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Association of *TBX21* polymorphisms in a Korean population with rheumatoid arthritis

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Abbreviations: PCR, polymerase chain reaction; RA, rheumatoid arthritis; SBE, single-base extension; SNP, single nucleotide polymorphism; T-box 21, TBX21

Abstract

TBX21 (T-bet) is a member of the T-box family of transcriptional factors that contain a conserved DNA binding domain. TBX21 is a critical regulator of the commitment to the Th1 lineage and IFN-γ production. Th1 and Th2 cells cross-regulate the differentiation of each other, and in this way TBX21 could be an attractive candidate gene for treating autoimmune disease such as rheumatoid arthritis (RA). In present study, we analyzed the genotypic frequencies of six polymorphisms of the *TBX21* gene between the 367 RA patients and the 572 healthy controls. We showed that the g.-1514T > C and c.99C > G polymorphisms are suggestively associated with RA susceptibility. It is interesting that the genotypic frequencies of the *TBX21* polymorphisms (g.-1514T > C and c.2103A > C) in the male RA patients were significantly different from the male control group (*P*=0.0016 and 0.045, respectively). We also found that the g.-1514T > C and c.2103A > C polymorphisms of the *TBX21* gene in the male RA patients have significant association with the levels of anti-CCP (*P* = 0.05) and rheumatoid factor (*P* = 0.03), respectively. These results suggest that the polymorphisms of the *TBX21* gene might be associated with the susceptibility to male RA patients.

Keywords: anti-CCP, haplotype; polymorphism; RA; RF; *TBX21*

Introduction

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases worldwide. RA is characterized by inflammation of synovial tissues and the formation of rheumatoid pannus, which is capable of eroding adjacent cartilage and bone and causing subsequent joint destruction. RA comes about through the complex interaction between multiple genetic factors as well as environmental factors (Gregersen, 1999). A characteristic feature of RA is the presence of rheumatoid factors (RFs) and RF-containing immune complexes in both the circulation and synovial fluid (Edwards and Cambridge, 1998). RFs are auto-antibodies 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 autoantibody system has been recently described for RA, in which the synthetic cyclic citrullinated peptide (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).

T-box 21 (TBX21, also know as T-bet) is a member of the T-box family of transcriptional factors that contain a conserved DNA binding domain. TBX21 was originally cloned both by virtue of its ability to bind to the T helper (Th)1-specific IL-2 promoter in a screened yeast hybrid, and by its expression in Th1 cells but not Th2 cells (Szabo *et al*., 2000). The regulation of interferon gamma (IFN-γ) is controlled by distinct transcriptional mechanisms within the T-cell lineage (Szabo *et al*.,

2002). There is a reduced expression of the TBX21 in T cells from the airways of asthma patients compared with that in T cells from the airways of non-asthmatic patients, suggests that the loss of TBX21 might be associated with the risk of asthma (Finotto *et al*., 2002). The absence of the TBX21 showed the enhanced protective functions in Th1-mediated colitis and exhibit increased TGF-β production and signaling (Neurath *et al*., 2002). The over-expression of TBX21 in developing Th2 cells results in a marked induction of the IFN-γ expression and a corresponding inhibition of Th2 cytokine production. These results indicate that TBX21 is necessary to induce helper T cells to differentiate into Th1 cells and for Th1 cells to produce IFN-γ. For these reasons, TBX21 is thought to be central to the feedback loops that regulate Th1 and Th2 cells, and in this way it could be an attractive candidate gene for autoimmune diseases such as RA. We recently identified twenty-three polymorphisms in the human *TBX21* gene, but we could not find any significant associations of the *TBX21* variants with the risk of asthma (Chung *et al*., 2003). The exonic single nucleotide polymorphisms (SNPs) discovered in our study were not located on the DNA binding domain to the IFN-γ opening frame, and the promoter SNPs are far from the binding sites of STAT1 or STAT4, which are induced by INF-γ or interleukin 12 (IL12), respectively.

To determine whether the SNPs of the *TBX21* gene are associated with the susceptibility of RA, we analyzed the allelic and genotypic frequencies between the RA patients and the healthy controls because the predominant of Th1 cells results in organ-specific autoimmune diseases such as RA.

Table 1. Genotype and allele analyses of the polymorphisms of *TBX21* gene in rheumatoid arthritis patients and healthy controls.

Position[a]	Genotype /Allele	Control n (%)	RA n (%)	Odds ratio[b] (95% CI)	*P*
g.-1514T > C	TT	546 (95.5)	332 (91.0)	1.00	0.022
(KN0002090)[c]	TC	26 (4.5)	33 (9.0)	2.09 (1.23-3.55)	
	CC	0 (0.0)	0 (0.0)	-	
	T	1,118 (97.7)	697 (95.5)	1.00	0.009
	C	26 (2.3)	33 (4.5)	2.04 (1.21-3.43)	
c.99C > G	CC	457 (84.0)	247 (76.7)	1.00	0.026
(His33Gln)	CG	81 (14.9)	71 (22.1)	1.62 (1.14-2.31)	
	GG	6 (1.1)	4 (1.2)	1.23 (0.35-4.41)	
	C	995 (91.5)	565 (87.7)	1.00	0.016
	G	93 (8.5)	79 (12.3)	1.50 (1.09-2.05)	
c.390A > G	AA	458 (82.5)	272 (81.4)	1.00	0.900
(rs2074190)	AG	93 (16.8)	59 (17.7)	1.07 (0.75-1.53)	
	GG	4 (0.7)	3 (0.9)	1.26 (0.28-5.69)	
	A	1,009 (90.9)	603 (90.3)	1.00	0.674
	G	101 (9.1)	65 (9.7)	1.08 (0.78-1.49)	
c.831C > T	CC	524 (97.4)	346 (95.8)	1.00	0.434
(Asp277Asp)	CT	14 (2.6)	15 (4.2)	1.62 (0.77-3.40)	
	TT	0 (0.0)	0 (0.0)	-	
	C	1,062 (98.7)	707 (97.9)	1.00	0.252
	T	14 (1.3)	15 (2.1)	1.61 (0.77-3.36)	
c.1455G > A	GG	518 (91.7)	301 (89.1)	1.00	0.421
(Pro485Pro)	GA	47 (8.3)	37 (10.9)	1.36 (0.86-2.13)	
	AA	0 (0.0)	0 (0.0)	-	
	G	1,083 (95.8)	639 (94.5)	1.00	0.206
	A	47 (4.2)	37 (5.5)	1.33 (0.86-2.08)	
c.2103A > C	AA	511 (95.7)	345 (95.6)	1.00	0.475
(rs7502875)	AC	23 (4.3)	15 (4.1)	0.97 (0.50-1.88)	
	CC	0 (0.0)	1 (0.3)	-	
	A	1,045 (97.8)	705 (97.6)	1.00	0.871
	C	23 (2.2)	17 (2.4)	1.10 (0.58-2.07)	

[a]Calculated from the translation start site (The reference sequence for *TBX21* was based on clone hCIT.211_P_7 or NM_013351).
[b]Logistic regression analyses were used for calculating OR (95% CI; confidence interval).
[c]From KSNP Database (http://ksnp.ngri.go.kr).

We further investigated the relationships between the genotypes of each polymorphism and the CCP or RF levels in the RA patients.

Results

We previously identified twenty-three genetic polymorphisms in the important *TBX21* gene, but no significant associations of the *TBX21* variants with the asthma phenotypes were detected (Chung *et al*., 2003). Among twenty-three identified variants, six were selected for larger scale genotyping for RA association study based on frequencies and location. To determine whether the SNPs of the *TBX21* gene are associated with the susceptibility of RA, we analyzed the genotype frequencies of six SNPs (g.-1514T > C, c.99C > G, c.390A > G, c.831C > T, c.1455G > A and c.2103A > C) between the RA patients and the healthy controls because of the predominance of Th1 cells results in organ-specific autoimmune diseases such as RA. The genotypes of these polymorphisms were determined in 367 unrelated RA patients and in 572 unrelated healthy controls (Table 1). All the genotype frequencies of these SNPs were in Hardy-Weinberg equilibrium (HWE), as determined by χ^2 tests (data not shown). The genotype and allele frequencies of the g.-1514T > C polymorphism in the RA patients were significantly different from those of the healthy control group (Table 1; P = 0.022 and 0.009, respectively). The genotype and allele frequencies of c.99C > G were also significantly different between the RA patients and the healthy controls (P = 0.026 and 0.016, respectively). When the data were adjusted for sex, the results were supported (data not shown).

We further analyzed the genotype and allele

Table 2. Genotype and allele analysis of the *TBX21* gene polymorphisms in the females of rheumatoid arthritis patients and controls.

Position[a]	Genotype /Allele	Control n (%)	R. A n (%)	Odds ratio[b] (95% CI)	*P*
g.-1514T > C	TT	208 (96.7)	276 (92.9)	1.00	0.173
	TC	7 (3.3)	21 (7.1)	2.26 (0.94-5.42)	
	CC	0 (0.0)	0 (0.0)	-	
	T	423 (98.4)	573 (96.5)	1.00	0.080
	C	7 (1.6)	21 (3.5)	2.21 (0.93-5.26)	
c.99C > G	CC	175 (85.0)	203 (78.1)	1.00	0.128
	CG	28 (13.6)	54 (20.8)	1.66 (1.01-2.74)	
	GG	3 (1.5)	3 (1.1)	0.86 (0.17-4.33)	
	C	378 (91.7)	460 (88.5)	1.00	0.102
	G	34 (8.3)	60 (11.5)	1.45 (0.93-2.26)	
c.390A > G	AA	175 (83.3)	221 (81.3)	1.00	0.811
	AG	34 (16.2)	49 (18.0)	1.14 (0.71-1.85)	
	GG	1 (0.5)	2 (0.7)	1.58 (0.14-17.61)	
	A	384 (91.4)	491 (90.3)	1.00	0.576
	G	36 (8.6)	53 (9.7)	1.15 (0.74-1.80)	
c.831C > T	CC	195 (98.5)	285 (96.9)	1.00	0.276
	CT	3 (1.5)	9 (3.1)	2.05 (0.55-7.69)	
	TT	0 (0.0)	0 (0.0)	-	
	C	393 (99.2)	579 (98.5)	1.00	0.380
	T	3 (0.8)	9 (1.5)	2.04 (0.55-7.57)	
c.1455G > A	GG	197 (92.9)	245 (89.1)	1.00	0.148
	GA	15 (7.1)	30 (10.9)	1.61 (0.84-3.07)	
	AA	0 (0.0)	0 (0.0)	-	
	G	409 (96.5)	520 (94.5)	1.00	0.169
	A	15 (3.5)	30 (5.5)	1.57 (0.84-2.96)	
c.2103A > C	AA	188 (95.9)	284 (96.6)	1.00	0.695
	AC	8 (4.1)	10 (3.4)	0.83 (0.32-2.14)	
	CC	0 (0.0)	0 (0.0)	-	
	A	384 (98.0)	578 (98.3)	1.00	0.809
	C	8 (2.0)	10 (1.7)	0.83 (0.33-2.12)	

[a]Calculated from the translation start site (The reference sequence for *TBX21* was based on clone hCIT.211_P_7 or NM_013351).
[b]Logistic regression analyses were used for calculating OR (95% CI; confidence interval).

frequencies between the females of the control group and the RA patients because the RA patients were predominantly female compared with the control subjects. Interestingly, the genotype and allele frequencies of the g.-1514T > C and c.99C > G polymorphisms were not significantly different from that of the female control group (Table 2). These results led us to compare the genotypes comparison between the males of the control group and the males of the RA patients. As we expected, the genotype frequencies of the SNPs in the male RA patients (g.-1514T > C and c.2103A > C) were significantly different from the males of the control group (Table 3; $P = 0.0016$ and 0.045, respectively). Therefore, we partially conclude that the association of the SNPs of *TBX21* could be affected by the gender of the RA patients.

On the other hand, to define a possible correlation between polymorphisms and the clinical features of RA, we further analyzed the difference of the anti-CCP and RF levels according to each genotype of the RA patients. We found that these SNPs in the RA patients have no significant association with the levels of RF and anti-CCP (Table 4). As showed in Table 3, the association of the SNPs of *TBX21* could be affected by the gender, and specifically the male gender, of the RA patients. So, we also analyzed the difference of the anti-CCP and RF levels according to each genotype in the RA male patients only, and we found that the c.831C > T and c.2103A > C polymorphisms of the *TBX21* gene in the RA patients have significant association with the levels of RF ($P = 0.05$ and 0.03, respectively), and the g.-1514T > C polymorphism have significant association with the levels of anti-CCP ($P = 0.05$) (Table 5).

We also evaluated the haplotype frequencies among the g.-1514T > C, c.99C > G, c.831C > T

Table 3. Genotype and allele analysis of the *TBX21* gene polymorphisms in the males of rheumatoid arthritis patients and controls.

Position[a]	Genotype /Allele	Control n (%)	R. A n (%)	Odds ratio[b] (95% CI)	*P*
g.-1514T > C	TT	338 (94.7)	56 (82.4)	1.00	0.0016
	TC	19 (5.3)	12 (17.6)	3.81 (1.75-8.28)	
	CC	0 (0.0)	0 (0.0)	-	
	T	695 (97.3)	124 (91.2)	1.00	0.002
	C	19 (2.7)	12 (8.8)	3.54 (1.68-7.48)	
c.99C > G	CC	282 (83.4)	44 (71.0)	1.00	0.067
	CG	53 (15.7)	17 (27.4)	2.06 (1.09-3.87)	
	GG	3 (0.9)	1 (1.6)	2.14 (0.22-20.99)	
	C	617 (91.3)	105 (84.7)	1.00	0.031
	G	59 (8.7)	19 (15.3)	1.89 (1.08-3.30)	
c.390A > G	AA	283 (82.0)	51 (82.3)	1.00	0.850
	AG	59 (17.1)	10 (16.1)	0.94 (0.45-1.96)	
	GG	3 (0.9)	1 (1.6)	1.85 (0.19-18.13)	
	A	625 (90.6)	112 (90.3)	1.00	0.869
	G	65 (9.4)	12 (9.7)	1.03 (0.54-1.97)	
c.831C > T	CC	329 (96.8))	61 (91.0)	1.00	0.101
	CT	11 (3.2)	6 (9.0)	2.94 (1.05-8.25)	
	TT	0 (0.0)	0 (0.0)	-	
	C	669 (98.4)	128 (95.5)	1.00	0.046
	T	11 (1.6)	6 (4.5)	2.85 (1.04-7.85)	
c.1455G > A	GG	321 (90.9)	56 (88.9)	1.00	0.608
	GA	32 (9.1)	7 (11.1)	1.25 (0.53-2.98)	
	AA	0 (0.0)	0 (0.0)	-	
	G	674 (95.5)	119 (94.4)	1.00	0.646
	A	32 (4.5)	7 (5.6)	1.24 (0.53-2.87)	
c.2103A > C	AA	323 (95.6)	61 (91.0)	1.00	0.045
	AC	15 (4.4)	5 (7.5)	1.77 (0.62-5.04)	
	CC	0 (0.0)	1 (1.5)	-	
	A	661 (97.8)	127 (94.8)	1.00	0.074
	C	15 (2.2)	7 (5.2)	2.43 (0.97-6.08)	

[a]Calculated from the translation start site (The reference sequence for *TBX21* was based on clone hCIT.211_P_7 or NM_013351).
[b]Logistic regression analyses were used for calculating OR (95% CI; confidence interval).

Table 4. Analyses of RF and anti-CCP levels among the genotypes of each SNP of *TBX21* gene in rheumatoid arthritis patients.

Position[a]	Genotype	RF			P^b	Anti-CCP			P^b
		n	Mean	SD		n	Mean	SD	
g.-1514T > C	TT	332	340.5	370.8	0.45	160	49.3	44.7	0.64
	TC	33	392.2	385.4		20	54.4	47.3	
	CC	0	-	-		0	-	-	
c.99C > G	CC	247	350.1	370.0	0.81	127	52.0	44.3	0.34
	CG	71	353.8	381.7		32	43.6	46.2	
	GG	4	231.9	225.7		1	-	-	
c.390A > G	AA	272	345.6	364.2	0.90	130	49.6	44.5	0.97
	AG	59	344.7	386.7		31	49.5	49.7	
	GG	3	245.6	365.7		3	41.2	51.9	
c.831C > T	CC	346	338.3	367.4	0.16	168	48.6	44.6	0.42
	CT	15	500.3	485.0		10	60.6	50.9	
	TT	0	-	-		0	-	-	
c.1455G > A	GG	301	349.4	373.9	0.94	151	49.3	45.0	0.53
	GA	37	344.0	369.5		16	41.8	46.3	
	AA	0	-	-		0	-	-	
c.2103A > C	AA	345	338.0	367.2	0.13	168	48.6	44.5	0.62
	AC	15	518.7	486.3		9	56.2	52.0	
	CC	1	-	-		1	-	-	

[a]Calculated from the translation start site (The reference sequence for *TBX21* was based onclone hCIT.211_P_7 or NM_013351).
[b]Values were analyzed by ANOVA or Kruskall-Wallis test.

Table 5. Analyses of RF and anti-CCP levels among the genotypes of each SNP of *TBX21* gene in the males of rheumatoid arthritis patients.

Position[a]	Genotype	RF			P^b	Anti-CCP			P^b
		n	Mean	SD		n	Mean	SD	
g.-1514T > C	TT	56	439.4	446.9	0.24	25	56.4	47.4	0.05
	TC	12	609.9	476.1		7	97.2	7.45	
	CC	0	-	-		0	-	-	
c.99C > G	CC	44	476.3	441.6	0.47	22	67.7	43.4	0.37
	CG	17	451.7	498.3		6	50.2	53.7	
	GG	1	-	-		0	-	-	
c.390A > G	AA	51	425.7	438.6	0.19	23	64.6	44.6	0.31
	AG	10	629.4	477.4		5	40.9	54.0	
	GG	1	-	-		1	-	-	
c.831C > T	CC	61	438.7	444.8	0.05	27	58.9	46.5	0.07
	CT	6	815.3	464.8		4	100	0.0	
	TT	0	-	-		0	-	-	
c.1455G > A	GG	56	460.6	450.9	0.69	28	64.6	45.8	0.49
	GA	7	533.0	484.4		2	40.8	55.9	
	AA	0	-	-		0	-	-	
c.2103A > C	AA	61	443.3	442.9	0.03	27	58.9	46.5	0.11
	AC	5	897.8	468.0		3	100	0.0	
	CC	1	-	-		1	-	-	

[a]Calculated from the translation start site (The reference sequence for *TBX21* was based on clone hCIT.211_P_7 or NM_013351).
[b]Values were analyzed by ANOVA or Kruskall-Wallis test.

and c.2103A > C polymorphisms in both the healthy controls and the RA patients (Table 6). While the major haplotype (Ht 1; TCCA) was identified and this explained more than 87.1% of the distribution in the controls, the frequency of the major haplotype in the RA patients was 82.1%, and this

Table 6. The haplotype frequencies in both rheumatoid arthritis patients and controls in *TBX21* polymorphisms

	Haplotypes				Frequency[a]		Chi-square	*P*[b]
	g.-1514T > C	c.99C > G	c.831C > T	c.2103A > C	RA	Control		
Ht 1	T	C	C	A	0.821	0.871	7.71	0.005
Ht 2	T	G	C	A	0.120	0.078	8.00	0.006
Ht 3	C	C	C	A	0.030	0.019	2.12	0.150
Ht 4	T	C	C	C	0.003	0.016	6.17	0.013
Others	-	-	-	-	0.026	0.016	-	-

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

showed a significant difference between both groups ($P = 0.005$). Further, the haplotype Ht 2 (TGCA) also had a significant association with RA ($P = 0.006$).

Discussion

The TBX21 has been the most extensively studied in Th cells, and it is known to play a critical role in the development and maintenance of Th1 cells (Murphy and Reiner, 2002). TBX21-deficient mice showed impaired Th1 differentiation, including defective IFN-γ production primarily in the CD4 and γδ T cells (Szabo *et al*., 2002; Yin *et al*., 2002). Glimcher and colleagues developed an animal model of spontaneous Th2-like, asthma-like syndrome, and they demonstrate increased susceptibility to experimental asthma that was likely due to an enhanced IL-13 expression (Finotto *et al*., 2005). Because the TBX21 expression in human Th cells correlates with the Th1 profiles, it is not surprising that a growing body of evidence has been accumulated for the pathogenic roles of TBX21 in autoimmune diseases such as RA. Kawashima and colleague reported that *TBX21* mRNA expression levels are no difference between the blood of RA patients and health controls (Kawashima *et al*., 2005). *TBX21* and *IFNγ* mRNA levels in the blood of RA patients are strongly correlated but not in the healthy control blood. However, the *TBX21* expression and serum C-reactive protein (CRP) levels in the blood of RA patients are negative correlated (Kawashima *et al*., 2005). Recently, Wang and colleague demonstrated that *TBX21* plays a critical function in dendritic cells (DCs) in controlling inflammatory arthritis (Wang *et al*., 2006). We previously suggested that the polymorphisms of the human *TBX21* gene were not associated with the susceptibility to asthma (Chung *et al*., 2003). Ylikoski and colleague also reported that *TBX21* polymorphisms were not related to a Finnish asthmatic population (Ylikoski *et al*., 2004). A study on a Japanese population demonstrated the associations of one *TBX21* polymorphism with aspirin-induced asthma, but not with other forms of asthma (Akahoshi *et al*., 2005). Raby and colleague recently demonstrated that *TBX21* polymorphisms were associated with asthma and airway hyperresponsiveness in a North American population (Raby *et al*., 2006).

The activated Th cells during the developing stage are differentiated into two phenotypically and functionally distinct types of cells, that is, the Th1 and Th2 (Mosmann and Coffman, 1989; Abbas *et al*., 1996). Th1 cells produce cytokines such as interferon-γ (IFN-γ), IL-12 and cytotoxic factor lymphotoxin. These cytokines are commonly associated with cell-mediated immune responses against intracellular pathogens and the 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 during 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 (Lack *et al*., 1994; Nicholson *et al*., 1995; Hofstra *et al*., 1998). We previously suggested that the exon 4 variations of the *Tim-1* gene (Chae *et al*., 2004) and the *eotaxin-3* polymorphisms (Chae *et al*., 2005) are associated with RA susceptibility.

In this study, we analyzed the genotype frequencies at six SNPs of the *TBX21* gene between the RA patients and the controls, and we showed that the g.-1514T > C and c.99C > G polymorphisms are strongly associated with RA susceptibility (Table 1). This is very interesting and led us to think that the polymorphisms of the *TBX21* gene might be associated with the development of helper T (Th) cell types according to the *TBX21*

expression level. A hallmark of RA is the presence of auto-antibodies; therefore, further evaluation was done to see if these SNPs have associations with the RF and anti-CCP levels according to each genotype in the RA patients. Although the genotypes of the *TBX21* gene polymorphisms in the RA patients have no significant association with the RF and anti-CCP levels (Table 4), as was shown that the association of the SNPs of the *TBX21* gene could be affected by the gender and specifically the male gender (Table 3) of the RA patients, the anti-CCP and RF levels, according to each genotype in the RA male patients only, have significant association with the g.-1514T > C, c.831C > T and c.2103A > C polymorphisms of the *TBX21* gene (Table 5). Although it will be need to be validated with using a large number of male RA samples, this result suggests that the g.-1514T > C, c.831C > T and c.2103A > C polymorphisms of the *TBX21* gene are associated with these factors in the RA male patients. The distribution of haplotype TCCA and TGCA by the g.-1514T > C, c.99C > G, c.831C > T and c.2103A > C polymorphisms of the *TBX21* gene were also significantly different between the healthy controls and the RA patients (Table 6). This result suggests that the haplotype, according to the *TBX21* gene polymorphisms, might be one of the most important genetic factors for the susceptibility to RA.

The polymorphisms within the binding site of the promoter region might have influence on the expression level by suppressing the binding between the specific transcriptional binding site and the transcription factor. Accordingly, it is interesting to know that the *TBX21* polymorphism (g.-1514T > C) might have some influence on the susceptibility to RA. Thus, our results will be important for future studies that will determine whether or not this polymorphism affects the levels of the *TBX21* gene expression and function. In summary, our results suggest that the polymorphisms of the *TBX21* gene might be associated with RA pathology.

Methods

Patients and DNA samples

After institutional review board approval and informed consent, blood samples were obtained from 367 RA patients (69 males and 298 females) and 572 healthy controls (357 males and 215 females). The mean age of the patients and controls was 54.3 years and 40.2 years, respectively. The clinical characteristics of RA patients are summarized in Suppl(Table1). Genomic DNA was extracted from the leukocytes in the peripheral blood by a standard phenol-chloroform method or by using a Genomic DNA Extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's directions. The RA patients were recruited from the outpatient clinic at Eulji University Hospital. RA was diagnosed according to the criteria of the American Rheumatism Association. Anti-CCP level in the RA patients was determined by enzyme-linked immunosorbent assay (ELISA) using DIASTAT anti-CCP kit (MBL Co, Nagoya, Japan) and read by automated EIA analyzer, CODA (Bio-RAD Co, Japan). Anti-CCP antibody was considered positive when the absorbance was higher than the cut-off value (5 U/ml). The concentration of anti-CCP antibody was estimated by interpolation from a dose-response curve based on standards. RF level in the RA patients was measured by the latex fixation test using Hitachi 7170S (Hitachi Co, Tokyo, Japan). The cutoff for positivity was 18 IU/ml for RF. The non-rheumatoid arthritis healthy controls were recruited from the general population who underwent comprehensive medical testing at Wonkwang University Hospital. All the subjects in this study were Korean.

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

Each region of the *TBX21* gene containing SNPs was partially amplified by PCR with using the two primer pairs Suppl(Table2). The PCR reactions were carried out in a 20 μl reaction volume containing 50 ng genomic DNA, 0.5 μM primers, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 10 mM TRIS-HCl (pH 8.3), and one unit of EF Taq polymerase (Solgent, Korea). Amplification was carried out in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystem) at 94°C for 5 min, followed by 30 cycles at 98°C for 10 s, 68°C for 30 s and 72°C for 3 or 2 min. The final extension was carried out at 68°C for 7 min. The PCR products for g.-1514T > C and c.1455G > A were digested with 1 unit of *AIW44 I* (Promega) for 3 h at 37°C and with 0.5 units of *Eco52 I* (Takara Co., Ltd., Japan) for 2 h at 37°C, respectively, and then they were separated on 1.5% agarose gel and visualized under UV light with ethidium bromide. After restriction enzyme digestion, the PCR products for g.-1514T > C (2,677 bp) and c.1455G > A (1,823 bp) took the form of two fragments, that is, 529 bp and 2,148 bp, and 1,086 bp and 737 bp, respectively.

Single-base extension (SBE)

Genotyping for the c.99C > G, c.390A > G, c.831C > T and c.2103A > C polymorphisms in the *TBX21* gene was performed by single-base extension (SBE) and, using the ABI Prism® SNaPshot™ Multiplex kit (Applied Biosystems). The PCR products purified by the PCR purification kit (Millipore) were used as the template DNA for the four SBE primers (Table 2). The SBE reaction mix was prepared according to a previously described method (Chae *et al*., 2006; Li *et al*., 2007). The primer extension reaction was performed at 96°C for 1 min, followed by 25 cycles at 96°C for 10 s 55°C for 40 s and 60°C for 30 s. To clean up the primer extension reaction, 1.5 units of CIP (New England BioLabs) were added to the reaction mixture, and the mixture was incubated at 37°C for 90 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 with 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 the biallelic loci and the haplotype frequencies of the *TBX21* gene polymorphisms for multiple loci were estimated using the expectation maximization (EM) algorithm with SNPAlyze software (DYNACOM, Japan). Logistic regression analyses (SPSS 11.5) were used to calculate the odds ratios (with the 95% confidence intervals), and ANOVA or Kruskall-Wallis test were applied in order to analyze differences between the each genotype and the RF or CCP levels in the RA patients. Cutoff value of RF is 18 IU/ml and that of anti-CCP antibody is 5 U/ml. The genotypes analysis was performed chi-square test and that the allele distribution was tested with Fisher's exact test. A *P*-value of less than 0.05 was considered to indicate statistical significance.

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