Effect of *RANTES* gene promoter genotypes in patients with ulcerative colitis

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Abstract. A complex interaction of genetic and environmental factors is closely associated with the development of inflammatory bowel disease. Previous studies reported that the expression of the regulated upon activation, normal T-cell expressed and secreted (*RANTES*) gene is enhanced in the colonic mucosa of ulcerative colitis (UC). Quantitative differences in *RANTES* gene expression among numerous promoter genotypes have also been reported. The aim of the present study was to clarify the effect of *RANTES* promoter polymorphism on the risk of UC, including its clinical phenotypes. A total of 150 UC patients and 372 healthy control (HC) subjects participated in the study. The UC patients were classified by disease behavior, severity and extent of disease. Restriction fragment length polymorphism analysis was performed for polymorphisms at -28 C/G in the *RANTES* gene promoter region. Although no significant difference of the *RANTES* promoter genotype distribution was observed between the HC and UC groups, the G/G genotype was significantly higher among female (OR=3.95, 95% CI=1.22-12.82, P=0.03), non-steroid dependent (OR=3.37, 95% CI=1.16-9.85, P=0.03) and non-refractory (OR=3.76, 95% CI=1.29-10.98, P=0.02) UC patients. The G carrier was also found to be associated with an increased risk of rectal colitis (OR=2.21, 95% CI=1.12-4.39, P=0.03). The data indicate that the polymorphism of the *RANTES* promoter is not directly associated with the susceptibility to UC, but the -28 G allele is associated with female UC patients and mild clinical phenotypes of UC, including non-steroid dependency, non-refractory and rectal colitis.

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Abbreviations: UC, ulcerative colitis

Key words: ulcerative colitis, *RANTES* promoter, polymorphism

Introduction

Ulcerative colitis (UC) is established by a chronic, relapsing colonic inflammation with an unknown cause. The inflammation affects the colon and rectum and usually involves the mucosa of the innermost lining, which are present as continuous areas of inflammation that have no areas of normal mucosa (1). It was previously suggested that an altered regulation of the immune system plays crucial roles in the pathogenesis of UC. UC is also a multifactorial, polygenic disease that has a possible genetic heterogeneity. Various genetic backgrounds may explain the numerous UC clinical patterns (2,3). The genetic predisposition to inflammatory bowel diseases has been well-established through epidemiological studies and genome-wide linkage analyses; however, the accountable genes require further analyses (4). Therefore, the association between the polymorphism of various genes and UC has been studied previously (5-11).

RANTES (regulated upon activation, normal T-cell expressed and secreted) is a member of the chemokine family, which is a large and growing family of immunoregulatory cytokines. Specifically, RANTES belongs to the C-C chemokine subfamily. RANTES is a strong chemotactic agent for T lymphocytes and monocytes (12) and is expressed following cellular activation in fibroblasts, T cells, monocytes, endothelial cells and certain epithelial cells.

It is well known that the expression of the pro-inflammatory cytokines, most notably interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α) and chemokines [IL-8, epithelial-derived neutrophil-activating peptide 78 (ENA-78), monocyte chemoattractant protein-1 (MCP-1) and RANTES is clearly increased in the intestinal mucosa of inflammatory bowel disease (IBD) patients. Previous studies have reported that the expression of *RANTES* is enhanced in the colonic mucosa of patients with UC (13,14). In addition, it was reported that treatment with 5-aminosalicylic acid (ASA) medication in IBD patients resulted in a lower plasma concentration of RANTES, indicating that this is a beneficial product of 5-ASA treatment (15).

Numerous polymorphisms have been found by genetic studies of the *RANTES* gene, including a polymorphism that results in a nucleotide substitution in the promoter region,

-28 C/G. This substitution may significantly alter the function of the *RANTES* promoter, and thus may influence its activity. The -28 G allele of the *RANTES* promoter is associated with a higher RANTES protein level compared with the C allele (16). With regards to the possible association between the *RANTES* promoter polymorphism and human diseases, previous studies have shown that the genotype of the *RANTES* promoter is linked with diabetic nephropathy in type 2 diabetic subjects (17), late onset asthma (16), atopic dermatitis (18) and progression of acquired immunodeficiency syndrome (19,20).

However, there have been no studies regarding the association between the *RANTES* promoter polymorphism and UC. The present study was performed to investigate the associations between the polymorphisms of the *RANTES* gene promoter and UC, including the clinical phenotypes in the Japanese population.

Materials and methods

Clinical samples and extraction of DNA. The studied population comprised 522 subjects, including patients with UC (UC group, n=150), who were enrolled at the Fujita Health University Hospital (Toyoake, Aichi, China), and unrelated healthy control (HC) subjects (HC group, n=372). The diagnosis of UC was based on standard clinical, endoscopic, radiological and histological criteria (21). According to the clinical courses of the patients, chronic UC cases were classified into chronic relapsing disease, chronic continuous disease and only one episode of the disease (22). The UC patients were also classified as extensive or distal colitis according to the location and extension of the inflammatory lesions judged by the endoscopic findings. In addition, the patients that required continuous intravenous or oral steroid therapy were identified as having the steroid-dependent phenotype. The Ethics Committee of Fujita Health University School of Medicine approved the protocol, and written informed consent was obtained from all the participating subjects.

Genotyping for the RANTES promoter. The genomic DNA was extracted from non-neoplastic colorectal biopsies or peripheral blood using the standard phenol/choloroform method. The polymorphisms of -28 C/G in the *RANTES* gene promoter region were investigated by polymerase chain reaction (PCR)-based restriction fragment length polymorphism analysis assays as previously described (19). In brief, DNA was amplified by the primers (5'-ACA GAG ACT CGA ATT TCC GGA-3' and 5'-CCA CGT GCT GTG TTG ATC CTC-3'). The 173 base pair (bp) PCR product (10 μl) was cleaved by *Mnl*I (New England Biolabs, Inc., Ipswich, MA, USA) in an appropriate buffer at 37˚C for 12 h, resulting in three fragments of 126, 27 and 20 bp for the C allele and two fragments of 146 and 20 bp for the G allele, on a 3.5% agarose gel by staining with ethidium bromide.

Statistical analysis. The genotype frequencies were calculated by direct counting. The allele counts were compared between two groups by a 2x2 table using a two-sided Fisher's exact test. Hardy-Weinberg equilibrium of the *RANTES* gene alleles in the control and UC subjects were assesed by χ^2 statistics. The strength of association was also assessed by calculating the odds ratio (OR) and 95% confidence intervals (CI). P<0.05 was considered to indicate a statistically significant difference.

Table I. Characteristics of UC patients.

Characteristics	Values
Subjects, n	150
Female/male, n (%)	79/71 (52.7/47.3)
Mean age ± SD, year	39.6±13.8

UC, ulcerative colitis; SD, standard deviation.

Results

RANTES promoter polymorphism and risk of UC. The polymorphisms of -28 C/G in the *RANTES* gene promoter region were genotyped in all 150 UC subjects and 372 unrelated HC subjects. The characteristics of the UC patients are presented in Table I. The frequency of the *RANTES* promoter polymorphism in the HC and UC groups did not deviate significantly from those expected under the Hardy-Weinberg equilibrium (P=0.85 and 0.10, respectively).

In the HC group, the *RANTES* promoter genotype distribution was 280 C/C (75.3%), 85 C/G (22.8%), and 7 G/G (1.9%), and in the UC group the distribution was 107 C/C (71.3%), 36 C/G (24.0%), and 7 G/G (4.7%).

No significant difference of the *RANTES* promoter genotype distribution was observed between the HC and UC groups (G/G vs. others, OR=2.55, 95% CI=0.88-7.40, P=0.13; and C/C vs. G carriers, OR=1.22, 95% CI=0.80-1.87, P=0.38; Table II).

RANTES promoter polymorphism and clinical phenotypes of UC. To investigate the association of the *RANTES* promoter polymorphism and different clinical phenotypes of UC, the subgroups of gender, age of onset, clinical type, extension of colitis and response to treatment were also included to stratify the analysis. Among these clinical phenotypes, the frequency of the G/G genotype was found to be significantly higher among female (OR=3.95, 95% CI=1.22-12.82, P=0.03), non-steroid dependent (OR=3.37, 95% CI=1.16-9.85, P=0.03) and non-refractory (OR=3.76, 95% CI=1.29-10.98, P=0.02) UC patients.

The G carrier was also found to be associated with an increased risk of rectal colitis (OR=2.21, 95% CI=1.12-4.39, P=0.03; Table III).

Discussion

Mucosal lesions that are present in IBD are established by a dense inflammatory cell infiltrate that is predominantly composed of neutrophils, macrophages and lymphocytes. The expression of the pro-inflammatory cytokines, most notably IL-1, IL-6, TNF-α and chemokines (IL-8, ENA-78, MCP-1 and RANTES), is also markedly enhanced.

Table II. Association between RANTES polymorphism and risk of UC.

Variables	Total, n	*RANTES* genotype, n (%)			OR (95% CI)		OR (95% CI)	
		C/C	C/G	G/G	G/G vs. others	P-value	C/C vs. G carriers	P-value
Control	372	280 (75.3)	85 (22.8)	7 (1.9)	Reference		Reference	
Overall UC	150	107 (71.3)	36 (24.0)	7 (4.7)	2.55 (0.880-7.40)	0.13	1.22 (0.80-1.87)	0.38

Statistical analysis was performed by the two-sided Fisher's exact test. *RANTES*, regulated upon activation, normal T cell expressed and secreted; UC, ulcerative colitis; OR, odds ratio; CI, confidence interval; n, number of samples; G carriers, GG+CG.

Table III. Association between *RANTES* polymorphism and clinical phenotypes of UC.

Variables	Total, n	*RANTES* genotype, n			OR (95% CI)		OR (95% CI)	
		C/C	C/G	G/G	G/G vs. others	P-value	C/C vs. G carriers	P-value
Control	372	280	85	7	References		References	
Gender								
Male	79	53	24	2	1.35 (0.28-6.65)	0.66	1.49 (0.88-2.52)	0.16
Female	71	54	12	5	3.95 (1.22-12.82)[a]	0.03[a]	0.96 (0.53-1.74)	1.00
Age of onset								
<20	26	19	6	1	2.09 (0.25-17.62)	0.42	1.12 (0.46-2.75)	0.82
≥20	109	76	27	6	3.04 (0.998-9.24)	0.08	1.32 (0.82-2.12)	0.26
Uncertain	25	12	13	0	NA	NA	NA	NA
Clinical type								
Only one episode	13	7	5	1	4.35 (0.49-38.16)	0.42	2.61 (0.85-7.96)	0.20
Chronic relapsing	68	53	13	2	1.58 (0.32-7.77)	0.63	0.86 (0.46-1.60)	0.76
Chronic continuous	64	44	16	4	3.48 (0.99-12.24)	0.06	1.38 (0.78-2.47)	0.28
Unknown	5	3	2	0	NA	NA	NA	NA
Extension of colitis								
Rectal colitis	38	22	13	3	4.47 (1.11-18.06)	0.06	2.21 (1.12-4.39)[a]	0.03[a]
Left side colitis	39	32	7	0	NA	NA	0.67 (0.28-1.56)	0.43
Total colitis	71	51	16	4	3.11 (0.89-10.93)	0.08	1.19 (0.678-2.11)	0.55
Unknown	2	2	0	0	NA	NA	NA	NA
Steroid-dependency								
(+)	32	28	4	0	NA	NA	0.43 (0.15-1.27)	0.76
(-)	115	76	32	7	3.37 (1.16-9.85)[a]	0.03[a]	1.56 (0.99-2.45)	0.06
Unknown	3	3	0	0	NA	NA	NA	NA
Refractory[b]								
(+)	44	37	7	0	NA	NA	0.58 (0.25-1.34)	0.26
(-)	104	68	29	7	3.76 (1.29-10.98)[a]	0.02[a]	1.61 (1.01-2.57)	0.06
Unknown	2	2	0	0	NA	NA	NA	NA
Hospitalization								
<2 times	111	76	29	6	2.98 (0.98-9.06)	0.09	1.40 (0.88-2.23)	0.18
≥2 times	31	26	5	0	NA	NA	0.59 (0.225-1.57)	0.38
Unknown	8	5	2	1	NA	NA	NA	NA

[a]Statistically significant; [b]?. Statistical analysis was performed by the two sided Fisher's exact test. *RANTES*, regulated upon activation, normal T cell expressed and secreted; UC, ulcerative colitis; OR, odds ratio; CI, confidence interval; n, number of samples; NA, not available; G carriers, GG+CG.

RANTES is a member of the chemokines family that belongs to the C-C chemokine subfamily, and it is a strong chemotactic agent for T lymphocytes and monocytes (12) that is expressed following cellular activation in fibroblasts, T cells, monocytes, endothelial cells and certain epithelial cells. Previous studies of enhanced expression of *RANTES* in the

colonic mucosa of patients with UC (13,14) and a lower plasma concentration of the RANTES chemokine following 5-ASA medication in patients with IBD suggests that this chemokine may play a significant role in the pathogenesis of IBD (15).

Although the *RANTES* gene is located on chromosome 17q11.2-q12, a position that is not included in the UC susceptible loci (4), the *RANTES* promoter polymorphism was investigated in patients with UC as *RANTES* may play an important role in the pathogenesis of this disease. In addition, the association between the *RANTES* promoter polymorphism and various clinical phenotypes of UC was also investigated. UC is a diverse in its clinical course, prognosis and response to treatment, and thus, it has been hypothesized that UC may be a syndrome that has various pathogenic mechanisms that result in numerous clinical phenotypes, and therefore, placing a greater emphasis on the disease heterogeneity may be required in studies of the genetic effect in UC.

In the present study, the association between the polymorphism of the *RANTES* promoter was investigated with regards to the risk of UC in a Japanese population. Although a direct association was not found between the *RANTES* promoter genotypes and UC, the -28 G/G genotype was associated with non-steroid-dependent and non-refractory UC patients. In addition, the -28 G carrier was associated with the rectal colitis phenotype. The frequency of the G/G genotype and G carrier were significantly higher among the non-steroid-dependent, non-refractory UC and rectal colitis compared with the HC group. This result indicates that various UC subgroups may have different genetic background and the *RANTES* promoter genotypes may not be associated with the risk of UC, but may modify the clinical phenotype in UC patients. The *RANTES* promoter -28 G allele may be of significance in the pathogenesis of relatively mild UC. This result may improve the information provided for the clinical implementation of UC patients reflecting the pathophysiology of individuals. It is possible that the *RANTES* promoter polymorphism plays a role in the development of the steroid-dependent and refractory phenotype. Further studies are required to evaluate the possible association of the UC phenotypes and various positions of the *RANTES* promoter gene polymorphisms. Modifications in human monocytes gene expression following the induction of the *RANTES* gene have been assessed by the oligonucleotide array method, demonstrating the activation of the transcription of cytokine genes (including MCP-1, pro IL-1β and IL-8), membrane receptors (such as oxidized low-density lipoprotein receptor), regulators of extracellular matrix proteins (including matrix metalloproteinase-9) and enzymes regulating intracellular signal transduction (including mitogen-activated protein kinase) by *RANTES* (23). It is known that there are four binding sites for nuclear factor-κB in the *RANTES* promoter that are critical for induction by the proinflammatory cytokines, TNF-α and IL-1β, and induction through the cluster of differentiation 28 co-stimulatory pathway (24). The *RANTES* promoter -28 C/G polymorphism is located directly downstream of the first of the aforementioned nuclear factor-κB sites (-40 to -31). A previous study has demonstrated that there are quantitative differences in the protein expression of RANTES between various *RANTES* promoter genotypes (16). Although the expression of RANTES in the serum or colonic mucosa of UC patients was not investigated, it is possible that the *RANTES* promoter polymorphism may influence the quantitative differences in RANTES expression and modify the risk of the clinical phenotype of UC by changing the immune response. Further studies are also required to resolve this issue.

From a functional perspective, the study by Hizawa *et al* (16) showed that the -28 G allele of the *RANTES* promoter had a higher level of mRNA and protein expression than those of the C allele, suggesting that the C allele has a more protective potential against inflammation. However, the finding of the association between the G allele with non-steroid dependency, non-refractory UC and rectal colitis phenotype indicates that the G allele is associated with relatively mild rather than severe UC. The reason for the high-producing genotype of the G allele increasing the susceptibility of mild UC remains to be clarified. Notably, the -28 G/G genotype has been shown to be associated with an increased risk of UC in females. Although it is known that the Th1/Th2 balance immune response is different between genders, the *RANTES* promoter polymorphism may be a factor that modifies the susceptibility of UC in females.

In conclusion, the present study has shown that the polymorphism of the *RANTES* promoter is not directly associated with UC susceptibility, but the -28 G allele is associated with non-steroid dependency, non-refractory, rectal colitis and female UC in the Japanese population. The *RANTES* promoter polymorphism was investigated in a select region of Central Japan. Previous studies have indicated that the *RANTES* promoter polymorphism shows variations in different ethnic groups (19,20,25). In addition, other polymorphisms have been reported in the human *RANTES* promoter that remain to be studied in UC (19,20). Further studies are required in a larger and more diverse population to confirm the effect of this gene on the pathogenesis of UC.

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