Host Genetic Factors, Related to Inflammatory Response, Influence the CpG Island Methylation Status in Colonic Mucosa in Ulcerative Colitis

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Abstract. Background: CpG island hypermethylation (CIHM) in gene promoter is frequently observed in the colonic mucosa in ulcerative colitis (UC), and is strongly involved in UC-associated colorectal carcinogenesis (CRC). The influence of common single nucleotide polymorphisms (SNPs) related to inflammatory immune response on the individual susceptibility to CIHM status in the non-neoplastic rectal mucosa in UC patients was evaluated. Patients and Methods: Ten candidate SNPs, multidrug resistance 1 (MDR1) 3435 (C>T), regulated upon activation, normal T-cell expressed and secreted (RANTES)-28 (C>G), heat-shock protein (HSP)70-2 1267 (B>A), NADPH oxidase p22PHOX 242 (C>T), Toll-like receptor (TLR)2-196 to -174 (ins >del), CD14-159 (C>T), Mannan-binding lectin (MBL)2 codon 54 (G>A), tumor necrosis factor-a(TNF-a) -857 (C>T), interleukin-1β(IL-1β)-511 (C>T), and IL-1β -31 (T>C) were genotyped in 58 UC patients without neoplastic lesions, in relation to CIHM in the rectal mucosa of three candidate CpG (p14, p16 and E-cadherin: CDH1) loci, assessed by methylation-specific-polymerase chain reaction (MSP). High CIHM was defined as two or more CpG islands methylated. Results: The CD14-159TT genotype held a significantly higher susceptibility to CIHM of the p16 promoter (OR=3.82, 95%CI=1.06-13.79, p=0.04) A significant association was also found between the IL-1β-31TC genotype and reduced susceptibility to high CIHM (OR=0.14, 95%CI=0.22-0.94, p=0.04). The p22PHOX 242CT genotype and MBL2 codon 54 A carrier (GA+AA) were significantly associated with a lower mean number of CIHM (p=0.029, 0.046, respectively). Conclusion: CD14 -159, IL-1β-31, p22PHOX 242 and MBL2 codon 54 SNPs may influence the CIHM status in the rectal mucosa of UC patients and may therefore be substantially involved in UC-associated carcinogenesis.

Ulcerative colitis (UC) affects the colon and rectum and typically involves the innermost lining mucosa, manifesting as continuous areas of inflammation, with no segments of normal mucosa (1). It is characterized by chronic, relapsing colonic inflammation with unknown etiology. UC is a multifactorial, polygenic disease with probable genetic heterogeneity. According to this hypothesis, different genetic backgrounds may explain the various clinical patterns of the disease (2, 3). Patients with UC are also predisposed to the development of colorectal carcinomas (CRC) compared with the average-risk population risk (4, 5). Important risk factors for developing CRC in UC are long-standing colitis (5), extensive colonic involvement (4), positive family history of CRC (6, 7), primary sclerosing cholangitis (8), and histologic evidence of active inflammation (9). The median age at diagnosis of CRC in patients with UC is significantly earlier than in sporadic CRC (10). Therefore, intensive CRC surveillance is strongly recommended for patients with UC and these risk factors. However, the accuracy of risk factors to predict neoplasias at an early stage is limited.

CpG island hypermethylation (CIHM) in gene promoters has been shown to be 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 (11, 12). In addition, it has been shown that gene methylation is closely associated with the inflammatory state of digestive organs (13-17), suggesting that gene methylation is a result
of chronic inflammation. In UC patients, a high degree of CIHM in nondysplastic mucosa has been reported in both the dysplastic and nondysplastic colonic mucosa (14), and was closely correlated with the risk of CRC (18). It is possible that CIHM is one of the mediators of UC-associated neoplasias and thus this molecular abnormality may allow the identification of effective markers to stratify the risk of progression to cancer in UC.

As exogenous carcinogens, generated reactive oxygen, and host genetic differences may influence the CHIM status (19), it is possible that continuous severe inflammation in the colonic musosa could accelerate CIHM in UC patients. However, there is considerable variation in the extent of colonic inflammation among UC patients and not all of them show same the CIHM status. This suggests that some genetic difference may contribute to this process, and may substantially reflect the future risk for developing CRC. To clarify this concept, the association between candidate single-nucleotide polymorphisms (SNPs), related to inflammatory immune response, oxidative stress, or the xenobiotic pathway, with the CIHM status of three tumor suppressor genes (p16, p14 and CDH1) in the rectal mucosa in UC were evaluated.

Patients and Methods

Patients and samples. A total of 58 patients, 34 male and 24 female with UC were enrolled in the study.

The average age was 40.7±14.2 years and the median clinical disease duration was 9.5±7.7 years. Rectal inflammatory mucosal specimens were obtained from all the patients, during colonoscopic biopsy, and preserved at –80˚C until use. All the diagnoses were confirmed by histopathological examinations and no evidence of dysplasia or neoplasia was found in any of the cases. The Ethics Committee in Fujita Health University approved the specimen collection procedures.

Bisulfite modification and methylation-specific PCR. DNA was extracted from the specimens by protein precipitation methods and bisulfite modification was performed according to the protocol recommended by the manufacturer (BisulFast Kit Toyobo Co., Osaka, Japan). Three candidate promoter CpG islands, p16, p14 and CDH1 were chosen to detect CIHM. Methylation-specific PCR (MSP) was carried out with the following primers: p16 methylated forward (MF), 5′-ttattaggggaggctgtg-3′ and p16 methylated reverse (MR), 5′-gacccgcacgctgtaa-3′, which amplified a 150-bp product; p16 unmethylated forward (UF), 5′-ttattaggggaggctgtt-3′ and p16 unmethylated reverse (UR), 5′-acacccaaacaccaaccataa-3′, which amplified a 151-bp product; p14 MF, 5′-ggtagaaggctgtgacg-3′ and p14 MR, 5′-aaaccccatcgcacgtaa-3′, which amplified a 122-bp product; p14 UF, 5′-tttattaggggaggctgtg-3′ and p14 UR, 5′-ccacccaaacaccaaccataa-3′, which amplified a 132-bp product (21); CDH1 MF, 5′-tttaggtaggggattctgg-3′ and CDH1 MR, 5′-taataaaatctccttgaca-3′, which amplified a 115-bp product and CDH1 UF, 5′-attattaggggattctttg-3′ and CDH1 UR, 5′-ccacccaaacaccaaccataa-3′, which amplified a 97-bp product (20). Annealing temperature and times were determined using DNA from the peripheral blood of a young individual and DNA was methylated with SssI methylase (New England BioLabs Inc., Beverly, MA, USA). The MSP chain reaction was carried out in a volume of 20 μl containing 20 ng of bisulfite-modified DNA.

The DNA was denatured at 95˚C for 5 min, followed by 33-35 cycles at 95˚C for 30 s, 57-69˚C for 1 min, and 72˚C for 1 min with a final extension at 72˚C for 5 min. The MSP reactions were conducted using EX Taq HS (Takara Bio, Inc., Shiga, Japan). The PCR products (10 μl) were separated by electrophoresis in 2.5% agarose gels and visualized by UV illumination using ethidium bromide staining. CIHM was defined as the presence of a positive methylation band showing signals approximately equal to or greater than that of the size marker (10 ng/μl: 100 bp DNA Ladder, Takara Bio) regardless of the presence of unmethylated bands. Samples giving faint positive signals were analyzed twice further and only samples with a consistent positive methylation band were considered as CHIM. In addition, the fluorescent intensities of the methylated bands of randomly selected CHIM samples were measured using a digital densitometer (Lane Analyzer, Atto, Tokyo, Japan) and confirmed that the fluorescent intensities of all the methylated bands were approximately equal to or greater than that of the size marker (data not shown). High CIHM was defined as the presence of two or more methylated CpG islands.

SNP selection and genotyping. To evaluate the association between CIHM status and host genetic factors, ten candidate common SNPs, that would be related to inflammatory immune response, oxidative stress or the xenobiotic pathway were selected. The candidate genotypes included multidrug resistance 1 (MDR1) 3435(C>T), regulated upon activation, normal T-cell expressed and secreted (RANTES) -28(G>C), heat-shock protein (HSP) 70-2 1267 (B>A), NADPH oxidase p22PHOX 242(C>T), Toll-like receptor (TLR)2 -196 to -174( ins >del), (TLR)4 -159(C>T), mannann-binding lectin (MBL)2 codon 54 (G>A), tumor necrosis factor-α(TNF-α)-857(C>T), interleukin-1β(IL-1β)-511(C>T), and IL-1β-31(T>C) SNPs. All the genotypes were determined by using DNA extracted from peripheral blood. Except for the TLR2 -196 to -174 (ins >del) polymorphism, genotyping was performed by the PCR–restriction fragment length polymorphism (PCR–RFLP) method (22-28). For the TLR2 -196 to -174 (ins >del) polymorphism, PCR was used (29).

Statistical analyses. CIHM and genotype frequencies were calculated by direct counting. Association between the CIHM status and individual genotypes was determined by the Chi-square test using a 2×2 contingency table, and the odds ratio (OR) with 95% confidence interval (CI) was calculated. The mean number of CIHM according to the TLR2 -196 to -174, tumor necrosis factor-α(TNF-α)-857, CD14 -159, mannan-binding lectin (MBL)2 codon 54, heat-shock protein (HSP) 70-2, interleukin-1β(IL-1β)-511, interleukin-1β-31, and NADPH oxidase p22PHOX 242 polymorphism, genotyping was performed by the PCR–restriction fragment length polymorphism (PCR–RFLP) method (22-28). For the TLR2 -196 to -174 (ins >del) polymorphism, PCR was used (29).

Results

All 58 rectal mucosa samples were available for MSP. Overall, CIHM was found in 30 (51.7%) for p16, 25 (43.1%) for p14, and 23 (39.7%) patients for CDH1. High CIHM was also found in 26 (44.8%) patients. The CHIM of all three genes and high CIHM were not significantly associated with age or gender (all p-values> 0.20, data not shown). Table I shows the associations of CIHM status of the three promoter CpG islands and high CIHM with the candidate genotypes.
HSP70-2, p22PHOX, TLR2, CD14, MBL2, IL-1β -511, and IL-1β -31 polymorphisms could not be genotyped for 1, 2, 5, 7, 1, 1 and 2 patients, respectively. Since age and gender were not associated with CIHM status, these associations were assessed by the Chi-square test using a 2×2 contingency table.

In the comparison of genotype frequencies, the CD14 -159T/T genotype had a significantly higher susceptibility to CIHM of the p16 promoter (OR=3.82, 95% CI=1.06-13.79, p=0.04), while the CC genotype was also weakly associated with higher susceptibility to p16 promoter CIHM. A weak
association between p22PHOX 242CT genotype and CIHM of the p16 promoter was also found. Although no association was found between the selected SNPs and CIHM of the p14, and CDH1 promoters, marginally significant association was found between the IL-1β-31TC genotype and reduced susceptibility to high CIHM (OR=0.14, 95% CI=0.22-0.94, p=0.04). A similar trend was also observed for the TT genotype compared to the CC genotype. IL-1β is an important proinflammatory cytokine (30) and MBL also plays a role in the innate immune response as a protein of the complement system (31). Both SNPs are associated with the activity or concentration of the respective protein (32-34). Since the inflammatory response is an important causative factor for gene methylation (19), both these SNPs may therefore influence the susceptibility to CIHM by altering an individual’s inflammatory immune responses.

The p22PHOX 242CT genotype was also significantly associated with a lower mean number of CIHM than the CC genotype (1.43±1.16 vs. 0.43±0.54, p=0.029).

In addition, the MBL2 codon 54 A carrier (GA+AA) was also significantly associated with a lower mean number of CIHM than the GG genotype (1.57±1.07 vs. 0.96±1.16, p=0.046). The CD14 -159CT genotype was weakly associated with a lower mean number of CIHM, when compared to the CC genotype.

**Discussion**

Some of the selected SNPs showed a marginally significant association with the CIHM status of the rectal mucosa in the UC patients.

The IL-1β-31TC were associated with reduced susceptibility to high CIHM. A similar trend was also found for the TT genotype compared to the CC genotype. MBL2 codon 54 A carrier status was also significantly associated with a lower mean number of CIHM. IL-1 is an important proinflammatory cytokine (30) and MBL also plays a role in the innate immune response as a protein of the complement system (31). Both SNPs are associated with the activity or concentration of the respective protein (32-34). Since the inflammatory response is an important causative factor for gene methylation (19), both these SNPs may therefore influence the susceptibility to CIHM by altering an individual’s inflammatory immune responses.

The p22PHOX 242CT genotype was also significantly associated with a lower mean methylation number, and showed a weak trend towards reduced susceptibility to CIHM of p16. p22PHOX is a component of the NADPH oxidase system, which transfers electrons within several subunits of NADPH oxidase and therefore plays an important role in the process of superoxide formation, as demonstrated by increases in reactive oxygen species being associated with increased levels of p22PHOX mRNA (35). The C242T polymorphism is thought to reduce the activation of NADPH oxidase and therefore confer protection against the superoxide related inflammatory state (36). Therefore, an association between lower methylation number and the 242CT genotype seems to be mechanistically reasonable.

As for CD14 -159 (C>T) SNP, the -159TT genotype had a significantly higher susceptibility to CHIM, while the CC...
genotype also weakly associated with CIHM of the \( p16 \) promoter. The \( CD14 \)-159CC and \( CD14 \)-159TT genotypes also showed a borderline association and a weak trend, respectively, towards increased susceptibility to high CIHM while the \( CD14 \)-159CT genotype was weakly associated with a lower mean number of CIHM, compared to the CC genotype. Thus both the CC and TT genotypes may increase and the CT genotype reduce the susceptibility to CIHM. Speculatively this polymorphism may be in linkage disequilibrium with other polymorphisms elsewhere in the \( CD14 \) gene, demonstrating biologically relevant variability for influencing the CIHM status, or the possibility exists that this polymorphism is in linkage disequilibrium with a genetic variation of another gene located near the \( CD14 \) gene that is related to susceptibility to CIHM.

In the present cohort of UC patients, CIHM were found at a relatively high frequency in the non-neoplastic colonic mucosa (51.7% for \( p16 \), 3.1% for \( p14 \), and 39.7% for \( CDH1 \)). This was possibly due to the long median clinical disease duration (9.5±7.7 years), suggesting that these individuals might be at especially high risk for developing CRC. The demonstrated associations indicate that some host genetic factors may influence the CIHM status and long-term outcome of UC and may meaningfully predict patients who may be at risk of developing CRC. However, whether the associations directly contribute to the risk of CRC is unclear, because the present cohort did not include CRC patients, therefore further well-designed study will be needed to confirm the result and evaluate the clinical utility in risk prediction of CRC in UC patients.

References


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