for adenocarcinoma and 0–2.48 for squamous cell carcinoma), the median ratio of hypermethylated APC/myoD DNA was 0.59 for adenocarcinoma, while the median ratio for squamous cell carcinoma was 0.0002. Among the adenocarcinoma tissues, 29 (56%) of 52 had hypermethylated APC/myoD DNA values greater than 0.5, whereas only seven (22%) of 32 squamous cell carcinomas had values greater than 0.5. This difference in distribution of hypermethylated APC/myoD DNA ratios between the two histologic subtypes was statistically significant by the Wilcoxon test (P = .001).

Hypermethylated APC DNA was also detected in 17 (39.5%) of 43 Barrett’s metaplasia tissues, a premalignant precursor lesion of esophageal adenocarcinoma (Fig. 2). A similar percent-
The quantity of methylated APC DNA was expressed as the ratio between polymerase chain reaction products amplified from the methylated APC gene and from the unmethylated myoD gene. Approximately one third of BE and NS specimens contained hypermethylated APC DNA (15% [41%] of 37). These two histologic subgroups (SCC and AdCa) were statistically different by the Wilcoxon test (p = .001). The mean methylated APC/myoD DNA ratio in AdCa was 0.488, with a range of 0–2.85 (n = 52). In contrast, the mean methylated APC/myoD DNA ratio in SCC was 0.0002, with a range of 0–2.48 (n = 32). These two histologic subgroups (SCC and AdCa) were statistically different by the Wilcoxon test (p = .001).

Age of normal gastric mucosal specimens, however, also manifested hypermethylated APC DNA (15% [41%] of 37). These normal gastric mucosal specimens were obtained from patients with Barrett’s esophagus. However, there was no relationship between Barrett’s and normal stomach methylation status. Among the 37 patients from whom both normal stomach and Barrett’s tissues were available, eight of 15 with hypermethylated APC DNA in normal stomach had no hypermethylated APC DNA in matching Barrett’s esophagus tissue; conversely, nine of 16 with hypermethylated APC DNA in Barrett’s esophagus tissue specimens had no hypermethylated APC DNA in matching normal gastric tissues (chi-square P value = .729 for the association between hypermethylated APC DNA status in normal stomach versus matching Barrett’s esophagus tissue). In contrast, normal esophageal epithelia did not contain hypermethylated APC DNA (none [0%] of 20). The maximal chi-square statistic dichotomized hypermethylated APC DNA/myoD DNA ratios into two groups: one with hypermethylated APC DNA/myoD DNA ratios above 0.5 and the other with ratios below 0.5 (Fig. 2).

Detection of Hypermethylation in the APC Gene Promoter Region in DNA Obtained From the Plasma of Patients With Esophageal Adenocarcinoma or Barrett’s Metaplasia

Results obtained in plasma contrasted sharply with those obtained in corresponding primary tissues. For example, in contrast to primary tissue results, none of the 54 noncancer patients contained hypermethylated APC DNA in their plasma (comprising none of 20 asymptomatic normal patients, none of 23 with gastritis, and none of 11 with Barrett’s metaplasia). However, 13 (25%) of 52 patients with untreated esophageal adenocarcinoma contained hypermethylated APC DNA in their plasma. All of these patients also showed hypermethylated APC DNA in their primary tumor tissues (Fig. 3). Moreover, among 32 squamous cell carcinoma patients, only two (6%) had detectable hypermethylated APC DNA in their plasma. This plasma rate was significantly lower than that observed in adenocarcinoma patients (p = .025; Fisher’s exact test; two-tailed).

There was a statistically significant association between hypermethylated APC DNA and advanced disease stage. The frequency of plasma-hypermethylated APC DNA was five (45%) of 11 patients with stage IV disease (active or recurrent disease), seven (47%) of 15 with stage III disease, one (4%) of 23 with stage II disease, and none (0%) of three with stage I disease (p = .002; Fisher’s exact test; Fig. 3).

Presence of Hypermethylated APC DNA in Plasma and Patient Prognosis

Fig. 4 displays a Kaplan–Meier plot of the estimated probability of survival versus plasma-hypermethylated APC/myoD DNA ratio in 52 patients from whom survival data were available. The stratified log-rank test was used to evaluate the association between plasma-hypermethylated APC DNA (patients classified as <0.5 versus >0.5) and survival. Patients were stratified by stage (stage I versus stage II versus stage III versus stage IV). The observed log-rank test statistic was 8.049. To determine the P value, we used bootstrap-like simulations to estimate the distribution of a maximal chi-square statistic, since the cut point of 0.5 had been chosen after examining the data. The resulting adjusted P value was .016.

Inspection of the relative hazard of dying (comparing patients...
In his primary tumor. APC DNA, one patient also lacked hypermethylated APC DNA with active or recurrent disease lacking plasma-hypermethylated the time his follow-up plasma was sampled. Of the three patients had not been checked for clinical recurrence for almost 1 year at group whose plasma was hypermethylated APC DNA positive.

DISCUSSION

The rate of APC promoter region hypermethylation (92%) in primary esophageal adenocarcinomas is somewhat higher than that described for hypermethylation of other genes in other tumor types. For example, reported hypermethylation frequencies for the glutathione S-transferase with plasma-hypermethylated APC DNA >0.5 with those with plasma-hypermethylated APC DNA <0.5: data not shown) suggested that the association between plasma-hypermethylated APC DNA level and survival was similar in the three stage groups.

Plasma-hypermethylated APC DNA positivity also showed a nonsignificant trend toward association with recurrent disease: Eight (73%) of 11 patients with recurrent disease had detectable plasma-hypermethylated APC DNA compared with one (25%) of four patients with no evidence of disease (NED) on follow-up (P = .14; Fisher’s exact test). In fact, the one patient in the NED group whose plasma was hypermethylated APC DNA positive had not been checked for clinical recurrence for almost 1 year at the time his follow-up plasma was sampled. Of the three patients with active or recurrent disease lacking plasma-hypermethylated APC DNA, one patient also lacked hypermethylated APC DNA in his primary tumor.

The significant association between high plasma levels of hypermethylated APC DNA and reduced survival seen in this study suggests the potential utility of plasma-hypermethylated APC DNA as a prognostic biomarker. Furthermore, these data suggest that the quantity of gene-specific methylated DNA in plasma, in addition to its presence or absence, may have prog-

![Graph](https://academic.oup.com/jnci/article-abstract/92/22/1805/2906015)

**Fig. 4.** Survival of esophageal adenocarcinoma patients as a function of the amount of methylated adenomatous polyposis coli (APC) DNA (the ratio between methylated APC and control myoD PCR products) in preoperative plasma specimens. High-methylated APC (HMAPC) DNA levels constituted ratios greater than 0.5, while low levels comprised ratios less than 0.5. To test for an association with HMAPC and survival, we first stratified patients on stage (I versus II versus III versus IV; see the “Materials and Methods” section). All patients alive at their last follow-up are indicated as tick marks on the plot; note that after 36 months only five patients remained alive. The 95% confidence intervals (CIs) for survival at 12 months were 0.0–0.71 for the HMAPC-high group and 0.61–0.88 for the HMAPC-low group. After 30 months, the survival in the HMAPC-low group leveled off at 0.57 (95% CI = 0.42–0.73).

with plasma-hypermethylated APC DNA >0.5 and with plasma-hypermethylated APC DNA <0.5: data not shown) suggests that the association between plasma-hypermethylated APC DNA level and survival was similar in the three stage groups.

Plasma-hypermethylated APC DNA positivity also showed a nonsignificant trend toward association with recurrent disease: Eight (73%) of 11 patients with recurrent disease had detectable plasma-hypermethylated APC DNA compared with one (25%) of four patients with no evidence of disease (NED) on follow-up (P = .14; Fisher’s exact test). In fact, the one patient in the NED group whose plasma was hypermethylated APC DNA positive had not been checked for clinical recurrence for almost 1 year at the time his follow-up plasma was sampled. Of the three patients with active or recurrent disease lacking plasma-hypermethylated APC DNA, one patient also lacked hypermethylated APC DNA in his primary tumor.

The more frequent occurrence of hypermethylated APC DNA seropositivity in patients with recurrent disease (25% for initial versus 73% for recurrent tumors) also suggests that patients can present as seronegative yet acquire seropositivity upon recurrence.

The substantially lower rate of tissue-hypermethylated APC DNA in Barrett’s metaplasia versus adenocarcinoma (39% versus 92% of patients) suggests that hypermethylated APC DNA usually occurs after the transition from normal to metaplastic epithelium (i.e., at the metaplasia–dysplasia or dysplasia–invasive cancer interfaces). However, further studies of dysplastic Barrett’s lesions will be required to more precisely pinpoint the timing of this event.

The rate of APC promoter region hypermethylation (92%) in primary esophageal adenocarcinomas is somewhat higher than that described for hypermethylation of other genes in other tumor types. For example, reported hypermethylation frequencies for the glutathione S-transferase (GSTP1) gene are 30% and 20% in breast and renal carcinomas, respectively (40), 30% for the p73 gene in acute lymphoblastic leukemia (41), 25%–40% for the O6-methylguanine–DNA methyltransferase (MGMT) gene in a series of cancers (42), and 40% for the p15 gene in leukemias (43). Similarly, in a series of non-small-cell lung cancers (NSCLCs), frequencies of hypermethylation were, respectively, 41% for p16, 23% for the death-associated protein kinase gene, 9% for the GSTP1 gene, and 27% for the MGMT gene (44). On the other hand, the 91% rate of hMLH1 gene hypermethylation in the subset of endometrial tumors showing frequent microsatellite instability (45) approximates that of hypermethylated APC DNA in esophageal adenocarcinomas. To our knowledge, the prevalence of APC hypermethylation has not yet been studied in NSCLC and small-cell lung cancer, but we and other investigators (46,47) have reported a high frequency of LOH at the APC gene locus in this cancer without evidence of APC mutation.

The prevalence of hypermethylated APC DNA in the plasma of patients with hypermethylated APC DNA-positive primary tumors (25%) is similar to that observed for other serologic markers in esophageal cancer patients, such as anti-p53 antibodies (48,49). However, in the study by Cawley et al. (48), anti-p53 antibodies tended to occur more frequently in squamous cell carcinoma (36% of patients) than in adenocarcinoma (27%). By contrast, in this study, plasma-hypermethylated APC DNA oc-

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**P = 0.016**
urred more frequently in esophageal adenocarcinoma (25% of patients) than in squamous cell carcinoma (6%). Furthermore, mean APC methylation levels in this study were substantially higher in primary adenocarcinoma tissues (0.59) than in primary squamous cell carcinoma tissues (0.0002), suggesting that adenocarcinoma tissues exhibit, on average, a higher quantity of methylated DNA per tumor than do squamous cell carcinoma tissues. This finding suggests that quantitative differences in degree of methylation exist between different tumor types.

One potential advantage of using gene methylation as a biomarker is the fact that its presence or absence is easily established by use of a single PCR (29–31,40–44). This ease of use contrasts with searching for gene mutations (e.g., of the tumor suppressor gene p53, also known as TP53), where each mutation must be identified by DNA sequencing or other methods impractical for routine clinical use (48–50).

In conclusion, the foregoing data suggest that 1) hypermethylated APC DNA occurs during the development or progression of most esophageal adenocarcinomas and 2) large-scale testing is warranted to confirm the potential value of plasma-hypermethylated APC DNA as a biomarker to help stage esophageal cancer, detect recurrent disease, and monitor disease progression or treatment response.

REFERENCES


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NOTES

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