RESEARCH NOTE

Polymorphisms of interleukin-31 are associated with anti-CCP levels in females with rheumatoid arthritis

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Introduction

Cytokines play important roles in a wide range of different cell types, tissues and organs. Interleukin-31 (IL-31) is a member of the IL-6 family of cytokines. IL-31 is a T helper type 2 (Th2) effecter cytokine that plays an important role in atopic and allergic diseases. It was found that genotype and allele frequencies of the *IL-31* SNPs in rheumatoid arthritis (RA) patients were not significantly different from those in the healthy controls. The haplotype frequencies of *IL-31* SNPs were also not significantly different between the RA patients and the healthy controls. The g.-1066G>A, g.586C>A and g.1449C>G polymorphisms of *IL-31* were significantly associated with the anti-CCP levels in the female RA patients ($P = 0.010$, 0.012 and 0.025, respectively). The results indicate that in RA patients, *IL-31* SNPs may play a role in anti-CCP production, and suggest that SNPs in the *IL-31* gene could be associated with susceptibility to RA.

RA is one of the most common autoimmune diseases worldwide and is characterized by the inflammation of synovial tissues and the formation of rheumatoid pannus, which is capable of eroding adjacent cartilage and bone and cause subsequent joint destruction (Goldring and Gravallese 2000). RA comes about through a complex interaction between multiple genetic and environmental factors (Gregersen 1999). The activated T helper (Th) cells during the developing stage are differentiated phenotypically and functionally into two distinct types of cells, Th1 and Th2 (Abbas *et al.* 1996). Th1 cells produce cytokines such as interferon-γ (IFN-γ), IL-12 and the 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 (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. Th1 and Th2 cells crossregulate 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 (Ho and Glimcher 2002).

Interleukin-31 (IL-31) is a member of the IL-6 family of cytokines (Dillon *et al.* 2004). IL-31 is mainly produced by the activated Th2 cells, and it interacts with a heterodimeric receptor consisting of IL-31 receptor A (IL-31RA) and the oncostatin M receptor (OSMR) that is constitutively expressed on epithelial cells and keratinocytes (Dillon *et al.* 2004). IL-31 mRNA is preferentially expressed by the activation of Th2 cells (Dillon *et al.* 2004). In mice, overexpression of IL-31 results in pruritus and skin dermatitis, which resembles human atopic dermatitis (Dillon *et al.* 2004). The serum levels of IL-31 in allergic asthmatic patients were significantly higher than those in normal healthy controls (Lei *et al.* 2008). IL-31 regulates the expressions of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1/CCL2) in human bronchial epithelial cells (Ip *et al.* 2007). In human alveolar epithelial cells, the binding of IL-31 to IL-31RA and OSMR activates signal transducer and activator of transcription factor 3 (STAT3), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and Akt signalling pathways (Chattopadhyay *et al.* 2007).

To determine whether the *IL-31* single-nucleotide polymorphisms (SNPs) are associated with susceptibility to RA, the genotype frequencies of the *IL-31* SNPs on genomic DNA samples that were isolated from RA patients and healthy

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controls were analysed. Further, the relationships between the SNPs and the rheumatoid factors (RFs) levels and anti-cyclic citrullinated peptide (anti-CCP) levels in RA patients were investigated. Finally, the haplotype frequencies defined by the SNPs in both groups were determined.

Material and methods

Patients and DNA samples

On the basis of approval and informed consent from the institutional review board, the genomic DNAs from 361 RA patients (70 males and 291 females) and 474 healthy controls (285 males and 189 females) were obtained. The mean age of the control and RA groups were 44.1 and 53.1 years, respectively. Genomic DNA was extracted from the peripheral blood leukocytes by using a standard phenol–chloroform method or by using a Genomic DNA Extraction kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's directions. RA was diagnosed according to the criteria of the American Rheumatism Association (Arnett *et al.* 1988). Anti-CCP level in RA patients was determined by the enzyme-linked immunosorbent assay (ELISA) using DIASTAT anti-CCP kit (MBL, Nagoya, Japan) and read by automated EIA analyser, CODA (Bio-RAD, Hercules, USA). RF level in the RA patients were measured by the latex fixation test using Hitachi 7170S (Hitachi, Tokyo, Japan). The standard deviation (SD) for both RF and anti-CCP levels observed in RA patients was very high. The nonrheumatoid arthritis healthy controls were selected from the general population who underwent comprehensive medical testing at Wonkwang University Hospital, South Korea. All study participants were Korean. The healthy controls ethnically matched with RA patients group.

Genotype analysis

The single-base extension (SBE) method was used for the genetic analysis of g.-1550T>C, g.-1066G>A, g.586C>A, and g.1449C>G in *IL-31* The SBE reaction was prepared according to a previously described method (Li *et al.* 2007). The primer extension reaction was also performed according to a previously described method (Yu *et al.* 2012).

Statistical analysis

RA patients and healthy controls were compared by case–control association analysis. χ^2 tests were employed to test for Hardy–Weinberg equilibrium (HWE). Pairwise comparison of the biallelic loci was employed for analyses of linkage disequilibrium (LD). Haplotype frequencies of *IL-31* for multiple loci were estimated using the expectation-maximization (EM) algorithm with SNPAlyze software (DYNACOM, Yokohama, Japan). Logistic regression analyses (SPSS 11.5: Statistical Package for Social Sciences, Chicago, USA) were used to calculate the odds ratios (with 95% confidence intervals). The ANOVA method was applied to define the RF and the anti-CCP levels of each genotype from individual RA patients. A P value less than 0.05 was considered to indicate statistical significance.

Results

Human *IL-31* gene is located on chromosome 12q24.31 and consists of three exons (http://www.ncbi.nlm.nih.gov/gene/386653). We previously identified five SNPs and four variation sites in the *IL-31* gene and showed the significant

Table 1. Genotype and allele analyses of *IL-31* polymorphisms in RA patients and healthy controls.

Position[a]	Genotype/ allele	Control (total/female) *n* (%)		RA (total/female) *n* (%)		Odds ratio[b] (total/female) (95% CI)		*P*[c]	
g.-1066G>A (rs11608363)	AA	234 (49.4)	100 (52.9)	183 (50.7)	145 (49.8)	1.00	1.00	0.883	0.474
	AG	238 (50.2)	87 (46.0)	177 (49.0)	145 (49.8)	0.95 (0.72–1.25)	1.15 (0.80–1.66)		
	GG	2 (0.4)	2 (1.1)	1 (0.3)	1 (0.4)	0.64 (0.06–7.11)	0.35 (0.03–3.85)		
	A	706 (74.5)	287 (75.9)	543 (75.2)	435 (74.7)	1.00	1.00	0.776	0.703
	G	242 (25.5)	91 (24.1)	179 (24.8)	147 (25.3)	0.96 (0.77–1.20)	1.07 (0.79–1.44)		
g.586C>A	CC	386 (81.4)	163 (86.2)	289 (80.1)	233 (80.1)	1.00	1.00	0.616	0.082
	CA	88 (18.6)	26 (13.8)	72 (19.9)	58 (19.9)	1.09 (0.77–1.55)	1.56 (0.94–2.58)		
	AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	–	–		
	C	860 (90.7)	352 (93.1)	650 (90.0)	524 (90.0)	1.00	1.00	0.675	0.103
	A	88 (9.3)	26 (6.9)	72 (10.0)	58 (10.0)	1.08 (0.78–1.50)	1.50 (0.93–2.43)		
g.1449C>G (rs7974857)	CC	386 (81.4)	163 (86.3)	289 (80.1)	233 (80.1)	1.00	1.00	0.401	0.117
	CG	83 (17.5)	25 (13.2)	64 (17.7)	51 (17.5)	1.03 (0.72–1.48)	1.43 (0.85–2.40)		
	GG	5 (1.1)	1 (0.5)	8 (2.2)	7 (2.4)	2.14 (0.69–6.60)	4.89 (0.60–40.1)		
	C	855 (90.2)	351 (92.9)	642 (88.9)	517 (88.8)	1.00	1.00	0.418	0.043
	G	93 (9.8)	27 (7.1)	80 (11.1)	65 (11.2)	1.15 (0.84–1.57)	1.63 (1.02–2.61)		

[a]Calculated from the translation start site.
[b]Logistic regression analyses were used for calculating OR (95% confidence interval).
[c]Values were determined by Fisher's exact test or χ^2 test from 2 × 2 contingency table.

Table 2. Analysis of RF and anti-CCP levels among each genotype of *IL-31* SNPs in RA patients.

Position[a]	Genotype	RF (total/female)						P^b (total/female)		Anti-CCP (total/female)						P^b (total/female)	
		n	Mean	SD	*n*	Mean	SD			*n*	Mean	SD	*n*	Mean	SD		
g.-1066G>A (rs11608363)	AA	176	65.9	68.5	139	62.6	63.9	0.73	0.85	89	64.6	58.7	69	65.0	57.5	0.100	0.010
	AG	175	68.5	72.5	144	61.1	67.1			89	51.0	52.6	76	42.2	48.1		
	GG	1	–	–	–	–	–			0	–	–	0	–	–		
g.586C>A	CC	280	68.7	71.2	226	64.1	66.6	0.42	0.24	137	61.6	57.1	111	59.3	55.4	0.093	0.012
	CA	72	61.1	67.2	58	52.7	60.1			41	44.9	50.7	34	32.9	43.1		
	AA	0	–	–	0	–	–			0	–	–	0	–	–		
g.1449C>G (rs7974857)	CC	280	68.7	71.2	226	64.1	66.6	0.61	0.33	137	61.6	57.1	111	59.3	55.4	0.220	0.025
	CG	64	62.7	68.7	51	55.6	62.6			36	46.3	49.8	30	36.3	44.8		
	GG	8	48.0	55.7	7	31.6	33.1			5	34.8	62.2	4	7.25	9.17		

[a]Calculated from the translation start site.
[b]Values were analysed by analysis of variance.

association of the *IL-31* polymorphism with IgE production in asthma patients (Yu *et al.* 2012). To determine whether the *IL-31* SNPs were associated with susceptibility to RA, the genotypes of four SNPs, g.-1550T>C (rs7312610), g.-1066G>A (rs11608363), g.586C>A, and g.1449C>G (rs7974857), between the RA patients and the healthy controls were analysed using the SBE method. We calculated the LD coefficients ($|D'|$) between all the SNP pairs and determined the absolute LD ($|D'| = 1$ and $r^2 = 1$) between g.-1550T>C and g.1449C>G (data not shown). Of the identified polymorphisms, three SNPs (g.-1066G>A, g.586C>A, and g.1449C>G) were selected for large-sample genotyping on the basis of their locations and LD coefficients. All the genotype frequencies in both the healthy controls and the RA patients were consistent with HWE, except for g.-1066G>A (data not shown). The genotype and allele frequencies of g.-1066G>A, g.586C>A and g.1449C>G polymorphisms of *IL-31* in RA patients were not significantly different from those in healthy controls (table 1). We further analysed the genotype and allele frequencies by gender between the healthy controls and the RA patients as the RA patients were predominantly female compared with the control subjects. The genotype and allele frequencies of g.-1066G>A, g.586C>A and g.1449C>G polymorphisms of *IL-31* in RA male patients were not significantly different from those in healthy male controls (data not shown). Although the genotype and allele frequencies of the g.-1066G>A and g.586C>A polymorphism and the genotype frequency of the g.1449C>G polymorphism were not significantly different between the female of the RA patients and the healthy controls, the allele frequency of g.1449C>G polymorphism in the female RA patients group was significantly different from that of the female control group ($P = 0.043$; table 1).

The features of RA are RF and anti-CCP levels; therefore, we further investigated the association between the *IL-31* SNPs and RF, and anti-CCP levels in the RA patients (table 2). The *IL-31* SNPs in the RA patients showed no significant association with the RF levels and anti-CCP levels (table 2). We also analysed the relationships between the *IL-31* SNPs and the RF levels, and anti-CCP levels in the female RA patients only (table 2), because as the allele frequency of g.1449C>G polymorphism in the female RA patients group was significantly different from that of the female healthy controls group (table 1). Interestingly, the levels of anti-CCP were significantly associated in g.-1066G>A, g.586C>A and g.1449C>G polymorphisms of *IL-31* ($P = 0.010$, 0.012 and 0.025, respectively). This result indicated that the *IL-31* SNPs may influence anti-CCP production in the female RA patients.

Finally, we estimated the haplotype frequencies of the g.586C>A and g.1449C>G SNPs of *IL-31* gene between the healthy controls and the RA patients (table 3). The major and minor haplotype frequencies were not significantly different between the two groups. These results suggest that the haplotypes of the *IL-31* polymorphisms are not associated with RA susceptibility.

Discussion

RA is one of the representative of autoimmune diseases, and most commonly detected in women. RA comes about through the complex interactions between multiple genetic factors and environmental factors. We previously suggested that the exon 4 variations of the *Tim-1* gene (Chae *et al.* 2004), the *eotaxin-3* polymorphisms (Chae *et al.* 2005), and

Table 3. Haplotype frequencies of *IL-31* SNPs in RA patients and healthy controls.

Haplotype		Frequency[a]			
g.586C>A	g.1449C>G	Control	RA	χ^2	P^b
C	C	0.902	0.889	0.715	0.408
A	G	0.093	0.100	0.225	0.598
C	G	0.005	0.011	1.799	0.167

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

TBX21 polymorphisms (Chae *et al.* 2009) are associated with RA susceptibility. In this study, we evaluated the associations between *IL-31* polymorphisms and the susceptibility to RA.

IL-31 is believed to play an important role in promoting allergic inflammation and inducing airway epithelial response such as allergic asthma (Chattopadhyay *et al.* 2007). It has been reported that nonatopic eczema is strongly associated with the following mutations in a common risk haplotype GAA of *IL-31*: *IL-31*-2057G>A (rs6489188), *IL-31*-1066G>A (rs11608363), and *IL-31*IVS2+12A>G of *IL-31*. Besides, the degree of *IL-31* expression was significantly higher in carriers of the risk haplotype as compared with noncarriers (Schulz *et al.* 2007) and *IL-31* SNP (rs7977932) were associated with increased risk of developing atopic eczema (Lan *et al.* 2011). McGrath and coworkers reported that the *IL-31* SNP (rs11608363) is not associated with susceptibility to epidermolysis bullosa pruriginosa (Nagy *et al.* 2010). We analysed the genotypes of the g.-1066G>A, g.586C>A, and g.1449C>G polymorphisms of *IL-31* in RA patients and healthy controls. The genotype and allele frequencies of the *IL-31* SNPs in RA patients were not significantly different from those in the control group (table 1). We also compared the genotype and allele frequencies between the female of RA patients and healthy controls. Although the allele frequency of g.1449C>G polymorphism in the female RA patients group was significantly different from that of the female control group, the genotype and allele frequencies of the *IL-31* SNPs were not associated with RA (table 1). These results suggest that the *IL-31* SNPs may not be associated with the susceptibility to RA.

A characteristic feature of RA is the presence of RFs and RF-containing immune complexes in both the circulation and synovial fluid (Edwards and Cambridge 1998). RFs are autoantibodies that recognize the Fc region of immunoglobulin G (IgG) antibodies and their isotypes. RF has been widely used as a screening test for patients with arthritis. RF is prognostically useful as it correlates with the function and outcomes of both RA and early inflammatory polyarthritis (Van der Heide *et al.* 1995; Harrison *et al.* 1999). A highly specific auto-antibody system has been recently described for RA, in which the synthetic CCP with deiminated arginines is used as the antigen for the anti-CCP antibodies (Schellekens *et al.* 2000). Anti-CCP antibodies are locally present at the site of inflammation in RA (Reparon-Schuijt *et al.* 2001) and citrullinated proteins are found in the RA synovium (Baeten *et al.* 2001). A feature of RA is the presence of auto-antibodies; therefore, further evaluation was carried out to see if the SNPs have associations with the RF and anti-CCP levels according to each genotype in the RA patients. Although, the genotypes of the *IL-31* gene polymorphisms in the RA patients have no significant association with the RF and anti-CCP levels (table 2), the g.-1066G>A, g.586C>A and g.1449C>G polymorphisms of the *IL-31* were significantly associated with the levels of anti-CCP in the female RA patients (table 2). This result indicated that *IL-31* polymorphism in the female RA patients may play a role in anti-CCP production.

In conclusion, the results suggest that *IL-31* may be a candidate gene associated with the pathogenesis of RA. Although it is not clear how the *IL-31* polymorphisms are related to the anti-CCP production in the female RA patients, the results provide useful information for further functional studies of the *IL-31* gene and RA. However, we still have not comprehensively captured all the diversity in *IL-31* gene, and other associations may ever reside at this locus; these associations could not be addressed in this study.

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