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ORIGINAL ARTICLE

# Usefulness of plasma epigenetic changes of five major genes involved in the pathogenesis of colorectal cancer

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**Abstract**
*Purpose* The purpose of present study was to investigate the methylation status of the promoter region in five genes (*mothers against decapentaplegic homolog 4*, *fragile histidine triad protein*, *death-associated protein kinase* 1, adenomatous polyposis coli (*APC*), and *E-cadherin*), which are known to be involved in the pathogenesis of colorectal cancer (CRC) and its clinicopathological significance.
*Methods* The study subjects were 60 CRC patients, 40 patients with adenomatous colorectal polyp and 60 healthy control individuals. We further enrolled a total of 16 patients (two patients with Crohn's disease, two patients with ulcerative colitis, one patient with serrated adenoma, and 11 patients with colorectal cancer). The methylation states of the five genes were determined in peripheral blood plasma using methylation-specific polymerase chain reaction single-strand conformation polymorphism analysis.
*Results* This study showed the most sensitive epigenetic markers, E-cadherin (60 %), followed by APC (57 %), for detecting CRC. E-cadherin and APC had similar specificities and amplified 84 and 86 %, respectively, of CRC patients compared to non-CRC patients. Additionally, APC was the only marker to be significantly increased (OR= 6.67, 95 % CI=1.19–23.4, $P$=0.045) and the most sensitive (57 %) and specific (89 %) marker in stage I CRC. Though we have not examined the paired cancer tissues and plasma, there was relatively high concordant rate (60–80 %) in our limited number of colorectal cancer patients.
*Conclusions* Five genes, promoter methylation, in plasma were statistically significant risk factors in CRC patients. In

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this study, E-cad and APC genes may be particularly useful epigenetic biomarkers in plasma for the detection of CRC. Additionally, APC may able to identify early potential CRC.

**Keywords** Colorectal cancer methylation · APC · E-cadherin · SMAD4 · FHIT · DAPK1

## Introduction

Despite the significant improvements in screening methods for the detection of colorectal cancer (CRC), only 30–40 % of patients are diagnosed at an early stage [1]. CRC develops as a result of progressive accumulation of genetic and epigenetic alterations, which lead to genetic instability resulting in malignant transformation [2]. Epigenetic modifications, particularly, DNA methylation in selected gene promoters, are recognized as common molecular alterations in human tumors. Hypermethylation of several gene clusters, termed CpG island methylator phenotype, appears to define a subgroup of CRC distinctly characterized by pathological, clinical, and molecular features [3]. CpG island DNA methylation represses gene transcription by excessive and aberrant methylation of CpG-rich regions, called "CpG islands", in the 5′ region of genes, which leads to transcriptional silencing of the promoter, and therefore, the gene is inactivated [4].

Based on the presence of certain genetic, epigenetic and related changes have been tempting to assess the diagnostic accuracy of the presence of these changes, in blood and stool, for the purpose of CRC screening [5]. Until recently, molecular colorectal testing has been based on the detection of genetic changes in stool. However, fecal DNA testing is expensive and the limited performance of these tests does not allow widespread use as a molecular CRC screening [6]. Another noninvasive approach is the detection of epigenetic changes in blood of patients with CRC. There have been previous reports of increased concentrations of circulating methylated DNA in the blood of cancer patients [7]. The development of blood-based cancer detection tests should improve patient compliance, and thereby, increasing the detection of such diseases at an earlier stage [8]. However, sensitive and specific blood-based tests for the determination of epigenetic alterations are difficult to apply them to clinical laboratory setting.

The present study was to investigate the methylation status of the promoter region in five genes which are known to be involved in the pathogenesis of CRC and its clinicopathological significance. The chosen genes are involved in cell cycle regulation [*fragile histidine triad protein* (*FHIT*)], signal transduction [*adenomatous polyposis coli* (*APC*), *mothers against decapentaplegic homolog* 4 (*SMAD4*)], metastasis/invasion [*epithelial caderin* (*E-cad*)] and apoptosis [*death-associated protein kinase* 1 (*DAPK*1)].

## Materials and methods

### Patients and healthy individuals

Five markers were selected for analysis in the plasma samples from 60 patients with CRC, 40 patients with adenomatous colorectal polyp (ACP) and 60 healthy control individuals. We further enrolled a total of 16 patients (two patients with Crohn's disease, two patients with ulcerative colitis, one patient with serrated adenoma, and 11 patients with colorectal cancer) in order to analyze methylation status in both cancer tissues and corresponding plasma samples (Table 5). Methylation analysis was performed in both plasma and cancer tissue from 10 patients with colorectal cancer. The methylation states of the *SMAD*4, *FHIT*, *DAPK*1, *APC*, and *E-cad* genes were measured in peripheral blood plasma from CRC patients.

Patients and healthy individuals were enrolled from the Chonnam National University Hwasun Hospital (Hwasun, Korea), Seonam University Namgwang Hospital (Gwangju, Korea), and Gwangju Christian Hospital (Gwangju, Korea). Colonoscopy and histological examination were utilized in diagnosing patients with CRC and ACP. Corresponding plasmas were collected after obtaining the appropriate Institutional Review Board permission and written informed consent from all patients. All 60 CRC patients were operated after stage work up and confirmed all adenocarcinoma by histopathology. The pathological staging was determined by the seventh edition of the American Joint Committee on Cancer tumor node metastasis grading system. Histopathological examination of 40 patients with polyp revealed all adenomatous polyps with low-grade dysplasia. Plasma samples were also obtained from 60 healthy individuals.

Total DNA isolation and methylation-specific polymerase chain reaction—single-strand conformation polymorphism (MSP–SSCP) analysis

Blood samples (3 ml each) were collected from CRC, ACP patients, and healthy control individuals in EDTA-containing tubes. DNA methylation patterns in the promoter region of five genes were determined by MSP–SSCP. MSP allows for the distinction between the unmethylated from the methylated alleles of a given gene, based on sequence alterations produced by bisulfate treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil and subsequent polymerase chain reaction (PCR) using primers specific to either methylated or unmethylated DNA. We used SSCP after bisulfite modification to assess the clonal expansion of differentially or partially methylated sequences reasoning that they should possess unique mobilities. Universal primers were used to amplify regions of the SMAD4, FHIT, DAPK1, APC, and E-cadherin gene. The primers for MSP–SSCP were adopted in the previously published works (Table 1). Briefly, 1 mg of genomic DNA

**Table 1** The primers for MSP–SSCP

| Gene | Sense (5′–3′) | Anti-sense (5′–3′) |
|---|---|---|
| APC | (U) GTGTTTTATTGTGGAGTGTGGGTT | CCAATCAACAAACTCCCAACAA |
| | (M) TATTGCGGAGTGCGGGTC | TCGACGAACTCCCGACGA |
| E-cadherin | (U) GGTGGGTGGGTTGTTAGTTTTGT | AACTCACAAATCTTTACAATTCCAAC |
| | (M) GTGGGCGGGTCGTTAGTTTC | CTCACAAATACTTTACAATTCCGACG |
| DAPK1 | (U) GGATAGTTGGATTGAGTTAATGTC | CAAATCCCTCCCAAACACCAA |
| | (M) GGATAGTCGGATCGAGTTAACGTC | CCCTCCCAAACGCCGA |
| FHIT | (U) TTGGGGTGTGGGTTTGGGTTTTTATG | CATAAACAACACCAACCCCACTA |
| | (M) TTGGGGCGCGGGTTTGGGTTTTTA CGC | CGTAAACGACGCCGACCCCACTA |
| SMAD4 | (U) GTAATAATATGGTTTTGGTTGTT | CTCCCACCCCCTAAACAACCACA |
| | (M) GTAATAATACGGTTTTGGTCGTC | CTCCCACCCCCTAAACGACCGCG |

was denatured by treatment with NaOH, which was modified by sodium bisulfite. DNA samples were then purified with the use of Wizard DNA purification resin, again treated with NaOH, precipitated with ethanol, subsequently resuspended in water. PCR was carried out using 50 ng of DNA, 10× PCR buffer with 1.5 mM $MgCl_2$, 2 μl mixture of 4 mM dNTPs, 20 pmol of each primer and 1 U of Tag DNA polymerase at final volume of 25 μl. The PCR conditions were 96 °C for 5 min, then 35 cycles, with each cycle consisting of 30 s at 94 °C, 1 min at the annealing temperature of the using primer (mostly around 56–59 °C) and 1 min at 72 °C, followed by one cycle at 72 °C for 10 min. PCR products were electrophoresed in 10 % nondenaturing polyacrylamide gels with an acrylamide/bisacrylaminde ratio of 30:0.8 at 70 V for 12 h at room temperature. DNA was detected by silver staining according to the methods described by others. Those single-stranded DNAs that took up an altered conformation appeared as aberrantly migrating bands on the electrophoresis gel.

The methylation status of the two genes was determined using MSP–SSCP, which are shown in Fig. 1. DNA from the peripheral blood lymphocytes of healthy subjects was used as a negative control for the MSP–SSCP. Lymphocyte DNA from healthy volunteers was treated with SssI 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.

### Methylation-specific direct sequencing

The bisulfite-treated DNAs were amplified by PCR for *E-cad* and *APC* gene with bisulfite sequencing specific primer pair (Table 1). The sequencing analysis was performed using an automatic genetic analyzer (model ABL 3130XL; Applied Biosystems, Foster City, CA, USA).

### Statistical analysis

All statistical analyses were performed using SPSS 12.0 (SPSS Inc. Chicago, IL, USA). CRC frequencies were compared according to each serum indicators, using the $\chi^2$ test or Fisher's exact test. Odds ratios were calculated using 95 % confidence intervals and $P$ value of <0.05 was considered statistically significant. To evaluate the validity of each serum indicators, sensitivity, specificity, positive predictability, and efficacy were used.

## Results

### Analysis of five markers methylation status by MSP–SSCP

The baseline characteristics of 160 patients are shown in Table 2. The mean age of this group of patients was 62 years, which ranged from 35 to 83 years, with 86 females and 74 males. Fourteen (23.3 %) of the patients had stage I disease. Among the 60 CRCs, 28 (46 %) were located in the proximal colon and 32 (54 %) in the distal colon, which included the rectum, sigmoid and descending colon below the splenic flexure. There was no statistical difference in age and gender between the healthy individuals and patients with CRC.

To validate performance of five markers, we examined odds ratio, confidential interval, sensitivity, and specificity of five genes promoter methylation in plasma. Comparison of odds ratio (OR), confidential interval (CI) for CRC indicator is shown in Table 3. All markers indices were significantly increased in CRC when compared to that of non-CRC (ACP and normal control): SMAD4 (OR=3.56; 95 % CI, 1.81–6.98), FHIT (OR=5.25; 95 % CI, 2.51–10.96), DAPK1 (OR=2.85; 95 % CI, 1.45–5.59), APC (OR=8.03;

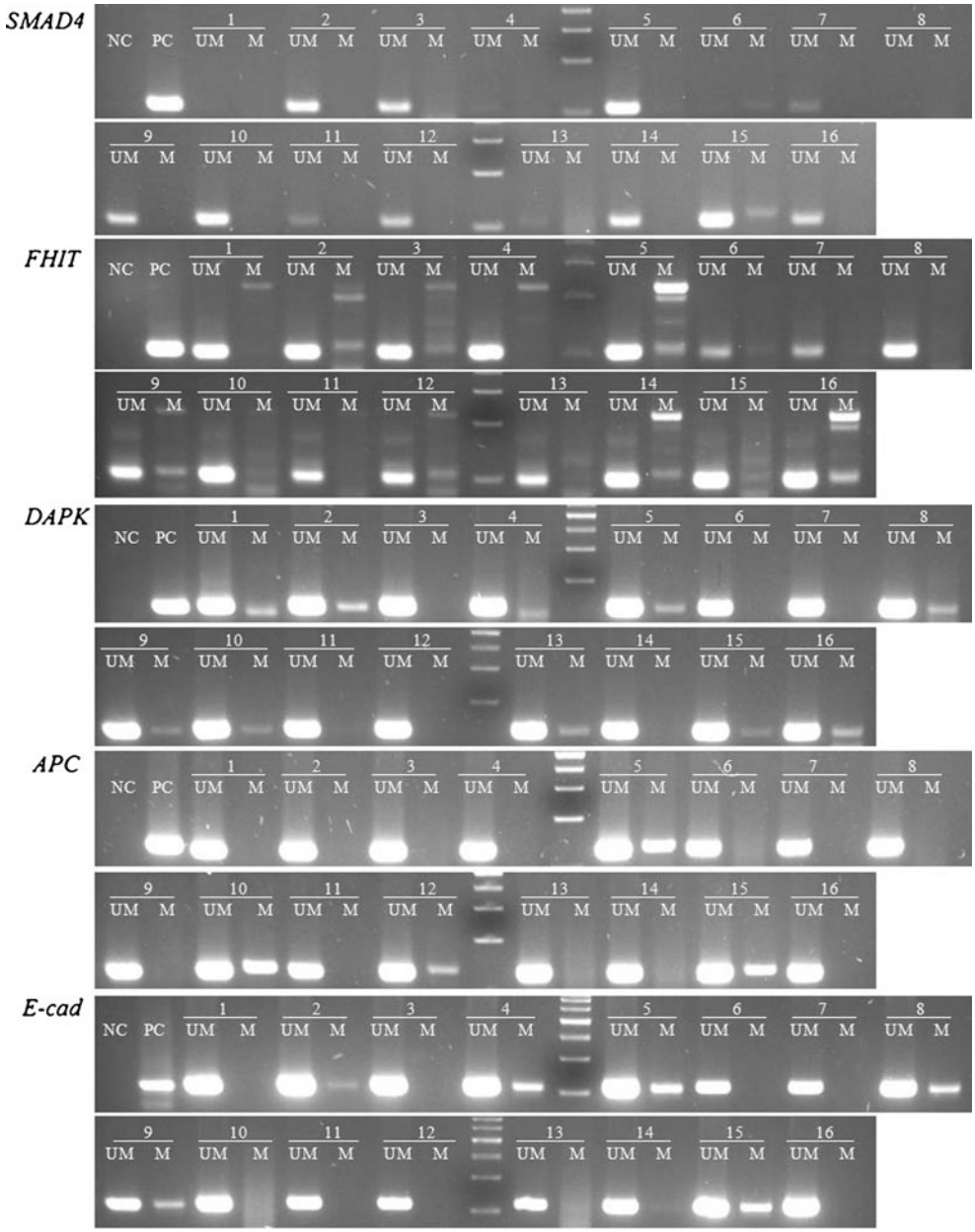


**Fig. 1** Methylation-specific polymerase chain reaction—single-strand conformation polymorphism analysis of SMAD4, FHIT, DAPK, APC, and E-cadherin (E-cad) gene (*PC* positive control, *NC* negative control, *1–16* patient)

95 % CI, 3.75–17.20), and E-cad gene (OR=7.88; 95 % CI, 3.75–16.56). However, all markers indices were not significantly different between ACP and normal control: SMAD4 (OR=1.33; 95 % CI, 0.58–3.06), FHIT (OR=1.63; 95 % CI, 0.56–4.76), DAPK1 (OR=0.58; 95 % CI, 0.23–1.51), APC (OR=0.56; 95 % CI, 0.16–1.91), and E-cad gene (OR=1.63; 95 % CI, 0.56–4.76). Additionally, APC is the only marker to be significantly increased in stage I CRC (OR=6.67; 95 % CI, 1.19–23.4). Methylation-specific sequencing was further performed to confirm the methylation status in 10 patients (Fig. 2).

Then we observed the sensitivity and specificity of the five genes to compare the methylation states of CRC patients with non-CRC patients. Validity of five markers is shown in Table 4 and Fig. 3. This study showed that the most sensitive marker for detection of CRC was E-cadherin, which amplified 60 %, followed by APC (57 %). E-cadherin and APC had similar specificities and amplified at 84 and 86 %, respectively, of CRC patients in comparison to that of non-CRC patients. Additionally, we evaluated positive predictive value and efficacy among the five markers. E-cadherin (69 %) and APC (71 %) had similar positive predictive value, E-cadherin (75 %) and APC (75 %) had same efficacy by CRC and non-CRC, respectively. Five genes promoter methylation in plasma were statistically

**Table 2** Clinical and demographic data of the patients and control group ($N$=160)

| Characteristic | Normal | Adenocarcinoma (%) | Adenomatous polyp with low grade dysplasia (%) | $p$ Value adenocarcinoma vs normal |
|---|---|---|---|---|
| Sex | | | | |
| Male | 30 | 24 | 20 | 0.436[a] |
| Female | 30 | 36 | 20 | |
| Age | | | | |
| | 61.4±8.97 | 63.4±10.1 | 62.4±10.75 | 0.491[b] |
| Stage | | | | |
| I | | 14 (23.3) | | |
| II | | 14 (23.3) | | |
| III | | 28(46.7) | | |
| IV | | 4 (6.7) | | |
| Location | | | | |
| Proximal | | 28 (46) | 16 (40) | |
| Distal | | 32 (54) | 24 (60) | |

[a]Chi-square test
[b]$T$ test

significant risk factor in colorectal adenocarcinoma patients and E-cad and APC gene were significantly accurate than other genes.

To compare the five genes methylation states of stage I CRC patients and non-CRC patients, the most sensitive marker was APC, 57 % of CRC patients, followed by E-cadherin (48 %) and SMAD4 (47 %; Table 4). The most specific marker was APC, 89 % of CRC patients, followed by E-cadherin (87 %) and SMAD4 (87 %). Positive predictive values of APC (44 %) and E-cad (43 %) were higher than the other markers; efficacy values of APC (78 %) and E-cad (78 %) were higher than the other markers. Consequently, APC predicted stage I CRC significantly accurate than the other markers.

In 10 cases of CRC patients, the corresponding CRC tissue samples were available for analysis. There was relatively high concordant rate in our limited number of colorectal cancer patients: concordant rate of methylation was 70 % for SMAD4, 70 % for FHIT, 80 % for DAPK1, 80 % for APC, and 60 % for E-cad. We further collected four patients with inflammatory bowel diseases (two Crohn's disease and two ulcerative colitis) and one patient with serrated adenoma. Aberrant methylation was mainly observed in *FHIT* and *DAPK1* gene from inflammatory bowel disease and serrated adenoma (Table 5).

## Discussion

In CRC, various genetic and epigenetic changes, including SMAD4, FHIT, DAPK1, APC, and E-cadherin alterations, are concurrently observed. These mutations and promoter hypermethylations are interconnected to generate diverse pathways of colorectal tumorigenesis.

Among those genes, which are subjected to epigenetic regulation, APC promoter hypermethylation occurs in all organs of GI cancers, both in hereditary and sporadic syndromes. This might indicate the importance of APC inactivation in tumorigenesis of these organs [9]. The major

**Table 3** Comparisons of odd ratio (OR) and confidential interval (CI) for epigenetic biomarkers in colorectal cancer

| Indicator | Adenomatous polyp vs normal | | | Adenocarcinoma vs non-adenocarcinoma[a] | | | Stage I adenocarcinoma vs normal | | |
|---|---|---|---|---|---|---|---|---|---|
| | CI | OR | $p$ value | CI | OR | $p$ value | CI | OR | $p$ value |
| SMAD4 | 0.58–3.06 | 1.33 | 0.63 | 1.81–6.98 | 3.56 | 0.008 | 0.81–8.74 | 2.67 | 0.39 |
| FHIT | 0.56–4.76 | 1.63 | 0.53 | 2.51–10.96 | 5.25 | 0.001 | 0.66–10.31 | 2.60 | 0.32 |
| DAPK1 | 0.23–1.51 | 0.58 | 0.43 | 1.45–5.59 | 2.85 | 0.029 | 0.53–5.76 | 1.75 | 0.66 |
| APC | 0.16–1.91 | 0.56 | 0.51 | 3.75–17.20 | 8.03 | <0.001 | 1.19–23.4 | 6.67 | 0.045 |
| E-cad | 0.56–4.76 | 1.63 | 0.53 | 3.75–16.56 | 7.88 | <0.001 | 1.34–17.78 | 4.88 | 0.11 |

[a]Non-adenocarcinoma, adenomatous polyp with low-grade dysplasia, and normal

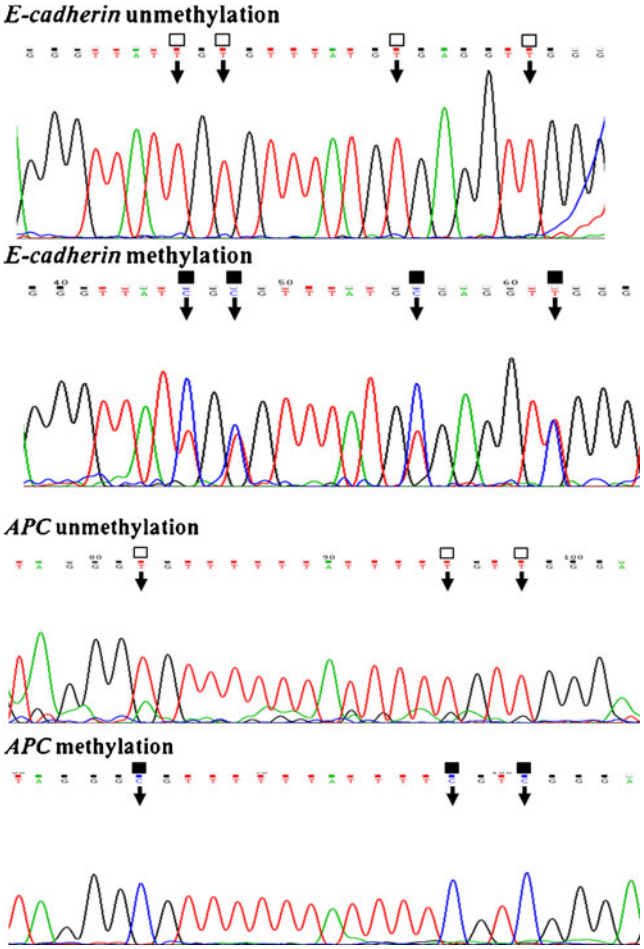


**Fig. 2** Sequencing chromatogram of the E-cadherin and APC gene in unmethylated DNA and methylated DNA obtained using direct sequencing analysis. The sequences represent CpG sites were shown by *arrows*. The nonmethylated cytosine (conversion of cytosine to thymine) are marked by *white squares* and methylated cytosines (non-conversion of cytosine to thymine) are marked by *black squares*

tumor suppressor function of APC is thought to be as a negative regulator of Wnt signaling, where it forms part of the β-catenin destruction complex, which is comprised of Axin, GSK3β, and CK1. Mutations in APC lead to the stabilization of β-catenin, and consequently, to the deregulation of the Wnt pathway through the activation of TCF/LEF target genes [10]. Inactivation of the APC gene is recognized as an early key event in the development of sporadic CRC with up to 80 % of CRC having mutations in the APC gene [10]. Moreover, APC along with several other hypermethylated genes play a prognostic indicatory role in squamous cell and adenocarcinoma of esophagus, bladder, and lung cancers [11]. In fact, in adenocarcinoma of esophagus, *APC* promoter hypermethylation has been observed in 92 % of cases [12]. Thus, *APC* could be considered as an appropriate predictive molecular marker, especially for digestive tract cancers. In our study, APC promoter hypermethylation had a sensitivity of 57 % and specificity of 86 % in CRC patients compared to those non-CRC patients in blood. APC also had 57 % sensitivity and 89 % specificity in stage I CRC patients compared to the normal control. APC was a useful epigenetic biomarker in blood for the detection of CRC. Particularly, APC may be a key component in identifying early potential CRC.

FHIT may be involved in a regulation of cell cycle and/or DNA replication [13]. FHIT gene has been proposed to be a promising candidate tumor suppressor gene involved in various types of human cancers [14]. Many authors found decreased expression of the FHIT gene in CRC [15]. Hao et al. and Mori et al. reported reduced expression of FHIT protein in 44 and 50 % of CRC patients, respectively [16, 17]. However, Mady and Mehlman found in the series of 100 CRC patients that the majority of cases (69 %) showed equal or higher expression of the FHIT protein in tumor tissue as compared to its adjacent normal mucosa, and only 8 % of patients FHIT protein was not detected [18].

SMAD4 has a central role in transducing receptor-mediated and SMAD-dependent signaling to the nucleus by all members of the TGF-β family. Loss of SMAD4 function, either due to loss of its expression or genetic mutation, has been reported in a significant proportion of colon and

**Table 4** Comparisons for validity of epigenetic biomarkers in colorectal cancer

| Indicator | | Sensitivity | Specificity | Positive predictability | Efficacy |
|---|---|---|---|---|---|
| CRC vs. non-CRC | SMAD4 | 0.52 | 0.64 | 0.53 | 0.65 |
| | FHIT | 0.50 | 0.84 | 0.65 | 0.71 |
| | DAPK1 | 0.50 | 0.74 | 0.54 | 0.65 |
| | APC | 0.57 | 0.86 | 0.71 | 0.75 |
| | E-cad | 0.60 | 0.84 | 0.69 | 0.75 |
| Stage I CRC vs. normal | SMAD4 | 0.47 | 0.87 | 0.29 | 0.65 |
| | FHIT | 0.29 | 0.67 | 0.33 | 0.76 |
| | DAPK1 | 0.43 | 0.70 | 0.33 | 0.65 |
| | APC | 0.57 | 0.89 | 0.44 | 0.78 |
| | E-cad | 0.48 | 0.87 | 0.43 | 0.78 |

*CRC* colorectal adenocarcinoma, *Non-CRC* adenomatous polyp with low-grade dysplasia and normal

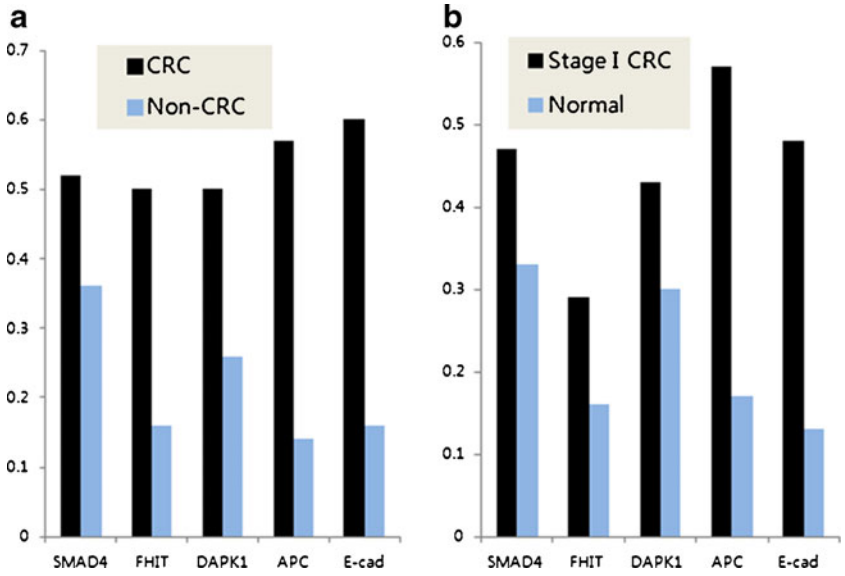


**Fig. 3** The sensitivity of five genes in the methylation states of colorectal cancer patients. **a** Compare to the methylation states of CRC patients and non-CRC patients, the most sensitive marker for detection of CRC was E-cadherin (60 %) followed by APC (57 %). The sensitivity ranged from 50 to 60 %. **b** Compare to the methylation states of stage I CRC patients and normal control, the most sensitive marker for detection of stage I CRC was APC (57 %) followed by E-cadherin (48 %) and SMAD4 (47 %). The sensitivity ranged from 29 to 57 % (*CRC* colorectal adenocarcinoma, *Non-CRC* adenomatous polyp with low-grade dysplasia and normal)

pancreatic cancer [19]. Frequent loss of SMAD4 expression in tumors that have acquired invasive and metastatic phenotype strongly implicates tumor-suppressive functions of SMAD4 during cancer progression [20].

mRNA and protein expressions of E-cadherin (encoded by CDH1) are frequently lost due to DNA methylation in multiple cancers, at the early stage of tumor progression and re-expressed at the metastatic foci of many cancers [21]. CDH1 plays a key role in the maintenance of tissue integrity by forming a complex with catenin and a loss of CDH1 gene expression results in the disruption of cellular clusters, indicating that E-cadherin functions as a tumor suppressor gene.

**Table 5** Additional analysis of epigenetic markers in inflammatory diseases, serrated adenoma and colorectal cancer tissues

| No. | Age/sex | Diseases | Epigenetic markers | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | SMAD4 | | FHIT | | DAPK1 | | APC | | E-cad | |
| | | | Plasma | Tissue | Plasma | Tissue | Plasma | Tissue | Plasma | Tissue | Plasma | Tissue |
| 1 | 24/M | Crohn's disease | NT | − | NT | − | NT | + | NT | − | NT | − |
| 2 | 27/M | Crohn's disease | NT | − | NT | + | NT | + | NT | − | NT | + |
| 3 | 52/F | Ulcerative colitis | NT | − | NT | + | NT | − | NT | − | NT | − |
| 4 | 54/F | Ulcerative colitis | NT | − | NT | − | NT | + | NT | − | NT | + |
| 5 | 38/M | Colorectal cancer | + | + | + | + | + | + | + | + | + | + |
| 6 | 52/M | Colorectal cancer | − | + | + | + | − | − | + | − | + | − |
| 7 | 76/M | Colorectal cancer | − | − | + | − | − | − | − | − | − | − |
| 8 | 60/M | Colorectal cancer | − | − | − | − | + | + | − | − | − | + |
| 9 | 77/F | Colorectal cancer | − | − | + | + | + | + | − | − | − | + |
| 10 | 68/F | Colorectal cancer | − | − | + | + | + | + | + | + | + | − |
| 11 | 51/M | Colorectal cancer | − | − | + | − | − | − | + | − | − | − |
| 12 | 77/M | Colorectal cancer | + | − | − | + | + | − | + | + | − | − |
| 13 | 46/M | Colorectal cancer | + | − | − | − | + | + | − | − | − | − |
| 14 | 41/F | Colorectal cancer | − | − | + | + | + | − | − | − | − | − |
| 15 | 77/F | Colorectal cancer | NT | + | NT | + | NT | + | NT | + | NT | + |
| 16 | 77/M | Serrated adenoma | NT | − | NT | + | NT | + | NT | − | NT | − |

Kinsella et al. found in the histological studies of colon carcinoma and colon adenoma that decreased E-cadherin expression in colorectal carcinoma was related to tumor differentiation, invasion and metastasis, as well as Duke's staging [22]. Hypermethylation of E-cadherin promoter region is a frequent event in CRC which may play an important role in the progression of carcinogenesis. In our study, E-cadherin promoter hypermethylation had a sensitivity of 60 % and specificity of 84 % in CRC patients compared to non-CRC patients in blood. Therefore, E-cadherin is useful epigenetic biomarker in blood for detection in CRC.

DAPK1 is commonly silenced in human cancers by methylation and has properties that suppress tumor and metastasis [23]. DAPK1 also induces autophagy and apoptosis through independent mechanisms [24]. By analyzing DAPK methylation status, it was possible to detect urinary bladder cancer from voided urine; head and neck cancer from saliva, mouth rinsing fluid or nasopharyngeal swab or serum; colon and breast cancer from serum; lung cancer from sputum, bronchial brush samples, or serum; and cervix cancer from cervical cytology specimens [25].

Several studies suggest that the failure to detect methylation in plasma or serum might be a result from the low amount of DNA present inside 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. In this study, a stringent selection process, incorporating MSP–SSCP analysis of plasma samples, was performed to further analyze the marker candidates. All five genes demonstrated sensitivity of over 50 % and specificity of over 64 % in plasma DNA from CRC patients. Examination of DNA-based markers in blood may improve diagnosis and monitoring of CRC patients. Because of its relative ease of performance, in a noninvasive approach, testing of DNA methylation markers may, 1 day, be easily applied during the regular health checkups.

It is known that cancer patients have higher levels of circulating DNA in their sera or plasma than either healthy people or those with nonmalignant diseases [2]. In general, a high concordance between epigenetic alterations in primary tumor specimens and in blood has been reported [26, 27]. In our study, all methylated genes found in the serum samples of six CRC patients were also methylated in the corresponding tissue sample. Several studies suggest that methylation of plasma DNA is increased among the high-risk patients and patients with chronic inflammation [28]. To decrease false positive results in our study, we excluded patients with symptomatic nonmalignant bowel diseases, patients with a history of polypectomy or family history for colorectal neoplasia and patients with chronic inflammatory diseases of the gastrointestinal tract.

This study was limited by the small number of plasma from CRC and ACP patients. Further validation is now focused on testing the markers, particularly APC and E-cadherin in larger studies that include plasma samples from CRC patients, individuals with preneoplastic disease, and other healthy and disease controls. In addition, the assay of two to four genes combination marker rather than a single marker may increase the sensitivity and specificity of DNA methylation in plasma for early detection of CRC patients.

In conclusion, our data demonstrates that five genes promoter methylation in plasma are statistically significant risk factors in CRC patients. In this study, E-cad and APC gene may be particularly useful epigenetic biomarkers in plasma for the detection of CRC. For detection stage I CRC, compared to that of the normal control, the most sensitive and specific marker was APC. Our exploratory study indicates that APC may be able to identify early potential CRC.

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