DNA hypermethylation of a selective gene panel as a risk marker for colon cancer in patients with ulcerative colitis

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Abstract. Patients with inflammatory bowel disease (IBD) which includes ulcerative colitis (UC) and Crohn's disease (CD) of the colon are at risk of developing colorectal cancer (CRC). Here, we analyzed the methylation status of selected genes as a risk marker in UC patients. We assessed methylation frequency of 4 genes [secreted frizzled-related protein 1 (*SFRP1*), transcription elongation regulator 1-like (*TCERG1L)*, fibrillin 2 (*FBN2)* and tissue factor pathway inhibitor 2 (*TFPI2*)] in biopsies of 36 UC patients. *SFRP1* and *TCERG1L* genes showed high methylation frequencies but *FBN2* and *TFPI2* genes showed methylation frequencies of 50% in UC patients which suggests that our sensitive selective markers could detect half of the UC patients. We also confirmed the methylation status in UC tissues by bisulfite sequencing analysis. We compared the levels of methylation in terms of quantification between UC patients and CRC tumors. Importantly, methylation levels of these 4 genes were found to be significantly higher in CRC compared to UC patients, even though we noted a frequent methylation pattern in UC patients. Our data suggest that sensitive DNA methylation markers are able to identify UC patients and this would implicate the risk of CRC. Therefore, assessing the methylation of these 4 genes in UC patients could contribute to prevent the progression of severe disease with regular colonoscopic surveillance.

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the intestines and includes two distinct disease categories: Crohn's disease (CD) and ulcerative colitis (UC). Both are associated with an increased risk of colorectal cancer (CRC) (1,2). UC is a disease that is characterized by chronic inflammation, rapid cell turnover and a substantial risk of colon cancer (1,3). However, UC-associated colon cancer differs from sporadic colon cancer in many ways (4,5).

In the case of UC patients, the risk of colon cancer has been reported to be 10- to 20-fold higher in patients with a disease duration of 20 years or more, even though current treatment may have modulated this risk (6-8). As a result of the recognition of this increased risk, annual colonoscopic surveillance with multiple biopsies is recommended for an early diagnosis of displasia in UC (9).

DNA methylation is one of the important epigenetic mechanisms that controls gene expression, chromatin structure, genome stability and X chromosome inactivation (10). Aberrant DNA methylation can lead to a serious imbalance in the normal function of cells and can promote pathological conditions (10). Promoter CpG island hypermethylation of tumor-suppressor genes has been known as a common hallmark of all human cancer (11). In addition, transcriptional silencing associated with promoter DNA hypermethylation of genes is an important and early event in CRC.

DNA methylation has been reported to be correlated with the development of colitis-associated cancer. DNA methylation level of the estrogen receptor 1 (*ESR1)* gene in non-neoplastic colorectal epithelium was higher in UC patients with neoplasia than in UC patients without neoplasia (12). In addition, E-cadherin (*CDH1*)/hyperplastic polyposis protein 1 (*HPP1*) in colon mucosa of UC was an early event in UC-associated carcinogenesis (13). However, chronic active inflammation is largely correlated with the occurrence of dysplasia or cancer in UC as well as *H. Pyrori*-associated gastritis (14,15). Therefore, active inflammation in UC may be correlated with accumulation of methylation, resulting in susceptibility to carcinogenesis.

In the present study, we selected 4 genes [secreted frizzled-related protein 1 (*SFRP1*), transcription elongation regulator 1-like (*TCERG1L)*, fibrillin 2 (*FBN2)* and tissue factor pathway inhibitor 2 (*TFPI2*)] which have recently been identified by cDNA microarray approach using DNA methyltransferase inhibitor (5-aza-2'-deoxycytidine) (16,17). They are cancer-specifically and frequently methylated in CRC

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tumors. In addition, all 4 genes have previously been identified as early DNA methylation biomarker candidates during colon cancer progression (18,19). Therefore, we hypothesized that these 4 genes are useful markers to detect, not only early-stage colon cancer but also chronic inflammation disease such as UC. The aim of the present study was to analyze the methylation status of selective genes (*SFRP1*, *TCERG1L*, *FBN2* and *TFPI2*) as a risk marker for colon cancer in UC patients.

Materials and methods

Patient samples. Enrolled in the study were 36 patients with UC, including 21 males and 15 females. The median age was 43.5 years and the median clinical disease duration was 24.6 months. The diagnosis of UC was based on standard clinical, endoscopic, radiological, and histological criteria (20). Rectal inflammatory mucosal specimens were obtained from all the patients during colonoscopic biopsy and were preserved at -80˚C until use. The histopathological examinations showed mild or moderate inflammation, with no evidence of dysplasia or neoplasia in all the cases. Clinicopathological characteristics such as gender, age of disease onset, clinical disease duration, lesion location and clinical type were investigated. UC patients were also classified as proctitis, left sided colitis or pancolitis according to the location and extension of the inflammatory lesions as judged by endoscopic findings. According to the clinical course, chronic UC cases were classified into chronic relapsing, chronic continuous or only one episode of the disease (21). Written informed consent was obtained from all participating subjects. The study protocol was approved by the Institutional Review Board (IRB). Clinical characteristics of the patients are documented in Table I.

CRC patient samples (n=8) were also evaluated to compare the DNA methylation level with UC patients. The mean age of the patients was 65.8 years. Of all the patients, 3 patients had well-differentiated and 5 had moderately differentiated carcinomas. Based on the tumor-node-metastasis classification, stage II (n=4), III (n=3) and IV (n=1) cases were noted.

Table I. Basic characteristics of the UC patient samples in this study.

Characteristics	
Total no. of patients	36
Age (years)	
Median (range)	43.5 (15-79)
Gender, n (%)	
Male	21 (58.3)
Female	15 (41.6)
Age at disease onset, n (%)	
≤20 years	6 (16.7)
21-40	12 (33.3)
>41	18 (50)
Duration of disease (median, months)	24.6
Lesion location, n (%)	
Proctitis	12 (33.3)
Left sided colitis	14 (38.9)
Pancolitis	10 (27.8)
Mayo endoscopic score, n (%)	
Normal or inactive	1 (2.8)
Mild disease	16 (44.4)
Moderate disease	13 (36.1)
Severe disease	6 (16.7)
Clinical type, n (%)	
Only one episode	21 (58.3)
Chronic relapsing	15 (41.7)
Chronic continuous	0 (0)

UC, ulcerative colitis.

DNA extraction and methylation analysis. Methylation analysis was performed using the methylation-specific polymerase chain reaction (MSP) strategy, as previously described (22). DNA was extracted following a standard phenol-chloroform extraction protocol. Bisulfite modification of DNA was performed using the EZ DNA Methylation™ Kit (Zymo Research) according to the manufacturer's instructions. Methylation-specific PCR was carried out in a 25-μl reaction containing 10X MSP buffer, 10 mM dNTPs, 10 pmol of each of the methylated or unmethylated primers, 1 unit of JumpStart™ REDTaq® DNA polymerase (Sigma) and 4 μl of bisulfite-treated DNA. Amplification cycles were as follows: one cycle at 95˚C for 5 min followed by 35 cycles of 95˚C for 30 sec, annealing temp for 30 sec, 72˚C for 30 sec and a final extension step at 72˚C for 5 min. *In vitro* methylated DNA (IVD) was used as a positive control for MSP. IVD was created by treating cell line DNA with Sassy methylate (NEB) as directed. DKO, which is a double knockout derivative of the CRC cell line HCT 116 with knockout of the major DNA methyltransferases (*DNMT1*$^{-/-}$ and *DNMT3b*$^{-/-}$) was used as an additional negative control. DKO lacks methylation at 95% of the known CpG sites (23). An amount of 7.5 μl of each amplification reaction was loaded and run on 2% agarose gel containing GelStar™ Nucleic Acid Gel Stain (Lonza) and visualized by ultraviolet illumination. All primers are listed in Table II.

Bisulfite sequencing analysis. Genomic DNA (1 μg) from each sample was bisulfite converted using the EZ DNA Methylation Kit following the manufacturer's protocol. PCR conditions and primer sequences are provided upon request. The PCR amplicons were gel-purified and subcloned into pCRII-TOPO vector (Invitrogen). At least 7 clones were randomly selected and sequenced on an ABI 3730xl DNA analyzer to ascertain the methylation patterns of each locus.

Quantitative methylation-specific PCR (MSP) using real-time PCR. Bisulfite modification of genomic DNA was carried out using the EZ DNA Methylation Kit. For quantitative real-time analyses, the Maxima SYBR-Green qPCR kit (Fermentas) was used, and the amplification conditions consisted of an initial 10-min denaturation step at 95˚C, followed by 40 cycles

Table II. Selected gene primers for MSP and bisulfite sequencing analysis.

Gene		Sense (5'-3')	Antisense (5'-3')	Ref.
SFRP1	Meth	TGTAGTTTTCGGAGTTAGTGTCGCGC	CCTACGATCGAAAACGACGCGAACG	(16)
	Unmeth	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT	CTCAACCTACAATCAAAAACAACACAAACA	
	BS	GTTTTGTTTTTTAAGGGGTGTTGAG	GCCTTTTGTCCCCGGAGGTCCCTGG	
TFPI2	Meth	GTTCGTTGGGTAAGGCGTTC	CATAAAACGAACACCCGAACCG	(18)
	Unmeth	CCCACATAAAACAAACACCCAAACCA	TGGTTTTGTTGGGTAAGGTGTTTG	
	BS	GGTTTATGGTGTAGGGG	CAATCACTAACAAATCATTTCC	
FBN2	Meth	GGGTTTTTAAAATTTTCGCGTCGC	CTACGAAACCGAACGAAAATACG	(31)
	Unmeth	GTTTTGTTGGGTTTTTAAAATTTTTGTGTTGTG	AAATAACAACTACAAAACCAAACAAAAATACA	
	BS	CTTCCAACCCYACCTTC	GTTTTTAGAAGAAGAGGAGGG	
TCERG1L	Meth	GGTCGTTTGCGTCGGATTC	CTACCCAACGCGAAACTAAAAACG	(31)
	Unmeth	TTTGGGGTTGTTTGTGTTGGATTTG	CATATCCCACTACCCAACACAAAACTAAAAAC	
	BS	AATTTGTTTGGTTTATTTGTGTAATAGAAAT	CTAATAACCTCTAACCCTCTAA	

MSP, methylation specific polymerase; *SFRP1*, secreted frizzled-related protein 1; *TFPI2*, tissue factor pathway inhibitor 2; *FBN2*, fibrillin 2; *TCERG1L*, transcription elongation regulator 1-like; Meth, methylated; Unmeth, unmethylated; BS, bisulfite.

of denaturation at 95˚C for 15 sec and annealing and extension for 30 and 60 sec, respectively. An CFX96 real-time PCR detection system (Bio-Rad) was used. For quantification, the comparative cycle threshold (Ct) method was used, normalizing the Ct values for the indicated gene to the Ct values of the unmethylated reaction relative to a methylated reaction sample. All primer sequences are listed in Table II.

Statistical analysis. All statistical analyses were conducted using the STATA 9.2 software package (Stata, College Station, TX, USA). Most analyses were conducted using a t-test, while continuous variables were analyzed using the Mann-Whitney U test. p-values of <0.05 were considered to indicate statistically significant results.

Results and Discussion

Detection of DNA promoter hypermethylation in UC patient samples. Hypermethylation of an increasing number of genes has been associated with human colorectal tumorigenesis (24-27). In UC, promoter methylation seems to precede dysplasia and occurs throughout the mucosa of colitis (27,28), reinforcing the link between chronic inflammation and DNA methylation (29,30).

We tested DNA methylation of selected genes, *TCERG1L*, *SFRP1*, *FBN2* and *TFPI2*. These genes have been previously reported to be cancer-specifically methylated and frequently methylated at the early stage of colon cancer progression such as in adenomas (16,31). Previously, DNA hypermethylation of these 4 genes have been identified from a gene expression microarray approach (17) using 5-aza-2'-deoxycytidine in colon cancer cells. These genes were highly methylated in CRC tumors as well as adenomas, which suggests that these 4 genes may be candidates for use as an early detection DNA methylation biomarker for CRC patients. To date, epigenetic silencing of *SFRP1* has been identified in a variety of malignancies, including cancers of the colon (32), bladder (33), prostate (34), lung (35), and breast (36). Hypermethylation of the *TFPI2* gene in CRC was identified by Glöckner *et al* (18), and *TFPI2* was found to have a methylation frequency of 80% in adenomas as well as in stool from CRC patient samples, suggesting DNA methylation of the *TFPI2* gene may be an early detection biomarker of CRC. Recently, promoter hypermethylation of the *TCERG1L* and *FBN2* genes was frequently noted in colon adenomas (31).

Here, we tested the methylation level of 4 genes in 36 patients with UC by MSP analysis since these 4 genes have shown high potential to detect early-stage disease such as cancer. Genomic DNA was extracted, and successful methylation analysis was performed in most of the samples. We assessed the methylation pattern of gene sets (*TCERG1L*, *SFRP1*, *FBN2* and *TFPI2*) in the UC samples by MSP analysis. Fig. 1 shows the methylation pattern in each UC sample tested in this study. *SFRP1* and *TCERG1L* genes were methylated in the majority of the samples tested (>94%). *FBN2* and *TFPI2* genes showed 53 and 50% frequencies in the UC samples, respectively (Fig. 1). Fifteen out of 36 samples (42%) showed methylation of all 4 genes. We assessed the clinical data to determine whether a correlated was present or not, but no correlation was noted between

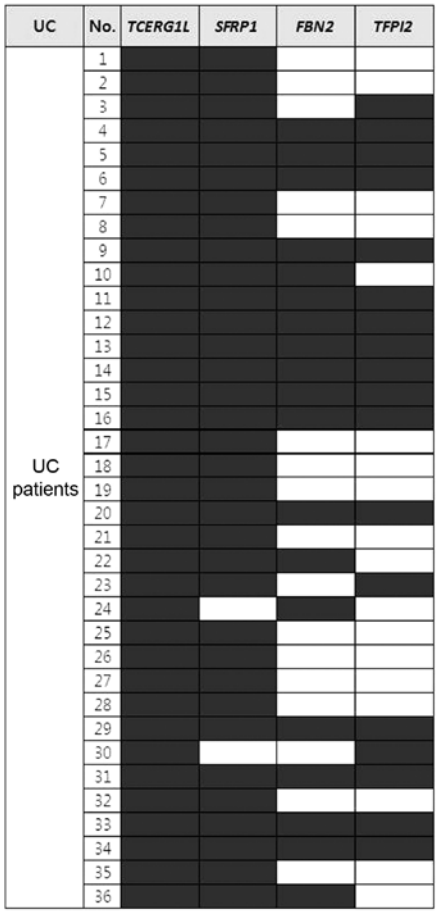

Figure 1. Summary of the methylation analysis of 4 genes in 36 patients with UC. Gene symbols are indicated at the top, and each row represents a UC patient sample. Filled boxes and open boxes indicate methylation and no methylation, respectively.

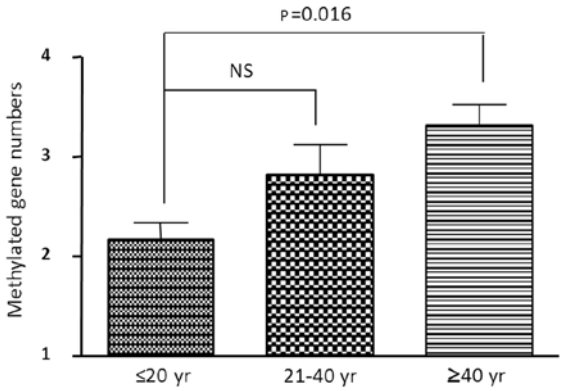

Figure 2. Percentage of methylated genes in the UC patients according to different age groups. The x-axis indicates the three different age groups [≤21 years (n=6), 21-40 years (n=11) and ≥41 years (n=19)]. The y-axis indicates the numbers of methylated genes. The number of methylated genes increased from the young group (≤21) to the old group (≥41) (young group vs. old group, p=0.02). No significant difference was noted between the mid-age (21-40) and old age (≥41) group. NS, statistically not significant.

clinical data particularly duration of disease with methylation of all 4 genes. In terms of the clinical correlation with DNA methylation level, we aimed to ascertain whether the number of DNA methylated genes was correlated with age-related methylation since all of the UC patient samples that we tested had no neoplasia. Table I indicates all of the UC patients categorized according to 3 age groups (≤21, 21-40 and ≥41 years). The number of methylated genes was statistically significant between the young (≤21) group and older (≥41) group (p=0.02, t-test) (Fig. 2). There was no significant difference between the young (≤21) and mid-age (21-40) group. In the older age group all 4 genes were methylated in 11 out of 19 (58%) samples (Fig. 2). These data suggest that age-related methylation occurred in a small number of UC patient samples, but we need to confirm this phenomenon using a larger sample size.

Epigenetic regulation of DNA hypermethylation in UC patient samples. Next, we confirmed the methylation pattern in CpG islands of the promoter regions of the 4 genes by bisulfite sequencing analysis (Fig. 3). *TCERG1L*, *FBN2*, *TFPI2* and *SFRP1* had 31, 46, 30 and 58 CpG sites, respectively, in the bisulfate sequencing region that we amplified. *TCERG1L* and *FBN2* genes were previously reported to have a dense CpG methylation pattern in CRC tumors (31). In this study, both genes showed a dense CpG methylation pattern in s UC patient (UC4) with 78% (methylation site per CpG site) and 71%, respectively. *TFPI2* and *SFRP1* genes also were previously reported to have a dense CpG methylation pattern in CRC tumors (18,32). Here, *TFPI2* and *SFRP1* showed a 71 and 84% CpG methylation frequency in UC patients, which confirmed that both genes were definitely present in UC patients. Our results suggest that the 4 genes are densely methylated in UC patients, and DNA methylation of the 4 genes is sensitive enough to detect inflammatory disease such as UC.

As previously mentioned, the 4 genes were previously reported to be highly methylated in CRC tumors. Here, we noted a high frequency of methylation of the 4 genes in UC patients. Therefore, we compared the level of methylation between UC patients and CRC tumors by quantitative real-time MSP analysis. Before comparing the methylation level in UC patients and CRC, we used MSP analysis on 8 CRC samples with the 4 genes to confirm methylation in CRC. The genes were methylated in all 8 CRC samples (data not shown).

We tested 12 UC and 8 CRC samples which were confirmed to be methylated by MSP analysis. Even though we noted frequent methylation levels in the UC patient samples by MSP analysis (Fig. 1), the methylation in UC and CRC samples was significantly different. Box plot indicates the methylation levels of all tested samples (n=13 for UC, n=8 for CRC) by real-time MSP. *TCERG1L* (mean, 0.81 for UC; mean, 4.28 for CRC) and *SFRP1* (mean, 1.39 for UC; mean, 4.96 for CRC) showed a significant increase in the methylation level in UC when compared to the level in the CRC patient samples ($p<0.05$) (Fig. 4). *FBN2* (mean, 0.32 for UC; mean, 3.85 for CRC) and *TFPI2* (mean, 0.31 for UC; mean, 7.08 for CRC) also showed a significant increase in the methylation level in UC when compared to the level in the CRC patient samples ($p<0.05$) (Fig. 4). This suggests that DNA methylation of *TCERG1L* and *SFRP1* may have more sensitivity

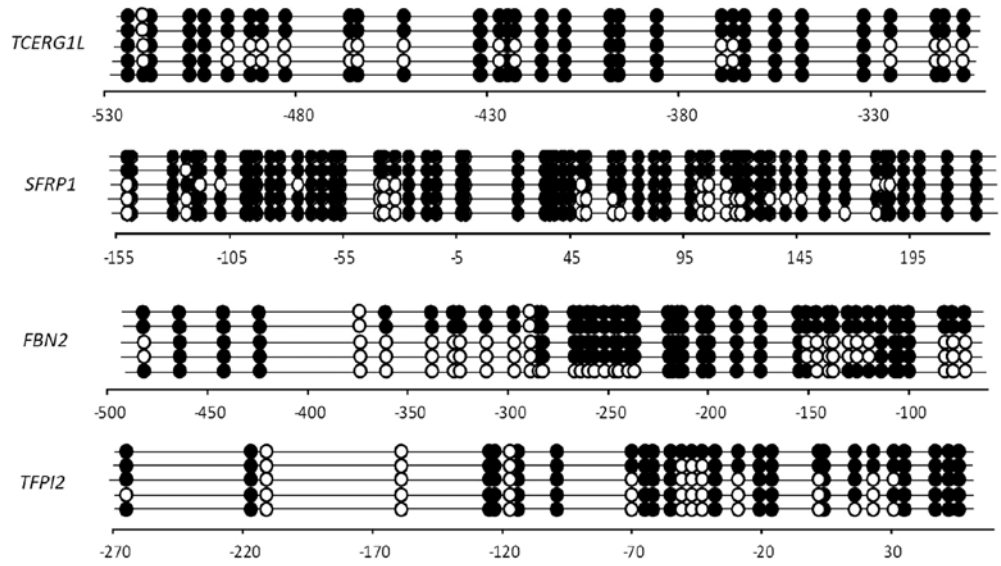

Figure 3. Representative bisulfite sequencing results for *SFRP1, TCERG1L, FBN2* and *TFPI2* genes in UC patient tissue samples. Primers for bisulfite sequencing are listed in Table II. The location of each CpG site relative to the transcription start site is shown at the bottom of the panels. Open and filled circles indicate methylation and no methylation, respectively.

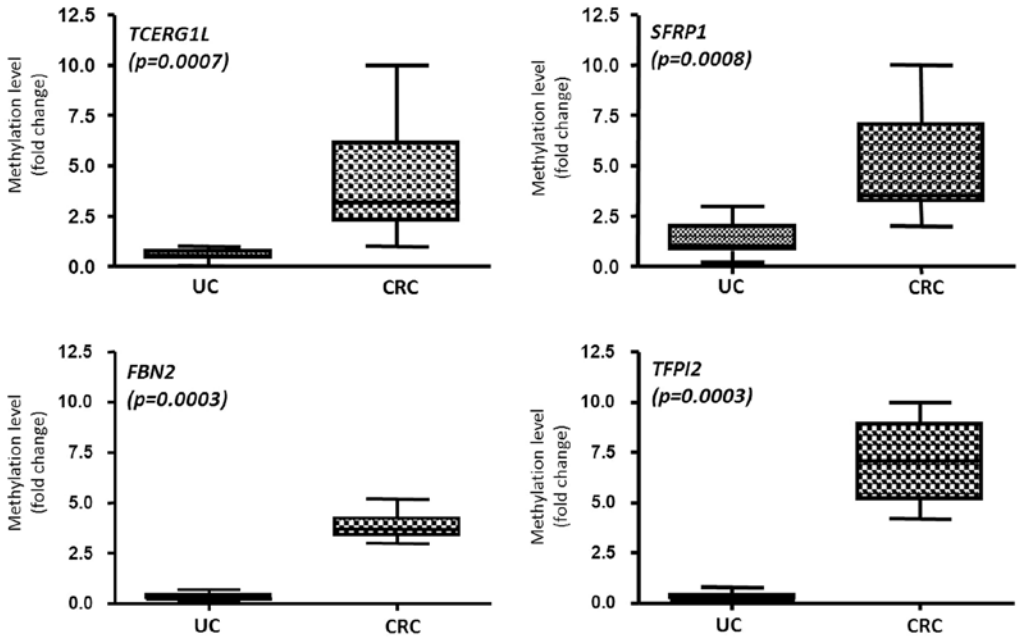

Figure 4. Quantitative MSP (qMSP) analysis for *SFRP1, TCERG1L, FBN2* and *TFPI2* genes in UC patient (n=13) and CRC samples (n=8). Box plot indicates the methylation levels of all tested samples (n=13 for UC, n=8 for CRC) by real-time qMSP.

in detecting not only CRC but also inflammatory disease. In contrast, DNA methylation of *FBN2* and *TFPI2* had a higher cancer-specific detection ability. Our data also suggest that DNA methylation can be triggered in UC patients, and accumulation of DNA methylation occurs during colon cancer progression. Our data also suggest that screening UC patients with the 4 genes may be useful to predict the risk for CRC.

Methylation of several gene-associated CpG islands was present in the normal-appearing epithelium from UC patients with high-grade dysplasia or cancer (25). *ESR1* methylation in different parts of the large intestine in UC patients with and without neoplasia confirmed that *ESR1* methylation is correlated with an increased risk of developing neoplasia (12). These reports suggest that the methylation levels of colonic mucosa of UC vary according to the presence or absence of neoplasia and that accumulation of methylation finally induces cancer development.

The present study had limitations. We could not conclude that detection of methylation in UC patients implies the process of neoplasia since we did not compare the methylation pattern of UC patients with and without cancer. In addition, the UC patients that were tested in this study did not have a

long duration of disease (Table I). However, Wang *et al* (28) revealed that promoter DNA methylation of *ER, p53, p14, p16, p21* and *hMLH1* genes was detected in UC patients without neoplasia, suggesting that these genes are useful for predicting cases at high risk of neoplasia. Our data support that detection of DNA methylation of the 4 genes in UC samples indicates a poor prognosis of UC patients who need routine colonoscopic surveillance which may prevent the progression to severe disease such as cancer.

In summary, we assessed the DNA methylation pattern in UC patients using very sensitive DNA methylation markers which are able to detect early-stage colon cancer such as adenomas. MSP analysis revealed that 2 genes (*TCERG1L* and *SFRP1*) showed a high (>95%) frequency of methylation while the other 2 genes (*FBN2* and *TFPI2*) showed a decreased (>45%) frequency of methylation in UC patients. We also confirmed a dense DNA methylation status of 4 genes in UC patients by bisulfite sequencing analysis. Notably, we compared the quantitative methylation level between UC patient and CRC tumors even though we noted a high frequency of methylation in UC patient by MSP analysis. The DNA methylation level was significant higher in CRC patients than in UC samples, which implies that DNA methylation may be triggered by inflammation and promote abnormal DNA hypermethylation in cancer. Our data suggest that sensitive methylation markers may be useful to detect inflammation diseases which have the potential risk of neoplasia. Therefore, examination of the methylation status of our markers could predict the progression of severe disease in UC patients.

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