SPANDIDOS PUBLICATIONS

Effect of MDR1 gene promoter methylation in patients with ulcerative colitis

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Abstract. Altered MDR1 expression and/or function contribute to the pathogenesis of inflammatory bowel disease (IBD). DNA methylation was shown as an important mechanism in gene silencing. We investigated DNA methylation of the MDR1 gene in ulcerative colitis (UC) and its relation to MDR1 C3435T genotypes. Eighty-three UC patients were enrolled. Methylation of MDR1 promoter was determined by methylation specific polymerase (MSP) for rectal inflammatory mucosa from all patients and normal terminal ileum from 17 patients. Promoter methylation of MDR1 gene was also quantified by digital densitographic analysis following MSP. MDR1 methylation was detected in 51 (61.4%) out of 83 patients in rectal inflammatory mucosa. Mean methylation level of MDR1 gene in rectal inflammatory mucosa was significantly higher than in normal terminal ileum (p=0.021). MDR1 methylation occurred more frequently in total colitis, and total + left side colitis, compared to rectal colitis (p=0.001, 0.013, respectively). Higher methylation levels were also associated with chronic continuous type (p=0.034) and earlier onset of disease (p=0.038). The 3435 CC+CT genotype of MDR1 was associated with more than 6-fold increased risk of MDR1 methylation, especially in UC patients with 9 years and shorter duration. Both frequency and level of MDR1 methylation were higher in UC onset at younger or in middle age with the same genotype. MDR1 methylation frequently occurred in inflammatory rectal mucosa from UC patients and was influenced by MDR1 C3435T polymorphism, especially in patients with shorter duration and younger onset.

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Introduction

P-glycoprotein (P-gp/MDR1), encoded by the ABCB1 gene, is a 170-kDa transmembrane protein highly expressed at the apical side of the intestinal epithelium (1). Its function is to mediate efflux of compounds from the mucosa to the gut lumen. There is increasing evidence that changes in MDR1 function and/or expression contribute to the pathogenesis of inflammatory disorders of the gastrointestinal tract (2).

Animal studies support a possible association of Mdr1 function and intestinal inflammation. Panwala *et al* showed that mdr1a$^{-/-}$ mice developed spontaneous intestinal inflammation (3,4), which is further aggravated by exposure to *Helicobacter bilis*. Some data suggest that inflammation also results in decreased levels of intestinal Mdr1 in rats (5) and mice (6). In addition, inflammatory bowel disease (IBD)-susceptibility loci were found on chromosome 7, where the MDR1 gene is located (7). Indeed, single nucleotide polymorphisms (SNPs) in the human MDR1 gene were reported to show an association with IBD (8-10). Moreover, the low mRNA levels of MDR1 in uninflamed intestinal tissue of ulcerative colitis (UC), was reported by Langmann *et al* (11). These data suggest a potential association between altered functions of MDR1 and IBD.

DNA methylation was shown as an important mechanism in gene silencing. In many kinds of cancer, some genes acquire aberrant methylation in their CpG islands. Meanwhile, some genes are methylated in non-neoplastic tissues by aging, and this alteration is known as age-related methylation (12,13). In addition, it was shown that gene methylation is present in non-neoplastic colorectal mucosa in patients with IBD (14,15), esophageal mucosa in patients with Barrett's esophagitis (16,17), and liver tissues in patients with chronic hepatitis (18), suggesting that gene methylation is a result of chronic inflammation.

Because loss of function of MDR1 is involved in the development of intestinal inflammation and DNA methylation accumulates during chronic inflammation, we hypothesized that DNA methylation in the MDR1 gene could be a major

pathogenesis of IBD. In the present study, we investigated the DNA methylation status of the MDR1 gene in colonic mucosa of UC patients and its relation to various clinical phenotypes of UC. In addition we assessed its association with MDR1 C3435T polymorphism, which is associated with expression of MDR1 (10,19,20), and susceptibility to UC (9).

Materials and methods

Patients and samples. The study cohort consisted of 83 UC patients enrolled at Fujita Health University Hospital (Aichi, Japan) from January 2005 to July 2007. The diagnosis of UC was based on standard clinical, endoscopic, radiological, and histo-logical criteria (21).

Clinico-pathological characteristics such as age of onset, clinical duration, and the number of times hospitalized were investigated. According to their clinical courses, chronic UC cases were classified into chronic relapsing, chronic continuous, and only one episode of the disease (22). UC patients were also classified as extensive or distal colitis according to the location and extension of the inflammatory lesions judged by endoscopic findings. In addition, patients needing continual intravenous or oral steroid therapy were identified as steroid dependent phenotype. The Ethics Committee of Fujita Health University School of Medicine approved the protocol and written informed consent was obtained from all participating subjects.

DNA isolation and bisulfite modification. Rectal inflammatory mucosal specimens were obtained from the patients and paired controls were taken from 17 patients at the normal terminal ileum. Specimens were taken during a colonoscopic biopsy and preserved in -80˚C until use. DNA was extracted from the specimens by blood using the standard phenol/chloroform method. To examine DNA methylation, genomic DNA was treated with sodium bisulfite using BislFast DNA Modification Kit for Methylated DNA Detection (Toyobo Co. Ltd., Osaka, Japan).

Methylation specific polymerase chain reaction. Promoter DNA methylation of MDR1 gene was determined by methylation specific polymerase chain reaction (MSP), as described previously (23). Using primers for the promoter region of MDR1 designed including six CpG dinucleotides that were linked to regulation of MDR1 expression (24). Primer sequences for amplification of unmethylated MDR1 were: forward 5'-GGGTGTGGGTTGAGTATAGTTGTTTT-3' reverse 5'-CCAACTTTACATACCCCTACCTCACA-3'; for methylated MDR1 were: forward 5'-GGGCGTGGGTTGAGTATAGT CGTTTC-3' reverse 5'-CGCTCCTTAAAACAACCACCA AAACG-3'. An annealing temperature and duration were determined using DNA from peripheral blood of a young individual and DNA methylated with SssI methylase (New England BioLabs Inc., Beverly, MA). The MSP was carried out in a volume of 20 μl containing 0.1 μg of bislufite-modified DNA. The DNA was denatured at 95˚C for 5 min, followed by 32 cycles at 95˚C for 30 sec, 56˚C for unmethylated DNA and 60˚C for methylated DNA for 1 min, and 72˚C for 1 min with a final extension at 72˚C for 7 min. The bands of MSP were detected by electrophoresis in 2.5% agarose gel stained with ethdium bromide.

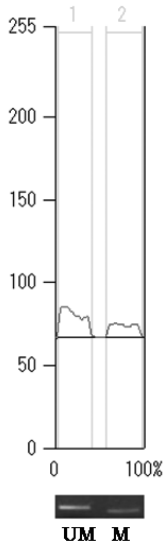

Figure1. The method of quantitive analysis of methylation levels is shown. The value of fluorescence intensities of methylated (M) and unmethylated (UM) bands of the MDR1 promoter were measured by a digital densitometer and the methylation levels were calculated as the ratio of value of methylated bands to methylated plus unmethylated bands.

Densitographic analysis of the MDR1 gene methylation levels. The value of fluorescence intensities of methylated and unmethylated bands of the MDR1 gene were measured by a digital densitometer. The methylation levels were calculated as the ratio of the value of methylated bands to methylated plus unmethylated bands (25,26) (Fig. 1).

Genotyping of MDR1 C3435T polymorphism. Genomic DNA was extracted from non-inflamed colorectal biopsy tissue or peripheral blood using the standard phenol/chloroform method in all patients. Then C3435T polymorphism of MDR1 was determined by PCR-RFLP, as previously described (27), using a forward primer (5'-tgttttcagctgcttgatgg-3') and a reverse primer (5'-aaggcatgtatgttggcctc-3'). PCR was carried out in a reaction volume of 25 μl containing 20 μg of genomic DNA, 1 x reaction buffer, 0.2 mmol/l deoxynucleotide triphosphates, 10 pmol of each primer and 0.6 units of Taq polymerase (Toyobo, Osaka, Japan). The DNA was denatured at 95˚C for 5 min, followed by 35 cycles of 95˚C for 30 sec, 63˚C for 30 sec, and 72˚C for 40 sec, with final extension at 72˚C for 7 min. Enzymatic digestion with 5 units of Mbo-I (New England Biolabs Inc., Beverly, MA) was done to analyze C3435T polymorphism, yielding products of 198 bp (3435T) and 158+39bp (3435C). After incubation overnight at 37˚C, the products were visualized by electrophoresis on 3% agarose gel and staining with ethidium bromide.

Statistical analysis. Statistical analysis was done with two-sided Fisher's exact test for the comparison of methylation frequencies between the two groups. Differences in methylation levels between the two groups of UC were examined by the Mann-Whitney U test. Differences in methylation levels between non-inflamed terminal ileum and inflamed rectal mucosa was assessed by paired Willcoxon's rank test. A $p<0.05$ was considered statistically significant.

haracteristics of UC patients.

Subjects (number)	83
Sex [male/female (%/%)]	48/35 (57.8/42.2)
Mean age ±SD (year)	40.5±13.4

MDR1 promoter methylation frequency. Promoter methylation of MDR1 gene was examined in rectal inflammatory mucosal specimens of all 83 UC patients and paired controls from 17 patients at the normal terminal ileum. The characteristics of the UC patients are summarized in Table I. First, we compared MDR1 methylation frequency between rectal inflammatory mucosa and normal terminal ileum in 17 UC patients. MDR1 methylation was detected in 16 (94.1%), and 12 (70.6%) out of 17 patients in rectal inflammatory mucosa and normal terminal ileum, respectively. Frequency of MDR1 methylation was higher in rectal inflammatory mucosa than in normal terminal ileum, but the difference was not significant.

In all 83 patients, MDR1 methylation was detected in 51 (61.4%) patients in rectal inflammatory mucosa. MDR1 methylation status was not associated with gender and age. We also investigated the association between MDR1 methylation status and various clinico-pathological phenotypes of UC. Promoter of MDR1 was frequently methylated in total colitis, and total + left side colitis, compared to rectal colitis (p=0.001, 0.013, respectively). Although the difference was not significant, the frequency of MDR1 methylation was relatively higher in chronic continuous type than chronic relapsing type (p=0.09) (Table II).

MDR1 promoter methylation levels in UC patients. Methylation levels of MDR1 gene were measured by digital densitographic analysis in all subjects. The methylation levels in rectal inflammatory mucosa and paired controls of the normal terminal ileum were compared in 17 patients (Fig. 2). Mean methylation level of MDR1 gene in rectal inflammatory mucosa was significantly higher than normal control (18.9±14.4% vs 9.7±15.5%, p=0.021).

Next, we investigated the association between methylation levels of MDR gene in rectal inflammatory mucosa and various clinico-pathological phenotypes of UC in all 83 subjects. The mean of methylation levels of the MDR1 gene was significantly higher in subjects with an onset before 20 years of age or younger than in subjects with an onset after 41 years of age or older (19.5±19.2 vs 6.4±9.8, p=0.038). The mean methylation levels of the MDR1 gene were also higher in chronic continuous type than chronic relapsing type (17.3±18.0 vs 10.0±14.7, p=0.034). Although the difference was not significant, the mean methylation levels of the MDR1 gene correlated with the higher number of hospitalizations (<2 vs ≥2; 11.7±15.7 vs 17.3±16.8, p=0.085) (Table III).

Association between MDR1 C3435T genotypes and MDR1 promoter methylation status. MDR1 C3435T polymorphism was genotyped in all 83 UC patients. The genotype distribution was 29 CC, 37 CT and 17 TT. In all 83 patients, frequency of MDR1 methylation tended to be higher in CT+CC genotype compared to TT genotype OR=2.86, 95% CI=0.96-8.52, p=0.0598. In various various clinico-pathological phenotypes, we found that the CC+CT genotypes held a significantly higher

Table II. MDR1 promoter methylation frequency in UC patients.

Variables (n)	MDR1 methylation (+)	MDR1 methylation (-)
Non-inflamed terminal ileum (17)	12	5
Inflammatory rectal mucosa (17)	16	1
Gender		
Male (48)	26	10
Female (35)	25	22
Age		
≤40 years (47)	32	15
≥41 years (36)	19	17
Extension of colitis[a]		
Total colitis (39)	29	10
Left side colitis (21)	13	8
Rectal colitis (23)	9	14
Age of onset		
≤20 (15)	12	3
21-40 (46)	26	20
≥41 (16)	9	7
Uncertain (6)		
Duration		
≥9 (47)	31	16
>10 (30)	16	14
Uncertain (6)		
Clinical type[b]		
Only one episode (5)	4	1
Chronic relapsing (49)	26	23
Chronic continuous (27)	20	7
Uncertain (2)		
Response to treatment		
Steroid-dependent or not		
(+) (17)	11	6
(-) (66)	40	26
Refractory or not		
(+) (24)	14	10
(-) (59)	37	22
Hospitalizations		
<2 (58)	32	26
≥2 (19)	14	5
Uncertain (6)		

[a]Total colitis vs rectal colitis, p=0.001; total colitis + left side colitis vs rectal colitis, p=0.013; [b]chronic relapsing vs chronic continuous p=0.09. Statistical analysis was performed by two-sided Fisher's exact test.

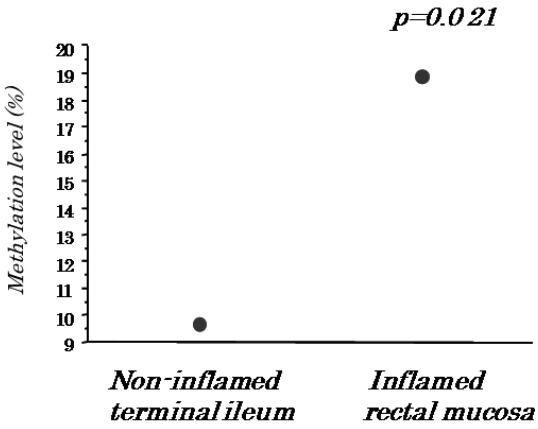

Figure 2. MDR1 methylation levels in rectal inflammatory mucosa and paired controls of the normal terminal ileum in 17 UC patients. Mean methylation level in rectal inflammatory mucosa was significantly higher than that in normal control (18.9±14.4% vs. 9.7±15.5%). Statistical analysis was performed by the paired Willcoxon's rank test.

risk of MDR1 methylation in patients with 9 years and shorter duration (OR=6.54, 95% CI=1.74-24.57, p=0.045) and patients with an onset at younger or in middle age (21-40 years, OR=6.27, 95% CI=1.41-27.87, p=0.017; ≥20+21-40 years, OR=6.54, 95% CI=1.74-24.57, p=0.005) (Table IV). A similar association was also observed between MDR1 methylation levels and CC+CT genotypes in patients that onset at younger or middle ages (21-40 years, CC+CT vs TT, p=0.049; ≥20+21-40 years, CC+CT vs TT, p=0.028, Table V). On the other hand, frequency or levels of MDR1 methylation were not associated with MDR1 genotypes in other clinico-pathological phenotypes of UC (data not shown).

Discussion

Issa *et al* (15) first reported that methylation of ER gene CpG island increased with age in non-neoplastic colorectal epithelium and highly methylated in colorectal epithelium from UC. Therefore, DNA methylation, as a mechanism of gene silencing, is increasing in importance contributing to the genetic alterations also in colitis associated cancer (CAC). On the other hand, Wang *et al* (28) showed that methylation of ER gene in rectal mucosa in UC patients was significantly higher in relapse-remitting type compared to one attack only type, and steroid-dependent cases also demonstrated a relatively higher methylated rate, suggesting the potential usefulness of DNA methylation as a molecular maker to predict disease behavior of UC.

In the present study, we investigated the promoter methylation frequency of MDR1 gene in UC patients. MDR1 methylation was observed in 61.4% from rectal inflammatory mucosal specimens of UC patients. Furthermore, methylation level of MDR1 gene in rectal inflammatory mucosa was significantly higher than in normal terminal ileum.

MDR1 is involved in the transmembrane transport of various substrates including toxic xenobiotics (29-31) and thus has a protective function in the intestinal epithelium. It was reported that mdr1a$^{-/-}$ mice developed spontaneous UC-like intestinal inflammation under specific pathogen-free conditions.

Table III. Association between MDR1 promoter methylation levels and clinical phenotypes of UC.

Variables (n)	Methylation levels (mean ±SD)
Extension of colitis	
Total colitis (39)	13.9±15.2
Left side colitis (21)	13.6±16.2
Rectal colitis (23)	11.6±17.7
Age of onset[a]	
≤20 years (15)	19.5±19.2
21-40 years (46)	12.8±15.8
≥41 years< (16)	6.4±9.8
Uncertain (6)	
Duration	
≤9 years (47)	12.8±15.6
≥10 years (30)	12.8±16.6
Uncertain (6)	
Clinical type[b]	
Only one episode (5)	22.4±12.6
Chronic relapsing (49)	10.0±14.7
Chronic continuous (27)	17.3±18.0
Uncertain (2)	
Response to treatment	
Steroid-dependent or not	
(+) (17)	15.0±17.7
(-) (66)	12.7±15.6
Refractory or not	
(+) (24)	13.0±17.5
(-) (59)	13.2±15.5
Hospitalizations[#]	
<2 (58)	11.7±15.7
≥2 (19)	17.3±16.8
Uncertain (6)	

[a]>20 vs >41 years, p=0.038; [b]chronic relapsing vs chronic continuous, p=0.034; only one episode vs chronic continuous, p=0.098; [c]<2 vs. ≥2; p=0.085. Statistical analysis was performed by the Mann-Whitney U test.

Since mdr1a$^{-/-}$ knockout mice are immunologically normal, the development of spontaneous colitis is presumably due to a defect in the barrier function of the intestinal epithelium. They also indicated that infection with *Helicobacter bilis* induced diarrhea, weight loss, and IBDs in mdr1a$^{-/-}$ knockout mice (3,4). There was also a report showing the down-regulation of MDR1 expression in colonic tissue of UC patients (11).

The MDR1 promoter region we examined by MSP includes six CpG dinucleotides that are linked to regulation of MDR1 expression (24). Although we did not investigate the quantitive difference of MDR1 in colonic mucosa of UC patients, it is possible that MDR1 methylation might affect the MDR1 expression. Our data suggest that MDR1 methylation occurs through chronic inflammation and further is involved in UC, altering the activity and expression of MDR1.

MDR1 C3435T genotypes in relation to MDR1 promoter methylation in UC patients.

Variables (n)	MDR1 genotypes (n)	MDR1 methylation(+)	MDR1 methylation(-)
Overall UC[a] (83)	CC (29)	19	10
	CT (37)	25	12
	TT (17)	7	10
Age of onset[b]			
≤20 years (15)	CC (5)	5	0
	CT (8)	6	2
	TT (2)	1	1
21-40 years (46)	CC (17)	11	6
	CT (17)	12	5
	TT (12)	3	9
≥41 years (16)	CC (4)	3	1
	CT (9)	4	5
	TT (3)	0	3
Duration[c]			
≤9 years (47)	CC (19)	13	6
	CT (19)	15	4
	TT (9)	3	6
≥10 years (30)	CC (7)	4	3
	CT (15)	8	7
	TT (8)	4	4

[a]CC+CT vs TT, OR (95% CI) p=2.86 (0.96-8.52) p=0.0598; [b]≥9 years, TT vs CC+CT, OR (95% CI) p=6.54 (1.74-24.57) p=0.045; [c]21-40 years, CC+CT vs TT, OR (95%CI) p=6.27 (1.41-27.87) p=0.017; ≥20+21-40 years, CC+CT vs TT, OR (95%CI) p=6.54 (1.74-24.57)p=0.005. Statistical analysis was performed by two-sided Fisher's exact test.

Table V. Association between MDR1 C3435T genotypes and MDR1 promoter methylation levels in UC patients.

Variables (n)	MDR1 genotypes (n)	MDR1 methylation levels (mean ±SD)
Overall UC (83)	CC (29)	14.5±14.4
	CT (37)	13.8±17.5
	CC+CT (66)	14.8±16.1
	TT (17)	9.7±15.7
Age of onset[a]		
≤20 years (15)	CC+CT (13)	20.7±19.9
	TT (2)	11.7±16.5
21-40 years (46)	CC+CT (34)	16.3±16.8
	TT (12)	8.4±15.7
≥41 years (16)	CC+CT (13)	4.3±6.6
	TT (3)	15.6±17.7

[a]21-40 years, CC+CT vs TT, p=0.049; >20+21-40 years, CC+CT vs TT, p=0.028. Statistical analysis was performed by Mann-Whitney U test.

We also investigated the association between MDR1 methylation status and various clinical phenotypes of UC. We found that the hypermethylation of MDR1 was associated with chronic continuous type. In addition, we found a positive association between MDR1 methylation and several severe disease phenotypes. Frequency of MDR1 methylation was significantly higher in extensive colitis phenotypes than in rectal colitis. Methylation levels of the MDR1 gene were higher in patients with an onset at 20 years of age or younger than in subjects with an onset after 41 years of age or older. Furthermore, higher methylation levels tended to correlate with higher number of hospitalizations.

UC is diverse in its clinical course, prognosis, and response to treatment, thus, it was hypothesized that UC is a syndrome in which different pathogenic mechanisms lead to various clinical phenotypes and it may be necessary to place greater emphasis on the disease heterogeneity. As we know, extensive colitis phenotypes and patients that developed the disease at younger age show more severe disease behavior and often need more intensive clinical treatment. This result indicates that different UC subgroups have different epigenetic backgrounds. Our result of association between MDR1 methylation status and certain clinical phenotypes of UC in the easily accessible rectum also indicates the potential usefulness of MDR1 methylation as a molecular marker to conduct more appropriate clinical implementation reflecting an individual's pathophysiology.

We also investigated whether the MDR1 methylation status in rectal mucosa might be affected by MDR1 C3435T

genotypes. We found that the CC+CT genotype was associated with more than 6-fold increased risk of MDR1 methylation, especially in UC patients with 9 years and shorter duration. In addition we also found that both frequency and level of MDR1 methylation were significantly higher in UC at an onset at younger or in middle age with the same genotypes.

The MDR1 gene is located on chromosome 7 at q21.1 (32), one of the loci of susceptibility to IBD is identified by a genome-wide analysis in a UK cohort (7). The linkage in this region was confirmed by genome scan meta-analysis (33). Finally, in 2003-2005, independent groups in Germany and Scotland indicated that a C3435T polymorphism in exon 26 is associated with susceptibility to UC but not to CD (9,34). In Japanese, 3435TT and T allele associated with UC only in patients who developed the disease at older age (19). Regarding the MDR1 expression in the gastrointestinal tract, it was reported that healthy, Japanese subjects with TT genotype showed a higher level of MDR1 expression in the duodenum compared to other genotypes (20). A similar result was also observed in rectal mucosa with an earlier onset UC patients (19).

The mechanism of gene methylation seems to be related to the chronic cycles of injury, inflammation, repair and rapid turnover of the colonic epithelia (35). Our data suggest that the CC and CT genotypes, associated with a low expression of MDR1, result in impairment for repair of MDR1, and is less protective against such conditions. Thus, these genotypes may be susceptible to MDR1 gene methylation. On the other hand, we did not find such an association in patients with longer duration and later onset. Other genetic or environmental factors may influence the susceptibility to gene methylation in such phenotypes.

In regard to the effect of MDR1 C3435T polymorphism on MDR1 expression, although TT genotype was reported to have higher expression of MDR1 in healthy, Japanese subjects (20), it should be emphasized that some of the studies have yielded contradictory results (10,20,36-38). Among five published studies, three reported decreased expression (10,36,37), and two found increased expression of MDR1 (20,38) in association with this polymorphism. Because the functional role of this polymorphism varies among the different studies and the possible functional effect of silent C3435T polymorphism is still unknown, further studies using *in vivo* and *ex vivo* systems will be needed to resolve this issue.

In conclusion, we showed that MDR1 methylation frequently occurs in inflammatory rectal mucosa from UC patients and correlated with chronic continuous type and severe disease phenotypes such as extensive colitis phenotypes and patients that developed the disease at 20 years of age or younger. Furthermore, we showed that the MDR1 methylation status in rectal mucosa was influenced by MDR1 C3435T poly-morphism especially in UC with shorter duration and an onset at younger and in middle age. This is the first investigation on the potential association between MDR1 methylation and UC in relation to MDR1 genotypes. Our results show that further longitudinal studies of aberrant DNA methylation are needed.

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