DNA methylation of colon mucosa in ulcerative colitis patients: correlation with inflammatory status

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Abstract

Background; Although DNA methylation of colonic mucosa in ulcerative colitis (UC) has been suggested, the majority of published reports indicate the correlation between methylation of colon mucosa and occurrence of UC-related dysplasia or cancer without considering the mucosal inflammatory status.

Aims; The aim of this study was to verify whether mucosal inflammation-specific DNA methylation occurs in the colon of UC.

Subjects and Methods; Of 15 gene loci initially screened, 6 loci (*ABCB1*, *CDH1*, *ESR1*, *GDNF*, *HPP1*, and *MYOD1*) methylated in colon mucosa of UC were analyzed according to inflammatory status using samples from 28 surgically resected UC patients.

Results; Four of six regions (*CDH1*, *GDNF*, *HPP1*, and *MYOD1*) were more highly methylated in the active inflamed mucosa than in the quiescent mucosa in each UC patient (p = 0.003, 0.0002, 0.02, and 0.048, respectively). In addition, when the methylation status of all samples taken from examined patients was stratified according to inflammatory status, methylation of *CDH1* and *GDNF* loci was significantly higher in active inflamed mucosa than in quiescent mucosa (p = 0.045 and 0.002, respectively). Multiple linear regression analysis revealed that active inflammation was an independent factor of methylation for *CDH1* and *GDNF*. DNA methyltransferase 1 and 3b were highly expressed in colon epithelial cells with active mucosal inflammation,

suggesting their involvement in inflammation-dependent methylation.

Conclusions; Methylation in colonic mucosa of UC was correlated with mucosal inflammatory status, suggesting the involvement of methylation due to chronic active inflammation in UC carcinogenesis.

Keywords: ulcerative colitis, inflammation, methylation

Introduction

DNA methylation is a powerful mechanism for the suppression of gene activity. The reciprocal relationship between the density of methylated cytosine residues and the transcriptional activity of a gene has been widely documented.[1] Gene hypermethylation, especially, is associated with the silencing of tumor suppressor genes in cancer and, therefore, many kinds of cancer are acquired by aberrant methylation of the CpG islands within promoter regions of gene loci.[2] Thus, DNA methylation is largely involved in the tumorigenesis of many kinds of cancer.

Chronic inflammation is also tightly associated with high levels of DNA methylation. Kanai et al. reported that DNA methylation alterations were involved in inflammation due to chronic hepatitis or liver cirrhosis resulting from infection with hepatitis B or C virus.[3] In patients with rheumatoid arthritis, CpG islands in the *DR3* gene promoter were specifically methylated to down-modulate the expression of *DR3* protein in rheumatoid synovial cells[4]. In the field of gastrointestinal tract, the correlation between methylation of gastric mucosa and inflammation caused by *Helicobacter pylori (H. pylori)* infection has particularly been highlighted. *H. pylori* infection potently induces aberrant DNA methylation in gastric mucosa, and methylation levels of a unique set of individual markers in gastric mucosa decreased after *H. pylori* eradication.[5]

Ulcerative colitis (UC) is a relapsing non-transmural inflammatory disease that is

restricted to the colon, with unknown etiology.[6] UC is characterized by mucosal ulceration, rectal bleeding, diarrhea, and abdominal pain. Also in UC, involvement of DNA methylation of colonic mucosa has been suggested. For example, it has been shown that the methylation level of the estrogen receptor 1 (*ESR1*) gene, which is known as an age-related methylation locus,[7] in nonneoplastic colorectal epithelium was higher in UC patients with neoplasia than in UC patients without neoplasia.[8] In addition, previous reports indicated that methylation of E-cadherin (*CDH1*) or hyperplastic polyposis protein 1 (*HPP1*) in colon mucosa of UC was an early event in UC-associated carcinogenesis.[9, 10] However, the majority of published reports correlate methylation of colonic mucosa with the occurrence of UC-related dysplasia or cancer without considering the inflammatory status of colonic mucosa. Chronic active inflammation is largely correlated with the occurrence of dysplasia or cancer in the field of UC as well as *H.pylori* associated gastritis.[5, 11] In this context, active inflammation in UC may be correlated with accumulation of methylation, resulting in susceptibility to carcinogenesis, as shown in gastric carcinogenesis correlated with *H.pylori*-induced methylation.

In this study, therefore, we investigated the methylation status of colon mucosa from UC patients who underwent colectomy, and correlated it with mucosal inflammatory status. The aim of this study was to verify whether DNA methylation that is specific to mucosal inflammation also occurs in the colonic mucosa of UC.

Material & Methods

Patients & tissue samples

Colonic mucosa from 28 patients with UC who underwent total proctocolectomy at Okayama University Hospital between July 2004 and March 2009 were collected and examined. Diagnosis of UC was ascertained according to the usual clinical criteria. The indications for surgery of the 28 cases were as follows: 23 medication-resistant, 1 toxic megacolon, 2 superveniences of other disease (1 appendicitis and 1 myelodysplastic syndrome), 1 fulminant disease with massive bleeding, and 1 side effect of corticosteroids. There were no cases with evidence of UC-associated dysplasia or cancer. Colorectal mucosal samples were collected from active inflamed mucosa in the distal portion (mainly sigmoid colon), and quiescent mucosa in the proximal portion (mainly ascending colon) of each patient. When available, samples from active mucosa in the proximal portion were also collected. In addition, peripheral blood buffy coat was collected from 15 of the 28 UC patients. These samples were stored at −80°C until analysis.

Clinical information about patients was obtained, including age, gender, disease duration, classification of disease as steroid-dependence or –refractory, and total amount of corticosteroids used. This study protocol was approved by the institutional review board of Okayama University Graduate School of Medicine, Dentistry, and

Pharmaceutical Sciences. All patients provided their informed consent.

Methylation analysis

The DNA in colonic tissue and peripheral blood buffy coat was extracted using the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Genomic DNA was modified by sodium bisulfite to convert unmethylated cytosines to uracil using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. The methylation status of bisulfite-converted DNA was determined by MethyLight, a fluorescence-based, real-time polymerase chain reaction (PCR) assay as described previously.[12-17]

The CpG loci examined were the promoter regions of glycoprotein/ATP binding cassette sub-family B member 1 (*ABCB1*), APEX nuclease (multifunctional DNA repair enzyme) 1 (*APEX*), *CDH1*, cyclin-dependent kinase inhibitor 2A (*CDKN2A, p16*), claudin 1 (*CLDN1*), β-catenin (*CTNNB1*), damage-specific DNA binding protein 1 (*DDB1*), *ESR1*, glial cell derived neurotrophic factor (*GDNF*), O-6-methylguanine-DNA methyltransferase (*MGMT*), myogenic differentiation 1 (*MYOD1*), *HPP1*, mut L homolog 1 (*MLH1*), SMAD family member 2 (*SMAD2*), and suppressor of cytokine signaling 1 (*SOCS1*). The promoter region of β-actin (*ACTB*) was also analyzed to normalize for input DNA. For the analysis of MethyLight, primers and probes, which generally span 7 to 10 CpG dinucleotides, were specifically designed to bind to bisulfite-converted DNA of these gene loci. The primer and probe sequences

for 6 genes that exhibited positive methylation results in the primary screening experiments are shown as follows (the forward PCR primer, TaqMan probe, and the reverse PCR primer, respectively):

ABCB1 (TCGGGTCGGGAGTAGTTATTTG, 6FAM5'-ACGCTATTCCTACCCAACCAATCAACCTCA-3'TAMRA, CGACTATACTCAACCCACGCC); *ACTB* (TGGTGATGGAGGAGGTTTAGTAAGT, 6FAM5'-ACCACCACCCAACACACAATAACAAACACA-3'TAMRA, AACCAATAAAACCTACTCCTCCCTTAA) *CDH1* (AGGGTTATCGCGTTTATGCG, 6FAM5'-ACTAACGACCCGCCCACCCGA-3'TAMRA, TTCACCTACCGACCACAACCA); *ESR1* (GGCGTTCGTTTTGGGATTG, 6FAM5'-CGATAAAACCGAACGACCCGACGA-3'TAMRA, GCCGACACGCGAACTCTAA); *GDNF* (CGGTAGTTGTCGTTGAGTCGTTC, 6FAM5'-CGCGCGTCGCGCTCTTAACTAAAA-3'TAMRA, AACAACCGCCGCTACTTTAAATA); *MYOD1* (GAGCGCGCGTAGTTAGCG, 6FAM5'-CTCCAACACCCGACTACTATATCCGCGAAA-3'TAMRA, TCCGACACGCCCTTTCC); *HPP1* (TTTTTTTTTCGGACGTCGTTG, 6FAM5'-AATTACCGAAAACATCGACCGA-3'TAMRA, CCTCTACATACGCCGCGAAT).[12, 15-18] The primer and probe sequences for the other 9 regions were described previously.[16] The specificity of the reactions for

methylated DNA was confirmed separately using SssI-treated genomic DNA as the methylated control.

For data presentation, the percentage of methylated references (PMR) was indicated. PMR at a specific locus was calculated by dividing the GENE:*ACTB* ratio of a sample by that of SssI-treated genomic DNA and multiplying by 100.[13, 14]

Immunohistochemistry

Immunohistochemistry of colonic mucosa for three types of DNA cytosine-5-methyltransferases (DNMT1, DNMT3a and DNMT3b), which play role**s** in the establishment and regulation of tissue-specific patterns of methylated cytosine residues, was performed. Sections (5 μm thick) of formalin-fixed, paraffin embedded tissues from three UC patients and one patient with colorectal cancer were deparaffinized in xylene and rehydrated in a series of graded ethanol. After washing three times with phosphate-buffered saline (PBS), slides were placed in a steam cooker filled with 10 mmol sodium citrate buffer, pH 6.0, for 12 min for antigen retrieval. After washing three times with PBS, endogeneous peroxidase activity was blocked by incubating sections with 3% H_2O_2. After treatment with a serum free dilution for 30 minutes to block nonspecific protein binding, mouse monoclonal antibody for Dnmt1 (IMG-261A mouse; Imgenex, San Diego, CA), Dnmt3a (39206 mouse; Active Motif, Carlsbad, CA) or Dnmt3b (52A1018 mouse; Abcam, Cambridge, UK) was applied and incubated at 4°C overnight. After washing three times with PBS and reaction with

biotinylated anti-mouse antibody for 5 minutes, antigen-antibody complexes were visualized using a streptavidin-horseradish peroxidase conjugate (LSAB kit; Dako, Carpenteria, CA) with diaminobenzidine as a chromogen. Slides were counterstained with Mayer's hematoxylin for 2 minutes. For the negative control, PBS replaced the primary antibody. Lymphocytes on the same slide were used as an internal positive control for DNMT immunoreactivity.[19, 20] We assessed DNMT immunoreactivity only in the nucleus using the staining intensity and the proportion of positive cells. The histological sections were independently evaluated, and relative intensities and proportions of the signals were estimated as weakly positive to strongly positive.

Statistical analysis

The differences in CpG island methylation levels (the PMR of *ABCB1*, *CDH1*, *ESR1*, *HPP1*, *MYOD1*, or *GDNF*) between proximal colon and distal colon mucosa of each UC patient was evaluated using the Wilcoxon signed-ranks test. The differences in methylation levels between quiescent and active colon mucosa of patients with UC was evaluated using the Mann-Whitney's U test. The association between PMR and clinical characteristics was evaluated using multiple linear regression analysis. These analyses were carried out using SAS version 9.1 (SAS Institute, Cary, NC, USA). All *P*-values were two-tailed and considered significant when less than 0.05.

Results

Clinical characteristics of patients

The methylation status of 28 UC patients who underwent colectomy was examined. Of these, 21 patients had active inflamed mucosa in the distal colon and quiescent mucosa in the proximal colon, and 13 patients had active inflamed mucosa both in the distal and proximal colon. All patients had active inflamed mucosa in the distal colon, and 6 patients had both active and quiescent portions in the proximal colon. Clinical characteristics of these patients are shown in Table 1.

Methylation status of colonic mucosa in each UC patient

To examine the methylation levels of colonic mucosa in UC patients according to the inflammatory status, we screened the methylation status of 15 gene loci (*ABCB1*, *APEX*, *CDH1*, *CDKN2A*, *CLDN1*, *CTNNB1*, *DDB1*, *ESR1*, *GDNF*, *MGMT*, *MYOD1*, *HPP1*, *MLH1*, *SMAD2*, and *SOCS1*) that were methylated in colon mucosa in previous reports.[7-10, 16, 21-25] Among these, 6 (*ABCB*, *CDH1*, *ESR1*, *GDNF*, *HPP1*, and *MYOD1*) loci exhibited methylation in colonic mucosa of UC and were examined according to inflammatory status. First, we compared methylation levels between distal active inflamed mucosa and proximal quiescent mucosa in each of 21 patients. Four genes (*CDH1*, *GDNF*, *HPP1*, and *MYOD1*) showed significantly higher methylation levels in active inflamed distal colon than in quiescent proximal colon (p = 0.003, 0.02, 0.048, and 0.0002, respectively) (Figure 1). Two other genes (*ABCB1* and *ESR1*) did not show significant differences between the proximal and distal colon mucosa, but the

methylation level of *ESR1* was slightly higher in the active inflamed distal colon than in quiescent proximal colon ($p = 0.06$).

After examining methylation changes in each patient separately, the patients were divided into two groups: patients with a large overall increase in methylation (n = 4) and patients with a small overall increase in methylation (n = 17). The group with a large increase in methylation showed more than two times the PMR values in the active mucosa compared to the values in the quiescent mucosa for three or more of the six loci examined. This group tended to have longer disease duration and a higher level of total corticosteroid use, although significant differences were not observed due to the small number of included patients ($p = 0.06$ and $p = 0.06$, respectively). Although no patients displayed increased methylation in all six loci, two patients exhibited very large increases (average 2.89 fold and 2.41 fold) in several loci. Both of these patients had disease duration of more than thirty years.

To examine the difference in methylation according to location, we then analyzed the methylation status of six genes between the proximal and distal colon of 13 patients, both of which exhibited active inflammation. In this case, only *CDH1* showed a significantly higher methylation level in distal colon than in proximal colon ($p = 0.03$) (Figure 2). The other five genes showed no differences between the proximal and distal colon mucosa.

Because many inflammatory cells, mainly comprised of white blood cells (WBC),

infiltrate active inflamed colonic mucosa of UC, their presence may inflate the methylation status of inflamed colonic mucosa. To estimate the contribution from infiltrating cells in UC colonic mucosa to the amount of methylation observed, we determined the methylation status of peripheral blood WBC DNA from buffy coat in 15 of the 28 patients. The PMRs of peripheral blood WBC DNA were much lower than those of colon tissue DNA in five loci except *ABCB1* (average PMRs of peripheral blood WBC DNA vs. colon tissue DNA; *CDH1*: 0.59 vs. 4.12, *ESR1*: 0.24 vs. 4.39, *HPP1*: 0.05 vs. 2.54, *MYOD1*: 0.22 vs. 3.30, *GDNF*: 0.15 vs. 4.77). In *ABCB1*, the PMRs of peripheral blood WBC were more than half that of colonic mucosa (average PMRs of peripheral blood WBC DNA vs. colon tissue DNA; 24.4 vs 42.4). Thus, the effects of migrating WBC on the methylation status of colonic mucosa were small except for the *ABCB1* locus. These results suggest that the methylation status of several gene loci in colon epithelial cells of UC differs according to the mucosal inflammatory status in each UC patient.

Specific methylation for active inflammation of UC

To identify specific methylation for active inflammation of UC, the methylation status of all samples was stratified according to mucosal inflammatory status. The methylation status of active inflamed colonic mucosal samples (41 samples) was compared with that of quiescent mucosal samples (21 samples) from all 28 patients (Figure 3). This analysis showed that methylation of *CDH1* and *GDNF* loci was

significantly higher in active inflamed mucosa than in quiescent colon mucosa (p = 0.045 and 0.002, respectively). There were no significant differences in the methylation levels of four other gene loci (*ABCB1*, *ESR1*, *HPP1*, and *MYOD1*) between active inflamed colon mucosa and quiescent colon mucosa.

Because it has been reported that six genes analyzed in this study are methylated due to various reasons, particularly in an age-dependent manner,[7, 26, 27] we examined whether methylation of *CDH1* and *GDNF* was unequivocally caused by mucosal inflammation via multiple linear regression analysis. Six parameters including age, gender, disease duration, total amount of corticosteroids, clinical course (steroid-dependence or –refractory), and inflammatory status (active or quiescent) were used for the analysis (Table 2). The results indicated that active inflammation was an independent factor for methylation of both *CDH1* and *GDNF* loci (p = 0.02 and 0.0006, respectively). In addition, disease duration and total amount of steroids were significant factors for methylation of *GDNF* (p = 0.02 and 0.01, respectively). Although a similar analysis was performed for both patient groups with a large overall increase in methylation and with a small overall increase in methylation, no other significant factors were identified. These results suggest that there are gene loci that are specifically methylated by active mucosal inflammation caused by UC.

Immunohistochemistry of DNA methyltransferases

Because we found that methylation specifically occurred in inflamed mucosa of

UC, we examined the expression of three types of DNA methyltransferases (DNMT1, DNMT3a and DNMT3b). DNMT1 is the main enzyme responsible for copying the methylation pattern after each round of DNA replication.[28] DNMT3a and DNMT3b encode DNA methyltransferases primarily involved in *de novo* methylation rather than maintenance of methylation.[29] Immunohistochemistry for DNMTs was performed in active and quiescent mucosa of three UC patients and in normal colon mucosa of one patient who had colorectal cancer without UC. DNMT1 was more highly expressed in active inflamed UC colon mucosa than in quiescent UC colon mucosa (Figures 4A and 4D). The DNMT1 expression level in normal colon mucosa from the colorectal cancer patient was similarly weak to that of quiescent UC mucosa (Figures 4G). DNMT3b showed an expression pattern similar to that of DNMT1 (Figures 4C, 4F and 4I), although the immunoreactiviy of DNMT3b was slightly weaker than that of DNMT1. In contrast, DNMT3a was negative in active inflamed mucosa, quiescent mucosa, and mucosa without UC (Figures 4B, 4E and 4H). These results suggest that active inflammation causes DNA methylation through activation of DNMT1 and DNMT3b.

Discussion

The data presented here demonstrate that methylation levels of several gene-associated CpG islands were substantially higher in active inflamed colon mucosa than in quiescent colon mucosa in UC patients. Particularly, *CDH1* and *GDNF* loci

were significantly more highly methylated in active colon mucosa. Finally, increased methylation in inflamed UC may result from the upregulation of DNMT1 expression.

The phenomenon that methylation in colon mucosa of UC is correlated with active inflammation supports the observations associated with natural history of UC. In contrast to mutation, methylation is prevalent in nonneoplastic cells and is reversible. In fact, methylation in gastric nonneoplastic mucosa caused by *H.pylori* can be reversed by eradication of the bacteria.[5] In UC, appropriate medication can allow recovery of actively inflamed mucosa into quiescent mucosa. In addition, the mucosal inflammatory status of different colonic locations at different times can vary in UC patients. Thus, these disease properties of UC may be correlated with the reversible character of DNA methylation.

Although there have been studies reporting increased DNA methylation in colonic mucosa of UC, the majority correlated methylation with the occurrence of UC-related dysplasia/cancer without considering the mucosal inflammatory status.[7-10, 24, 30, 31] Issa et al. reported that methylation of several gene-associated CpG islands was present in the normal-appearing epithelium from UC patients with high-grade dysplasia or cancer.[7] Tominaga et al.[8] analyzed *ESR1* methylation in different parts of the large intestine in UC patients with and without neoplasia and proved that *ESR1* methylation was correlated with an increased risk of developing neoplasia. 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, as shown in *H.pylori* related gastritis[11, 32-35] and termed ‘epigenetic field cancerization’.[34] In addition to this evidence, we demonstrate that methylation of colonic mucosa is largely correlated with active inflammation of the mucosa. A clinical study indicated that the severity of microscopic inflammation over time is an independent risk factor for developing advanced colorectal neoplasia among patients with long-standing UC.[36] This clinical feature may be due to the accumulation of methylation caused by active inflammation, culminating as epigenetic field cancerization of UC.

We selected 15 candidate gene loci and found methylation in 6 loci in colonic mucosa of UC patients. Expression of *ABCB1* in the intestinal tract has an important role in the pharmacokinetics of drugs used to treat UC, such as corticosteroids, and methylation of this locus was reported in colonic mucosa of UC.[21] E-cadherin is encoded by the *CDH1* gene, which is located on chromosome 16q22. Azarschab et al. found methylation of *CDH1* to be associated with dysplasia of UC.[22] Issa et al.[7] reported that *ESR1* and *MYOD1*, which are affected by age-related methylation in colorectal epithelium, were extensively methylated not just in the neoplastic mucosa but also in the normal-appearing epithelium from UC patients with high-grade dysplasia or cancer. Methylation of *HPP1*, found by screening hyperplastic polyps and normal mucosa, was shown to be a relatively common early event in UC-associated

carcinogenesis.[9] *GDNF* expression is up-regulated in inflammatory bowel disease and experimental colitis, and this neurotrophic factor has strong anti-apoptotic effects on colonic epithelial cells.[23] Methylation of *GDNF* was observed in a subset of colorectal cancer.[16] These loci were methylated to some extent in colon mucosa of UC, whereas the other 9 loci (*APEX*, *CDKN2A*, *CLDN1*, *CTNNB1*, *DDB1*, *MLH1*, *MGMT*, and *SOCS1*, *SMAD2*) were not methylated. The results suggest that methylation caused by inflammation of UC is locus specific, although the mechanism is unknown. However, such locus specific methylation has also been reported in *H. pylori*-related gastric inflammation.[11, 35]

Our results indicate that active inflammation was the strongest determinant for the PMRs of both *CDH1* and *GDNF*. E-cadherin (encoded by *CDH1*) mediates cell-cell adhesion in colon epithelium. A previous report observed a substantial decrease in membranous E-cadherin expression, with a concomitant increase in cytoplasmic E-cadherin expression, in UC-related cancer.[37] The decrease in membranous E-cadherin may be an early event in UC-related tumorigenesis because decreased membranous E-cadherin expression can also be detected in UC-affected, nondysplastic colonic mucosa.[38] In addition, a previous study showed that *CDH1* promoter methylation might be involved in neoplastic progression from chronic inflammation to colorectal cancer in patients with long-standing UC.[22] Taken together, methylation of *CDH1* correlates with active inflammation of colonic mucosa and may be correlated

with a decrease in membranous E-cadherin. As a result, accumulation of methylation at this locus may be involved in UC-related carcinogenesis.

On the other hand, *GDNF* family ligands and their receptors activate the Ret signaling pathway and regulate cell survival and proliferation.[39] In addition, Ret expression compromises neuronal cell survival in the colon.[40] Therefore, it has been considered that *GDNF* is a novel member in the set of protective mucosal factors.[23] In this context, dysregulation of *GDNF* can lead to down-regulation of Ret expression and may finally result in failure of colonic mucosal protection. Thus, methylation of the *GDNF* locus may itself be correlated with severe inflammation in colon mucosa of UC through loss of mucosal protection. Moreover, neoplastic progression may be facilitated by the loss of mucosal protection involving *GDNF*.

The deregulation of DNMT expression (mainly DNMT1 and DNMT3b) has been shown to contribute to tumorigenesis and tumor suppressor gene hypermethylation[41]. Therefore, our results that DNMT1 and DNMT3b were highly expressed in active inflamed mucosa suggest the correlation between active inflammation of UC and occurrence of dysplasia/cancer. In particular, DNMT1 functions as a maintenance DNMT enzyme and is responsible for accurately replicating genomic DNA methylation patterns during cell division.[42] DNMT1 is necessary and sufficient to maintain global methylation and aberrant CpG island methylation in human cancer cells.[43] A previous study reported that oxidative stress, caused by anticancer agents and ionizing radiation,

increased both DNMT1 protein levels as well as overall global DNA methylation in colon cancer cells.[42] In turn, inflammation in active UC was strongly correlated with oxidative stress.[45-47] Thus, high expression of DNMT1 in accordance with active mucosal inflammation may be induced by oxidative stress, and this aberrant expression of DNMT1 may increase methylation of several gene loci.

There are limitations to this study. First, we analyzed methylation of fifteen candidate genes and did not perform a comprehensive analysis. To more rigorously examine methylation correlated with inflammation of UC, a comprehensive approach is required. Second, we could not directly investigate the influence of infiltrating WBC on the analysis of methylation of colonic mucosa. To compensate for this limitation, we determined the methylation status of peripheral blood WBC in this study, following a protocol similar to that in a report examining the methylation of gastric mucosa with infiltrating WBC due to *H. pylori* infection.[48] Although the precedent indicated that the influence of WBC could be ignored, the effect of a small fraction of infiltrating cells other than WBC may exist. Third, we could not confirm the expression of DNMTs by PCR or Western blotting analysis. Because DNMTs are expressed in the infiltrating inflammatory cells, such methods were considered inappropriate for evaluating the expression of DNMTs in colonic epithelium. Finally, the analyzed specimens were all taken from patients who underwent colectomy. These patients usually have a more

severe clinical course and may be different from UC patients who go through a usual clinical course.

In conclusion, we showed that there are loci methylated in accordance with mucosal inflammation in colonic mucosa of UC patients. In particular, increased methylation of *CDH1* and *GDNF* is specific to mucosal active inflammation. Our results may indicate a potential epigenetic mechanism underlying mucosal inflammation and occurrence of dysplasia/cancer with chronic inflammation in UC patients.

Figure Legends

Figure 1

The differences in methylation levels between the quiescent proximal and active distal colon mucosa of each UC patient. (n = 21)

Wilcoxon signed-ranks test

Figure 2

The differences in methylation levels between active proximal and active distal colon mucosa of each UC patient. (n = 13)

Wilcoxon signed-ranks test

Figure 3

The differences in methylation levels between the quiescent (n=21) and active (n=41) colon mucosa from all examined UC patients. Mann-Whitney's U test

Figure 4

Immunohistochemical staining of DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3a (DNMT3a) and DNA methyltransferase 3b (DNMT3b). DNMT1 expression was strong in active inflamed mucosa of UC patients (A), weak in quiescent mucosa of UC (D), and weak in normal appearing mucosa from a colorectal cancer patient (G). DNMT3b showed an expression pattern similar to that of DNMT1, although the intensity of DNMT3b in active inflamed mucosa was slightly weaker than that of DNMT1 (C, F, and I). DNMT3a was negative in active inflamed mucosa of UC,

quiescent mucosa of UC, and normal appearing mucosa from a colorectal cancer patient (B, E, and H). Magnification of each figure was ×200. The scale bars indicate 100 μm.

References

1. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet.* 1999;21:163-7.

2. Baylin SB, Herman JG, Graff JR, et al. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res.* 1998;72:141–96.

3. Kanai Y. Genome-wide DNA methylation profiles in precancerous conditions and cancers. *Cancer Sci.* 2010;101:36-45.

4. Takami N, Osawa K, Miura Y, et al. Hypermethylated promoter region of DR3, the death receptor 3 gene, in rheumatoid arthritis synovial cells. *Arthritis Rheum*. 2006;54:779-87.

5. Nakajima T, Enomoto S, Yamashita S, et al. Persistence of a component of DNA methylation in gastric mucosae after Helicobacter pylori eradication. *J Gastroenterol*. 2010;45:37-44.

6. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet.* 2007;369:1641-57.

7. Issa JP, Ahuja N, Toyota M, et al. Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res*. 2001;61:3573-7.

8. Tominaga K, Fujii S, Mukawa K, et al. Prediction of colorectal neoplasia by quantitative methylation analysis of estrogen receptor gene in nonneoplastic epithelium from patients with ulcerative colitis. *Clin Cancer Res.* 2005;11:8880-5.

9. Sato F, Shibata D, Harpaz N, et al. Aberrant Methylation of the *HPP1* Gene in

Ulcerative Colitis-associated Colorectal Carcinoma. *Cancer Res*. 2002;62:6820-2.

10. Wheeler JM, Kim HC, Efstathiou JA, et al. Hypermethylation of the promoter region of the E-cadherin gene (CDH1) in sporadic and ulcerative colitis associated colorectal cancer. *Gut.* 2001;48:367-71.

11. Maekita T, Nakazawa K, Mihara M, et al. High levels of aberrant DNA methylation in Helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. *Clin Cancer Res.* 2006;12:989-95.

12. Eads CA, Danenberg KD, Kawakami K, et al. MethyLight: a highthroughput assay to measure DNA methylation. *Nucleic Acids Res.* 2000;28:E32.

13. Eads CA, Lord RV, Wickramasinghe K, et al. Epigenetic patterns in the progression of esophageal adenocarcinoma. *Cancer Res.* 2001;61:3410-8.

14. Trinh BN, Long TI, Laird PW. DNA Methylation Analysis by MethyLight Technology. *Methods.* 2001;25:456–462.

15. Widschwendter M, Siegmund KD, Müller HM, et al. Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. *Cancer Res.* 2004;64:3807–13.

16. Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet*. 2006;38:787-93

17. Weisenberger DJ, Campan M, Long TI, et al. Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res.* 2005:33:6823-36.

18. Ebert MP, Mooney SH, Tonnes-Priddy L, et al. Hypermethylation of the TPEF/HPP1 gene in primary and metastatic colorectal cancers. *Neoplasia.* 2005;7:771-8.

19. Choi MS, Shim YH, Hwa JY, et al. Expression of DNA methyltransferases in multistep hepatocarcinogenesis. *Hum Pathol*. 2003;34:11-7.

20. Zhu YM, Huang Q, Lin J, et al. Expression of human DNA methyltransferase 1 in colorectal cancer tissues and their corresponding distant normal tissues. *Int J Colorectal Dis*. 2007;22:661-6.

21. Tahara T, Shibata T, Nakamura M, et al. Effect of MDR1 gene promoter methylation in patients with ulcerative colitis. *Int J Mol Med*. 2009;23:521-7.

22. Azarschab P, Porschen R, Gregor M, et al. Epigenetic control of the E-cadherin gene (CDH1) by CpG methylation in colectomy samples of patients with ulcerative colitis. *Genes Chromosomes Cancer*. 2002;35:121-6.

23. Steinkamp M, Geerling I, Seufferlein T, et al. Glial-derived neurotrophic factor regulates apoptosis in colonic epithelial cells. *Gastroenterology.* 2003;124:1748-57.

24. Konishi K, Shen L, Wang S, et al. Rare CpG island methylator phenotype in ulcerative colitis-associated neoplasias. *Gastroenterology*. 2007;132:1254-60.

25. Kawakami K, Ruszkiewicz A, Bennett G, et al. DNA hypermethylation in the

normal colonic mucosa of patients with colorectal cancer. *Br J Cancer* 2006;94:593-8.

26. Gravina S, Vijg J. Epigenetic factors in aging and longevity. *Pflugers Arch.* 2010;459:247-58.

27. Fraga MF. Genetic and epigenetic regulation of aging. *Curr Opin Immunol.* 2009;21:446-53.

28. Robertson KD, Uzvolgyi E, Liang G, et al. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res.* 1999;27:2291-8.

29. Schmidt WM, Sedivy R, Forstner B, et al. Progressive Up-Regulation of Genes Encoding DNA Methyltransferases in the Colorectal Adenoma-Carcinoma Sequence. *Mol Carcinog.* 2007:46:766–72

30. Fujii S, Katsumata D, Fujimori T. Limits of diagnosis and molecular markers for early detection of ulcerative colitis-associated colorectal neoplasia. *Digestion.* 2008;77:2-12.

31. Moriyama T, Matsumoto T, Nakamura S, et al. Hypermethylation of p14 (ARF) may be predictive of colitic cancer in patients with ulcerative colitis. *Dis Colon Rectum.* 2007;50:1384-92.

32. Mikami T, Yoshida T, Numata Y, et al. Low frequency of promoter methylation of O6-methylguanine DNA methyltransferase and hMLH1 in ulcerative colitis-associated tumors: comparison with sporadic colonic tumors. *Am J Clin Pathol.* 2007;127:366-73.

33. Suzuki H, Iwasaki E, Hibi T. Helicobacter pylori and gastric cancer. *Gastric Cancer*. 2009;12:79-87.

34. Shen L, Kondo Y, Rosner GL, et al. MGMT promoter methylation and field defect in sporadic colorectal cancer. *J Natl Cancer Inst*. 2005;97:1330-8.

35. Kang GH, Lee HJ, Hwang KS, et al. Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation. *Am J Pathol.* 2003;163:1551-6.

36. Gupta RB, Harpaz N, Itzkowitz S, et al. Histologic inflammation is a risk factor for progression to colorectal neoplasia in ulcerative colitis: a cohort study. *Gastroenterology.* 2007;133:1099-105.

37. Aust DE, Terdiman JP, Willenbucher RF, et al. Altered Distribution of beta-Catenin, and Its Binding Proteins E-Cadherin and APC, in Ulcerative Colitis–Related Colorectal Cancers. *Mod Pathol.* 2001;14:29–39.

38. Karayiannakis AJ, Syrigos KN, Efstathiou J, et al. Expression of catenins and E-cadherin during epithelial restitution in inflammatory bowel disease. *J Pathol*. 1998;185:413-8.

39. Takahashi M. The GDNF/RET signaling pathway and human diseases. *Cytokine Growth Factor Rev.* 2001;12:361-73.

40. Uesaka T, Nagashimada M, Yonemura S, et al. Diminished Ret expression compromises neuronal survival in the colon and causes intestinal aganglionosis in mice.

J Clin Invest. 2008;118:1890-8.

41. Lin RK, Hsu HS, Chang JW, et al. Alteration of DNA methyltransferases contributes to 5'CpG methylation and poor prognosis in lung cancer. *Lung Cancer.* 2007;55:205-13.

42. Yamagata Y, Asada H, Tamura I, et al. DNA methyltransferase expression in the human endometrium: down-regulation by progesterone and estrogen. *Hum Reprod*. 2009;24:1126-32.

43. Robert MF, Morin S, Beaulieu N, et al. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat Genet.* 2003;33:61-5.

44. Mishra MV, Bisht KS, Sun L, et al. DNMT1 as a molecular target in a multimodality-resistant phenotype in tumor cells. *Mol Cancer Res.* 2008;6:243-9.

45. Oshitani N, Kitano A, Okabe H, et al. Location of superoxide anion generation in human colonic mucosa obtained by biopsy. *Gut.* 1993;34:936-8.

46. Seril DN, Liao J, Yang GY, et al. Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. *Carcinogenesis.* 2003;24:353-62.

47. Roessner A, Kuester D, Malfertheiner P, et al. Oxidative stress in ulcerative colitis-associated carcinogenesis. *Pathol Res Pract.* 2008;204:511-24.

48. Nakajima T, Maekita T, Oda I, et al. Higher methylation levels in gastric mucosae significantly correlate with higher risk of gastric cancers. *Cancer Epidemiol Biomarkers Prev*. 2006;15:2317-21.