RANTES promoter polymorphism as a genetic risk factor for rheumatoid arthritis in the Chinese

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Abstract

Objectives

There is increasing evidence suggesting a role of RANTES in the pathogenesis of rheumatoid arthritis (RA) and we evaluated the possible effect of RANTES gene on the susceptibility to RA in Chinese patients.

Methods

We examined the polymorphisms at the promoter positions -28 and -403 of this gene in 151 Chinese RA patients and 149 ethnically matched healthy controls.

Results

The genotypic frequencies in this study were in Hardy-Weinberg equilibrium. RA patients had significantly higher frequencies of the A allele (36.1% vs. 27.5%, p = 0.024) and A/A genotype (odds ratio [OR] = 3.3, 95% confidence interval [CI] = 1.4-7.9, p = 0.005) at the promoter -403 position. Differences in allele and genotype frequencies at the promoter -28 position between patients and controls were not statistically significant (for G allele, p = 0.103 and for genotype, p = 0.106). RA patients also had a significantly higher frequency of the -28 C/G with -403 A/A compound genotype (OR = 4.6, 95% CI = 1.5-14.5, p = 0.005), and a higher frequency of the -28 G/-403 A haplotype with marginal statistical significance (OR = 1.7, 95% CI = 1.0-3.1, p = 0.059).

Conclusion

Our results indicate that polymorphism in the promoter region of RANTES gene is associated with the susceptibility to RA in the Chinese population.

Key words

RANTES, genetic polymorphism, rheumatoid arthritis, Chinese.

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Introduction

Chemokines are a superfamily of small, inducible, secreted, pro-inflammatory cytokines, which are building blocks of the most versatile, coherently functioning system of intercellular sophisticated communications used by all human cell types, including immune cells (1, 2). Chemokines act primarily on leukocytes, regulating their trafficking (3). Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease with synovial tissue invasion by inflammatory cells. These cells and associated products such as chemokines play an essential role in synovitis, pannus formation and, eventually, joint destruction (4). Both synovial-lining cells and infiltrating leukocytes are sources of these chemokines (5-7). The battery of secreted chemokines accounts for the recruitment of the leukocytes responsible for initiating and maintaining the inflammatory response within rheumatoid joint.

The cDNA for human regulated upon activation, normal T cell expressed and presumably secreted (RANTES), a CC chemokine subfamily, generates a 68-aminoacid residue mature protein (8). RANTES can chemoattract monocytes, degranulated eosinophils, basophils, unstimulated $CD4^+$ memory T cells and stimulated $CD4^+$ and $CD8^+$ T cells (9-12). The *in vitro* biological activity of RANTES suggests that it may have an important role in T cells-mediated immune and inflammatory processes (13). The RANTES molecules have been observed in T cells of peripheral blood and synovial fluid, as well as in sites of extensive T cell infiltration in synovial tissue samples from RA patients (14, 15). RA synovial fibroblasts produce RANTES mRNA upon stimulation by TNFα and IL-1 (16). There is increasing evidence suggesting a role for RANTES in the pathogenesis of RA (14-16). A considerable body of evidence associates the susceptibility to RA with several human leukocyte antigen (HLA) class II alleles; however, the contribution of HLA class II molecules to RA has been estimated at 30% to 50% of the total genetic component (17). Additional genetic contribution is necessary for the occurrence of RA.

The purpose of this study was to evaluate the possible effect of the RANTES gene on the susceptibility to RAby examining the polymorphism of promoters at positions -28 and -403 of this gene in clinically well characterized Chinese RA patients and ethnically matched healthy controls.

Materials and methods

Patients and controls

We recruited 151 RA patients (121 women and 30 men, with a mean age of 49.0 ± 12.1, from 26 to 77), with diagnosis according to the diagnostic criteria set by the American College of Rheumatology (18). There were 42 seronegativity and 109 seropositivity in these patients. Extraarticular clinical manifestations were found in 60 patients. They were regularly followed up at the Rheumatology Section, Internal Medicine Department, National Cheng Kung University Hospital. Another 149 ethnically matched healthy controls (113 women and 36 men, with a mean age of 47.8 ± 13.8, from 23 to 80) were recruited for comparisons. A venous blood sample was drawn and informed consent was obtained from each study participant. The Ethics Committee of the National Cheng-Kung University Hospital approved this study.

DNA preparation

Peripheral blood mononuclear cells were isolated from heparin-anticoagulated venous samples by Histopaque (Sigma Diagnostics, St. Louis, MO) gradient centrifugation. The cells were washed three times with HBSS medium and stored at –70°C until use. Genomic DNA was prepared from these cells using a PUREgene DNAisolation kit (Gentra, Minneapolis, MN) and purified DNA was dissolved in TE solution.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

300 ng of genomic DNA was used in the routine PCR-RFLP genotyping of the RANTES -28 and -403 polymorphism as reported previously (19, 20). Primer sequence for RANTES -28 was forward (5,-ACTCGAATTTCCG-

GAGG CTA-3,) and reverse (5,-TCT-GCAGCTCAGGCTGGCCCTTTAT-3,), and that for RANTES -403 was forward (5,-GCCTCAATTTACAGT-GTG-3,) and reverse (5,-TGCT-TATTCATTA CAGATGTT-3,). The G base (underlined) at the 3' end of the reverse primer for RANTES -403 was mutated from a C to introduce an enzyme cutting site for *Mae* III. PCR cycles for RANTES -28 were as follows: 94°C for 5 min followed by 35 cycles each of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min. A final extension step was carried out at 72°C for 10 min. PCR cycles for RANTES -403 were as follows: 94°C for 5 min followed by 35 cycles each of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min. A final extension step was carried out at 72°C for 10 min. The amplification with the RANTES -28 specific primers yielded a 149 bp PCR product that was subsequently digested with restriction enzyme *Mnl* I (New England Biolab, Beverly, MA) yielding 110 and 39 bp DNA fragments as a diagnostic determinant for the mutant allele. A 136 bp PCR product was amplified by the specific primers of RANTES -403 and digestion with restriction enzyme *Mae* III (Roche Diagnostics GmbH, Penzberg, Germany) gave rise to 112 and 24 bp DNAfragments in the mutant allele. The PCR products for the genotyping of these alleles were selected for automated nucleotide sequence analysis (ABI Prism 377 DNA Sequencer; Perkin Elmer, Foster City, CA) to ensure the accuracy of the PCR-RFLPdeterminations.

Statistical analysis

Data analyses were performed using the SAS statistical package (version 8.2, SAS Institute, Cary, NC), and statistical tests were conducted at the two-sided significant level of 0.05. Differences in allele, genotype and haplotype frequencies between RA patients and controls were evaluated using the chi-square test and Fisher's exact test. Differences in the allele, genotype and haplotype frequencies between the presence of rheumatoid factor and extraarticular involvement of RA patients were evaluated using the chi-square test and Fisher's exact test. The odds ratio (OR) and 95% confidence interval (CI) were calculated, and the trend in OR was evaluated by the chi-square test for trend. The genotype distributions of the -28 and -403 polymorphisms tested in the study population were found to be in Hardy-Weinberg equilibrium.

Results

RA patients and controls had similar distributions of promoter -28 genotype polymorphisms (Table I). Slight increases in the frequencies of G carrier and G allele of promoter -28 were noted in patients, but the differences were not statistically significant (p= 0.107 and p= 0.103, respectively). Among the promoter -403 genotype polymorphisms, patients had a significantly higher frequency of A/A genotype (OR 3.3, 95% 1.4-7.9, p = 0.005), and there was a significant trend in the ORs from genotypes G/G, G/A, to A/A(p = 0.025 for the trend test). In addition, the frequency of A allele was significantly higher in patients (36.1% vs. 27.5%, p = 0.024), although the carrier rate was similar between patients and controls (57.0% vs. 50.0%, p = 0.206).

We further analyzed the distribution of the RANTES promoter -28 and -403 compound genotypes, and observed only six genotypes in both RA patients and controls (Table II). The following compound genotypes were not observed: C/G at -28 with G/G at -403, G/G at -28 with G/G at -403, and G/G at -28 with G/A at -403. A significant increase in the frequency of the -28 C/G with -403 A/A compound genotype was found in RApatients (OR 4.6, 95% CI 1.5- 14.5, p = 0.005). We found that all individuals with a genotype of G/G at -28 also had an A/A at -403 and that all of those with a genotype of G/G at –403 also had a C/C at -28. These data indicated the absence of a haplotype containing a G at both -28 and -403 positions. There was a significant increasing trend in the ORs from the haplotypes -28 C/-403 G, -28 C/-403 A, to -28 G/-403 A (p = 0.025 for the trend test), and RApatients had a higher frequency of the haplotype -28 G/-403 A with a marginal statistical significance (OR 1.7, 95% CI 1.0- 3.1, p = 0.059).

Additionally, we examined if the genotype and allele of promoter -28 or -403 or the compound genotype and haplotype of promoter -28 and -403 were associated with clinical features of RA. However, we did not find any statistical

Table I. Genotype and allele frequencies of RANTES gene polymorphism for rheumatoid arthritis patients and healthy controls of Chinese.

Polymorphism	Patient	(%)	Control	(%)	OR	[95% CI]	p
-28 C/G							
Genotype							0.106[1]
C/C	119	(78.8)	128	(85.9)	1.0		
C/G	30	(19.9)	20	(13.4)	1.6	[0.9, 3.0]	0.127
G/G	2	(1.3)	1	(0.7)	2.2	[0.2, 24.0]	0.612[2]
G carrier	32	(21.2)	21	(14.1)	1.6	[0.9, 3.0]	0.107
G allele	34	(11.3)	22	(7.4)	–		0.103
-403 G/A							
Genotype							0.025[1]
G/G	65	(43.1)	75	(50.3)	1.0		
G/A	63	(41.7)	66	(44.3)	1.1	[0.7, 1.8]	0.693
A/A	23	(15.2)	8	(5.4)	3.3	[1.4, 7.9]	0.005
Acarrier	86	(57.0)	74	(50.0)	1.3	[0.9, 2.1]	0.206
Aallele	109	(36.1)	82	(27.5)	–		0.024
Total	151		149				

OR: odds ratio; CI: confidence interval.
[1] p value for test for trend;
[2] p value for Fisher's exact test.

Table II. RANTES promoter compound genotype and haplotype frequency in rheumatoid arthritis patients and healthy individuals of Chinese.

RANTES promoter site		Patient	(%)	Control	(%)	OR[1]	[95% CI]	p[2]
-28	-403							
Genotype								
C/C	G/G	65	(43.0)	75	(50.3)	1.0		
C/C	G/A	49	(32.5)	50	(33.6)	1.1	[0.7, 1.9]	0.640
C/C	A/A	5	(3.3)	3	(2.0)	1.9	[0.4, 8.4]	0.477[3]
C/G	G/A	14	(9.3)	16	(10.7)	1.0	[0.5, 2.2]	0.981
C/G	A/A	16	(10.6)	4	(2.7)	4.6	[1.5, 14.5]	0.005
G/G	A/A	2	(1.3)	1	(0.7)	2.3	[0.2, 26.0]	0.600[3]
Total		151		149				
Haplotype								0.025[4]
C	G	193	(63.9)	216	(72.5)	1.0		
C	A	75	(24.8)	60	(20.1)	1.3	[0.9, 2.0]	0.120
G	A	34	(11.3)	22	(7.4)	1.7	[1.0, 3.1]	0.059
Total		302		298				

OR: odds ratio; CI: confidence interval.
[1]OR using the -28 C/C with -403 G/G as the reference group (OR=1) in the analysis of genotype and the -28 C with -403 G as the reference group in the analysis of haplotype.
[2]p value for chi-square test, unless otherwise labeled; [3]p value for Fisher's exact test; [4]p value for chi-square test for trend.

association of RANTES promoter polymorphism with the presence of rheumatoid factor or extraarticular involvement in the present study (data not shown).

Discussion

CCR5 is used by the R5 human immunodeficiency virus-1 (HIV-1) strains as coreceptor in the early stage of viral infection (21). Mutations at the CCR5 promoter might down-regulate the CCR5 expression at the transcription level and further decrease the surface expression levels (22). A 32-nucleotide deletion in CCR5 coding region (Δ 32 CCR5) produces a truncated protein that failed to reach the cell surface and, consequently, Δ 32 CCR5 homozygotes resist HIV infection despite repeated exposures (23). A nucleotide transition from C to T at position 59653 in the CCR5 promoter pCCR5-59653 has been shown to slow the progression of HIV infection (24). Furthermore, a polymorphic change at position 59029 in the CCR5 promoter pCCR5-59029 has also been shown to affect the rate of progression of HIV-infected patients to AIDS (25).

Both *in vitro* and *in vivo* findings have argued persuasively that the CCR5 ligands, including RANTES, MIP-1α and MIP-1β, are inhibitors of cell entry and replication of HIV-1 (23, 26). Among these natural CCR5 ligands, RANTES showed the highest potency to suppress *in vitro* replication of the R5 strains (27). Two single nucleotide polymorphism sites, -28 C/G and -403 G/A, in the promoter region of RANTES have been identified, and it has been demonstrated that -28 G variant or the -403 A variant could increase the transcriptional activity and subsequent expression of RANTES in human cells (28, 29). In addition, the *In1.1C* regulatory allele was reported to be responsible for the diminished transcription of RANTES (19). These SNP sites in RANTES gene have been reported to influence the *in vivo* HIV-1 spread and the disease progression to AIDS (19, 23, 26, 28).

An analysis of population genetics might provide additional support to the hypothesis that the chemokine signaling through the CCR5 receptor contributes to the recruitment of T cells and monocytes and thus to the development of RA(30,31). A study found homozygous knockout existed in 7 out of 815 Caucasian controls, whereas none was homozygous for a Δ 32 CCR5 polymorphism in 673 RA patients that rendered CCR5 receptor inactive (30). However, another study in Mexican Amerindian population did not support an important role of Δ 32 CCR5 in the pathogenesis of RA (32). No Δ 32 CCR5 mutant allele was found in Chinese including 155 healthy controls and 155 RA patients; however, an increased frequency of CCR5 promoter 59653 T/T genotype was found in Chinese RApatients (5).

A marginal increase ($p=0.07$) in the allele A frequency of RANTES promoter -403 was observed in 99 Spanish patients with RA as compared with 65 ethnically matched healthy controls (33). The gene polymorphism of promoter -28 was not simultaneously assessed in that study. The present study observed more patients and controls, and we also looked into the compound genotype and haplotype frequencies of both -28 and -403 positions. As a result, we found RApatients had significantly higher frequencies of the A allele and A/A genotype at the promoter -403 position, and in addition, we found they had a significantly increased frequency of the -28 C/G with -403 A/A compound genotype and a higher frequency of the -28 G/-403 A haplotype with marginal statistical significance. The RANTES -28 G and -403 G alleles were reported to be in complete linkage disequilibrium with -403 A and -28 C alleles, respectively, in different racial groups including non-Indian Asians (24, 28). In the present study, the findings that all individuals with a genotype of G/G at -28 also had an A/A at –403 and that all individuals with a genotype of G/G at -403 also had a C/C at -28 indicate the absence of a haplotype containing a G at both -28 and –403 positions. In addition, we found an increased frequency of the -28 G/–403 A haplotype with marginal statistical significance.

We did not find any statistically significant difference in serum levels of RANTES among healthy controls of different haplotypes (unpublished observation). In a cohort study, the RANTES -28 G/-403 A haplotype was re-

ported to enhance promoter activity in $CD4^+$ Jurkat T and U937 monocytic cell lines and, similar to our finding, no statistically significant difference in serum RANTES levels was observed between healthy individuals with and without the -28 G/-403 A haplotype (28). Nevertheless, in that study, RANTES levels in culture supernatants of the activated $CD4^+$ lymphocytes were higher in subjects with the -28 G/-403 A haplotype than those without this haplotype. These data suggested that, in HIV-infected individuals, the -28 G/-403 A haplotype did not affect the HIV-1 transmission but did delay the disease progression to AIDS. In attracting Th1 cells from the circulation toward the inflamed joint, synovial tissue cell-derived RANTES was shown to play a critical role in the recruitment (34). The *in vitro* migration of mononuclear cells toward synovial fluid from RApatients was reported to be correlated with synovial RANTES levels and to clinical disease activity (35). For individuals with the RANTES -28 G/-403 A haplotype, the enhancement of gene transcription and the subsequent increase in production levels within the synovial environment could be involved in the susceptibility to RA.

In conclusion, we found that polymorphism in the promoter region of RANTES gene is associated with RA susceptibility in Chinese. RA patients had significantly higher frequencies of the -403 A/Agenotype and the -28 C/G with -403 A/Acompound genotype.

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