ORIGINAL RESEARCH

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Association of Interleukin-10 gene polymorphisms with ankylosing spondylitis

Abstract

Objective: Genetic polymorphisms of the Interleukin-10 (IL-10) promoter have been implicated in several autoimmune diseases, including seronegative spondyloarthropathies. This study investigated whether single nucleotide polymorphisms (SNPs) and haplotypes of IL-10 are associated with ankylosing spondylitis (AS), a common subtype of spondyloarthritis (SpA).

Methods: The serum levels of IL-10 were measured with an enzyme-linked immunosorbent assay (ELISA). The single nucleotide polymorphisms (SNPs) at positions -1082A/G, -819C/T and -592C/A in the IL-10 gene promoter were analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: 110 AS patients and 120 ethnic-matched healthy controls were included in this study. The serum levels of IL-10 were significantly higher in AS patients than healthy controls ($Z=-10.9$, $P<0.001$). Single SNP analysis showed no significant differences in the allelic and genotypic frequencies of -592A/C between the AS patients and healthy controls. No -1082GG genotype was found in this study. An increased frequency of -1082G allele was noted in AS patients ($P=0.047$). In a logistic regression analysis, the -1082AG genotype was associated with an odds ratio of 1.993 (95%CI, 1.046-3.800, $P=0.034$) for AS. And the -819CC genotype was associated with an odds ratio of 3.125 (95%CI, 1.246-7.836, $P=0.015$) for AS. Furthermore, haplotype analysis revealed that GCC haplotype was associated with a significantly increased risk of AS as compared with the ATA haplotype (OR=2.19; 95% CI, 1.13-4.26; $P=0.02$).

Conclusion: Our results indicate that the gene haplotype of IL-10 can contribute to the susceptibility to AS in a Chinese population.

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Ankylosing spondylitis (AS) is a complex multifactorial disease characterized by inflammatory back pain, peripheral arthritis, enthesitis and specific organ involvement such as anterior uveitis [1-3]. The prevalence of AS was estimated to be 0.2-1.2% [4,5]. Symptomatic AS causes loss of productivity, a substantial use of healthcare resources and compromised quality of life [6-8]. The pathogenesis of AS remains incompletely understood. It is generally accepted that inflammatory cellular infiltrates, cytokines, environmental and genetic factors play key roles [4,6,9]. Although human leucocyte antigen (HLA)-B27 is the dominant gene associated with AS, only 1%-5% of HLA-B27-positive individuals develop the disease, and the contribution of HLA-B27 to the overall genetic predisposition has been estimated at only 20%-30% [10]; therefore, there is increasing evidence to suggest the roles of non-major histocompatibility complex (non-MHC) genes in AS [4,11,12].

The cytokine secretion pattern in AS has a Th2 pattern with low production of IFN- γ and TNF- α as well as high production of Interleukin-10 [13]. Previous studies have suggested that production of Interleukin-10 is significantly higher in AS patients than healthy controls [14]. In addition, expression levels of many cytokines and immune response regulators are genetically determined [15,16]. Accordingly, it is very likely that genetic polymorphisms would influence cytokine secretion pattern in AS. The human IL-10 gene is located on chromosome 1 at q31-32, and is composed of five exons and four introns. Three common SNPs are at positions -1082A/G, -819T/C and -592A/C, and are closely linked and related to inter-individual differences in IL-10 secretion [17]. In a study on Finnish patients with reactive arthritis (ReA), the IL-10. G10 and G12 microsatellites were underrepresented in ReA patients compared with healthy controls, and the G8 allele was associated with a lower likelihood of developing chronic arthritis [18]. A recent study on Taiwanese patients showed a tendency toward a difference in the allelic distribution of IL-10-819T/C between AS patients and healthy controls [19]; however, that study did not evaluate the effect of interleukin-10 gene haplotype on AS.

In the present study, the -1082A/G, -819C/T and -592C/A common polymorphisms were measured in order to determine the effect of the IL-10 gene haplotype on the susceptibility to AS in Chinese population.

Materials and Methods

Study population

In a preliminary experiment, 45 patients and 45 healthy controls were randomly selected to be genotyped. Eight samples

with -819CC genotype were found in the patient group (P1=17.8%) and three samples with -819CC genotype were found in the control group (P2=6.7%). The number of AS patients considered for the study ($n = 213$) was determined according to the formula:

$$n = 1641.6 \left(\frac{u_{\alpha} + u_{\beta}}{\arcsin \sqrt{P1} - \arcsin \sqrt{P2}} \right)^2,$$

where $\alpha = 0.05$, $\beta = 0.20$, $u_{\alpha} = 1.960$, $u_{\beta} = 1.282$, $P1 = 17.8\%$, $P2 = 6.7\%$.

Only Han Chinese were eventually included in the study, thus limiting the chance of population stratification due to ethnicity. In addition, as the experiment progressed, the P1 became larger. Finally, 110 AS patients (56 males/54 females; mean age 35.4 ± 11.3 years) and 120 healthy controls (62 males/58 females; mean age 34.8 ± 10.6 years) were included in the study. The patients were diagnosed according to the modified New York criteria for AS [20]. And the exclusion criteria were the following [21]: (1) hypertension (HT), (2) diabetes mellitus (DM), (3) hyperlipidemia, (4) smoking and obesity, or (5) treated with SZP, MTX or anti-TNF during the previous four weeks. The healthy controls were recruited among relatives of health professionals and blood donors. No significant differences were noted in sex and age between AS patients with healthy controls. To eliminate ethnic biases within the population studied, all enrolled subjects were from Han Chinese. Written informed consent was obtained from each participating subject or a close relative, and research protocols were approved by the Ethical Committee of our hospital.

Determination of serum IL-10

Peripheral blood was collected from patients and healthy controls. The serum levels of IL-10 were measured with a specific enzyme-linked immunosorbent assay (ELISA) using the R&D DuoSet human IL-10 ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

DNA extraction

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood leukocytes using the Wizard Genomic DNA Purification kit (Promega, Madison WI) according to the manufacturer's instructions.

Determination of IL-10 gene polymorphisms

The IL-10 -1082A/G, -819C/T and -592C/A genotypes were determined by using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, and the

FIGURE 1. PCR-RFLP assay for analyzing the -1082A/G polymorphism of IL-10. Lanes 2, 3, 4, 5, 6, 7, 8 and 9 homozygous AA genotype, A allele cut with EcoNI generating 280 bp and 97 bp fragments; Lane 1, 10 heterozygous AG genotype, A allele cut with EcoNI generating 280 bp and 97 bp fragments, G allele cut with EcoNI generating 253 bp, 97 bp and 27 bp (not shown) fragments; and Lane M, bp marker.

PCR primers were designed based on described previously. In addition, 30% of samples were randomly selected to be genotyped a second time to ensure the quality of genotyping.

IL-10-1082A/G

The primers were as follows [22] (Shanghai Sangon Biological Engineering Technology & Services Co., Shanghai, China):
Forward: 5'CCAAGACAACACTACTAAGGCTCCTTT3'
Reverse: 5'GCTTCTTATATGCTAGTCAGGTA3'

PCR condition consisted of 94°C 5 min, followed by 35 cycles of 94°C 30 sec, 56°C 45 sec and 72°C 1 min, with a final extension at 72°C for 10 min. PCR products were 377 bp and digested with 5 units of restriction enzyme EcoNI (New England Biolabs, Ipswich, MA) at 37°C overnight. Digestion fragments of 280 bp + 97 bp for allele A or 253 bp + 97 bp + 27 bp for allele G, visualized by electrophoresis on a 3% agarose gel stained with 0.1% ethidium bromide (Fig.1).

IL-10-819C/T

The primers were as follows [23] (Shanghai Sangon Biological Engineering Technology & Services Co.):

Forward: 5'TCATTCTATGTGCTGGAGATGG3'
Reverse: 5'TGGGGGAAGTGGGTAAGAGT3'

PCR condition consisted of 94°C 5 min, followed by 35 cycles of 94°C 30 sec, 56°C 45 sec and 72°C 1 min, with a final

FIGURE 2. PCR-RFLP assay for analyzing the -819C/T polymorphism of IL-10. Lane 4, homozygous CC genotype, C allele cut with Mae III generating 125 bp and 84 bp fragments; lane 1, 2, 6, 7 and 9 heterozygous CT genotype, C allele cut with Mae III generating 125 bp and 84 bp fragments, while T allele does not cut; Lane 3, 5, 8 and 10 homozygous TT genotype, T allele does not cut, only 209 bp fragment; Lane M, bp marker.

extension at 72°C for 10 min. PCR products were 209 bp and digested with 5 units of restriction enzyme Mae III (Roche, Basel, Switzerland) at 55°C for 1h. Digestion fragments of 125 bp + 84 bp for allele C or 209 bp for allele T, visualized by electrophoresis on a 3% agarose gel stained with 0.1% ethidium bromide (Fig. 2).

IL-10-592C/A

The primers were as follows [23] (Shanghai Sangon Biological Engineering Technology & Services Co.):

Forward: 5'GGTGAGCACTACCTGACTAGC3'
Reverse: 5'CCTAGGTCACAGTGACGTGG3'

FIGURE 3. PCR-RFLP assay for analyzing the -592C/A polymorphism of IL-10. Lane 2, 4, 5, 6 and 9 homozygous AA genotype, A allele cut with RsaI generating 176 bp and 236 bp fragments; Lane 3, 7 and 8 heterozygous AC genotype, A allele cut with RsaI generating 176 bp and 236 bp fragments, while C allele does not cut; Lane 1, 10, homozygous CC genotype, C allele does not cut, only 412 bp fragment; Lane M, bp marker.

PCR condition consisted of 94°C 5 min, followed by 35 cycles of 94°C 30 sec, 64°C 45 sec and 72°C 1 min, with a final extension at 72°C for 10 min. PCR products were 412bp and digested with 5 units of restriction enzyme RsaI (MBI Fermentas) at 37°C for 4 h. Digestion fragments of 176 bp + 236 bp for allele A or 412 bp for allele C, visualized by electrophoresis on a 3% agarose gel stained with 0.1% ethidium bromide (Fig.3).

Statistical analysis

The serum IL-10 levels were expressed as mean $\pm$ SD and compared using the Mann-Whitney U test. A chi-square test and a 2-sample t-test were used to compare the basic demographic data between groups. A chi-square test was used to compare allele and genotype frequencies among groups and Hardy-Weinberg equilibrium of genotype distribution. Odds ratios (OR) with 95% confidence intervals (CIs) was calculated using logistic regression. To provide separate odds ratios (ORs) for each genotype, the most common genotype was considered as the reference group. Haplotypes and linkage disequilibrium (LD) were determined based on the expectation-maximization algorithm using the SNPStats program (available at <http://bioinfo.iconcologia.net/SNPstats/> provided in the public domain by the Biostatistics and Bioinformatics Unit, Catalan Institute of Oncology, Barcelona, Spain).

All statistical analyses were performed with the SPSS statistical software version 12.0. *P* values were two-sided and *P*<0.05 was considered statistically significant.

Results

The basic demographics of the subjects enrolled in this study are shown in Table 1. There were no significant differences between the AS patients and healthy controls for the mean age and gender distribution (*P*>0.05).

Serum IL-10 levels in patients and healthy controls

The serum levels of IL-10 in AS patients were significantly higher than those in healthy controls (591.3 $\pm$ 40.6 pg/mL vs. 532.2 $\pm$ 38.9 pg/mL; *Z*=-10.9, *P*<0.001).

The association between IL-10 gene polymorphism and serum IL-10 levels

The IL-10-1082AG genotype was associated with higher IL-10 levels as compared with the -1082AA genotype (*P*<0.001). The -819CC genotype was associated with higher IL-10 levels as compared with the -819TT genotype (*P*=0.041) (Fig.4).

FIGURE 4. The association between IL-10 gene polymorphism and serum IL-10 levels.

The allele and genotype frequencies of IL-10 in patients and healthy controls

The allele and genotype frequencies of the IL-10 gene -1082A/G, -819C/T and -592C/A polymorphisms were counted, calculated and summarized in Table 2. Within each study group, the genotype distributions were in accordance with the Hardy-Weinberg equilibrium.

An increased frequency of -1082G allele was noted in AS patients (*P*=0.047). In addition, there were significant differences in the genotype frequencies of the IL-10 gene -1082A/G and -819C/T polymorphisms between patients and healthy controls (*P*=0.034, 0.028 respectively). To estimate the association between the genotype and AS disease, odd ratio (OR) and 95% confidence interval (CI) were calculated, and the results suggest that the -1082AG and -819CC genotypes were associated with an increased risk of AS (-1082AG OR=1.993, 95%CI, 1.046-3.800, *P*=0.034; -819CC OR=3.125, 95%CI, 1.246-7.836, *P*=0.015).

No significant differences were found in the distribution of the IL-10 -592 A/C polymorphisms between patients and healthy controls.

Haplotype analysis of the IL-10 gene

Haplotype analyses were performed and the possible five haplotype frequencies are shown in Table 3. LD was observed between allele A at locus -1082 and allele T at locus -819 [*D'*=0.9989] and allele A at locus -1082 and allele A at locus -592 [*D'*=0.9989] and allele T at locus -819 and allele A at locus -592 [*D'*=0.9427]. Haplotype analysis revealed major ATA haplotype accounted for 58.1% and 66.2% of these five haplotypes in both patients and healthy controls, respectively. Using haplotype analyses, GCC haplotype was found to be associated

TABLE 1. Demographics of AS patients and healthy controls

	Healthy controls (n=120)	AS patients (n=110)	P
Gender (M/F)	62/58	56/54	0.909 ³
Age (years)	34.8±10.6 ¹	35.4 ±11.3 ¹	0.686 ²

¹Mean ± SD (all such values).²2-sample Student's *t* test.³Pearson's chi-square test.

TABLE 2. The allele and genotype frequencies of IL-10 polymorphism in patients and healthy controls

	Healthy controls n=120 (%)	AS patients n=110 (%)	χ ²	P
IL-10-1082A/G				
AA	101 (84.2)	80 (72.7)		
AG	19 (15.8)	30 (27.3)	4.480	0.034
A allele	221 (92.1)	190 (86.4)		
G allele	19 (7.9)	30 (13.6)	3.945	0.047
IL-10-819C/T				
TT	50 (41.7)	40 (36.4)		
CT	62 (51.7)	50 (45.5)		
CC	8 (6.7)	20 (18.2)	7.118	0.028
T allele	162 (67.5)	130 (59.1)	3.501	0.061
C allele	78 (32.5)	90 (40.9)		
IL-10-592C/A			3.461	0.177
AA	61 (50.8)	51 (46.4)		
AC	49 (40.8)	41 (37.3)		
CC	10 (8.3)	18 (16.4)		
A allele	171 (71.3)	143 (65.0)	2.07	0.15
C allele	69 (28.8)	77 (35.0)		

TABLE 3. IL-10 gene haplotypes distribution in the patients and healthy controls

Haplotype	Healthy controls 2n = 240 (%)	AS patients 2n = 220 (%)	Odds ratio (95%confidence interval)	P
ATA	159 (66.2)	128 (58.1)	1.00	
ACC	47 (19.5)	45 (20.4)	1.19 (0.74-1.91)	0.48
GCC	19 (7.9)	30 (13.6)	2.19 (1.13-4.26)	0.02
ACA	12 (5.1)	15 (6.9)	1.64 (0.76-3.53)	0.21
ATC	3 (1.2)	3 (1.3)	0.84 (0.18-4.02)	0.83

with a significantly increased risk of AS as compared with the ATA haplotype (OR=2.19; 95% CI, 1.13-4.26; *P*=0.02).

Discussion

AS, a common subtype of SpA, is associated with chronic inflammation of the axial skeleton, sacroiliac and peripheral joints and entheses [24] and it has been confirmed that genetic and environmental factors play important roles in its pathogenesis [9,25]. With respect to the Th2 cytokine secretion pattern in AS, there has been some interest in the role of IL-10 in the pathogenesis of AS. Baeten *et al.* [13], reported that IL-10 production was increased in SpA patients compared with healthy controls. Rudwaleit *et al.* [14], reported that the percentage of IL-10+/CD8+ T cells was higher in AS patients than in healthy controls. In contrast, a study conducted by Vazquez-Del Mercado [26] showed no differences in levels of IL-10 between AS patients and healthy controls (*P*>0.17). A study conducted by Kozaci [21] reported that IL-10 levels were significantly lower in the AS patients than in healthy controls. It should be noted that all of those studies had very small sample sizes.

In our study, serum levels of IL-10 in 110 AS patients and 120 healthy controls were measured and found to be significantly higher in AS patients than in healthy controls. Therefore, our findings are in agreement with those of Baeten *et al.* and Rudwaleit *et al.*, with all three research groups showing higher IL-10 levels produced by peripheral blood mononuclear cells (PBMCs) in AS patients compared with healthy controls.

Few studies have reported the association between IL-10 gene polymorphisms and AS. A study conducted by Goedecke [27] reported that levels of IL10-597 and -824 SNPs were weakly associated with age of AS onset, but failed to find an association between IL-10 gene polymorphisms and susceptibility to AS. Kaluza *et al.* [18] reported that the IL-10.G10 and G12 microsatellites were underrepresented in ReA patients compared with healthy controls, and that the G8 allele was associated with a lower likelihood of developing chronic arthritis; however, no similar study has been reported for Chinese patients.

In this study, the IL-10 gene polymorphisms and -1082A, -819T and -592A were investigated in Chinese population with AS. There were no significant differences in the distribution of the IL-10-592C/A polymorphisms between the AS patients and healthy controls; however, an increased frequency of -1082G allele was noted in AS patients. In addition, the frequencies of -1082AG and -819CC genotypes were significantly increased in AS patients. The logistic regression analysis suggested that the -1082AG and -819CC genotypes were asso-

ciated with an increased risk of AS in Chinese population. In agreement with our results, a recent study [19] on Taiwanese patients reported that IL-10-819C/T allelic frequencies between AS patients and healthy controls showed a trend toward significant association ($P=0.054$).

In the present study, the association between haplotypes of IL-10 gene and the susceptibility to AS was analyzed. Results suggested that GCC haplotype was associated with a significantly increased risk of AS as compared with the ATA haplotype. Additionally, it has been demonstrated that GCC haplotype was associated with increased IL-10 production [17]. In addition, the present study found that -1082AG and -819CC genotypes, which are associated with increased risk of AS, were also found to be associated with higher IL-10 production. Thus, it is possible that the genetic variations of IL-10 can influence the susceptibility to AS through changing the expression of IL-10. Few other studies have reported the association between haplotypes of IL-10 gene and AS.

Previous studies of European populations have only observed three haplotypes (GCC, ACC and ATA) of IL-10 gene promoter region [28]; however, in this study, at least five haplotypes (ATA, ACC, GCC, ACA and ATC) were found in the Chinese Han population, consistent with previous studies of the Chinese population [29,30]. The reason may be that the genotype/haplotype frequencies of IL-10 promoter SNP in different populations have wide differences according to ethnicity [31,32].

In conclusion, our results suggest that IL-10 -1082AG, -819CC genotypes and the GCC haplotype have significant association with the susceptibility of ankylosing spondylitis in Chinese population. The results of our study are limited because of the relatively small numbers of cases and controls and additional studies with larger sample sizes will be needed to further elucidate the genetic effects of the IL-10 polymorphisms on ankylosing spondylitis.

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