Aberrant Methylation of *APC, MGMT, RASSF2A*, and *Wif-1* Genes in Plasma as a Biomarker for Early Detection of Colorectal Cancer

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Abstract

Purpose: To identify epigenetic molecular makers in plasma for the early detection of colorectal cancer.

Experimental Design: We retrospectively analyzed the methylation status of 10 genes in fresh-frozen tissues and corresponding plasma samples from 243 patients with stage I and II sporadic colorectal cancer, 276 healthy individuals, and plasma from 64 colorectal adenoma patients using methylation-specific PCR. The methylation score (M score) was used to find molecular markers with high sensitivity and specificity.

Results: Of the 243 colorectal cancer tissues, methylation was detected in 18% for *p14*, 34% for *p16*, 27% for *APC*, 34% for *DAPK*, 32% for *HLTF*, 21% for *hMLH1*, 39% for *MGMT*, 24% for *RARβ2*, 58% for *RASSF2A*, and 74% for *Wif-1*. Receiver operator characteristic curve analysis in plasma from 243 patients with cancer and 276 healthy individuals showed that the M score of any single gene had a sensitivity of <40% after controlling for age, sex, and tumor location. The specificity of the M score was not different between multigene and single gene analyses, but the sensitivity of the M score was significantly increased by multigene analysis. For all patients, the M score in a model including *APC, MGMT, RASSF2A*, and *Wif-1* genes had a sensitivity of 86.5% and a specificity of 92.1% when 1.6 was used as a cutoff. In this model, the M score had a positive predictive value of 90.6% and a negative predictive value of 88.8%.

Conclusion: The present study suggests that tumor-specific methylation of *APC, MGMT, RASSF2A*, and *Wif-1* genes might be a valuable biomarker in plasma for the early detection of colorectal cancer. (Clin Cancer Res 2009;15(19):6185–91)

Colorectal cancer is one of the most common malignancies in the world. The prognosis for colorectal cancer has significantly improved because of advances in the treatment and detection of the disease. Despite significant improvements in screening methods for colorectal cancer, however, only 30% to 40% of patients are diagnosed at an early stage (1). Accordingly, an effective screening for premalignant adenomas and early stage cancers would have substantial clinical benefits, and would reduce the mortality of patients with colorectal cancer. Recent advances in molecular genetics have shown that colorectal cancer arises as a multistep process involving the progressive accumulation of genetic and epigenetic alterations, which could be surrogate markers for the monitoring of colorectal cancer.

Aberrant methylation of CpG islands at the promoter region of a gene is an epigenetic change that induces the transcriptional silencing of tumor suppressor genes (2). Hypermethylation of CpG islands in tumor suppressor genes has been reported for several human cancers, including colorectal cancers (3). Changes in the methylation pattern of CpG islands at the promoter region have obvious applications in both clinical diagnostics and therapeutics, as predictors of the response to therapy. It is known that patients with cancer have higher levels of circulating DNA in their sera or plasma than either healthy people or those with nonmalignant diseases. Analysis of the circulating cell-free DNA in the plasma or serum might be a promising noninvasive diagnostic tool for the early detection of cancer, as well as for monitoring the prognosis during follow-up. Recent reports have found that aberrant methylation of tumor suppressor genes in the plasma or serum of patients with colorectal cancer could be used as a surrogate marker for monitoring colorectal cancer (4–7).

Methylation-specific PCR (MSP) is most often used for detecting aberrant methylation of a CpG island in the plasma or serum from patients with colorectal cancer. Although the MSP is appropriate for detecting a few cell-free DNA molecules from

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Translational Relevance

Despite significant advances in screening methods for colorectal cancer, only 30% to 40% of patients are diagnosed at an early stage. Accordingly, more effective techniques for screening are needed to reduce the mortality for patients with colorectal cancer. The methylation statuses of 10 genes was retrospectively analyzed in fresh-frozen tissues and corresponding plasma samples from 243 patients with stage I and II sporadic colorectal cancer, 276 healthy individuals, and plasma from 64 patients with colorectal adenoma to identify tumor-specific methylation in plasma as a molecular marker for the early detection of colorectal cancer. The studies described in this article suggest that the analysis of methylation statuses of *APC, MGMT, RASSF2A*, and *Wif-1* genes in plasma will allow us to detect colorectal cancer at the earliest stage.

a heterogeneous cell population including a background of contaminating normal cells, the early detection of colorectal cancer may require a panel of markers due to the molecular heterogeneity of colorectal cancer. To this end, we analyzed the frequencies and patterns of promoter methylation in 10 genes, which are known to be involved in the pathogenesis of colorectal cancer and are frequently silenced by hypermethylation in colorectal cancer, in tissue and plasma from 243 patients with stage I and II colorectal cancers and 276 healthy individuals. The genes chosen are involved in cell cycle regulation (*p14* and *p16*), DNA repair or protection (*hMLH1* and *MGMT*), signal transduction (*APC, RARβ2, RASSF2A*, and *Wif-1*), apoptosis (*DAPK*), and chromatin remodeling (*HLTF*).

Materials and Methods

Study population. A total of 243 patients who underwent curative surgical resection for colorectal cancer at the Department of Surgery in the Samsung Medical Center, Seoul, Korea between September 2004 and November 2007 were enrolled in this study. The surgically removed tumor tissues and corresponding plasmas were collected after obtaining the appropriate Institutional Review Board permission and written informed consent from all of the patients. Plasma samples were also obtained from 276 healthy individuals and 64 patients that had visited the hospital for regular health checkups; these 64 patients had histologically confirmed colorectal adenoma including tubular, villous, or serrated adenoma. Normal colonic mucosas were obtained by colonoscopic biopsy from 64 of 276 healthy individuals. Pathologic stage was determined by tumor-node-metastasis staging.

DNA extraction from fresh tissue and plasma. The tumors were snap-frozen in liquid nitrogen and stored at -80°C until needed. Before DNA was extracted from the fresh-frozen tissues, the sections were placed on slides and stained with H&E to evaluate the admixture of tumorous and nontumorous tissues. Areas corresponding to the tumor were microdissected manually under a microscope. Tumors containing at least 75% neoplastic tissue were used in this study. The microdissected tissues were digested with proteinase K and resuspended in lysis buffer ATL (DNeasy Tissue kit; Qiagen), and the genomic DNA was isolated according to the instructions of the manufacturer. Venous blood samples were collected in vacutainer plastic tubes coated with potassium EDTA (Becton Dickinson) for plasma preparation. The plasma was immediately separated from the cellular fraction by centrifugation at 2,500 rpm for 10 min and frozen at -80°C. The DNA was extracted from 400 μL of plasma using a QIAamp Blood Kit (Qiagen), dissolved in 50 μL of sterile distilled water and stored at -20°C.

MSP. The methylation status of the 10 genes was determined using MSP (Fig. 1), using two pairs of primers: one for the unmethylated promoter and the other for the methylated promoter, as described by Herman et al. (8). Briefly, 1 μg of genomic DNA from the fresh-frozen tissues or 40 μL of DNA extracted from the plasma was bisulfite-modified, using a CpGenome DNA modification kit (Chemicon) according to the protocol described by the manufacturer, then dissolved in 20 μL of TE (10 mmol/L Tris and 1 mmol/L EDTA; pH 8.0). The PCR mixture consisted of 1× PCR buffer [50 mmol/L KCl, 67 mmol/L Tris (pH 8.8), 1.5 mmol/L $MgCl_2$], deoxynucleotide triphosphates (each 1.25 mmol/L), primers (300 ng of each per reaction), 2.5 units of Taq polymerase, and bisulfite-modified DNA (50 ng of modified DNA from fresh-frozen tissue, or 1 μL of modified plasma DNA) as a template for MSP. Primer sequences and annealing temperatures for the MSP have been previously described by our group and others (9–12). DNA from the peripheral blood lymphocytes of healthy subjects was used as a negative control for the methylation-specific assays. Lymphocyte DNA from healthy volunteers was treated with *Sss*I methyltransferase (New England BioLabs), and then with bisulfite. The resulting product was used as a positive control for methylated alleles. Bisulfite-modified DNA from normal lymphocytes was used as a positive control for unmethylated alleles, and the unconverted DNA from normal lymphocytes was used as a negative control for the methylated alleles. When a gene is amplified by a primer specific for methylated DNA in the presence of amplification of unmethylated DNA, the gene is considered methylated.

Statistical analysis. The Wilcoxon rank sum test (or *t* test) and Fisher's exact test (or the χ^2 test) were used for univariate analysis of the continuous and categorical variables, respectively. Kappa agreement statistics was done to assess the concordance of methylation between tumor tissue and plasma. We calculated the methylation score (M score) to find molecular markers with high sensitivity and specificity for each sample as previously described (12). For overall patients, the M score was defined

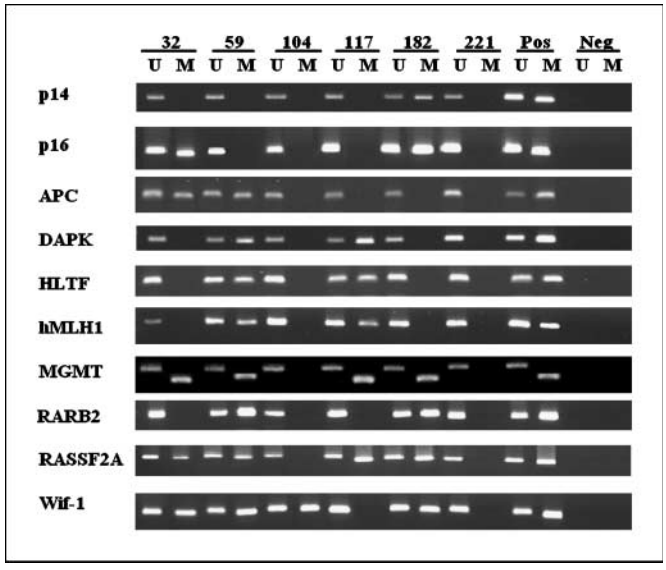

Fig. 1. MSP of 10 genes. Values are sample identification numbers (*top*). Pos, positive control for the unmethylated (*U*) and methylated (*M*) alleles. Two positive controls came from different sources of DNA: the bands at the "M" column were produced by amplification of *in vitro* translated DNA but those at the "U" column were produced by amplification of bisulfate-modified DNA from normal lymphocyte without *in vitro* translation. Neg, negative control.

Table 1. Clinicopathologic characteristics ($N = 243$)

Variables	Results (%)
Age*	61 ± 11
Sex	
Men	139 (57)
Women	104 (43)
Stage	
I	44 (18)
II	199 (82)
Histology	
Adenocarcinoma	204 (84)
Mucinous adenocarcinoma	22 (9)
Others	17 (7)
Location	
Proximal	56 (23)
Distal	187 (77)
Differentiation	
Well	27 (11)
Moderately	211 (87)
Poorly	5 (2)

*Mean ± SD.

as the sum of the corresponding log odds ratio coefficients for the genes, derived from multivariate logistic regression analyses in plasma. The optimal sensitivity and specificity of the M score for diagnosis of colorectal cancer was determined by receiver operator characteristic (ROC) curve analysis using MedCalc Software. All data, except for ROC curve analysis, were analyzed by the StatView V statistical package (SAS Institute, Inc.). All statistical analyses were two-sided with a type I error rate of 5%.

Results

Clinicopathologic characteristics. The baseline characteristics of 243 patients with colorectal cancers are shown in Table 1. The patients ranged in age from 22 to 86 years, with a mean age of 61 years. The patients consisted of 139 (57%) men and 104 (43%) women. Forty-four (18%) of the patients had stage I disease and 199 (82%) had stage II disease. The histologic subtypes were adenocarcinoma (84%), mucinous adenocarcinoma (9%), and other (7%). Among the 243 colorectal cancers, 56 (23%) were located in the proximal colon and 187 (77%) in the distal colon, which included the rectum, sigmoid, and descending colon up to the splenic flexure. Twenty-seven (11%) of the colorectal cancers were well-differentiated, 211 (87%) were moderately differentiated, and 5 (2%) were poorly differentiated. The 276 healthy individuals ranged in age from 28 to 76 years with a mean age of 58 years. There was no statistical difference between the ages of healthy individuals and patients with stage I and II colorectal cancer ($P = 0.28$; Wilcoxon rank sum test). The healthy individuals consisted of 167 men and 109 women, and no gender difference was found between the healthy individuals and the patients with cancer ($P = 0.44$).

Prevalence of methylation in normal tissues from cancer-free individuals and in cancer tissues. For the early detection of colorectal cancer, it is important to differentiate age-related methylation and tumor-specific methylation. To do this, we analyzed the methylation status in normal colorectal mucosa from healthy individuals. Among 276 healthy individuals, normal colonic mucosa was available from 148 individuals that had normal colonoscopy and who did not have any known diagnosis of malignant disease. Aberrant methylation was not detected in *DAPK*, *RARβ2*, and *RASSF2A* genes in normal colonic mucosa from 148 healthy individuals, but methylation of *p14*, *p16*, *APC*, *HLTF*, *hMLH1*, *MGMT*, and *Wif-1* genes was found in 2%, 3%, 3%, 2%, 3%, 4%, and 2%, respectively. The methylation status of the 10 genes studied was not associated with patient age, whether age was treated as a continuous or categorical variable (data not shown). This indicated that the methylation status of the 10 genes was tumor-specific, so all genes were considered in further analyses. The prevalence of genetic methylation in 243 cancer tissues ranged from 18% to 74%. *Wif-1* methylation was the highest (74%) and *p14* methylation was lowest (18%; Fig. 2A). The mean number of methylated genes was 3.8 in cancer tissues, and 5 (2%) showed concurrent methylation in seven or more genes.

Analytic sensitivity and specificity of tumor-specific methylation in plasma. Before building a model to find candidate plasma markers with high sensitivity and specificity, we examined analytic sensitivity and specificity in tumor tissues and matched plasma samples collected from 243 patients with colorectal cancer to differentiate genes showing false-positive methylation in plasma. Analytic sensitivity in plasma was defined as the ratio of the number of individuals with a true-positive result to those with a true-positive or false-negative result in plasma, whereas analytic specificity referred to the proportion of individuals without methylation in tumor tissue having a negative result in plasma (13). Analytic sensitivity ranged from 27% to 60% (Fig. 2B), and analytic specificity was 100% in all genes except *HLTF*. The analytic sensitivity and specificity of *HLTF* methylation in the plasma were 41% and 93%, respectively.

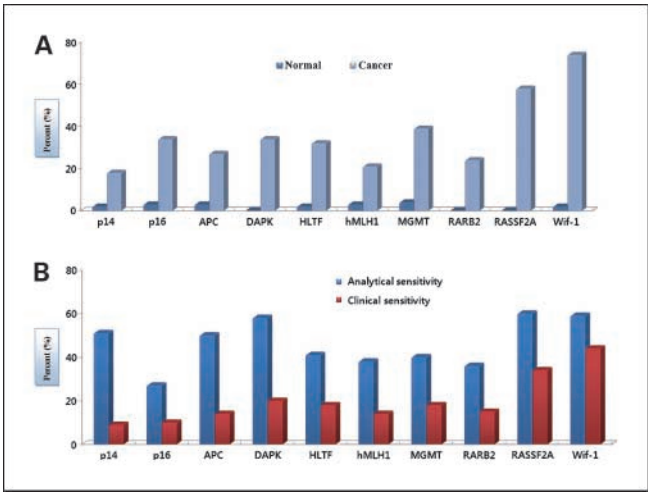

Fig. 2. Prevalence of methylation in tissues and the analytic and clinical sensitivity in plasma. *A*, aberrant methylation of 10 genes was analyzed in normal colonic mucosa from 148 healthy individuals and in colonic tissue from 243 cancer patients. In normal colonic mucosas and cancer tissues, the prevalence of methylation for the genes ranged from 0% to 4% and from 18% to 74%, respectively. *B*, analytic and clinical sensitivity of 10 genes was analyzed in tissues and matched plasma samples collected from 243 patients with colorectal cancer. Analytic sensitivity ranged from 27% to 60%, and clinical sensitivity ranged from 9% to 44% for 10 genes. The "clinical sensitivity" used here has the same meaning as "sensitivity" that refers to the proportion of patients with colorectal cancer who test positive for the methylation assay in plasma.

Table 2. Prevalence of *HLTF* methylation in tumor and matched plasma

	Plasma	Tumor		*P**
		U	M	
HLTF	U	153	46	
	M	12	32	<0.001

Abbreviations: U, unmethylated; M, methylated.
**P* value; kappa-statistic.

Out of 243 patients, 185 (76%) showed identical methylation changes in the *HLTF* gene in both the tumor and matched plasma DNA, but 12 out of 165 patients who did not show *HLTF* methylation in tumor tissue showed *HLTF* methylation in matched plasma (Table 2). The methylation status of the *HLTF* gene between tumor and plasma showed a statistically significant discordance ($P < 0.0001$; Kappa statistics). Accordingly, the *HLTF* gene was excluded in further analyses due to the false-positive results in plasma DNA. Here, the term "clinical sensitivity" was defined as the probability that a patient with colorectal cancer has a positive methylation result in plasma and has the same meaning as the commonly used definition of "sensitivity." The clinical sensitivity for methylation in plasma ranged from 9% to 44% (Fig. 2B).

Evaluation of methylation score. We used the M score to find an epigenetic marker for the early detection of colorectal cancer in plasma, applying ROC curve analysis to determine the optimal sensitivity and specificity of the score. First, we conducted multivariate logistic regression analyses on plasma data from 243 patients with colorectal cancer and 276 healthy individuals to determine which genes were independent predictors of colorectal cancer. The multivariate analysis revealed that methylation status of nine genes studied was a significant, independent predictor of colorectal cancer, after adjusting for age, sex, and tumor location (data not shown).

We next analyzed the sensitivity and specificity of the M score for each gene, using an optimal cutoff value to evaluate if the gene could be used as a single reliable biomarker. *Wif-1* methylation was found to be the most sensitive indicator in the single gene analysis, but the M score of *Wif-1* had a sensitivity of 36.7% and a specificity of 90.6% by ROC curve analysis, with an area under the curve (AUC) of 0.641, using the optimal cutoff point (>0; Table 3; Fig. 3A). The low sensitivity suggests that the assay of multiple DNA markers rather than a single marker was necessary to increase the sensitivity of DNA methylation in plasma.

Multigene analysis was therefore done to find groups of genetic methylation that showed high sensitivity and specificity by M scores of multigene, using recursive partitioning analysis. The overall M score in a model with *APC*, *MGMT*, *RASSF2A*, and *Wif-1* genes was higher than that in any model using other genes, and showed a sensitivity of 86.5% and a specificity of 92.1% (AUC, 0.927; Table 3A) as a diagnostic biomarker when a cutoff value of 1.6 was used for ROC curve analysis (Fig. 3A). The addition of other genes (*p14*, *p16*, *hMLH1*, *DAPK*, and *RARβ2*) did not significantly improve the ROC curve (data not shown). The sensitivity and specificity, and their 95% confidence intervals (95% CI) against the different cutoff values, are plotted in Fig. 3B and C. The sensitivity in a model including only *Wif-1* was significantly low compared with that in a model including four genes (*APC*, *MGMT*, *RASSF2A*, and *Wif-1*) at any cutoff point, but the specificity was similar between the two models.

Of the 243 tumors and matched plasmas, 91% of tissues and 86% of plasmas showed methylation at one or more loci. Methylation at two or more genes was found in 35% (85 of 243) of tumor tissues and in 20% (49 of 243) of plasma samples. Concurrent methylation in three or more genes was found in 14% (34) of tissues and in 7% (17) of plasmas. Methylation of four genes was found in two tumor tissues. Finally, we evaluated the M score as a predictive biomarker for colorectal cancer in plasma and analyzed the positive predictive value and negative predictive value of colorectal cancer detection using the model. At a cutoff value of 1.6, the positive predictive value was 90.6% and the negative predictive value was 88.8% using the model with four genes (Table 3A).

Validation of four markers in the plasma of patients with colorectal adenoma. Methylation of the four genes was reassessed in plasma from 64 patients with colorectal adenoma for validation of the predictive markers. Methylation of the *APC*, *MGMT*, *RASSF2A*, and *Wif-1* genes was found in 18%, 14%, 37%, and 32% of plasma from 64 patients with colorectal adenoma, respectively. The overall M score in a model with *APC*, *MGMT*, *RASSF2A*, and *Wif-1* genes was found to show a

Table 3. Sensitivity and specificity of M score in colorectal adenomas ($N = 64$) and cancers ($N = 243$)

Target genes	AUC	Cutoff (M score)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
(A) Colorectal cancer						
APC + MGMT + RASSF2A + Wif-1	0.927	1.6	86.5 (81.7-90.8)	92.1 (88.2-95.0)	90.6 (86.0-94.1)	88.8 (84.4-92.2)
Wif-1	0.641	>0	36.7 (30.6-43.7)	90.6 (86.4-93.2)	77.5 (68.6-84.7)	62.1 (56.9-66.1)
(B) Colorectal adenoma						
APC + MGMT + RASSF2A + Wif-1	0.864	1.8	74.6 (62.6-85.0)	91.3 (87.5-94.6)	71.6 (57.3-80.1)	93.9 (90.2-96.5)

NOTE: Age, sex, and tumor location were adjusted for colorectal cancers, and age and sex for colorectal adenomas.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

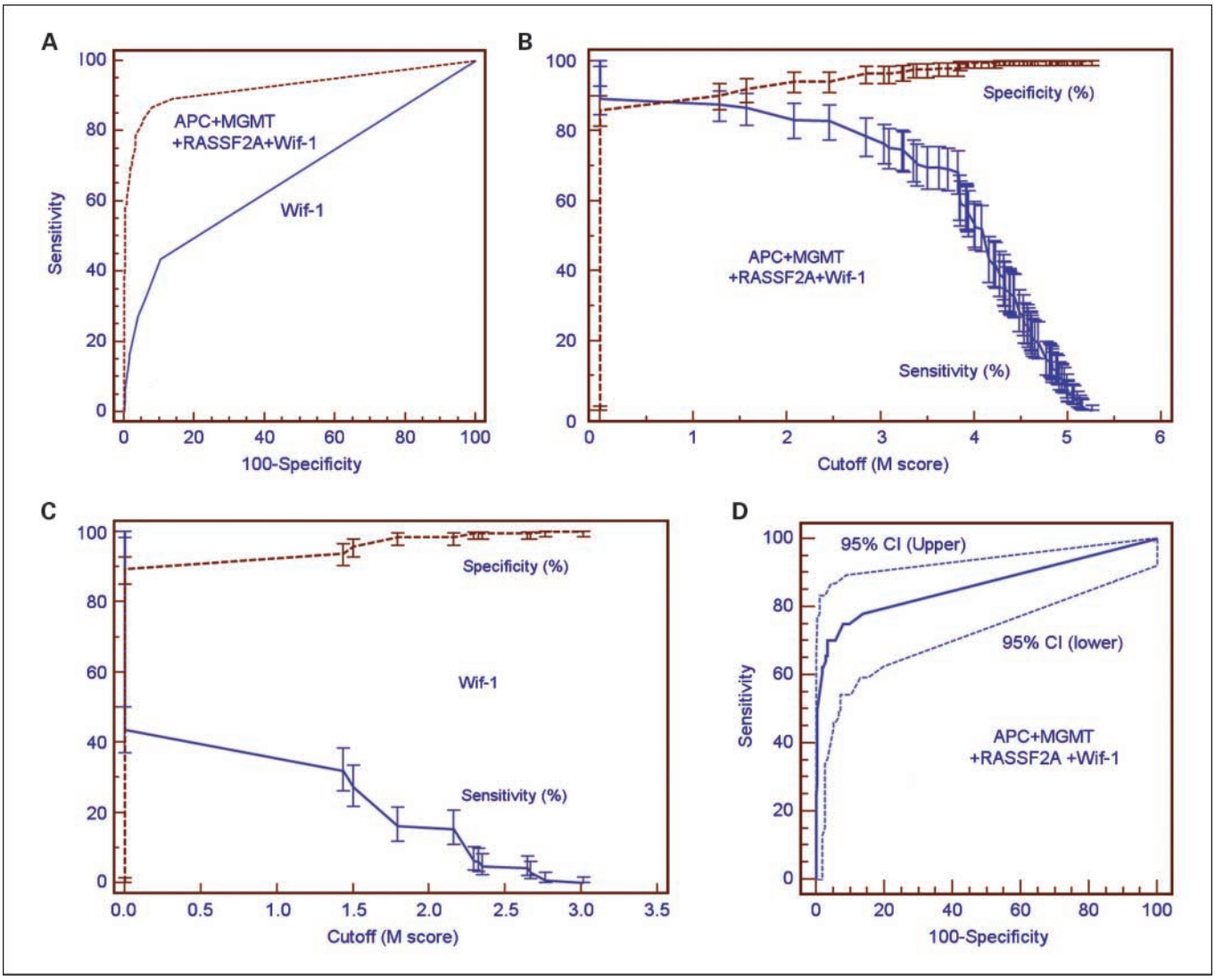

Fig. 3. Representatives of ROC curve and a plot versus cutoff values in colorectal cancer (*A, B,* and *C*) and colorectal adenoma (*D*). *A,* ROC curve analysis showed that a model including four genes (*APC, MGMT, RASSF2A*, and *Wif-1*) was significantly different from a model with only *Wif-1* ($P < 0.001$). *B* and *C,* the sensitivity at different cutoff values showed a significant difference between two models but the specificity was similar. *D,* methylation of the four genes was reassessed in plasma from patients with colorectal adenoma for validation of the predictive marker. The overall M score showed a sensitivity of 74.6% and a specificity of 91.3% (AUC, 0.864) when 1.8 was used as a cutoff value.

sensitivity of 74.6% and a specificity of 91.3% (AUC, 0.864; Table 3B; Fig. 3D), when 1.8 was used as the cutoff value, after adjusting for age and sex. The positive predictive value was 71.6% (95% CI, 57.3-80.1) and the negative predictive value was 93.9% (95% CI, 90.2-96.5) using the model at a cutoff value of 1.8.

Discussion

The critical factor for the early detection of colorectal cancer is to differentiate age-or environment-related methylation in patients without cancer, from tumor-specific methylation in patients with cancer, because aberrant methylation of some tumor suppressor genes occurs after the onset of neoplastic evolution, and others become hypermethylated in normal epithelial cells from environmental factors such as exposure to folate and aging. Thus, such factors could be the source of false-positives in the study of tumor-specific methylation in colorectal cancer. In the present study, we did not observe statistically significant age-related methylation for the 10 genes studied. The lack of association of *p14*, *p16*, *DAPK*, and *hMLH1* methylation with age in this study is contradictory to previous studies showing age-related methylation in normal colonic mucosa (14–17).

This may be due to the very small number ($n = 148$) of normal tissues, or differences in age distributions, tumor distributions, or CpG sites analyzed. Nakagawa et al. (15) reported that the prevalence of *hMLH1* methylation was significantly higher in patients ≥80 years of age than in those <60 years of age (83% versus 44%, respectively), but in this study, the mean age was 61 years and only two patients were >80 years of age. They also found that full methylation of *hMLH1* occurred at a high frequency in patients with MSI+ tumors, which are mainly found

on the right side of the colon. In this study, 77% of colorectal cancers occurred in the left colon. Analysis of different CpG sites may also be the source of the conflicting results. Issa et al. (14) found substantial levels of *p16* methylation in the exon 1 region, but not in the upstream region in nonneoplastic mucosa with ulcerative colitis and high-grade dysplasia. This suggests that age-related methylation might be limited to the edges of the CpG islands with protection against the spreading of methylation to CpG island from methylation center. Other possible explanations for the discrepant results between aging and methylation are (*a*) different rates and sites of aberrant methylation between populations because of genotypic variation (17), (*b*) application of different criteria (qualitative versus quantitative) in determining hypermethylation in normal colonic tissue, (*c*) differing exposure to environmental factors, such as diet or microorganisms, or (*d*) different methods of assay. In this study, the methylation statuses of all genes were analyzed by qualitative assay. Age-related methylation needs to be reassessed in quantitative values of methylation.

The *APC*, *MGMT*, *RASSF2A*, and *Wif-1* genes are known to be frequently methylated in colorectal adenoma, which is generally considered to be a precursor lesion of colorectal cancer. The defective APC protein is not able to bind and degrade β-catenin, and the resultant increased β-catenin levels lead to the activation of growth-promoting genes such as c-myc via the action of increased β-catenin/Tcf-4 transcription complexes. Although mutation is the most common cause of APC inactivation, *APC* methylation was also detected in normal colon mucosa and colorectal adenoma (18–21). Aberrant methylation of the DNA repair gene O^6-methylguanine-DNA methyltransferase (*MGMT*) impairs the ability of the MGMT protein to remove alkyl groups from the O^6-position of guanine and thereby increases the risk of cancer. *MGMT* methylation was reported at a high frequency in colorectal adenoma (20, 22). In addition, Shen et al. (23) found *MGMT* methylation at adjacent and distal mucosas of sporadic colorectal cancer, and reported that *MGMT* methylation could play a role as one of the mediators of field cancerization in colon mucosa.

The RAS association domain family 2 (RASSF2) is a novel tumor suppressor that regulates the Ras signaling pathway and is involved in actin cytoskeleton organization and apoptosis (24). The *RASSF2* gene contains 11 exons that span ~43 kb at chromosome 20p13, and three isoforms have been identified: *RASSF2A*, *RASSF2B*, and *RASSF2C*. *RASSF2B* and *RASSF2C* do not have CpG islands, but *RASSF2A* has a CpG island between -105 bp and +1075 bp relative to the transcription start site (NM_014737; ref. 11). *RASSF2A* methylation was found in 16 of 16 adenomas (25), and in 7 of 8 colon adenomas (11). Wnt-inhibitory factor 1 (WIF-1) is a secreted extracellular molecule that blocks the Wnt signaling pathway primarily by directly binding to Wnt proteins and preventing their access to cell surface receptors. Belshaw et al. (26) reported significantly higher median levels of *Wif-1* methylation in mucosal samples from patients with adenomatous polyps than those from patients with cancer (19.18% versus 1.75%, respectively). Taniguchi et al. (27) also observed the down-regulation of Wif-1 expression in 32 of 44 colorectal adenoma tissues and found *Wif-1* methylation in all tumor samples with Wif-1 down-regulation. Based on these observations, aberrant methylation of *APC*, *MGMT*, *RASSF2A*, and *Wif-1* genes is considered to be related to tumor initiation, but not to tumor progression.

Several studies suggest that the failure to detect methylation in plasma or serum might result from the low amount of DNA present in plasma or serum, with a consequent diminished sensitivity of the assay. In addition to low plasma DNA levels, the sensitivity of the methylation assay may be affected by the fragmentation of cell-free circulating DNA. Small pieces of fragmented DNA, ~100 bp in size, were found with the cell-free DNA from pancreas, colon, ovarian, and prostate cancer patients, but not in normal cell-free DNA (28, 29). The MSP in this study produced PCR products of 200 to 400 bp for the methylated and unmethylated segments, respectively. Accordingly, fragmentation of the cell-free DNA might have reduced the sensitivity in this study. DNA degradation by bisulfate modification may be another source of low sensitivity. DNA is usually incubated in a high concentration of bisulfite salt at high temperature and low pH for the complete conversion of unmethylated cytosines. These harsh conditions may lead to a high degree of DNA fragmentation during conversion, and DNA loss during purification, which subsequently affects the detection of methylation in plasma.

In this study, false-positive *HLTF* methylations were found in 14 of 162 matched plasma samples. The mispriming of methylation-specific primers may be a technical source of false-positives, and might pose a greater problem when higher numbers of PCR cycles with a small amount of methylated DNA, or two-stage nested primers, are used. Recently, Shaw et al. (30) reported that MSP using a low concentration of methylated DNA and a high number of PCR cycles resulted in the mispriming of methylation-specific primers in ~10% of cases, with subsequent false-positives. Although the number of PCR cycles used for MSP in this study was not high, the possibility that the cycle number might play a role in producing false-positives in a minute amount of cell-free DNA cannot be ruled out. Accordingly, further evaluation for mispriming is needed using assays such as methylation enrichment pyrosequencing or bisulfite DNA sequencing (31).

This study was limited by the small number of normal tissues from healthy individuals and plasma from patients with colorectal adenoma. Although *HLTF* methylation was not included in the final model because it gave false-positives in plasma, *HLTF* methylation is known to be associated with early stages of colorectal cancer (10). Therefore, additional work is needed to evaluate the role of HLTF methylation as a biomarker in plasma. Additionally, the present results need to be validated using quantitative real-time methods such as MethyLight for methylation analysis. In conclusion, the present study suggests that methylation of *APC*, *MGMT*, *RASSF2A*, and *Wif-1* genes may be a valuable molecular marker in plasma for the early detection of colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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