Prediction of Colorectal Neoplasia by Quantitative Methylation Analysis of Estrogen Receptor Gene in Nonneoplastic Epithelium from Patients with Ulcerative Colitis

Keiichi Tominaga,1,2 Shigehiko Fujii,1 Kenichiro Mukawa,1,2 Mikio Fujita,1 Kazuhito Ichikawa,1 Shigeki Tomita,1 Yasuo Imai,1 Kazunari Kanke,2 Yuko Ono,1 Akira Terano,2 Hideyuki Hiraishi,2 and Takahiro Fujimori1

Abstract

Purpose: The incidence of colorectal neoplasia has increased among patients with longstanding and extensive ulcerative colitis (UC). Therefore, surveillance colonoscopy has been widely recommended. However, there is controversy about the impact of cancer surveillance, and ways to improve its effectiveness are being sought. The estrogen receptor (ER) gene shows age-related methylation in the colorectal epithelium and is frequently methylated in colorectal neoplasia, suggesting that ER methylation occurs early in the process of colorectal tumorigenesis.

Experimental Design: To clarify whether methylation analysis of the ER gene in nonneoplastic epithelium can help predict an increased risk for UC-associated neoplasia, a total of 105 non-neoplastic colorectal epithelia from 18 patients with longstanding and extensive UC, including 8 patients with neoplasia and 10 patients without neoplasia, were analyzed. In all patients, multiple samples were taken from six regions of the colorectum. The combined bisulfite restriction analysis method was used to determine the methylation status of the ER gene.

Results: The mean methylation level of the ER gene was 25.4% in the nonneoplastic epithelia from UC patients with neoplasia, whereas it was only 4.0% in those without neoplasia (P < 0.001). The methylation level of the ER gene in UC patients with neoplasia was significantly higher than in UC patients without neoplasia throughout the colorectum except for the cecum. In UC patients with neoplasia, the mean ER methylation level in the distal colon (36.1%) was significantly higher than in the proximal colon (14.6%; P < 0.001).

Conclusions: These results suggest that the analysis of ER gene methylation in nonneoplastic colorectal epithelium could have the potential to be a useful adjunct for identifying individuals with longstanding and extensive UC who are at increased risk of neoplasia and contribute to more effective cancer surveillance.

Ulcerative colitis (UC) is a chronic inflammatory bowel disease of unknown etiology. It is well established that colorectal neoplasia is the most dreaded complication of UC (1–4). The incidence risk of colorectal neoplasia increases with the duration of disease and is greater in patients with extensive colitis (5–8). In order to detect UC-associated dysplasia and the early stages of cancer, regular surveillance colonoscopy with multiple-step biopsy at intervals of 1 to 2 years has been recommended for patients with longstanding and extensive UC (9–12). However, studies examining the efficacy of surveillance in UC have produced conflicting results and have suggested that surveillance detects the early stage of neoplasia in only a minority of patients and cannot guarantee cancer detection at the curable stage (13–18). Thus, in order to improve the efficacy of surveillance, there is a great need for objective and reliable markers to identify individuals at an increased risk of neoplastic transformation among patients with longstanding and extensive UC.

DNA methylation is a powerful mechanism for the suppression of gene activity. In many kinds of cancer, some genes seem to acquire aberrant methylation in their CpG islands. In nonneoplastic colorectal epithelium, some genes are methylated with aging, and this alteration is known as age-related methylation (19, 20). Issa et al. (19) reported that methylation of the estrogen receptor (ER) CpG island increased with age in nonneoplastic colorectal epithelium, and that the same methylation occurred in most sporadic colorectal neoplasia. They concluded that methylation of the ER gene in aging colorectal epithelium could represent one of the earliest events predisposing to sporadic colorectal tumorigenesis.

Detection of DNA methylation has recently attracted considerable attention. Combined bisulfite restriction analysis (COBRA), one of the methods for detecting methylation, relies on the use of restriction enzyme digestion to detect sequence
In this study, to clarify whether methylation analysis of the ER gene in nonneoplastic epithelium could contribute to the prediction of an increased risk for UC-associated neoplasia, we quantitatively investigated the methylation status in nonneoplastic epithelium obtained from longstanding and extensive UC patients with and without colorectal neoplasia using the COBRA method.

Materials and Methods

**Patient samples.** We studied nonneoplastic colorectal epithelia from 18 patients with longstanding (>7 years) and extensive (proximal to the splenic flexure) UC. The 18 patients included 8 UC patients with neoplasia and 10 patients without neoplasia. In all patients, whenever possible, multiple samples were taken from six regions (rectum, sigmoid colon, descending colon, transverse colon, ascending colon, and cecum) of the colorectum. Nonneoplastic samples of UC patients with neoplasia were retrieved from colectomy specimens. The samples of UC patients without neoplasia were obtained from biopsy specimens in surveillance colonoscopy. All samples were embedded to the splenic flexure) UC. The 18 patients included 8 UC patients with neoplasia and 10 patients without neoplasia. In all patients, whenever possible, multiple samples were taken from six regions (rectum, sigmoid colon, descending colon, transverse colon, ascending colon, and cecum) of the colorectum. Nonneoplastic samples of UC patients with neoplasia were retrieved from colectomy specimens. The samples of UC patients without neoplasia were obtained from biopsy specimens in surveillance colonoscopy. All samples were embedded in optimal cutting temperature compound (Nikon Instech, Tokyo, Japan) and stored at −80°C until study. The Ethical Committee of Dokkyo University School of Medicine approved all protocols, and informed consent for tissue procurement was obtained from all patients.

**Histologic evaluation.** Histologically, we classified the inflammatory activity of each specimen into the following categories: no inflammation, mild to moderate inflammation, or severe inflammation. We also confirmed that all of the samples were negative for neoplasia in accordance with the Riddell classification of gastrointestinal epithelial neoplasia (22).

**Combined bisulfite restriction analysis.** The methylation status of the ER promoter was analyzed quantitatively by the COBRA method as has been previously described (21). In brief, modified DNA was amplified using PCR, which was carried out using the following amplification profile: once for 10 minutes at 95°C, 30 seconds at 94°C, 1 minute at 55°C, 1 minute at 72°C for 40 cycles, and 10 minutes at 72°C. The primers: 5'-GGTTTTTGAGTTTTTTGTTTTG-3' (forward primer) and 5'-AAGTTTACTATCCAAATACCTC-3' (reverse primer) were used to amplify a 206-bp fragment of the ER promoter. After amplification, the PCR products were digested with restriction enzyme BstU1 at 60°C for 4 hours. All restriction products were visualized by 6% PAGE followed by staining with ethidium bromide, and the methylation-specific bands were quantified as methylation levels by densitometry using the Kodak

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**Table 1. Clinicopathologic features**

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*M, male; F, female.
†total, total colitis; left side, left-sided colitis.
*HGD, high-grade dysplasia; Ca, invasive carcinoma.
*IR, rectum; S, sigmoid colon; T, transverse colon; blanks, not applicable.
Digital Science one-dimensional image analysis software (Eastman Kodak Company, Rochester, NY).

**Statistical analysis.** The age, disease duration, and methylation level of UC patients with and without neoplasia were expressed as mean ± SD, and the differences between groups were analyzed using Welch’s t test; differences at $P < 0.05$ were considered significant. Welch’s t tests were used to compare the inflammatory activity of colitis and the methylation level of the ER gene, and to determine the difference of the methylation level by colorectal region, differences at $P < 0.05$ were considered significant.

**Results**

**Clinicopathologic features.** The clinicopathologic features of the 18 patients with UC are shown in Table 1. The mean age of the UC patients with neoplasia group was 55.3 ± 13.9 years (range, 45-74 years), whereas in the UC patients without neoplasia group, this was 48.1 ± 10.1 years (range, 33-69 years). On the other hand, the mean duration of disease in the UC patients with neoplasia group was 16.9 ± 5.3 years (range, 7-22 years), whereas in the UC patients without neoplasia group, it was 17.9 ± 7.4 years (range, 10-31 years). There were no significant differences in age or disease duration between UC patients with and without neoplasia. In eight patients with neoplasia, six patients had resections due to cancer-related complications, and two patients had resections due to dysplasia progression.

**Methylation analysis of estrogen receptor gene using combined bisulfite restriction analysis.** Methylation levels of the ER gene in all samples are shown in Table 2, and examples of electrophoresis are shown in Fig. 1. A total of 105 samples were available for COBRA analysis. In some regions, due to the lack of an available tissue specimen, methylation analysis could not be done. The mean (±SD) methylation level of the ER gene was 25.4 ± 17.8% in the nonneoplastic epithelia from UC patients with neoplasia, whereas it was only 4.0 ± 6.4% in patients without neoplasia. Of note, the mean of the ER methylation in the nonneoplastic epithelium was higher in patients with neoplasia than in patients without neoplasia ($P < 0.001$). The ER gene was highly methylated in both nonneoplastic epithelia from UC patients with carcinoma and dysplasia.

The mean (±SD) of the ER gene methylation level in each region of the colorectum is shown in Fig. 2. In UC patients with neoplasia, the ER gene showed increased methylation levels over a widespread area of nonneoplastic colorectal epithelium, and the ER gene methylation in the UC patients with neoplasia was significantly higher than in the UC patients without neoplasia in all regions throughout the colorectum except for the cecum (rectum, 42.2 ± 16.9% versus 6.9 ± 8.9%, $P < 0.001$; sigmoid colon, 33.2 ± 13.6% versus 3.8 ± 4.9%, $P < 0.001$; descending colon, 32.4 ± 14.1% versus 5.4 ± 7.5%, $P < 0.001$; transverse colon, 15.0 ± 9.6% versus 3.6 ± 7.8%, $P < 0.05$; ascending colon, 15.2 ± 11.3% versus 1.7 ± 2.6%, $P < 0.05$; cecum, 13.6 ± 20.4% versus 2.3 ± 3.3%, not significant).

In UC patients with neoplasia, the mean of the ER gene methylation level in the distal colon was higher than in the proximal colon: 36.1 ± 15.1% of ER methylation in the distal colon versus 14.6 ± 13.6% in the proximal colon ($P < 0.001$). In UC patients without neoplasia, there was no significant difference in the ER gene methylation levels between the proximal and the distal colon (Fig. 3).

<table>
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*R, rectum; S, sigmoid colon; D, descending colon; T, transverse colon; A, ascending colon; C, cecum; blanks, not done.

**The relationship between estrogen receptor gene methylation and inflammatory activity of colitis.** An examination of the inflammatory activity in the 46 samples from UC patients with neoplasia revealed that 16 (34.8%) had no inflammation, 18 (39.1%) had mild inflammation, 7 (15.2%) had moderate inflammation, and 5 (10.9%) had severe inflammation. Examining inflammatory activity in the 59 samples from UC patients without neoplasia revealed that 16 (34.8%) had no inflammation, 27 (45.8%) had mild inflammation, 14 (23.7%) had moderate inflammation, and 9 (15.3%) had severe inflammation. There were no significant differences in inflammatory activity between patients with and without neoplasia.

The relationship between ER gene methylation status and the inflammatory activity of colitis is shown in Table 3. There were no significant differences between ER gene methylation and inflammatory activity in UC patients with or without neoplasia.

**Discussion**

In this study, we have shown that the ER gene methylation level in nonneoplastic epithelium is higher in UC patients with neoplasia than in UC patients without neoplasia. In UC patients with neoplasia, high ER gene methylation levels were detected not only in regions in which neoplasia was located, but also in other regions widely scattered throughout the colorectum. Furthermore, these epigenetic alterations occurred in both nonneoplastic epithelia of UC patients with carcinoma and UC patients with dysplasia. These results suggest that an increase in age-related methylation may precede or be a relatively early event in UC-associated carcinogenesis. Therefore,

**Table 2. Percentage of ER methylation in all samples**
an analysis of ER gene methylation in the nonneoplastic colorectal epithelium of UC patients may have the potential of being a useful adjunct for identifying individuals at an increased risk of neoplasia among patients with longstanding and extensive UC, and contribute to more effective cancer surveillance.

Since the association between UC and colorectal neoplasia was first reported in 1925 (24), it has been confirmed in many studies from several countries. A recent meta-analysis reviewed 116 studies of UC-associated colorectal cancer and found that the prevalence of colorectal cancer in patients with UC was 2% at 10 years, 8% at 20 years, and 18% at 30 years (25). In order to detect neoplasia at a surgically curative stage, and preferably in the preinvasive stage, periodic colonoscopy combined with extensive biopsy sampling throughout the colorectum is widely recommended as a surveillance program for UC patients with longstanding and extensive colitis (9–12). However, according to several studies that have analyzed the efficacy of surveillance, an appreciable number of cancers are detected at an advanced stage despite colonoscopic surveillance, and many of these cases have less-than-ideal outcomes (13–18). Thus, it remains questionable whether surveillance colonoscopy with multistep biopsy effectively enables the early detection of UC-associated neoplasia.

The unsatisfactory efficacy of current surveillance colonoscopy for the early detection of UC-associated neoplasia can be attributed to difficulties in the endoscopic and histologic diagnosis of UC-associated neoplasia at an early stage. In order to overcome these difficulties, adjunctive diagnostic modalities such as chromoendoscopy that identifies neoplasia in nonneoplastic inflamed epithelium and analysis for the p53 alteration that distinguishes neoplastic lesions from regenerative epithelium have been reported. Endoscopically, Kiesslich et al. (26) have reported that methylene blue aided chromoendoscopy in UC surveillance, and detected about three times more dysplastic lesions than with conventional colonoscopy in a randomized trial. Subsequently, several reports dealing with the diagnosis of UC-associated neoplasia using chromoendoscopy and magnifying colonoscopy have been published (27–29). Histologically, the usefulness of p53 alteration analysis for distinguishing neoplastic lesions from regenerative epithelium has also been reported (30–34). However, it would be unrealistic to perform these adjunctive modalities for the surveillance of all UC patients with longstanding and extensive colitis because of their labor-intensive nature and expense. If the identification of the high- and low-risk subgroups of UC patients with longstanding and extensive colitis were possible, it would enable physicians to conduct more intensive surveillance using these modalities, chromoendoscopy and analysis of p53 alteration, in patients at higher risk of developing colorectal neoplasia.

Recently, numerous studies have revealed higher frequencies of molecular alterations in the nonneoplastic epithelium of UC patients with neoplasia than in the nonneoplastic epithelium of UC patients without neoplasia. Thus, these molecular alterations may be useful as new markers for identifying individuals...

**Fig. 1.** COBRA for the ER gene in each region of the colorectum. N, the unmethylated breast cancer cell line MCF-7; P, the methylated colon cancer cell line DLD-1; R, rectum; S, sigmoid colon; D, descending colon; T, transverse colon; A, ascending colon; C, cecum. A, representative samples of nonneoplastic epithelia from patient with neoplasia (case 5). B, representative samples of nonneoplastic epithelia from patient without neoplasia (case 18).

**Fig. 2.** The average of ER methylation in nonneoplastic epithelium from UC patients with and without neoplasia in each region of the colorectum. In the rectum, sigmoid colon, descending colon, transverse colon, and ascending colon, the average ER methylation in UC patients with neoplasia was significantly higher than in those without neoplasia. *, P < 0.01; **, P < 0.05; ***, not significant. R, rectum; S, sigmoid colon; D, descending colon; T, transverse colon; A, ascending colon; C, cecum.
with UC at increased risk of neoplasia (30, 33, 35–38). In the present study, we analyzed age-related methylation of the ER gene in nonneoplastic epithelium throughout the entire colorectum of patients with longstanding and extensive UC for the purpose of evaluating the usefulness of this new marker. Age-related methylation has been shown for several genes, including ER, insulin-like growth factor II, MYOD, N33, and E-cadherin in the colorectal epithelium, and patients with a high level of methylation in their colorectal epithelium may be at higher risk of developing sporadic colorectal neoplasia (19, 20, 39). In addition, age-related methylation in neoplastic or nonneoplastic tissue has been reported in many types of organs, such as ER in the prostate, E-cadherin in the bladder, ER and N33 in the brain, human telomerase reverse transcriptase in the ovary, and DAP-kinase and E-cadherin in the stomach (40–44). Furthermore, these previous studies showed that age-related methylation is an early event in tumorigenesis, and higher levels of age-related methylation would be expected to indicate an increased risk of tumor formation.

In UC-associated neoplasia, Issa et al. (45) first reported that the ER, MYOD, CSPG2 genes, and the p16 gene exon 1, all of which exhibit similar behavior to the age-related methylation in colorectal epithelium, were intensively methylated in neoplastic epithelium from high-grade dysplasia/cancer patients with UC. Furthermore, they showed that these genes were highly methylated in nonneoplastic epithelium from UC patients with high-grade dysplasia/cancer compared with nonneoplastic epithelium from UC patients without dysplasia. Therefore, they suggested that age-related methylation could be used as a molecular marker for identifying UC patients at increased risk of developing neoplasia. However, they made no mention of methylation status by region in the colorectum.

In our present study, we quantitatively analyzed ER gene methylation in multiple samples taken from six regions (rectum, sigmoid colon, descending colon, transverse colon, ascending colon, and cecum) throughout the colorectum. We found that ER methylation levels in nonneoplastic epithelia from UC patients with neoplasia were higher than in UC patients without neoplasia. Furthermore, the ER gene was highly methylated in samples of nonneoplastic epithelium throughout the colorectum except for the cecum in UC patients with neoplasia as compared with UC patients without neoplasia. From these findings, it is expected that an analysis of a single biopsy sample, such as a rectal biopsy, may have the possibility of identifying UC patients at particularly high risk of developing neoplasia, in contrast to the large number of biopsy samples currently needed with surveillance colonoscopy.

Considering that several genes such as MYOD, CSPG2 genes, and p16 gene exon 1 have been reported to belong to a group of age-related genes, we may have obtained similar results if we analyzed other age-related methylation genes (45). Thus, similar to ER methylation, age-related methylation in other genes may provide an attractive biomarker for the identification of patients at high risk for the development of colorectal neoplasia, in other words, age-related methylation itself may be more important for the biomarker. Further study on the methylation of other genes in nonneoplastic epithelium throughout the colorectum from UC patients would be needed to evaluate the advantage of other genes as biomarkers.

Recent reports have suggested that UC-associated neoplasia arises from the distal to the transverse colon in 73% to 87% of cases (46, 47). In our study, seven of the eight neoplastic lesions were located in the distal colon. If a higher level of ER gene methylation in nonneoplastic epithelium predisposes to an increased risk of future neoplastic progression, one would expect that such epigenetic alteration would be prone to occur in the distal colon. In patients with UC-associated neoplasia, we showed that there was a significantly higher level of ER gene methylation in the nonneoplastic epithelium of the distal colon compared with the level seen in the proximal colon. On the other hand, in UC patients without neoplasia, the methylation level was low throughout the colorectum. These findings are consistent with the expectancy made above.

Although the mechanism of age-related methylation is not well known, several factors may contribute to this epigenetic alteration, including exogenous carcinogens, reactive oxygen species, and host genetic differences. The chronic inflammation seen in UC would be associated with an elevated level of exogenous carcinogens and reactive oxygen species, and UC

### Table 3. Relationship between the percentage of ER methylation and inflammatory activity of colitis

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<td>22.6 ± 15.4 (n = 16)</td>
</tr>
<tr>
<td>UC without neoplasia</td>
<td>2.9 ± 2.7 (n = 9)</td>
</tr>
</tbody>
</table>
colonocytes would be subject to a high level of epigenetic damage. In the present study, the inflammatory activity of colitides did not have any relationship with the level of ER gene methylation, suggesting that ER gene methylation does not simply reflect transient inflammation, and that cumulative and repetitive inflammation may lead to the progression of methylation.

In this preliminary study, due to the small sample size (only 18 samples), it was not possible to accurately determine the precise level of ER gene methylation that would identify UC patients at high risk for neoplasia. However, our results show that further longitudinal studies of ER methylation are needed to explore its potential as a molecular biomarker and its clinical utility in the surveillance of UC patients. It is anticipated that the analysis of ER gene methylation using rectal biopsy specimens may make it possible to identify UC patients at high risk for having neoplasia elsewhere in the colorectum or future neoplastic progression.

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References


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Keiichi Tominaga, Shigehiko Fujii, Kenichiroh Mukawa, et al.


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