GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

CTLA-4 and *CD86* genetic variants and haplotypes in patients with rheumatoid arthritis in southeastern China

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Genet. Mol. Res. 12 (2): 1373-1382 (2013)
Received August 21, 2012
Accepted January 27, 2013
Published April 25, 2013
DOI http://dx.doi.org/10.4238/2013.April.25.8

ABSTRACT. The cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4*) and costimulatory molecule (*CD80*/*CD86*) genes are important susceptibility genes associated with autoimmune diseases. *CTLA-4* polymorphisms have been found to be associated with various autoimmune diseases. However, the association data are inconsistent for rheumatoid arthritis (RA). We investigated the genetic association of *CTLA-4* and *CD86* polymorphisms with RA in a Chinese population. Four single nucleotide polymorphisms (SNPs) (rs5742909 and rs231775 in *CTLA-4*, and rs17281995 and rs1129055 in *CD86*) were genotyped in 213 patients with RA and 303 healthy controls using polymerase chain reaction-restriction fragment length polymorphism. The genotype and

allele distributions of rs5742909 differ significantly between RA patients and controls ($P < 0.05$) and the dominant model was found to be the best inheritance model. Stratification studies showed that *CTLA-4* gene polymorphisms were more significantly associated with rheumatoid factor-negative and anti-cyclic citrullinated peptide-negative subgroups in the southeastern Han Chinese population. We also found that the haplotype of 2 *CTLA-4* SNPs showed significant association with the disease ($P = 0.0025$) with the T-A (OR = 1.88, 95%CI = 1.12-3.15) and T-G (OR = 3.45, 95%CI = 1.10-10.87) haplotypes being observed more frequently in cases than in controls. We failed to find any significant association of the 2 *CD86* SNPs with RA. These results indicate that the polymorphisms of *CTLA-4* (rs5742909) may be important genetic factors for RA risk in the southeastern Han Chinese population.

Key words: Rheumatoid arthritis; Single nucleotide polymorphisms; *CTLA-4*; *CD86*

INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic inflammatory autoimmune disease characterized by significant disability and early mortality. It affects ~1% of the adult population worldwide. It is generally accepted that RA is a complex autoimmune disorder characterized by a chronic T-cell response that evades normal control mechanisms (Arnett et al., 1988; Fox, 2005). Therefore, the genes involved in the regulation of T-cell responses may be primary determinants of the susceptibility to RA.

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a CD28-family receptor expressed on T-cells. The B7-CD28/CTLA-4 costimulatory pathway has a dominant role in regulating T-cell activation. Although B7 molecules provide the major signal for augmenting and sustaining T-cell responses by interacting with CD28, they can also deliver inhibitory signals when interacting with CTLA-4 (Sharpe and Freeman, 2002). Deficiency of CTLA-4 results in massive fatal lymphoproliferation and autoimmune disease in mice. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role for CTLA-4 (Tivol et al., 1995). Several reports strongly suggest that molecular variants in *CTLA-4* are involved in T-cell-mediated autoimmune and inflammatory diseases such as type 1 diabetes, autoimmune thyroid disease, multiple sclerosis, systemic lupus erythematosus, and RA (Scalapino et al., 2008; Kusztal et al., 2010; Liu et al., 2010).

CD86 is one of the essential costimulatory molecules expressed in antigen-presenting cells. The gene encoding CD86 was mapped to 3q21 with locus consisting of receptor proteins, cytokines, and associated factors. CD86 activates T-cells and regulates the immune response, CD86 deficiency led to impairments in dendritic cells, B-lymphocytes, and macrophages (Yadav and Sarvetnick, 2007). The expression of the CD86 molecule was upregulated in RA synovia (Liu et al., 1996). Peripheral B-cells from RA patients have an altered expression of CD86 (Catalan et al., 2010). Associations with *CD86* polymorphisms were reported in systemic sclerosis and several cancers (Abdallah et al., 2006; Howson et al., 2009; Landi et al., 2011).

However, the investigation of *CTLA-4* and *CD86* polymorphisms associated with RA

has yielded variable and inconsistent results, suggesting that the genetic backgrounds of different ethnic groups should be taken into account while performing association studies of these polymorphisms with RA. The aim of this study was to investigate the influence of *CTLA-4* and *CD86* polymorphisms on the susceptibility to RA in the Han Chinese population.

MATERIAL AND METHODS

Subjects

This study was approved by the Ethics Committee of Soochow University and all subjects gave informed consent for the genetic analyses. A total of 213 unrelated Chinese RA patients were recruited from the Outpatient Departments of Rheumatology in the First and the Third Affiliated Hospitals of Soochow University. This population, whose mean age was 53.3 years (±10.6 years), was composed of 48 men and 165 women. All patients, who self-reported as being Han Chinese, have resided in southeastern China for at least 3 generations and fulfill the revised criteria for the classification of RA published by the American Rheumatism Association in 1987 (Arnett et al., 1988). Their families did not have any recorded history of RA. The control group consisted of 303 gender-, age-, and ethnicity-matched unrelated healthy individuals who had medical checkups at the above 2 hospitals (Table 1).

Table 1. Characteristics of rheumatoid arthritis (RA) patients and controls.

Characteristic	RA patients	Controls	P value
Total number	213	303	
Female	165 (77.5%)	234 (77.2%)	>0.05[a]
Male	48 (22.5%)	69 (22.8%)	
Age (means ± SD years)	53.3 ± 10.6	51.3 ± 10.1	>0.05[b]
Duration of disease (years)	12.1 ± 8.0		
RF+[c]	125 (81.6%)		
RF-[c]	30 (19.4%)		
Anti-CCP+[c]	107 (76.4%)		
Anti-CCP-[c]	33 (27.1%)		

RF = rheumatoid factor; anti-CCP = anti-cyclic citrullinated peptide. [a]P value calculated by the Pearson chi-square test (all frequency >0.05) or the Fisher exact test (any frequency <0.05). [b]P value calculated by the Student *t*-test. [c]Clinical data were not available for some cases.

Single nucleotide polymorphism (SNP) selection and genotyping

A peripheral venous blood sample of 2 mL was drawn from each individual by standard venipuncture and the sample was stored at -20°C. Genomic DNA was isolated from peripheral blood leukocytes by standard procedures. The *CTLA-4* -318C/T (rs5742909) promoter polymorphism, the *CTLA-4* +49A/G (rs231775) exon 1 polymorphism, the *CD86* +1057G/A (rs1129055) (Ala304Thr) exon 8 polymorphism, and the *CD86* +2379G/C (rs17281995) 3'-UTR polymorphism were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. PCR primers, conditions, and restriction enzymes are shown in Table 2. DNA fragments were visualized on 2.5% agarose gels stained with ethidium bromide. The PCR-RFLP-based genotyping results were confirmed by direct sequencing of 20 randomly selected samples from each genotype group using an ABI Prism, Model 3100, Avant sequencer (Applied Biosystems, USA).

Table 2. Primers, annealing temperatures, and restriction endonucleases used for genotyping.

Polymorphism	Sense primer	Antisense primer	Annealing temperature (°C)	Restriction enzyme
rs5742909	5'-AGTCTCCACTTAGTTATCCAGATCCT-3'	5'-AAAAGACAACCTCAAGCACTCA-3'	58	*Mnl*I
rs231775	5'-CACGGCTTCCTTTCTCGTAA-3'	5'-CCCTGGAATACAGAGCCAGC-3'	63	*Bbv*I
rs1129055	5'-TGGGATGAATGGAAGGAGGCTT-3'	5'-CCTTGAGCAAAAACAATCATCCGA-3'	62	*Mly*I
rs17281995	5'-TCCATATACCTGAAAGATCTGATGCA-3'	5'-GAGCTGGAGTTACAGGGAGGCT-3'	60	*Bbv*I

Statistical analysis

Deviation from Hardy-Weinberg equilibrium (HWE) was examined in the controls by the χ^2 test. The following statistical analyses were performed using the SNPstats software (http://bioinfo.iconcologia.net/SNPstats) (Sole et al., 2006). Based on the logistic regression method, the case-control association of genotypes was tested and odds ratios (OR) and 95% confidence intervals (95%CI) were calculated. D' and r^2 were calculated to evaluate the magnitude of linkage disequilibrium (LD). Haplotype frequencies were estimated using the expectation maximization algorithm coded into the haplo.stats package (http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm). The association analysis of haplotypes was performed with logistic regression similarly to that of genotypes, and the results are presented as OR and 95%CI. The most frequent haplotype was automatically selected as the reference category and rare haplotypes were pooled together in a group. The log-additive inheritance model was assumed by default. The significance level of all these tests was $P < 0.05$.

RESULTS

SNP analysis

A total of 4 SNPs were successfully genotyped in 213 RA patients and 303 healthy controls. Table 3 shows the allele and genotype distributions of these 4 SNPs. P values for Hardy-Weinberg proportions of the SNPs are shown in Table 3 as well. For all 4 SNPs, the genotypic distribution in controls conformed to HWE. Among the 4 SNPs, the genotype and allele distributions of SNP1 (rs5742909) differed significantly between RA patients and controls ($P < 0.05$). When logistic regression was used for association analysis by modeling the effect of the SNPs in the additive, dominant, and recessive models, SNP1 showed a significant difference between cases and controls in the dominant (OR = 2.70; 95%CI = 1.56-4.68), over-

Table 3. Single nucleotide polymorphisms (SNPs) of *CTLA-4* and *CD86* genes investigated in the cases (N = 213) and controls (N = 303).

SNP	dbSNP ID and position		Frequency (%)							HWE P
			Allele		P	Genotype			P	
CTLA-4			C	T		C/C	C/T	T/T		
1	rs5742909	Cases	70.5	29.5		47.7	45.6	6.7		
	promoter (-318C/T)	Controls	83.2	16.8	0.0015*	70.5	25.3	4.2	0.002*	0.34
			A	G		A/A	A/G	G/G		
2	rs231775	Cases	37.7	62.3		11.7	52.1	36.2		
	exon 1 (+49A/G)	Controls	36.4	63.6	0.76	15.9	41.1	43.0	0.19	0.24
CD86			A	G		A/A	A/G	G/G		
3	rs1129055	Cases	59.3	40.7		32.3	54.0	13.7		
	exon 8 (+1057G/A)	Controls	54.2	45.8	0.25	25.4	57.7	16.9	0.44	0.07
			C	G		C/C	C/G	G/G		
4	rs17281995	Cases	92.0	8.0		84.0	16.0	0.0		
	3'-UTR (+2379G/C)	Controls	93.9	6.1	0.48	87.9	12.1	0.0	0.47	0.6

HWE = Hardy-Weinberg equilibrium. *P < 0.05.

dominant (OR = 2.55; 95%CI = 1.45-4.48), and log-additive (OR = 2.17; 95%CI = 1.36-3.49) models (Table 4). The dominant model was accepted as the best inheritance model because it showed the smallest Akaike information criterion value (316.3). The other 3 SNPs did not show association with RA in any of the 5 inheritance models (data not shown).

Table 4. Association analysis of rs5742909 with rheumatoid arthritis (adjusted by gender) by using logistic regression.

Model	Genotype	Cases (%)	Controls (%)	OR (95%CI)	P value	AIC	BIC
Codominant	C/C	47.7	70.5	1	0.0013*	318.2	328.7
	C/T	45.6	25.3	2.75 (1.55-4.88)			
	T/T	6.7	4.2	2.43 (0.73-8.12)			
Dominant	C/C	47.7	70.5	1	<0.001*	316.3	323.2
	C/T-T/T	52.3	29.5	2.70 (1.56-4.68)			
Recessive	C/C-C/T	93.3	95.8	1	0.39	328.5	335.5
	T/T	6.7	4.2	1.66 (0.51-5.46)			
Overdominant	C/C-T/T	54.4	74.7	1	0.001*	318.4	325.4
	C/T	45.6	25.3	2.55 (1.45-4.48)			
Log-additive	-	-	-	2.17 (1.36-3.49)	<0.001*	318.2	325.2

OR = odds ratio; 95%CI = 95% confidence intervals; AIC = Akaike's information criterion; BIC = Bayesian information criterion; *P < 0.05.

Stratified analysis of rs5742909 in subgroups based on the presence of rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies

Since SNP rs5742909 exhibited the strongest association with RA in the present study, we analyzed the allele and genotype frequencies of rs5742909 in RA patients stratified by autoantibody status (Table 5). The stratification results showed that the rs5742909 T allele was significantly associated with the RF-negative (P < 0.001, OR = 4.608, 95%CI = 2.430-8.740) and anti-CCP-negative subgroups (P = 0.001, OR = 2.904, 95%CI = 1.486-5.678). This allele also showed a significantly association with RF-positive (P = 0.006, OR = 2.024, 95%CI = 1.224-3.345) and anti-CCP-positive subgroups (P = 0.032, OR = 1.757, 95%CI = 1.047-2.950). The homozygous TT genotype of rs5742909 was only associated with the RF-negative and anti-CCP-negative subgroups (P < 0.001, OR = 13.400, 95%CI = 2.555-70.279 and P = 0.036, OR = 5.025, 95%CI = 0.977-25.847, respectively), but not with the RF-positive and anti-CCP-positive subgroups (Table 5).

Table 5. Genotype frequencies of rs5742909 in rheumatoid arthritis patients, stratified by rheumatoid factor and anti-CCP autoantibody status.

Groups	Genotype frequency (%)			T *vs* C		TT *vs* CC	
	CC	CT	TT	P	OR (95%CI)	P	OR (95%CI)
Control (N = 303)	0.71	0.25	0.04				
RF+ (N = 125)[a]	0.48	0.47	0.06	0.006	2.024 (1.224-3.345)	0.299	2.043 (0.519-8.047)
RF- (N = 30)[a]	0.17	0.69	0.14	0.000	4.608 (2.430-8.740)	0.000	13.400 (2.555-70.279)
Anti-CCP+ (N = 121)[a]	0.54	0.40	0.06	0.032	1.757 (1.047-2.950)	0.333	1.948 (0.495-7.660)
Anti-CCP- (N = 45)[a]	0.37	0.52	0.11	0.001	2.904 (1.486-5.678)	0.036	5.025 (0.977-25.847)

P values were calculated by the Pearson chi-square test. Odds ratio (OR) and 95% confidence interval (95%CI) were calculated in the indicated patient subgroups in comparison to controls. [a]Clinical data were not available for some cases.

LD and haplotype association analysis

Pairwise LD between the SNPs in *CTLA-4* (rs5742909, rs231775) and *CD86* (rs17281995, rs1129055) was calculated for the cases and controls in the Han Chinese. The LD analysis of the *CTLA-4* (rs5742909/rs231775, D' = 0.55) and *CD86* (rs17281995/rs1129055, D' = 0.11) SNPs revealed incomplete LD for each pair of SNPs in controls and RA patients.

The analysis of the association of haplotypes with RA was performed using logistic regression, similarly to that of genotypes with RA (Table 6). We found that the rs5742909/rs231775 haplotype showed significant association with the disease (P = 0.0025). The T-A (OR = 1.88, 95%CI = 1.12-3.15) and T-G haplotypes (OR = 3.45, 95%CI = 1.10-10.87) were more frequently observed in cases than in controls. The rs17281995/rs1129055 haplotype showed no association with the disease (Table 6). The association analysis of the haplotypes was adjusted by gender.

Table 6. Estimated haplotype frequencies and the association analysis with rheumatoid arthritis.

Haplotype	Sequence	Cases	Controls	Total	OR (95%CI)	P value
SNP1/SNP2	CG	0.5382	0.6084	0.5641	1	-
	CA	0.1631	0.2301	0.1915	0.82 (0.49-1.37)	0.45
	TA	0.2086	0.1291	0.1747	1.88 (1.12-3.15)	0.018*
	TG	0.0901	0.0323	0.0697	3.45 (1.10-10.87)	0.035*
Global haplotype association P value: 0.0025*						
SNP3/SNP4	AC	0.5279	0.5225	0.5208	1	-
	GC	0.3924	0.4092	0.4033	1.08 (0.71-1.65)	0.72
	AG	0.0630	0.0212	0.0467	0.33 (0.05-2.22)	0.26
	GG	0.0167	0.0471	0.0293	2.90 (0.40-21.27)	0.29
Global haplotype association P value: 0.40						

OR = odds ratio; 95%CI = 95% confidence interval. *P < 0.05.

DISCUSSION

RA is a chronic inflammatory disease that may involve extra-articular organs in addition to involving joints. Genetic and environmental factors are implicated in the pathogenesis of RA. CTLA-4 is an important costimulatory molecule that blocks interleukin-2 (IL-2) production and the expression of IL-2 receptors and inhibits the function of cytotoxic T-lymphocytes. The *CTLA-4* gene yields at least 2 major mRNA transcripts in humans. One transcript encodes a transmembrane protein and the other encodes a soluble form of CTLA-4 that lacks a transmembrane domain (Magistrelli et al., 1999). A variety of studies found elevated levels of the soluble CTLA-4 protein in the plasma of patients with a variety of immunologically mediated diseases (Magistrelli et al., 1999). Polymorphisms in *CTLA-4* have been associated with several autoimmune diseases including type 1 diabetes, inflammatory bowel disease, RA, celiac disease, multiple sclerosis, and systemic lupus erythematous (Haimila et al., 2009; Plant et al., 2010; Rai and Wakeland al., 2011). Two SNPs may influence the cell surface expression of the CTLA-4 molecule: -318C/T in the promoter region (rs5742909) and +49A/G in the first exon (rs231775). The T allele of -318C/T had been found to be associated with greater promoter activity than the C allele, and with significantly increased expression of both CTLA-4 mRNA in unstimulated cells and cell-surface CTLA-4 on activated T-cells (Ligers et al., 2001; Wang et al., 2002). The +49A/G SNP resulting in an amino acid substitution (Thr17Ala) in

the leader peptide causes altered processing in the endoplasmic reticulum and lower surface expression in transected cells (Ligers et al., 2001). In fact, studies found that the *CTLA-4* -318C/T polymorphism is associated with the risk of developing several autoimmune diseases and malignancies (Chang et al., 2004; Almasi et al., 2006; Su et al., 2007; Jones et al., 2009). In this study, we demonstrated an association between the -318C/T *CTLA-4* polymorphism and RA. A higher frequency of the -318C/T genotype (45.6 *vs* 25.3%) was observed in RA patients than in the control group. This association was similar to that reported previously in Graves' disease and chronic obstructive pulmonary disease (COPD) (Kouki et al., 2002; Liu et al., 2010).

The +49A/G variant of the *CTLA-4* gene is another commonly studied polymorphism that alters IL-2 production and the intracellular distribution of *CTLA-4* and, as a result, alters T-cell proliferation (Maurer et al., 2002). However, association results for this variant are often contradictory in different populations. Associations of this SNP with RA have been reported in German, Japanese, British, Chinese, and Irish populations. Conversely, several other reports showed no association of this SNP with RA in Japanese, Spanish, British, Tunisian, and Korean populations (Orozco et al., 2006). In our study, no significant association was found between the +49A/G SNP and RA. We were unable to explain this discrepancy; genetic heterogeneity or ethnic and geographic variations may cause variable frequencies of specific polymorphisms in different populations causing these variants to have dissimilar genetic and etiologic contributions to different diseases.

In the *CD86* gene, the +1057G/A polymorphism in exon 8 (rs1129055) results in an alanine to threonine substitution at codon 304 located in the cytoplasmic tail of CD86, which contains putative phosphorylation sites for protein kinase C. This substitution may modify the phosphorylation level in this region and influence the antigen-presenting cell-signal transduction pathway (Pawlak et al., 2010). Recently, the +2379C/G polymorphism (rs1721995) in the 3'-UTR of *CD86* was reported to be located in the seed region of an miRNA binding site and to have a functional role in regulating the expression of *CD86* (Landi et al., 2011). Liu et al. (2010) found that the A allele of rs1129055 might increase the risk of COPD by 1.3-fold. Genotype analysis demonstrated that the AA and GA genotypes were almost 10% higher than the GG genotype in individuals with COPD (Liu et al., 2010). Marin et al. (2005) also reported that the rs1129055 polymorphism was involved in liver transplant acceptance and may decrease acute rejection frequency and increase graft survival. In this research, we failed to find a significant association of these 2 SNPs with RA. This is consistent with a previous report by Matsushita et al. (2000).

Being a complex disease, the clinical heterogeneity of RA may be due to many additional factors such as the absence or presence of RF and anti-CCP antibodies. Our stratification study showed that the rs5742909 T allele and the TT genotype were more significantly associated the anti-CCP-negative and RF-negative subgroups than with the RF-positive and anti-CCP-positive subgroups. These data suggest that the *CTLA-4* polymorphisms may not be associated with an increased risk of producing antibodies against citrullinated proteins as other genetic association studies have also revealed (Liang et al., 2011).

In our study, we found incomplete LD between the *CTLA-4* -318C/T and +49A/G SNPs, which is contrary to previous reports (Ligers et al., 2001; Kouki et al., 2002; Zaletel et al., 2006). The 2q33 chromosomal region harbors a cluster of genes. It is possible that *CTLA-4* is also in linkage with other gene polymorphisms in this cluster. Haplotype analysis revealed the presence of predisposing and protective haplotypes. In this study, we investigated

the haplotypes of *CTLA-4* and *CD86* (Table 5). We showed that the haplotype consisting of 2 *CTLA-4* SNPs (rs5742909/rs231775) was significantly associated with RA in the Han Chinese population. In conclusion, our results confirm a genetic association of *CTLA-4* with RA in the southeastern Han Chinese population, strongly supporting the hypothesis that *CTLA-4* may be a common RA susceptibility marker across different ethnic groups.

ACKNOWLEDGMENTS

Research supported by grants from the National Natural Science Foundation of China (#81001337 and #31170834), the National Science Foundation for Postdoctoral Scientists of China (#20090461140), and the Research and Innovation Project for College Graduates of Jiangsu Province (#CXLX11_0077). We also gratefully acknowledge the numerous sample donors for making this study possible.

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