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NRAMP1 Gene Polymorphisms in Patients with Rheumatoid Arthritis In Koreans

Natural resistance-associated macrophage protein 1 (*Nramp1*) is a genetic locus associated with innate resistance or susceptibility of murine hosts to infection with intracellular pathogens such as *Salmonella, Leishmania* and *Mycobacterium*. The human homologue of the *Nramp1* gene, designated *NRAMP1*, has been investigated as a candidate gene for genetic susceptibility to autoimmune diseases as well as infections. This study tries to determine whether *NRAMP1* polymorphisms are associated with susceptibility to rheumatoid arthritis in Koreans. The nine NRAMP1 polymorphisms (1 microsatellite, 1 variation in 3′ UTR, 5 silent substitution, 2 amino acid substitution) were typed by PCR-RFLP in 74 patients with rheumatoid arthritis (RA) and 53 healthy controls in Koreans. The distribution of allele and genotype frequencies were compared between patients and controls. Three *NRAMP1* polymorphisms (823C/T, D543N and 1729+55del4) were significantly associated with RA. In addition, there were significant differences in the genotype frequencies for 823C/T, D543N and 1729+55del4 polymorphisms between RA patients and controls. Genotypes of A/A homozygote for D543N and TGTG deletion homozygote for 1729+55del4 were only detected in the patient group. These data indicate that genetic polymorphisms of *NRAMP1* might be associated with the susceptibility to rheumatoid arthritis in Koreans.

Key Words: *Polymorphism (Genetic); Arthritis, Rheumatoid; Natural Resistance-Associated Macrophage Protein 1*

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INTRODUCTION

Genetic factors strongly influence susceptibility to infection or autoimmune disease (1, 2). Innate resistance or susceptibility of murine hosts to infection with intracellular pathogens such as *Salmonella, Leishmania* and *Mycobacterium* is under the control of a single dominant genetic locus expression designated (Natural resistance-associated macrophage protein (*Nramp1*) (3-6). The human homologue of the *Nramp1* gene, designated *NRAMP1*, has been cloned and mapped to human chromosome 2q35 (7-9). Numerous studies have established *NRAMP1* as an encoding gene for important regulatory element in the pathways of macrophage activation and differentiation (5, 10, 11). Functional studies indicate that NRAMP1 affects macrophage function, including TNFα, ILβ1 expression, MHC class II expression, nitric oxide-mediated antimicrobial activity, bactericidal and tumocidal activity. This suggests human *NRAMP1* is a candidate gene for genetic susceptibility to autoimmune diseases as well as infections involving macrophage response.

Among the genetic factors of rheumatoid arthritis (RA), HLA-DRB1 has been established as a susceptibility factor. However, HLA-DRB1 has been estimated to partly contribute, and the other possibility of non-HLA gene determining the susceptibility to RA has not yet been identified (12, 13). Also, infectious etiology as a trigger for RA has not been established (14, 15).

To determine whether *NRAMP1* polymorphisms are associated with susceptibility to RA, we analyzed the frequency of several polymorphisms in the *NRAMP1* gene in RA patients and healthy controls in Koreans.

MATERIALS AND METHODS

Patients and controls

Seventy-four patients with rheumatoid arthritis were identified at Rheumatology Clinics in Samsung Medical Center in Seoul, Korea. The diagnosis of RA was made by the revised criteria developed by American College of

Rhematology in 1987. Their mean (±SD) age was 50±14 years, and 12 males and 62 females were included. Fifty-three healthy individuals who visited Samsung Medical Center for periodic checks, were selected as controls. Their mean (±SD) age was 50±9 years, and 24 males and 29 females were included.

NRAMP1 genotyping

Nine *NRAMP1* polymorphisms typed were a (GT)n microsatellite in the 5' to exon 1, denoted here (GT)n; single nucleotide change in exon 3 (274C/T), intron 4 (469+14G/C), intron 5 (577-18G/A), exon 8 (823C/T), exon 9 (A318V), intron 13 (1465-85G/A), and exon 15 (D543N) were denoted 274C/T, 469+14G/C, 577-18G/A, 823C/T, A318V, 1465-85G/A and D543N, respectively; and a TGTG deletion in the 3' untranslated region (1729+55del4) denoted at 1729+55del4 (16) (Table 1).

DNA was extracted from whole venous blood with the use of Wizard™ Genomic DNA Purification System (Promega, U.S.A.). The *NRAMP1* polymorphisms were typed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), in which primers and restriction enzymes were used as described by Liu et al. (16). Restriction enzyme digestion products were resolved by electophoresis on 5% NuSieve agarose (FMC, U.S.A.) gel stained with ethidium bromide. For (GT)n microsatellite, standard sequencing gel with silver stain was used.

Statistical analysis

Comparisons of allele and genotype frequencies were made using chi-square analysis or Fisher's exact test when appropriate. $p<0.05$ was considered significant.

RESULTS

NRAMP1 allele frequencies

NRAMP1 allele frequencies for 823C/T, D543N and 1729+55del4 differed significantly between patient and control groups. The three polymorphisms were each significantly associated with rheumatoid arthritis ($p<0.05$): Allele 1 for 823C/T, silent nucleotide substitution (C to T) at nucleotide 823 at codon 249 (glycine) in exon 8, allele 1 for D543N, an aspartic acid to asparagine substitution at codon 543 in exon 15 and allele 1 for 1729+55del4, a TGTG deletion in the 3' untranslated region. There was no significant difference in the allele frequencies of the six polymorphisms for (GT)n, 274C/T, 469+14G/C, 577-18G/A, A318V and 1465-85G between patient and control groups (Table 2).

NRAMP1 genotypes

The distribution of genotypes in the control group and

Table 1. Identification of *NRAMP1* polymorphisms

Name	Primers (5' to 3')	PCR product size (bp)	Restriction enzyme	Allele
(GT)n	GAC ATG AAG ACT CGC ATT AG TCA AGT CTC CAC CAG CCT AGT	800	*Nco*I	Allele 1: 286 bp Allele 2: 288 bp Allele 3: 290 bp
274C/T	TGC CAC CAT CCC TAT ACC CAG TCT CGA AAG TGT CCC ACT CAG	216	*Mnl*I	Allele 1 (T): 167, 37, and 12 bp Allele 2 (C): 102, 65, 37, and 12 bp
469+14G/C	TCT CTG GCT GAA GGC TCT CC TGT GCT ATC AGT TGA GCC TC	624	*Apa*I	Allele 1 (G): 624 bp Allele 2 (C): 455 and 169 bp
577-18G/A	CTG GAC CAG GCT GGG CTG AC CCA CCA CTC CCC TAT GAG GTG	146	*Msp*I	Allele 1 (A): 146 bp Allele 2 (G): 125 and 21 bp
823C/T	CTT GTC CTG ACC AGG CTC CT CAT GGC TCC GAC TGA GTG AG	234	*Nar*I	Allele 1 (T): 234 bp Allele 2 (C): 135 and 99 bp
A318V	TCC TTG ATC TTC GTA GTC TC GGC TTA CAG GAC ATG AGT AC	232	*BsoF*I	Allele 1 (Val): 232 bp Allele 2 (Ala): 171 and 61 bp
1465-85G/A	GCA AGT TGA GGA GCC AAG AC ACC TGC ATC AAC TCC TCT TC	241	*Bsr*I	Allele 1 (A): 142, 75, and 24 bp Allele 2 (G): 102, 75, 40, and 24 bp
D543N	GCA TCT CCC CAA TTC ATG GT AAC TGT CCC ACT CTA TCC TG	240 or 244	*Ava*II	Allele 1 (Asn): 201 and 39 bp Allele 2 (Asp): 126, 79, and 39 bp
1729+55del4	Same as for D543N		*Fok*I	Allele 1 (-TGTG): 240 bp Allele 2 (-TGTG): 211 and 33 bp

Table 2. *NRAMP1* allele frequencies of nine polymorphisms in Koreans

NRAMP1 polymorphisms	Allele type	Allele frequency*				$p^{\dagger}$
		Controls (n=53[§])		Patients (n=74[‖])		
(GT)n	Allele 1 (286)[†]	80	(80)	115	(78)	
	Allele 2 (288)	18	(18)	25	(17)	NS
	Allele 3 (290)	2	(2)	8	(5)	
274C/T	Allele 1 (T)	21	(20)	24	(16)	NS
	Allele 2 (C)	85	(80)	124	(84)	
469+14G/C	Allele 1 (G)	92	(88)	127	(87)	NS
	Allele 2 (C)	12	(12)	19	(13)	
577-18G/A	Allele 1 (A)	3	(3)	8	(5)	NS
	Allele 2 (G)	103	(97)	138	(95)	
823C/T	Allele 1 (T)	1	(1)	14	(10)	0.006
	Allele 2 (C)	95	(99)	132	(90)	
A318V	Allele 1 (Val)	0		0		NS
	Allele 2 (Ala)	106	(100)	148	(100)	
1465-85G	Allele 1 (A)	22	(21)	48	(32)	NS
	Allele 2 (G)	84	(79)	100	(68)	
D543N	Allele 1 (Asn)	4	(4)	22	(15)	0.006
	Allele 2 (Asp)	98	(96)	126	(85)	
1729+55del4	Allele 1 (-TGTG)	4	(4)	23	(14)	0.006
	Allele 2 (+TGTG)	98	(96)	125	(86)	

*Data are n(%), [†]NS: not significant

[†]Names of polymorphisms and allele types are the same as described in Table 1.

[§]The total numbers of alleles counted in control group were variable due to unidentified cases.

[‖]The total numbers of allele for 577-18G/A and 823C/T polymorphism counted in patient group were 146 in 73 patients.

patients with RA is shown in Table 3. There was significant differences in the frequencies of the genotypes for 823C/T, D543N and 1729+55del4 ($p<0.05$). As compared with C/C (allele 2/allele 2) homozygote, C/T (allele 2/allele 1) heterozygote for 823C/T were significantly increased in patients with RA.

For D543N, G/G homozygote was represented in 92%, G/A heterozygote in 8% and A/A homozygote in 0% in control group, compared with patient group, of which 22% and 4% patients had the G/A and A/A genotypes, respectively. Similarly, for 1729+55del4, genotypes, including TGTG deletion, significantly increased in the patient group and TGTG deletion homozygote was only detected in the patient group (Table 3).

DISCUSSION

Many studies have established mouse Nramp1 as an important regulatory factor in the pathways of macrophage differentiation (5, 10, 11). This suggests human homologue, *NRAMP1* may be a candidate gene for many diseases involving the macrophage, most notably infectious diseases or autoimmune diseases. There is evidence that show macrophages are directly involved in the inflammatory process in RA (17, 18). Also, NRAMP1 as a candidate for susceptibility to RA is interesting in relation to a bacterial/mycobacterial etiology for the disease (14, 15). Then, we investigated nine *NRAMP1* polymorphisms in patients with RA to determine the possibility of *NRAMP1* as a susceptibility gene. Five of these polymorphisms were silent nucleotide substitution, one variant was a microsatellite located in 5' to exon 1, and one variation in 3' UTR. Only two of the polymorphisms were predicted to cause amino acid substitution in A318V, an alanine to valine substitution, and in D543N, an aspartic acid to asparagine substitution, which could affect protein function by substituting a negatively charged amino acid with an uncharged residue in the predicted cytoplasmic carboxyl terminal end of the protein (16).

Our study showed that *NRAMP1* might be associated with susceptibility to RA at least in some genetic variants influencing the function of the NRAMP1 protein. There were significant differences in the allele and genotype frequencies for 823C/T, D543N and 1729+55del4 polymorphisms between RA patients and controls. There was no difference in results between MTX-treated and un-

Table 3. Relation between *NRAMP1* genotypes and rheumatoid arthritis in Koreans

NRAMP1 genotype	Controls* (n=53§)		Patients* (n=74‖)		$p^{\dagger}$
5'(GT)n†					
286/286	32	(64)	43	(58)	NS
286/other	16	(32)	29	(39)	NS
other/other	2	(4)	2	(3)	NS
274C/T					
C/C	33	(62)	52	(70)	NS
C/T	19	(36)	20	(27)	NS
T/T	1	(2)	2	(3)	NS
469+14G/C					
G/G	40	(77)	55	(75)	NS
G/C	12	(23)	17	(23)	NS
C/C	0	(0)	1	(1)	NS
577-18G/A					
G/G	50	(94)	65	(89)	NS
G/A	3	(6)	8	(11)	NS
A/A	0	(0)	0	(0)	
823C/T					
C/C	47	(98)	59	(81)	0.005
C/T	1	(2)	14	(19)	0.005
T/T	0	(0)	0	(0)	
A318V					
C/C	53	(100)	74	(100)	
1465-85G					
G/G	28	(53)	29	(39)	NS
G/A	24	(45)	42	(57)	NS
A/A	1	(2)	3	(4)	NS
D543N					
G/G	47	(92)	55	(74)	0.02
G/A	4	(8)	16	(22)	0.05
A/A	0		3	(4)	NS
1729+55del4					
TGTG+/+	47	(92)	54	(73)	0.01
TGTG+/del	4	(8)	17	(23)	0.03
TGTGdel/del	0		3	(4)	NS

*Data are n(%), †NS: not significant
†Names of polymorphisms and allele types are the same as described in Table 1 and Table 2.
§The total numbers of alleles counted in control group were various due to unidentified cases.
‖The total numbers of allele for 577-18G/A and 823C/T polymorphism counted in patient group were 146 from 73 patients.

treated patient group (data not shown). Among these polymorphisms, the variant for D543N could possibly alter the macrophage function by amino acid substitution, an aspartic acid to asparagine substitution at codon 543 in the predicted cytoplasmic terminal end of NRAMP1 protein. The physiological effect of sequence polymorphisms in the 3' untranslated region (3'UTR) containing the 1729+55del4 locus is not fully understood, although it has been already known that there are regulatory elements for several genes within the 3'UTR. However, our results for association of 3'UTR polymorphism with RA support the importance of this region and useful genetic marker for testing the role of NRAMP1 in susceptibility to other autoimmune disorders.

It is also possible that several associations presented here are due to linkage disequilibrium between *NRAMP1* and other nearby susceptible genes. To prove this problem, a family study on a large scale may be necessary. Shaw et al. (19) provided preliminary evidence for a gene near 2q35 region, contributing to susceptibility to RA by typing for a dinucleotide repeat in the NRAMP1 promoter region and four other marker genes near 2q35 (TNP1, IL8R, VIL1, DES) with 61 RA families.

In rheumatoid arthritis, MHC class II has been shown to be a major susceptibility gene (12, 13), that has been estimated to contribute 37% (13), but the remaining non-MHC factor for genetic susceptibility has not been identified yet. As a candidate gene for susceptibility to RA, further study for the possible interaction between NRAMP1 and MHC class II is of particular interest. In addition, macrophage function studies of the populations expressing different NRAMP1 genotypes would also be of interest.

Human NRAMP1-related studies have been focused on the relationship to intracellular pathogen infection, particularly mycobacterial infection (20, 21). Among autoimmune disease, insulin-dependent diabetes mellitus (IDDM) shows linkage to the region on chromosome 2 (22). In the mouse, a major determinant of IDDM, Idd-5, was identified and mapped to the *Nramp* region (23). Thus, further analyses of genetic polymophisms and functional activity of human *NRAMP1* gene would provide new insight into the pathophysiological mechanism of polygenic autoimmune diseases like IDDM or rheumatoid arthritis.

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