Association of *FOXJ1* polymorphisms with systemic lupus erythematosus and rheumatoid arthritis in Korean population

Chun-Shi Li[1,3]**, Qinggao Zhang**[4]**, Mi-Kyoung Lim**[5]**,**
Dong-Hyuk Sheen[5]**, Seung-Cheol Shim**[5]**,**
Ji-Young Kim[5]**, Shin-Seok Lee**[6]**, Ki-Jung Yun**[2]**,**
Hyung-Bae Moon[2]**, Hun-Taeg Chung**[1,7]
and Soo-Cheon Chae[2,7]

[1]Genome Research Center for Immune Disorders
[2]Department of Pathology
School of Medicine, Wonkwang University
Iksan 570-749, Korea
[3]Department of Pharmacology
[4]Department of Microbiology and Immunology
Yanbian University Medical College
133000 Yianji, Jilin, China
[5]Division of Rheumatology
Department of Internal Medicine
Eulji University School of Medicine
Daejeon 301-831, Korea
[6]Division of Rheumatology
Department of Internal Medicine
Chonnam National University Medical School
Gwangju 501-746, Korea
[7]Corresponding authors: Tel, 82-63-850-6793;
Fax, 82-63-852-2110; E-mail, chaesc@wonkwang.ac.kr (for S.C.C.) and
Tel, 82-63-850-6762; Fax, 82-63-851-5066;
E-mail, htchung@wonkwang.ac.kr (for H.T.C.)

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Abbreviations: FOXJ1, forkhead-box J1; RA, rheumatoid arthritis; SBE, single-base extension; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism

Abstract

The forkhead-box J1 (FOXJ1) transcription factor could suppress a spontaneous activation of T cells and B cells through an induction of IκBβ that results in repression of NF-κB activity. In Foxj1 deficiency mice, systemic autoimmune inflammation is quite common symptom. Therefore, deregulated Foxj1 is supposed to be associated with autoimmune diseases and/or other inflammatory diseases. Previously, we identified that polymorphisms of human *FOXJ1* gene (*g.−460C > T, g.1805G > T* and *g.3375G > C*) are associated with allergic rhinitis in a Korean population. In present study, we compared the genotype and allele frequencies of these SNPs between healthy controls and systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) patients. We also investigated the relationships between each genotype and the expression levels of antinuclear antibodies in SLE patients, and rheumatoid factor and anti-cyclic citrullinated peptide in RA patients. The frequencies of haplotypes constructed by these *FOXJ1* SNPs were compared between controls and SLE (or RA) patients. The results of genotype and allele analysis showed that the prevalence of polymorphism *g.3375G > C* was associated with the susceptibility of SLE (*P* = 0.0072 and 0.0042, respectively). But no significant association was found with RA. In the haplotype analysis, however, the main *CGG* showed a weak association between controls and RA patients (*P* = 0.048).

Keywords: autoantibodies; FOXJ1 protein, human; polymorphism, genetic; lupus erythematosus, systemic; rheumatoid arthritis

Introduction

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are the representative autoimmune diseases. These diseases are arisen by the complex interaction between multiple genetic factors and environmental factors (Kotzin, 1996; Gregersen, 1999). The clinical manifestations of RA are thought to be triggered by over-activation of helper T1 (Th1) cell (Simon *et al.,* 1994; Dolhain *et al.,* 1996) while SLE is characterized by elevation of both Th1 and Th2 cells. The predominance of Th1 and/or Th2 cells depends on the stage of disease development (Akahoshi *et al.,* 1999; Chang *et al.,* 2002). RA and SLE are most commonly presented in women. Particularly, the frequency of SLE between female and male is 9 to1 (Hochberg, 1997). Rheumatoid factor (RF) has been widely used as a screening test for patients with arthritis. RF is prognostically useful to correlates with functional (Van der Heide *et al.,* 1995) and outcomes in both RA and early inflammatory polyarthritis (Harrison *et al.,* 1999). More recently a highly specific autoantibody system has been described for RA, in which the synthetic cyclic citrullinated peptide (CCP) with deiminated arginines is used as the antigen for anti-CCP antibodies (Schellekens *et al.,* 2000).

Foxj1 (also known as HFH-4 and FKHL-13) is a member of the forkhead-box (FOX) gene family, which encodes transcription factors composed of with a conserved DNA binding motif known as the forkhead domain (Weigel and Jackle, 1990). Recent studies suggest more than 100 forkhead transcription factors classified into 17 subgroups, from FOXA to FOXQ, of FOX family (Kaestner *et al.,* 2000). Fox family takes part in diverse biological functions including development, metabolism, aging, and cancer (Carlsson and Mahlapuu, 2002; Tran *et al.,* 2003; Coffer and Burgering, 2004). *Foxj1* is expressed mainly in tissues of ciliated respiratory cells, reproductive and central nervous systems. Furthermore, it is regarded as having association with ciliated cell development. In most cases, the mutation of *foxj1* gene resulted in an absence of cilia in the tissues of mice models (Hackett *et al.,* 1995; Lim *et al.,* 1997; Chen *et al.,* 1998; Blatt *et al.,* 1999). *Foxj1* also plays critical roles in immune system (Lin *et al.,* 2004, 2005). *Foxj1* is a repressor of NF-κB, a transcription factor responsible for a great variety of pro-inflammatory cytokines production. *Foxj1*-deficient mice showed multi-organ inflammation. And the expression of *foxj1* was decreased in lupus prone mice (Lin *et al.,* 2004). On the basis of the rationale, *foxj1* has been postulated as a potential candidate gene that is associated with auto-immune and (or) inflammatory diseases.

As we previously identified, the seven SNPs, [*g.−460C>T* (rs880213) and *g.−342G>C* (rs-880212) in promoter region, *g.1164G>C*, *g.1805G >T* (rs1868823), *g.1824C>G* and *g.1849G>C* (rs1868824) in intron1, and *g.3375G>C* (rs319-2453) in 3'-UTR region, in human FOXJ1 gene and reported that the *g.−460C>T*, *g.1805G>T* and *g.3375G>C* polymorphisms in *FOXJ1* gene were associated with susceptibility to allergic rhinitis (Li *et al.,* 2006). To determine whether these polymorphisms are associated with the susceptibility of autoimmune diseases such as SLE and RA, we compared the genotype and allele frequencies between healthy controls and patients having RA or SLE. We also investigated the relationships of each genotype with some clinical characteristics, such as RF and CCP antibody for RA, and anti-nuclear antibodies (ANA) for SLE. We further analyzed the differences of haplotype distributions by these SNPs between controls and patients with RA or SLE.

Materials and Methods

Patients and genomic DNA

Blood samples were obtained from 418 controls (260 males and 158 females), 204 SLE patients (13 males and 191 females) and 376 RA patients (70 males and 306 females). The mean age of the control, SLE and RA groups were 38.3 years, 34.8 years and 53.8 years, respectively. Healthy controls were recruited from individuals who took comprehensive examination in Wonkwang University Hospital. The RA and SLE patients were recruited from outpatient clinic at Eulji University Hospital and Chonnam National University Hospital. RA and SLE were diagnosed according to the criteria of the American Rheumatism Association, and the American College of Rheumatology (ACR), respectively (Arnett *et al.,* 1988; Hochberg, 1997). CCP and RF levels in RA patients and ANA levels in SLE were determined in a routine laboratory at the Eulji University Hospital and Chonnam National University Hospital. Genomic DNA was extracted from leukocytes in peripheral blood by a standard phenol-chloroform method or by Genomic DNA Extraction kit (iNtRON Biotechnology, Korea) according to manufacturer's directions.

PCR

The previously used three primer pairs were used for amplification of the FOXJ1 genes containing *g.−460C>T*, *g.1805G>T* and *g.3375G>C* polymorphisms (Li *et al.,* 2006). PCR was performed in a 20 μl reaction volume and reaction mixture were prepared by previously described condition (Chae *et al.,* 2004). Amplification was carried out in a GeneAmp PCR system 9700 thermocycler (Applied Biosystem) at 95°C for 5 min to pre-denature the template DNA, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 68°C for 30 s and extension at 72°C for 2.0 or 2.5 min, finally, extension for another 10 min at 72°C to end the reaction. The PCR products were purified by PCR Purification Kit (Millipore) and were used as template DNA for direct sequencing and single base extension.

Genotyping

Genotyping was performed by single-base extension (SBE) using the ABI Prism® SNaPshot™ Multiplex kit (Applied Biosystems, Foster city, CA). The previously used three SBE primers were used in this study for genotyping of *FOXJ1* SNPs in RA patients, SLE patients and controls (Li *et al.,* 2006). The SBE reaction mixture was prepared according to the manufacturer's instructions. The primer extension reaction was performed at 96°C for 1 min, followed by 25 cycles at 96°C for 5 s, 55°C for 30 s, and 60°C for 30 s. To clean up the primer

extension reaction, 1.5 U of calf intestinal alkaline phosphatase (CIP, New England BioLabs) was added to the reaction mixture, and the mixture was incubated at 37°C for 60 min, followed by 15 min at 72°C, for purposes of enzyme inactivation. The purified extension products were added to Hi-Di formamide (Applied Biosystems) according to the recommendations of the manufacturer. The mixture was incubated at 95°C for 5 min, followed by 5 min on ice, and then electrophoresis was performed, using the ABI Prism 3100 Genetic Analyzer. The results were analyzed using the ABI Prism GeneScan and Genotyper software (Applied Biosystems).

Statistic analysis

χ^2 tests were used to estimate the Hardy- Weinberg equilibrium (HWE). Linkage Disequilibrium (LD) analyses by pair-wise comparison of biallelic loci and the haplotype frequencies of *FOXJ1* for multiple loci were estimated using the expectation maximization (EM) algorithm with SNPAlyze software (DYNACOM, Japan). Logistic regression analyses were used to calculate odds ratios (95% confidence interval), and ANOVA was applied in order to analyze differences among the each genotypes, and RF, CCP levels for RA patients or ANA and anti-RNP levels for SLE patients. Cutoff value of RF is 18 IU/ml and that of anti-CCP antibody is 5 U/ml. A *P*-value of less than 0.05 was considered to indicate statistical significance.

Results

We previously identified seven SNPs in human *FOXJ1* gene and the three polymorphisms (*g.−460C>T*, *g.1805G>T* and *g.3375G>C*) were associated with susceptibility to allergic rhinitis (Li *et al.,* 2006). In our present study, we attempted to find out whether these polymorphisms were further associated with genetic predisposition of SLE and RA. The genotypes of these polymorphisms were determined in 204 unrelated SLE patients, in 376 unrelated RA patients and in 418 unrelated controls without SLE and RA. All genotype frequencies were in Hardy-Weinberg equilibrium (HWE) by χ^2 tests (data not shown). In comparison between SLE (or RA) patients and the controls, the genotype and allele frequencies of each polymorphism were analyzed (Table 1). While the genotype and allele frequencies of these SNPs, in RA patients, were no significant association. The genotype and allele frequencies of *g.3375G>C* polymorphism, in SLE patients, were significantly different from those of controls group (*P* = 0.007 and 0.004, respectively). Among female, we further analyzed the *P* values for *g.−460C>T*, *g.1805G>T* and *g.3375G>C* polymorphisms between the controls and RA (or SLE) patients, because both RA and SLE patients are predominantly female in compare with control subject (Table 2). As we expected, however, our results indicate that gender is a minor factor in the association of RA patients. On the whole, in SLE female patients, the genotype and

Table 1. Genotype and allele analysis of the polymorphisms in *FOXJ1* gene between patients with SLE or RA and controls.

Position[a]	Genotype	Control n (%)	RA n (%)	SLE n (%)	P[b] vs. RA	P[b] SLE
g.−460C>T (rs880213)	CC	321 (76.8)	288 (77.0)	137 (71.7)	0.996	0.284
	CT	90 (21.5)	80 (21.4)	48 (25.1)		
	TT	7 (1.7)	6 (1.6)	6 (3.1)		
	C	732 (87.6)	656 (87.7)	322 (84.3)	0.939	0.125
	T	104 (12.4)	92 (12.3)	60 (15.7)		
g.1805G>T (rs1868823)	GG	294 (70.5)	269 (71.7)	132 (65.3)	0.881	0.283
	GT	114 (27.3)	97 (25.9)	62 (30.7)		
	TT	9 (2.2)	9 (2.4)	8 (4.0)		
	G	702 (84.2)	635 (84.7)	328 (80.7)	0.835	0.146
	T	132 (15.8)	115 (15.3)	78 (19.3)		
g.3375G>C (rs3192453)	GG	318 (77.0)	289 (77.7)	135 (67.2)	0.760	0.007
	GC	88 (21.3)	79 (21.2)	65 (32.3)		
	CC	7 (1.7)	4 (1.1)	1 (0.5)		
	G	724 (87.7)	657 (88.3)	335 (83.3)	0.699	0.004
	C	102 (12.3)	87 (11.7)	67 (16.7)		

[a]Calculated from the translation start site.
[b]Value was determined by Fisher's exact test or χ^2 test from 2 × 2 contingency table.

allele frequencies of *g.3375G>C* polymorphism were not significantly different from that of female control groups (*P* = 0.273 and 0.185, respectively). On the contrary, in SLE male patients, the polymorphisms of SLE (*g.1805G>T* and *g.3375G>C*) are significantly different from the male control group (*P* = 0.023 and 0.003 respectively, data not shown). Therefore, we partially suggest that the association of the SNPs of *FOXJI* (*g.1805G>T* and *g.3375G>C*) could be affected by the gender of patients. On the other hand, to define a possible correlation between polymorphisms and clinical features of SLE and RA, we further analyzed each

genotype of these SNPs with ANA in SLE patients and anti-CCP and RF levels in RA patients (Table 3). We found that these SNPs in RA and SLE patients have no significant association with the levels of RF and anti-CCP, and ANA, respectively (Table 3). We analyzed each genotype of these SNPs according to positivity of RF and anti-CCP in RA patients RA, and found that there are no significant associations (data not shown). We also evaluated the haplotype frequencies among *g.−460C>T*, *g.1805G>T* and *g.3375G>C* polymorphisms in both healthy controls and RA patients or SLE patients (Table 4). While three

Table 2. Genotype and allele analysis of the polymorphisms in *FOXJ1* gene between the female of RA or SLE patients and controls.

Position[a]	Genotype	Control n (%)	RA n (%)	SLE n (%)	P[b] vs. RA	SLE
g.−460C>T	CC	116 (74.4)	227 (76.9)	131 (72.8)	0.736	0.629
	CT	38 (24.4)	63 (21.4)	44 (24.4)		
	TT	2 (1.2)	5 (1.7)	5 (2.8)		
	C	270 (86.5)	517 (87.6)	306 (85.0)	0.675	0.583
	T	42 (13.5)	73 (12.4)	54 (15.0)		
g.1805G>T	GG	110 (70.5)	213 (72.2)	126 (66.7)	0.623	0.446
	GT	44 (28.2)	75 (25.4)	57 (30.2)		
	TT	2 (1.3)	7 (2.4)	6 (3.1)		
	G	264 (84.6)	501 (84.9)	309 (81.7)	0.922	0.359
	T	48 (15.4)	89 (15.1)	69 (18.3)		
g.3375G>C	GG	119 (76.8)	228 (77.8)	130 (69.1)	0.729	0.273
	GC	35 (22.6)	61 (20.8)	57 (30.3)		
	CC	1 (0.6)	4 (1.4)	1 (0.5)		
	G	273 (88.1)	517 (88.2)	317 (84.3)	1.00	0.185
	C	37 (11.9)	69 (11.8)	59 (15.7)		

[a]Calculated from the translation start site.

[b]Value was determined by Fisher's exact test or χ^2 test from 2 × 2 contingency table.

Table 3. Analysis of RF and anti-CCP levels in RA patients, and ANA levels in SLE patients among the each genotype of *FOXJ1* SNPs.

Position[a]	Genotype	RF (1 U/ml)			P[b]	Anti-CCP			P[b]	ANA			P[b]
		n	Mean	SD		n	Mean	SD		n	Mean	SD	
g.−460C>T	CC	270	70.0	71.0	0.37	142	57.4	57.2	0.33	90	2.40	0.41	0.36
	CT	78	57.3	66.1		32	58.3	53.1		35	2.31	0.36	
	TT	6	69.5	90.2		4	99.9	33.4		4	2.20	0.35	
g.1805G>T	GG	253	69.6	70.8	0.18	131	59.0	57.3	0.26	87	2.38	0.40	0.90
	GT	93	58.8	65.3		41	52.1	52.8		46	2.38	0.40	
	TT	9	98.9	105.0		6	92.0	46.3		6	2.30	0.37	
g.3375G>C	GG	271	70.9	71.8	0.12	143	57.4	57.0	0.87	91	2.38	0.38	0.92
	GC	77	56.4	66.0		32	59.1	54.0		47	2.38	0.43	
	CC	4	40.5	31.7		2	−	−		0	−	-	

[a]Calculated from the translation start site.

[b]Values were analyzed by ANOVA.

Table 4. The haplotype frequencies between RA or SLE patients and controls in *FOXJ1* SNPs.

Haplotype			Frequency[a]			P[b]	
g.−460C > T	g.1805G > T	g.3375C > T	Control	RA	SLE	*vs.* RA	SLE
C	G	G	0.802	0.840	0.804	0.048	0.945
T	T	C	0.088	0.113	0.118	0.103	0.106
C	T	G	0.050	0.034	0.014	0.103	0.010
T	G	G	0.014	0.004	0.000	0.033	0.025
T	G	C	0.014	0.001	0.000	0.017	0.042
C	T	C	0.012	0.005	0.002	0.004	0.130
C	G	C	0.011	0.003	0.027	0.086	0.088
T	T	G	0.009	0.000	0.035	0.404	0.001

[a]Values were constructed by EM algorithm with genotyped SNPs.
[b]Values were analyzed by permutation test.

major haplotypes were identified explaining more than 94.0% of distribution in controls, two major types were identified in SLE (92.1%) and RA (95.3%) patients out of eight possible haplotypes. And the main haplotype, *CGG*, represented a weak association with RA ($P = 0.048$), the haplotype *CTG* also showed a significant difference between SLE patients and controls ($P = 0.01$).

Discussion

The activated Th cells during the developing stage are differentiated into phenotypically and functionally two distinct types of cells, Th1 and Th2 (Mosmann and Coffman, 1989; Abbas *et al.,* 1996). Th1 cells produce cytokines such as IFN-γ, IL-12 and cytotoxic factor lymphotoxin. They are commonly associated with cell-mediated immune responses against intracellular pathogens and induction of organ-specific autoimmune diseases such as RA (Kuchroo *et al.,* 1995; Abbas *et al.,* 1996). On the other hand, the Th2 cell related cytokines IL-4, IL-5 and IL-10, are known to be associated with atopic and allergic diseases. The balance between Th1 and Th2 cells is critical in the immune response to pathogens, tumor antigens and allergens. Th1 and Th2 cells cross-regulate the differentiation of each other. The predominant induction of Th2 cells inhibits autoimmune diseases such as RA and the predominant induction of Th1 cells inhibits induction of asthma and allergic diseases (Lafaille *et al.,* 1997; Ho and Glimcher, 2002).

Foxj1 is a presumed transcription factor containing DNA binding domain. Foxj1 induces the production of IκBβ and subsequently suppresses the activity of NF-κB, the potent proinflammatory cytokine inducer. Down-regulated expression of Foxj1 was observed in a lupus prone mice model. Further more, systemic autoimmune inflammation was demonstrated in Foxj1 (−/−) mice (Lin *et al.,* 2004). Under the neutralizing condition, the CD4$^+$ T cells from *Foxj1* (−/−) mice produced more Th1 cytokines than that from *Foxj1* (+/+) mice. The phenomena, probably, designates a Th1 bias state (Lin *et al.,* 2004). It suggests that *Foxj1* is a possible gene of candidate in autoimmune and/or inflammatory diseases. These result let us to think that the gene variation of *FOXJ1* might be associated with susceptibility to allergic and/or autoimmune diseases. Through the results of our previous study, we confirmed that the polymorphisms of *g.−460C>T*, *1805G>T*, *g.3375G>C* in human *FOXJ1* may be associated with the susceptibility to allergic rhinitis in a Korean population (Li *et al.,* 2006). Moreover, our present studies indicate that *g.3375G>C* could be specifically associated with the susceptibility to SLE (Table 1). Interestingly, the genome-wide screen study from three ethnic groups indicates that the human *FOXJ1* gene is located on chromosome 17q22-25 region linked with SLE (Cantor *et al.,* 2004). The polymorphism of *g.3375 G>C* falls on 3'-UTR region that is particularly considered to be engaged in the control of translation, mRNA stability and poly-adenylation of signaling molecules (Kuersten and Goodwin, 2003). The genotype frequencies of FOXJ1 gene polymorphisms are significantly different between SLE male patients and the male control group, but no difference between both female groups. These results indicated that the male with g.3375G>C polymorphism in FOXJ1 are more significantly associated with the susceptibility of SLE than female, and suggest that the association of the SNPs of *FOXJI* (*g.1805G>T* and *g.3375G>C*) could be affected by the gender of SLE patients. However, it

will be need to validate using large male's SLE samples by other research group. It is difficult to enroll SLE male patients. Because of SLE are very rare disease and the sex ratio between female and male in SLE patients is 9:1.

A hallmark of SLE and RA is the presence of autoantibodies; therefore further evaluation was made to see these SNPs have associations with ANA, RF and anti-CCP. The association levels were measured by ANOVA and compared the relationship. The genotype of *FOXJ1* gene poly-morphisms have no significant association with the RF and anti-CCP levels in RA patients and also no significant association with the ANA levels in SLE patients (Table 3). We also analyzed each geno-type of these SNPs with RNP levels and hemolytic anemia in SLE patients. But there are also no significant associations by each genotype (data not shown). These results suggest that the polymor-phisms of the *FOXJ1* are not associated with these factors in RA or SLE patients. The haplotype distribution, specifically main haplotype *CGG*, by *g.−460C>T*, *g.1805G>T* and *g.3375C>T* poly-morphisms of *FOXJ1* gene between the healthy controls and RA patients were significantly dif-ferent, and the haplotype *CTG* also showed the significant difference between SLE patients and controls (Table 4). This result suggests that the haplotype by *FOXJ1* gene polymorphisms might be one of the most important genetic factors in RA and SLE susceptibility.

In summary, the *g.3375G>C* polymorphisms of *FOXJ1* might be contribute to the genetic predis-position to autoimmune diseases. These findings demonstrate that SLE and RA may have not a common genetic background in the case of human *FOXJ1* gene. Because some *FOXJ1* variants appears to be unique only for SLE. It is interesting to know that *FOXJ1* polymorphism (*g.3375G>C*) might have some influence on susceptibility to autoimmune diseases. Thus, our results will be important in future studies, to determine whether or not this polymorphism affects the *FOXJ1* gene translation, mRNA stability and function.

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