Increased Expression of DNA Methyltransferase-1 in Non-Neoplastic Epithelium Helps Predict Colorectal Neoplasia Risk in Ulcerative Colitis

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Key Words

Ulcerative colitis · Colorectal neoplasia · DNA methyltransferase · Molecular marker

Abstract

Aims: To clarify the possible significance of increased expression of DNA methyltransferase (DNMT)-1 in the tumorigenesis of colorectal neoplasia in ulcerative colitis (UC) and to clarify whether analysis of DNMT1 expression in non-neoplastic epithelium can contribute to the prediction of increased risk for UC-associated neoplasia. **Methods:** Sixty-two patients with long-standing and extensive UC were included in this study: 31 with colorectal neoplasia (dysplasia in 11 and invasive cancer in 20) and 31 without. Immunohistochemical analysis and quantitative RT-PCR were performed to determine the expression of DNMT1 in rectal epithelium of UC patients without neoplasia, and in non-neoplastic rectal epithelium and colorectal neoplasia of UC patients with neoplasia. **Results:** The immunoreactive DNMT1 expression gradually increased from rectal epithelium of UC patients without neoplasia (0.13 ± 0.07) to non-neoplastic rectal epithelium of UC patients with neoplasia (0.32 ± 0.12 , $p < 0.001$), and to colorectal neoplasia (0.54 ± 0.20 , $p < 0.001$). Among 31 neoplasias, there was no difference in the immunoreactive DNMT1 expressions between

dysplasia and invasive cancer (0.47 ± 0.52 vs. 0.58 ± 0.63). The expression level of DNMT1 mRNA tended to increase gradually from rectal epithelium of UC patients without neoplasia (0.53 ± 0.34) to non-neoplastic rectal epithelium of UC patients with neoplasia (0.88 ± 0.57 , $p = 0.06$), and to colorectal neoplasia (1.38 ± 0.64 , $p = 0.07$). **Conclusion:** Increased expression of DNMT1 in non-neoplastic epithelium may precede or be a relatively early event in UC-associated tumorigenesis, and may help predict the risk of colorectal neoplasia in UC.

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Introduction

Patients with ulcerative colitis (UC) show an increased incidence of colorectal neoplasia, and UC-associated colorectal neoplasia represents a major cause of increased mortality in such patients [1, 2]. The risk of UC-associated colorectal neoplasia increases with disease duration and extent [1, 3]. Therefore, surveillance colonoscopy with multiple-step biopsies has been widely recommended for patients with long-standing and extensive UC in

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order to diagnose UC-associated neoplasia at an early or precancerous stage [4, 5]. However, because UC-associated neoplasia is often difficult to detect endoscopically and to discriminate from inflammatory regenerative epithelium histologically, it remains a matter of contention whether conventional surveillance colonoscopy is effective for early detection [6–8]. In order to improve the efficacy of surveillance, there is an urgent need for sensitive and specific markers to identify individuals at increased risk of neoplasia among patients with long-standing and extensive UC.

Neoplastic progression in UC occurs in a histologically stepwise manner, from chronic epithelial inflammation to dysplasia, and to carcinoma, and the process of neoplastic progression involves accumulation of genetic and epigenetic alterations [9, 10]. Some of these alterations are known to occur in both the neoplastic and non-neoplastic epithelium of UC patients with neoplasia, and are considered to be widespread and to occur early in the process of neoplastic progression, suggesting that they could be applicable as markers for identifying patients who are likely to have, or to develop, neoplasia [11–21].

In several types of neoplasia, aberrant methylation of promoter-region CpG islands, as an epigenetic modification of DNA, is associated with transcriptional inactivation of tumor suppressor genes and plays a crucial role in the development and progression of neoplasia. DNA methylation results from a methyl transfer reaction performed by the three active DNA methyltransferases (DNMTs): DNMT1, DNMT3a and DNMT3b [22, 23]. Of these, DNMT1 is the most abundant DNMT targeted to replication foci and has a preference for hemimethylated DNA substrates [24]. It seems to be the main enzyme responsible for maintaining the methylation pattern after each round of DNA replication. Recent investigations have shown that DNMT1 is overexpressed in tumorigenic cells and several types of human tumors, and that increased expression of DNMT1 is dependent on cell proliferation [25–29]. However, to our knowledge, there are no reported data on DNMT1 expression in inflamed epithelium and neoplasia of UC.

In this study, to clarify the possible significance of increased expression of DNMT1 in the tumorigenesis of UC-associated neoplasia and to clarify whether analysis of DNMT1 expression in non-neoplastic epithelium can contribute to the prediction of increased risk for UC-associated neoplasia, we assessed the expression of DNMT1 in non-neoplastic rectal epithelium and colorectal neoplasia in patients with long-standing and extensive UC.

Materials and Methods

Patient Samples and Histological Evaluation

Sixty-two UC patients were included in this study: 31 (16 men, 15 women) with colorectal neoplasia and 31 (21 men, 10 women) without. The clinicopathological features of the UC patients are shown in table 1. All patients had long-standing (≥ 7 years) and extensive (proximal to the splenic flexure) UC. Of the 31 UC patients with neoplasia, 20 had the highest histological grade of invasive cancer, 10 had high-grade dysplasia, and 1 had low-grade dysplasia. All UC patients without neoplasia were diagnosed as neoplasia-free by endoscopic and histological assessments throughout periodic surveillance colonoscopy on the basis of multiple-step biopsy samples. The mean ($\pm$ SD) age of the UC patients with neoplasia was 49.9 ± 10.8 years (range 39–79), and that of UC patients without neoplasia was 46.9 ± 7.5 years (range 33–66). On the other hand, the mean ($\pm$ SD) duration of disease in the UC patients with neoplasia was 17.2 ± 6.3 years (range 7–33), and that in the UC patients without neoplasia was 18.1 ± 7.3 years (range 10–36). There were no significant differences in age, sex, disease duration, disease extent, medication for UC between UC patients with, and those without, neoplasia.

Thirty-one paired samples of colorectal neoplastic tissue and corresponding non-neoplastic rectal epithelium from the 31 UC patients with neoplasia were retrieved from total colectomy specimens. The rectal epithelium samples from the 31 UC patients without neoplasia were obtained from step biopsy specimens during surveillance colonoscopy. Histologically, we confirmed that all of the 62 non-neoplastic samples from UC patients with and without neoplasia were negative for neoplasia in accordance with the Riddell classification of gastrointestinal epithelial neoplasia [9]. We also classified the inflammatory activity of each specimen into three categories: mild, moderate and severe inflammation. Histological evaluations were confirmed by two experienced gastrointestinal pathologists. The Ethics Committee of Dokkyo University School of Medicine approved all protocols, and informed consent for tissue procurement was obtained from all patients.

Immunohistochemical Analysis of DNMT1 Protein

As described previously, immunohistochemical analysis was carried out with a goat anti-human polyclonal antibody for DNMT1 (N16, dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) in formalin-fixed paraffin-embedded tissue sections using an LSAB2 kit (Dako, Carpinteria, Calif., USA) [27, 30]. Finally, the immunoreactivity was visualized with 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 , followed by counterstaining with Carazzi's hematoxylin. In each specimen, lymphocytes and macrophages on the same slide were used as an internal positive control for DNMT1 immunoreactivity.

The immunoreactivity DNMT1 was assessed in areas showing the highest density of cells with positively staining nuclei. A minimum of 500 nuclei in selected areas was counted under $\times 300$ magnification. The level of immunoreactive DNMT1 expression was expressed as the percentage of positive cells relative to the total number of cells counted. Assessment of immunoreactive DNMT1 expression was done without knowledge of patient source.

Laser Capture Microdissection and RNA Extraction

Thirty-seven samples of fresh-frozen tissue were embedded in optimal cutting temperature (OCT) compound (Sakura Fine Technical, Tokyo, Japan) and stored at -80°C until study. These included 8 samples of neoplastic tissue, 15 samples of non-neoplastic epithelium from UC patients with neoplasia, and 14 samples of rectal epithelium from UC patients without neoplasia. Frozen sections $20\text{ }\mu\text{m}$ thick were cut in a cryostat, and then mounted on a clear polyethylene membrane attached to an aluminum frame slide (Molecular Machines & Industries AG, Zurich, Switzerland). To obtain only epithelial crypts for RNA extraction, laser capture microdissection was performed using a UV laser microdissection system (Molecular Machines & Industries AG) and the dissected epithelium samples were each collected on a plastic cap. The caps were placed in a microcentrifuge tube, and the RNA was extracted using an RNeasy Micro kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol.

Quantitative RT-PCR of DNMT1 mRNA

Total RNA was reverse transcribed using an oligo-dT primer (Applied Biosystems, Foster City, Calif., USA), and quantitative real-time PCR of DNMT1 and an internal reference gene (β -actin) was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each amplification was done in $25\text{ }\mu\text{l}$ of a reaction mixture containing forward and reverse primer ($5\text{ }\mu\text{mol/l}$ each), cDNA ($2.5\text{ }\mu\text{l}$) and $2\times$ SYBR Premix Ex TaqII (Takara, Shiga, Japan). Forward primers $5'$ -GGCCTTGCCGCC-TCATA- $3'$ and $5'$ -GGCCTTGCCGCCTCATA- $3'$ and reverse primers $5'$ -GGCCTTGCCGCCTCATA- $3'$ and $5'$ -GGCCTTGCCGCCTCATA- $3'$ were used for DNMT1 and β -actin, respectively. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or in a different exon. The PCR cycling conditions were 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The Human Universal Reference total RNA (Clontech, Palo Alto, Calif., USA) was used to construct a standard curve. Five serial 10-fold dilutions of the reference RNA were amplified to create standard curves for DNMT1 and β -actin. The relative amount of the mRNA was determined using the ratio of the target gene concentration relative to that of β -actin. The real-time PCR reactions were performed in triplicate for each sample-primer set, and the average value of triplicate determinations was used as the relative mRNA expression level in each sample.

Statistical Analysis

Age, disease duration, and expression levels of DNMT1 were expressed as mean $\pm$ SD, and the differences were analyzed using the t test. The correlation analyses were assessed using Pearson's correlation coefficient. For all tests, differences of $p < 0.05$ were considered statistically significant, and all p values were two-sided.

Results

Immunoreactivity of DNMT1

The immunoreactivities of DNMT1 in samples of rectal epithelium from UC patients without neoplasia, non-neoplastic rectal epithelium from UC patients with neo-

Table 1. Clinicopathological features of the patients

	UC with neoplasia (n = 31)	UC without neoplasia (n = 31)	
Mean age at study, years	49.9 ± 10.8	46.9 ± 7.5	NS
Male/female	16/15	21/10	NS
Mean disease duration of UC, years	17.2 ± 6.3	18.1 ± 7.3	NS
Disease extent (total/left)	27/4	24/7	NS
Medication for UC			NS
SASP/5-ASA	20	18	
Prednisolone	9	8	
Immunosuppressive drug	2	5	
Location of neoplasia			
Rectum	20		
Left colon	5		
Right colon	6		
Histology of neoplasia			
LGD	1		
HGD	10		
Carcinoma ^a	20		
Grade 1	13		
Grade 2	3		
Grade 3	4		
UICC stage			
UICC I	10		
UICC II	1		
UICC III	9		
UICC IV	0		

Total = Total colitis; left = left-sided colitis. SASP = salicylazopyridine; 5-ASA = 5-aminosalicylic acid. LGD = low-grade dysplasia; HGD = high-grade dysplasia.

^aHistological grading and staging of carcinoma were done according to UICC (Union International Against Cancer) and WHO criteria.

plasia, and colorectal neoplastic tissues are shown in figure 1a. Examples of immunohistochemical staining for DNMT1 are shown in figure 2. The mean ($\pm$ SD) levels of immunoreactive DNMT1 expression were 0.13 ± 0.07 in rectal epithelium from UC patients without neoplasia, 0.32 ± 0.12 in non-neoplastic rectal epithelium from UC patients with neoplasia, and 0.54 ± 0.20 in colorectal neoplastic tissues. The immunoreactive DNMT1 expressions increased gradually from rectal epithelium of UC patients without neoplasia to non-neoplastic rectal epithelium of UC patients with neoplasia ($p < 0.001$ compared with rectal epithelium from UC patients without neoplasia), and to colorectal neoplastic tissues ($p < 0.001$ compared with rectal epithelium from UC patients without neoplasia, $p < 0.001$ compared with non-neoplastic rectal epithelium from UC patients with neoplasia).

Fig. 1. a Immunoreactive DNMT1 expressions in rectal epithelium from UC patients without neoplasia (UC without neoplasia), non-neoplastic rectal epithelium from UC patients with neoplasia (UC with neoplasia), and colorectal neoplastic tissues (neoplasia). The levels of immunoreactive DNMT1 expression increased gradually from UC without neoplasia, to UC with neoplasia, and to neoplasia. **b** DNMT1 mRNA expression in rectal epithelium from UC patients without neoplasia (UC without neoplasia), non-neoplastic rectal epithelium from UC patients with neoplasia (UC with neoplasia), and colorectal neoplasia (neoplasia). The expression level of DNMT1 mRNA in neoplasia was significantly higher than that in UC without neoplasia. The expression level of DNMT1 mRNA tended to increase gradually from UC without neoplasia to UC with neoplasia, and to neoplasia. Paired samples are joined by straight lines.

Fig. 2. Immunohistochemical staining for DNMT1 protein in rectal epithelium from UC patients without neoplasia (a), non-neoplastic rectal epithelium from UC patients with neoplasia (b) and colorectal neoplasia (c). $\times 300$.

Among the 31 samples of non-neoplastic rectal epithelium from UC patients with neoplasia, there was no significant difference in the immunoreactive DNMT1 expressions between non-neoplastic rectal epithelium from UC patients with dysplasia and those with invasive cancer (0.33 ± 0.38 vs. 0.32 ± 0.34 , $p = 0.90$). Among the 31 samples of neoplasia including 11 dysplasias and 20 invasive cancers, the mean ($\pm$ SD) levels of immunoreactive DNMT1 expression were 0.47 ± 0.52 and 0.58 ± 0.63 , respectively. There was no statistically significant difference between them ($p = 0.63$).

Correlation of DNMT1 Immunoreactivity Level in Rectal Epithelium with Patient Age, Disease Duration and Inflammatory Activity of Colitis

In both UC with and without neoplasia, there were no significant correlations between the immunoreactive DNMT1 expression in rectal epithelium and patient age ($r = 0.315$, $p = 0.102$; $r = -0.027$, $p = 0.885$, respectively). Similarly, there were no significant correlations between the immunoreactive DNMT1 expression in rectal epithelium and disease duration ($r = 0.169$, $p = 0.390$; $r = 0.062$, $p = 0.741$, respectively).

The correlation between the immunoreactive DNMT1 expression and inflammatory activity of colitis was eval-

uated in the 62 samples of rectal epithelium from patients with and without neoplasia (38 mild, 16 moderate, 8 severe inflammation). The immunoreactive DNMT1 expression in rectal epithelium with mild, moderate, severe inflammation were 0.23 ± 0.13 , 0.20 ± 0.14 and 0.26 ± 0.17 , respectively. There were no significant differences in immunoreactive DNMT1 expressions among the different severities of inflammation.

DNMT1 mRNA Expression

The expression levels of DNMT1 mRNA in samples of rectal epithelium from UC patients without neoplasia, non-neoplastic rectal epithelium from UC patients with neoplasia, and colorectal neoplastic tissues are shown in figure 1b. The mean ($\pm$ SD) expression levels of DNMT1 mRNA were 0.53 ± 0.34 in rectal epithelium from UC patients without neoplasia, 0.88 ± 0.57 in non-neoplastic rectal epithelium from UC patients with neoplasia, and 1.38 ± 0.64 in colorectal neoplastic tissues. The expression level of DNMT1 mRNA in samples of colorectal neoplasia was significantly higher than that in rectal epithelium from UC patients without neoplasia ($p < 0.001$). Although the difference was not statistically significant, the expression level of DNMT1 mRNA tended to increase gradually from rectal epithelium of UC patients without neoplasia to non-neoplastic rectal epithelium of UC patients with neoplasia ($p = 0.06$ compared with rectal epithelium from UC patients without neoplasia), and to colorectal neoplasia ($p = 0.07$ compared with non-neoplastic rectal epithelium from UC patients with neoplasia).

Correlation of Immunoreactivity with mRNA Expression of DNMT1

As shown in figure 3, when the immunoreactive and mRNA expressions were compared on an individual basis, immunoreactive DNMT1 expression correlated well with mRNA expression from the same tissue ($r = 0.576$, $p < 0.001$).

Discussion

In this study, we have shown that expression of DNMT1 increased with progression from rectal epithelium of UC patients without neoplasia to non-neoplastic rectal epithelium of UC patients with neoplasia, and then to UC-associated colorectal neoplasia. Furthermore, the immunoreactive expression of DNMT1 in dysplasia was not significantly different from that in invasive cancer.

Fig. 3. DNMT1 mRNA expression were significantly correlated with immunoreactive DNMT1 expression in the same tissue. DNMT1 expression in rectal epithelium from UC patients without neoplasia, non-neoplastic rectal epithelium from UC patients with neoplasia, and colorectal neoplasia is shown by circle, triangle and square symbols, respectively.

These results suggest that an increase in the expression of DNMT1 may precede, or be a relatively early event in UC-associated tumorigenesis, and that analysis of DNMT1 expression in non-neoplastic rectal epithelium may be of potential use for identifying individuals at increased risk of neoplasia among patients with long-standing and extensive UC.

In order to detect neoplasia at a surgically curative and preferably preinvasive stage, periodic colonoscopy combined with extensive biopsy sampling throughout the colorectum is recommended [4, 5]. However, according to several studies that have assessed the efficacy of surveillance, an appreciable number of neoplasia were detected at an advanced stage despite surveillance colonoscopy, and many of these cases had less-than-ideal outcomes [6–8]. Thus, it still remains questionable whether surveillance colonoscopy with multiple-step biopsy effectively enables early detection of UC-associated neoplasia.

The unsatisfactory efficacy of current surveillance colonoscopy for early detection of UC-associated neoplasia can be attributed to difficulties in endoscopic and histological diagnosis of UC-associated neoplasia at an early stage. Endoscopically, early-stage UC-associated

neoplasias show various macroscopic changes. These changes are not clear, and are sometimes missed in areas of chronically inflamed epithelium [31]. Therefore, detection of UC-associated neoplasias at the precancerous and early stages is difficult by endoscopy, and physicians have no choice but to depend on histological evaluation of multiple-step biopsies obtained by surveillance colonoscopy. Histologically, similarly to sporadic colorectal neoplasia, diagnosis of UC-associated neoplasia is based on a combination of architectural and cytological alterations. However, because these alterations are often unremarkable and are limited to the lower half of the crypt or a few crypts in the inflamed epithelium, it is not unusual for pathologists to experience difficulty in discriminating between a neoplastic lesion and inflammatory regenerative epithelium using biopsy specimens stained with hematoxylin and eosin [9]. Furthermore, different pathologists may use different diagnostic criteria for determining UC-associated neoplasia [32].

In order to overcome such difficulties, adjunctive diagnostic modalities such as chromoendoscopy and narrow-band imaging endoscopy for identifying neoplasia in chronically inflamed epithelium, and analysis of p53 alteration for distinguishing neoplastic lesions from regenerative epithelium have been reported [30, 33–37]. However, it would be impractical to perform these adjunctive modalities for surveillance of all UC patients with long-standing and extensive colitis. If refinement of high- and low-neoplasia risk subgroups of UC patients with long-standing and extensive colitis were possible, it would enable physicians to conduct more intensive surveillance using the adjunctive modalities, chromoendoscopy and narrow-band imaging endoscopy and analysis of p53 alteration, for patients at higher risk of developing colorectal neoplasia, and to improve the effectiveness of surveillance colonoscopy.

Numerous studies have revealed molecular alterations (e.g. p53 mutation, chromosomal instability, p16 hypermethylation, p14 hypermethylation, microsatellite instability, age-related methylation, telomere length shortening, mitochondrial DNA mutation, gene expression using DNA microarray) of non-neoplastic epithelium in UC patients with neoplasia [11–19]. Several of these studies have indicated higher frequencies of molecular alterations in non-neoplastic epithelium of UC patients with neoplasia than in that of UC patients without neoplasia, suggesting that these molecular alterations may be applicable as new markers for identifying individuals with UC at increased risk of neoplasia.

Recently, we evaluated the methylation status of the estrogen receptor (ER), which is affected by age-related methylation in the colorectal epithelium, in samples of non-neoplastic colorectal epithelium from UC patients with and without neoplasia, and we demonstrated that the ER gene was highly methylated in non-neoplastic epithelium from UC with neoplasia compared to that without neoplasia [20, 21]. In addition, methylation of the ER gene was detected not only in regions of neoplasia, but also in other regions widely scattered throughout the colorectum. These findings implied that analysis of ER gene methylation in non-neoplastic colorectal epithelium of UC patients may be a useful molecular marker for helping predict the risk of colorectal neoplasia. However, analysis of methylation involves a great deal of time and expense, and would be difficult to put to practical use as a molecular marker. Therefore, a new molecular marker that is readily available in clinical laboratories should be established.

Although conflicting results between the DNA methylation status of specific genes and DNMT1 expression have been described in the literature, increased expression of DNMT1 potentially results in hypermethylation of CpG islands [25–28]. In the present study, therefore, we investigated whether analysis of DNMT1 expression in non-neoplastic epithelium is a candidate molecular marker for predicting increased risk of UC-associated neoplasia, similarly to methylation of the ER gene. The most noteworthy result of this study was that the immunoreactive DNMT1 expression in non-neoplastic rectal epithelium from UC patients with neoplasia was significantly higher than that in rectal epithelium of UC patients without neoplasia, even though there was no remarkable difference histologically between the two types of epithelium. Similarly, although the difference determined by quantitative RT-PCR assay did not reach statistical significance, the expression level of DNMT1 mRNA tended to be higher in non-neoplastic rectal epithelium from UC patients with neoplasia than in rectal epithelium from UC patients without neoplasia. These results imply that the increased expression of DNMT1 in non-neoplastic rectal epithelium may be indicative of concurrent or future neoplasia somewhere in colorectum.

Although the mechanism responsible for increased expression of DNMT1 in UC is not well known, the previous reports that DNMT1 protein overexpression was significantly associated with the PCNA-labeling index in bladder cancer suggests that the expressions of DNMT1 immunoreactivity and mRNA we observed in UC may be attributable to increased cell proliferation [38]. In addi-

tion, previous studies have demonstrated increased DNMT1 expression in non-neoplastic liver tissue with chronic hepatitis or liver cirrhosis and non-neoplastic pancreatic tissue with chronic pancreatitis, suggesting that the increase in expression may be related to chronic inflammation [27, 29]. In the present study, the inflammatory activity of colitis was unrelated to the immunoreactive DNMT1 expression, suggesting that such expression does not simply reflect transient inflammation, and that cumulative and repetitive inflammation may lead to an increase of DNMT1 expression.

Because immunostaining using formalin-fixed, paraffin-embedded sections from the biopsy specimens used for routine histopathological diagnosis is simple, reliable, and reproducible, immunohistochemical analysis of DNMT1 may be sufficiently acceptable as a routine procedure. Therefore, DNMT1 expression would become a readily available molecular marker for identifying UC patients at high risk of neoplasia elsewhere in the colorectum or future neoplastic progression. In this preliminary study, due to the small sample size, it was not possible to determine with accuracy the precise expression level of DNMT1 that would identify UC patients at high risk for neoplasia. Although further longitudinal studies with large numbers of subjects are needed to clarify the role of DNMT1 in the pathogenesis of UC-associated neoplasia, and to validate the predictive power and clinical utility of

analysis of DNMT1 expression, these preliminary results could allow, on the basis of DNMT1 expression status, for the adjustment of a patient's surveillance interval and to select UC patients who should undergo intensive surveillance.

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Disclosure Statement

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References

- Ekblom A, Helmick C, Zack M, Adami HO: Ulcerative colitis and colorectal cancer. A population-based study. *N Engl J Med* 1990; 323:1228–1233.
- Langholz E, Munkholm P, Davidsen M, Blinder V: Colorectal cancer risk and mortality in patients with ulcerative colitis. *Gastroenterology* 1992;103:1444–1451.
- Eaden JA, Abrams KR, Mayberry JF: The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001;48:526–535.
- Kornbluth A, Sachar DB: Practice Parameters Committee of the American College of Gastroenterology: ulcerative colitis practice guidelines in adults (update): American College of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol* 2004; 99:1371–1385.
- Carter MJ, Lobo AJ, Travis SP: Guidelines for the management of inflammatory bowel disease in adults. *Gut* 2004;53(suppl 5):V1–V16.
- Lynch DA, Lobo AJ, Sobala GM, Dixon MF, Axon AT: Failure of colonoscopic surveillance in ulcerative colitis. *Gut* 1993;34:1075–1080.
- Karlén P, Kornfeld D, Broström O, Löfberg R, Persson PG, Ekblom A: Is colonoscopic surveillance reducing colorectal cancer mortality in ulcerative colitis? A population-based case control study. *Gut* 1998;42:711–714.
- Fujii S, Fujimori T, Chiba T, Terano A: Efficacy of surveillance and molecular markers for detection of ulcerative colitis-associated colorectal neoplasia. *J Gastroenterol* 2003; 38:1117–1125.
- Riddell RH, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC, Sommers SC, Yardley JH: Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. *Hum Pathol* 1983;14:931–968.
- Itzkowitz SH, Yio X: Inflammation and cancer. IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol* 2004;287:G7–G17.
- Lashner BA, Shapiro BD, Husain A, Goldblum JR: Evaluation of the usefulness of testing for p53 mutations in colorectal cancer surveillance for ulcerative colitis. *Am J Gastroenterol* 1999;94:456–462.
- Rabinovitch PS, Dziadon S, Brentnall TA, Emond MJ, Crispin DA, Haggitt RC, Bronner MP: Pancolonic chromosomal instability precedes dysplasia and cancer in ulcerative colitis. *Cancer Res* 1999;59:5148–5153.
- Hsieh CJ, Klump B, Holzmann K, Borchard F, Gregor M, Porschen R: Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. *Cancer Res* 1998;58:3942–3945.
- Sato F, Harpaz N, Shibata D, Xu Y, Yin J, Mori Y, Zou TT, Wang S, Desai K, Leytin A, Selaru FM, Abraham JM, Meltzer SJ: Hypermethylation of the p14 (ARF) gene in ulcerative colitis-associated colorectal carcinogenesis. *Cancer Res* 2002;62:1148–1151.

- 15 Brentnall TA, Crispin DA, Bronner MP, Chorian SP, Hueffed M, Rabinovitch PS, Rubin CE, Haggitt RC, Boland CR: Microsatellite instability in non-neoplastic mucosa from patients with chronic ulcerative colitis. *Cancer Res* 1996;56:1237–1240.
- 16 Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA: Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res* 2001;61:3573–3577.
- 17 O'Sullivan JN, Bronner MP, Brentnall TA, Finley JC, Shen WT, Emerson S, Emond MJ, Gollahon KA, Moskovitz AH, Crispin DA, Potter JD, Rabinovitch PS: Chromosomal instability in ulcerative colitis is related to telomere shortening. *Nat Genet* 2002;32:280–284.
- 18 Watanabe T, Kobunai T, Toda E, Kanazawa T, Kazama Y, Tanaka J, Tanaka T, Yamamoto Y, Hata K, Kojima T, Yokoyama T, Konishi T, Okayama Y, Sugimoto Y, Oka T, Sasaki S, Ajioka Y, Muto T, Nagawa H: Gene expression signature and the prediction of ulcerative colitis-associated colorectal cancer by DNA microarray. *Clin Cancer Res* 2007;13:415–420.
- 19 Nishikawa M, Oshitani N, Matsumoto T, Nishigami T, Arakawa T, Inoue M: Accumulation of mitochondrial DNA mutation with colorectal carcinogenesis in ulcerative colitis. *Br J Cancer* 2005;93:331–337.
- 20 Fujii S, Tominaga K, Kitajima K, Takeda J, Kusaka T, Fujita M, Ichikawa K, Tomita S, Ohkura Y, Ono Y, Imura J, Chiba T, Fujimori T: Methylation of the oestrogen receptor gene in non-neoplastic epithelium as a marker of colorectal neoplasia risk in long-standing and extensive ulcerative colitis. *Gut* 2005;54:1287–1292.
- 21 Tominaga K, Fujii S, Mukawa K, Fujita M, Ichikawa K, Tomita S, Imai Y, Kanke K, Ono Y, Terano A, Hiraishi H, Fujimori T: Prediction of colorectal neoplasia by quantitative methylation analysis of estrogen receptor gene in non-neoplastic epithelium from patients with ulcerative colitis. *Clin Cancer Res* 2005;11:8880–8885.
- 22 Li E, Bestor TH, Jaenisch R: Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 1992;69:915–926.
- 23 Okano M, Bell DW, Haber DA, Li E: DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999;99:247–257.
- 24 Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, Jones PA: The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res* 1999;27:2291–2298.
- 25 Etoh T, Kanai Y, Ushijima S, Nakagawa T, Nakanishi Y, Sasako M, Kitano S, Hirohashi S: Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. *Am J Pathol* 2004;164:689–699.
- 26 Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW: CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res* 1999;59:2302–2306.
- 27 Peng DF, Kanai Y, Sawada M, Ushijima S, Hiraoka N, Kitazawa S, Hirohashi S: DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multi-stage carcinogenesis of the pancreas. *Carcinogenesis* 2006;27:1160–1168.
- 28 Kim H, Kwon YM, Kim JS, Han J, Shim YM, Park J, Kim DH: Elevated mRNA levels of DNA methyltransferase-1 as an independent prognostic factor in primary non-small cell lung cancer. *Cancer* 2006;107:1042–1049.
- 29 Sun L, Hui AM, Kanai Y, Sakamoto M, Hirohashi S: Increased DNA methyltransferase expression is associated with an early stage of human hepatocarcinogenesis. *Jpn J Cancer Res* 1997;88:1165–1170.
- 30 Fujii S, Fujimori T, Chiba T: Usefulness of analysis of p53 alteration and observation of surface microstructure for diagnosis of ulcerative colitis-associated colorectal neoplasia. *J Exp Clin Cancer Res* 2003;22:107–115.
- 31 Fujii S, Katsumata D, Fujimori T: Limits of diagnosis and molecular markers for early detection of ulcerative colitis-associated colorectal neoplasia. *Digestion* 2008;77(suppl 1):2–12.
- 32 Eaden J, Abrams K, McKay H, Denley H, Mayberry J: Inter-observer variation between general and specialist gastrointestinal pathologists when grading dysplasia in ulcerative colitis. *J Pathol* 2001;194:152–157.
- 33 Kiesslich R, Fritsch J, Holtmann M, Koehler HH, Stolte M, Kanzler S, Nafe B, Jung M, Galle PR, Neurath MF: Methylene blue-aided chromoendoscopy for the detection of intraepithelial neoplasia and colon cancer in ulcerative colitis. *Gastroenterology* 2003;124:880–888.
- 34 Rutter MD, Saunders BP, Schofield G, Forbes A, Price AB, Talbot IC: Pancolonic indigo carmine dye spraying for the detection of dysplasia in ulcerative colitis. *Gut* 2004;53:256–260.
- 35 East JE, Suzuki N, von Herbay A, Saunders BP: Narrow band imaging with magnification for dysplasia detection and pit pattern assessment in ulcerative colitis surveillance: a case with multiple dysplasia associated lesions or masses. *Gut* 2006;55:1432–1435.
- 36 Harpaz N, Peck AL, Yin J, Fiel I, Hontanosas M, Tong TR, Laurin JN, Abraham JM, Greenwald BD, Meltzer SJ: p53 protein expression in ulcerative colitis-associated colorectal dysplasia and carcinoma. *Hum Pathol* 1994;25:1069–1074.
- 37 Brentnall TA, Crispin DA, Rabinovitch PS, Haggitt RC, Rubin CE, Stevens AC, Burner GC: Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis. *Gastroenterology* 1994;107:369–378.
- 38 Nakagawa T, Kanai Y, Saito Y, Kitamura T, Kakizoe T, Hirohashi S: Increased DNA methyltransferase-1 protein expression in human transitional cell carcinoma of the bladder. *J Urol* 2003;170:2463–2466.