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Identification of Human *LRG1* Polymorphisms and Their Genetic Association with Rheumatoid Arthritis

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Abstract

Human leucine-rich alpha-2-glycoprotein 1 (*LRG1*) was first identified as a trace protein in human serum. The primary sequence of *LRG1* includes repeated leucine residues and putative membrane-binding domains. But, there is no published information on the genetic variation of this gene. In this study, *LRG1* was identified as one of several upregulated genes in RA patients. We examined the expression levels of *LRG1* between an RA patient and a healthy control by RT-PCR and validated that *LRG1* was highly expressed in RA patients compared with controls. We identified the possible variation sites and single nucleotide polymorphisms (SNPs) in the human *LRG1* gene by direct sequencing and analyzed the association of genotype and allele frequencies between RA patients and a control group without RA. We further investigated the relationship between these polymorphisms and the level of RF or anti-CCP in RA patients. We identified a total of three SNPs (g.-678A>G, g.-404C>T and g.1427T>C) and two variation sites (g.-1198delA and g.-893delA) in the *LRG1* gene. Our results suggest that polymorphisms of the *LRG1* gene are not associated with the susceptibility of RA in the Korean population.

Keywords: HRM, *LRG1*, polymorphism, rheumatoid arthritis

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Introduction

RA is a common systemic autoimmune disease that is characterized by chronic inflammation of the synovium, which can lead to progressive joint destruction, and is a complex disease that is a combination of multiple genetic factors and environmental contributions (Gregerin *et al*., 1999). RA is a disease that has a prevalence of 0.3% to 1% worldwide. RA is accompanied by the presence of many autoantibodies, such as rheumatoid factors (RFs), anti-cyclic citrullinated peptide (anti-CCP) antibody, and antibodies to immunoglobulin binding protein (BiP). RFs and anti-CCP antibody are used in clinical practice (Blass *et al*., 1999; Schellekens *et al*., 2000; Blass *et al*., 2001). RFs were the first described human autoantibodies and are directed to the Fc region of IgG and are usually of the IgM isotype (Waaler *et al*., 1940). They are detectable in up to 10% of normal individuals and 70% to 80% of patients with RA, and anti-CCP can be detected in up to 80% of patients with RA (Tighe *et al*., 2001). The RA susceptibility loci were identified, including 1p36, 5q31, and 21q22, by whole-genome linkage analysis (genome-wide association studies), and many polymorphisms also have been identified at these loci (Suzuki *et al*., 2003; Tokuhiro *et al*., 2003).

Human leucine-rich alpha-2-glycoprotein 1 (*LRG1*) was first identified as a trace protein in human serum (Haupt & Baudner, 1977). The *LRG1* gene is located on chromosome 19p13.3, and the primary sequence of LRG includes repeated leucine residues and also has putative membrane-binding domains. Serum *LRG1* is the first extracellular ligand for cytochrome *c* (Cyt *c*). Cyt *c* is a ubiquitous, heme-containing protein that normally resides in the space between the inner and outer mitochondrial membranes (Newmeyer *et al*., 2003). Extracellular Cyt *c* may play a role in inflammation, as it has been reported to cause arthritis when it is injected into mice. Its levels in RA patients' sera are significantly lower than those of healthy controls (Pullerits *et al*., 2005). At least eight repeating 24-amino acid segments that have a notable consensus sequence were identified in a large family of LRG proteins. The function of LRG has not been elucidated, although the functions of many of the other members of the LRR (leucine-rich repeat)-containing superfamily are known (Kobe & Deisenhofer, 1994; Buchanan & Gay, 1996). Plasma LRG expression levels are lower in liver cancer patients who are treated with radiofrequency ablation (Kawakami

et al., 2005). The expression levels of plasma LRG are high in idiopathic normal pressure hydrocephalus (INPH) and lung and pancreatic cancer patients (Kakisaka *et al*., 2007; Li *et al*., 2007; Okano *et al*., 2006). Li *et al*. reported that LRG is a specific biomarker in idiopathic normal pressure hydrocephalus (Li *et al*., 2006). Genetic studies of *LRG1* have not been reported.

We identified several candidate genes and proteins for RA patients by DNA microarray and 2-D gel analysis between RA patients and healthy controls (unpublished data). *LRG1* was identified as one of several upregulated genes in RA patients. In this study, we identified the possible variation sites and single nucleotide polymorphisms (SNPs) in the human *LRG1* gene by direct sequencing of genomic DNA that was isolated from 48 individuals (24 non-RA patients and 24 RA patients) and analyzed the association of genotype and allele frequencies between RA patients and a control group without RA. We further investigated the relationship between these polymorphisms and molecular markers (RF levels and anti-CCP) in RA patients.

Methods

Subjects and DNA samples

Blood samples and records were obtained from 520 RA patients (102 males, 418 females) and 567 controls (355 males, 212 females) without RA. The mean age of RA patients and controls was 53.2 years and 40.3 years, respectively. Genomic DNA was extracted from leukocytes in peripheral blood by a standard phenol-chloroform method or by using the Invisorb Spin Blood Maxi kit (Invitek, Germany) according to the manufacturer's instructions. Patients with RA were recruited from the outpatient clinic at Eulji University Hospital. RA was diagnosed according to criteria set forth by the American Rheumatism Association. CRP and RF levels in patients with RA were determined in a routine laboratory at Eulji University Hospital. The non-RA controls were recruited from the general population and had received comprehensive medical testing at Wonkwang University Hospital. All subjects in this study were Korean.

Polymerase chain reaction (PCR) and DNA sequencing

PCR was performed using 50 ng of genomic DNA, Taq DNA polymerase (EF Taq, SolGent, Korea), and 0.5 μM of each primer (Table 1) under the following conditions: 30 cycles of PCR consisting of denaturation for 10 sec at 98°C, annealing for 30 sec at 65°C, extension for 1 min at 72°C, and a final extension for 10 min at 72°C in a thermocycler (Gene Amp PCR system 9700, PE Applied Biosystems, USA). After purification using a PCR purification kit (Millipore, Ireland), the PCR products were used as template DNA for sequencing analysis (ABI Prism BigDye Terminator cycle sequencing system; ABI 3100 automatic sequencer, PE Applied Biosystems, USA). For sequencing, the same PCR pri-

Table 1. Primer sequences used for amplification, sequence analysis, and genotyping of the *LRG1* gene

Application	Primer name	Primer sequence (5'→3')	Region
PCR analysis	LRG1-PF1	ACCCGCCACCACGCCCCGCT	Promoter
	LRG1-PR1	TCCCCACCCCCGTCCAGTTCCTGA	
	LRG1-PF2	AGCCAAGTCCATGAGATGCAGCCA	Promoter and Exon1
	LRG1-PR2	AGCAAATCCGCCCACTTCTTCCCA	
	LRG1-PF3	ACACACACCCCTACAGAAGCCCA	Exon2
	LRG1-PR3	ACCATATCCCACACCCCAGGGCCT	
DNA sequencing analysis	LRG1-SF1	CCAGGAGTTTGAGACCAGCCT	Promoter
	LRG1-SR1	TCAGTTTCCCCATCTATGAAGCTGA	Promoter
	LRG1-SF2	GCATGGTGGCTCACACCTGT	Promoter
	LRG1-SF3	ACAGGTGAGAGATGAGGTAGCAT	Exon2
	LRG1-SF4	ACCTGCCAGCCAACCTCCT	Exon2
	LRG1-SF5	TCCTGAGGGGTCCGCTGCA	Exon2
	LRG1-SF6	TGGTAGAACACTGCAACCCGCT	Exon2
RT-PCR	LRG1-RF1	ACCAGGGGCTTGGGTTGAGGGT	3'- UTR
	LRG1-RR1	TCTGGGAAACAGGGAACGGCA	
HRM analysis	LRG1-HF1	TGGAAACAATAGCATGGATGTCA	g.-678A>G
	LRG1-HR1	CTTTGTGACCTTTGGCAAGTGAC	
	LRG1-HF2	CAACATACCATGAAATCAAGACGAGT	g.-404T>C
	LRG1-HR2	TGCAAGGCACTATTCCTGGAGA	
	LRG1-HF3	GCTGGAAAGCCTCTCGCCCGA	g.1427C>T
	LRG1-HR3	TCAATACCAGGGTGTCCAGGGT	

mers and four additional primers were used (Table 1). The reference sequence of *LRG1* was based on human chromosome 19 clone CTB-50L17.

RNA extraction and RT-PCR

For total RNA, human peripheral blood mononuclear cells (PBMC) from four pairs of RA patients and healthy controls were isolated from the buffy coats by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Sweden), washed with phosphate-buffered saline (GIBCO BRL, Invitrogen, USA), and lysed in TRIzol reagent (Invitrogen, USA) according to the instructions of the manufacturer. Approximately 1-2 μg of total RNA was used in the first-strand cDNA synthesis with a sequence-specific primer using M-MLV reverse transcriptase (Bioneer, Korea) for RT-PCR. The expression assay for several tissues was performed using multiple human tissue cDNAs (Clontech, USA).

Genotype analysis

Genotyping was performed by high resolution melting (HRM) analysis. The intercalating dye that was used was EvaGreen (Biotium, USA). The 10-μL reaction mixture was made up using the 1 x QuantiTect Probe PCR Kit (Qiagen, USA) and consisted of 50 ng of genomic DNA, 100 nM of each primer, and 1 x Evagreen solution. PCR cycling and HRM analysis was carried out using the Rotor-Gene thermal cycler RG6000 (Corbett Research, Australia). The 138-bp, 127-bp, and 133-bp amplicons were produced according to the following conditions; one cycle at 95°C for 15 min; 45 cycles at 95°C for 15 sec, 68°C for 10 sec, and 72°C for 30 sec. Optical measurements in the green channel (excitation at 470 nm and detection at 519 nm) were recorded during the extension step. After a completion of 45 cycles, melting curve data were generated by increasing the temperature from 77°C to 95°C at 0.1°C per second and recording fluorescence. HRM curve analysis was performed using the software Rotor-Gene 1.7.40 and the HRM algorithm that was provided.

Statistical analysis

The RA patients and controls without RA were compared by case-control association analyses. χ^2 tests were used to estimate the Hardy-Weinberg equilibrium (HWE). Pair-wise comparison of biallelic loci was employed for the analyses of linkage disequilibrium (LD). The haplotype frequencies of *LRG1* for multiple loci were estimated using the expectation maximization (EM) algorithm with SNPAlyze software (DYNACOM, Japan). ANOVA was applied to analyze differences between the genotype and anti-CCP or RF levels in RA patients. A P value of less than 0.05 was considered to indicate statistical significance.

Results

We have studied cDNA microarrays with mRNAs from four RA patients and four controls and 2-DE analysis to examine the expression level of mRNAs as well as the difference in protein levels between RA patients and controls (unpublished data). Human leucine-rich alpha-2-glycoprotein 1 (*LRG1*) was identified as one of many upregulated genes in RA patients in both the cDNA microarray and 2-D gel analysis. We confirmed the expression levels of *LRG1* between RA patients and healthy controls by RT-PCR and validated that *LRG1* was highly expressed in RA patients compared with controls (Fig. 1A). We also examined the expression pattern of *LRG1* in 15 normal human tissues and peripheral leukocytes. Our results showed that the expression level of *LRG1* mRNA was highest in peripheral leukocytes, while the expression level of *LRG1* mRNA in

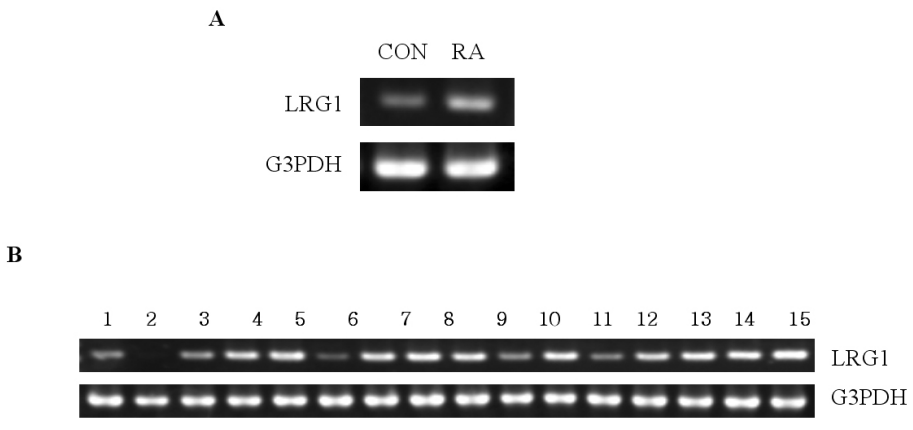

Fig. 1. Expression levels and patterns of *LRG1* mRNA by three independent tests. (A) The expression levels of *LRG1* mRNA between RA patients and healthy controls, (B) the expression patterns of *LRG1* mRNA in various human tissues. Lanes: 1, heart; 2, brain; 3, placenta, 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; and 16, peripheral leukocytes.

brain and skeletal muscle was only barely detected (Fig. 1B).

To determined the possible variation sites in entire sequences of the *LRG1* gene, including about 2.0 kb of the promoter region, we first scanned the genomic DNA that was isolated from 24 unrelated controls and 24 RA patients by a direct sequencing method. We identified three SNPs-g.-678A>G (novel SNP) and g.-404T>C (rs4806985) in the promoter region, g.1427T>C (rs966384) in exon2-and two novel variation sites (g.-1198 delA and g.-893 delA) (Fig. 2). Linkage disequilibrium (LD) coefficients (|D'|) were calculated between all SNP pairs. Although an absolute LD (|D'|=1 and r^2=1) was not found, strong LDs were found between *g.-404C>T* and *g.1427C>T*, and between *g.-678A>G* and *g. 1427T>C* (|D'| = 0.91 and 0.90, respectively). A single nucleotide transition (T to C) at g.1427T>C resulted in an amino acid change to p.Pro133Ser. We analyzed the genotype of these SNPs in RA patients and in controls by the HRM method and compared the genotype frequencies between these groups. The genotype frequencies of all loci were in HWE (p>0.05, data not shown). The genotype and allele frequencies of g.-678A>G, g.-404C>T, and g.1427T>C were not significantly different between RA patients and controls. These results suggest that the SNPs of *LRG1* are not associated with susceptibility to RA (Table 2).

On the other hand, to define a possible correlation between polymorphisms and clinical features of RA, we further analyzed each genotype of these SNPs with anti-CCP and RF levels in RA patients (Table 3). We found that these SNPs in RA patients have no significant as-

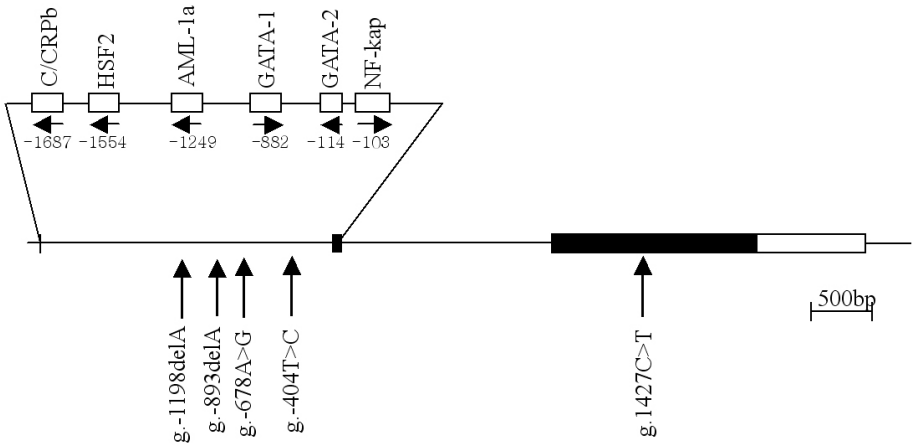

Fig. 2. Location of each single nucleotide polymorphism (SNP) and sites of variation in LRG1. Coding exons are marked by filled blocks and 3'-untranslated regions (UTR) by open block. The positions of SNPs were calculated from the translation start site. Putative transcription factor sites at http://www.cbrc.jp/research/db/TFSERCH.html. The reference sequence for LRG1 was based on the sequence of human chromosome 19 clone CTB-50L17.

Table 2. Genotype and allele frequencies in the SNPs of *LRG1* between RA patients and controls

Position*	Genotype/Allele	Control, n (%)	RA, n (%)	p value†
g.-678 A>G	AA	422 (75.8)	394 (75.3)	0.985
	AG	131 (23.5)	125 (23.9)	
	GG	4 (0.7)	4 (0.8)	
	A	975 (87.5)	913 (87.3)	0.897
	G	139 (12.5)	133 (12.7)	
g.-404 C>T (rs4806985)	CC	225 (40.1)	216 (41.6)	0.701
	CT	261 (46.5)	243 (46.7)	
	TT	75 (13.4)	61 (11.7)	
	C	711 (63.4)	675 (64.9)	0.473
	T	411 (36.6)	365 (35.1)	
g.1427 T>C (rs966384)	TT	138 (24.7)	141 (27.0)	0.677
	TC	295 (52.8)	272 (52.0)	
	CC	126 (22.5)	111 (21.0)	
	T	571 (51.1)	554 (53.0)	0.414
	C	547 (48.9)	494 (47.0)	

*Calculated from the translation start site.
†Value was determined by χ^2 test from 2×3 contingency table.

Table 3. The levels of rheumatoid factors (RF) and anti-CCP among the genotypes of polymorphisms of the *LRG1* gene in RA

Position*	Genotype	RF			p†	anti-CCP			p†
		n	Mean	SD		n	Mean	SD	
g.-678 A>G	AA	328	73.7	77.1	0.18	176	53.6	51.0	0.57
	AG	116	62.8	68.3		71	57.7	51.7	
	GG	1	-	-		1	-	-	
g.-404 C>T	CC	189	70.6	73.3	0.66	111	52.6	51.7	0.81
(rs4806985)	CT	206	69.8	76.2		114	56.1	50.0	
	TT	55	80.0	80.1		25	58.8	58.0	
g.1427 T>C	TT	118	76.5	76.4	0.65	66	50.3	52.0	0.77
(rs966384)	TC	227	68.5	75.1		131	56.0	50.1	
	CC	102	70.7	76.1		51	54.7	53.4	

*Calculated from the translation start site.
†Values were analyzed by ANOVA.

sociation with the levels of RF and anti-CCP (Table 3). We also calculated and compared the haplotype frequencies among the g.-678A>G, g.-404C>T, and g.1427T>C SNPs of *LRG1* between controls and RA patients. Three major haplotypes out of eight possible haplotypes were identified that revealed more than 93.3% and 97.8% distribution in the controls and RA patients, respectively (Table 4). The distribution rate of the haplotype *ACC* was significantly different between the controls and RA patients ($p < 0.0003$).

Table 4. The haplotype frequencies in both RA patients and controls of *LRG1* polymorphisms

Haplotype			Frequency*		p†
g.-678 A>G	g. -404 C>T	g. 1427 T>C	Control	RA	
A	C	T	0.492	0.518	0.238
A	T	C	0.333	0.342	0.663
G	C	C	0.108	0.118	0.478
A	C	C	0.037	0.012	<0.0003
A	T	T	0.01	5.1E-3	0.176
G	T	C	9.4E-3	9.5E-4	0.0075
G	C	T	7.3E-3	2.9E-3	0.154
G	T	T	3.8E-3	2.0E-3	0.44

*Values were constructed by the expectation maximization (EM) algorithm with genotyped SNPs.
†Values were analyzed by the permutation test.

Discussion

RA is a chronic, systemic inflammatory disorder that affects approximately 1% of the population, at a female-to-male ratio of 3:1 (Schena *et al.*, 1995). In this study, the genotype and allele frequencies of *LRG1* SNPs were not significantly different between RA patients and controls (Table 2). This result indicates that the *LRG1* SNPs are not associated with a susceptibility to RA.

LRG has putative membrane-binding domains, which is suggestive of the possibility of protein-protein and protein-deoxyribonucleic acid interaction (Takahashi *et al.*, 1985; Kobe & Deisenhofer, 1994). *LRG1* is a novel ligand for Cyt *c*. The observation of Cyt *c* in normal serum and its binding to *LRG1* suggests that these two molecules may have physiological relevance. Cyt *c* could serve as an adaptor by altering the activity of *LRG1*. Pullerits *et al.* reported that Cyt c levels in the synovial fluid of RA patients were low (Pullerits *et al.*, 2005). Several studies have reported that serum LRG expression levels were high in INPH and cancers. In the present study, the expression levels of plasma *LRG1* mRNA were higher in RA patients than in healthy controls. Our result also showed that the expression level of *LRG1* mRNA was the highest in peripheral leukocytes compared with various human tissues (Fig. 1B). Our novel approach might suggest that *LRG1* may be related to inflammatory responses in peripheral blood.

cDNA microarray technology allows for expression monitoring, identification, and quantification of thousands of genes simultaneously (Schena *et al.*, 1995; Shalon *et al.*, 1996). Using the cDNA microarray method, expression analysis in diseased RA tissue was conducted and demonstrated its utility to analyze complex diseases, such as RA, systemic lupus erythematosus (SLE), and inflammatory bowel disease (Heller *et al.*, 1997). Recently, gene expression profiles have been identified from peripheral blood cells of patients with SLE, RA, and multiple sclerosis (MS) (Baechler *et al.*, 2003; Batliwalla *et al.*, 2005; Bomprezzi *et al.*, 2003). We previously performed cDNA microarray and 2-D gel

analysis to screen RA-related specific genes. About 144 and 62 genes were identified as having more than 5.0-fold higher or lower mRNA levels in RA patients (unpublished data). Even though the expression levels of *LRG1* were higher in RA patients than in controls, the genetic polymorphisms of *LRG1* might not be associated with the susceptibility of this immune disorder.

A hallmark of RA is the presence of autoantibodies; therefore, we evaluated whether these SNPs have associations with RF and anti-CCP. But the genotypes of *LRG1* gene polymorphisms have no significant association with the RF and anti-CCP levels in RA patients (Table 3). These results suggest that the polymorphisms of *LRG1* are not associated with these factors in RA patients.

In conclusion, the g.-678A>G, g.-404C>T, and g.1427T>C polymorphisms of the *LRG1* gene do not confer genetic susceptibility to RA in Koreans.

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