RESEARCH ARTICLE

Determination of IL-23 receptor gene polymorphism in Iranian patients with ankylosing spondylitis

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ABSTRACT. ***Introduction:*** **The result of recent genome-wide association studies revealed that, in addition to *HLA-B27*, a few non-*HLA* genes are associated with susceptibility to ankylosing spondylitis (AS) in Caucasian populations. According to these studies, *IL-23R* is one of the genes that is associated with AS. In this study, we evaluated five important single nucleotide polymorphisms (SNPs) of the *IL-23R* gene which confers susceptibility to AS, and its effects on the severity of the disease in *HLA-B27* positive and negative patients and several subtypes of *HLA-B27*.** ***Materials and methods:*** **The study population consisted of 294 AS patients and 352 age-, sex-, and ethnicity-matched healthy controls. All patients were examined by rheumatologists, and met modified, New York criteria for the disease. Five SNPs (rs1004819, rs11209032, rs1495965, rs11465804, and rs1004819) of the IL-23R gene were genotyped using the Real-Time PCR TaqMan genotyping method.** ***Results:*** **We found that only rs1004819 has a significant association with AS, and that the remaining four SNP alleles are not associated with AS. Also, there was no association between these five polymorphisms and BASDAI, BASFI, and BASMI indices. Two haplotypes, ACGAT and ACGAG, were found to be associated with the heritability of AS. In addition, two significant, protective diplotypes (D8, $\frac{\text{GCGAG}}{\text{GTGGG}}$; and D9, $\frac{\text{ACGAG}}{\text{GCGAG}}$) were discovered.** ***Conclusion*****: This study supported our previous findings regarding the differences between the genetic patterns of AS in Iranian patients compared with those in other parts of the world.**

Key words: ankylosing spondylitis, HLA-B27, IL23R, SNP

Ankylosing spondylitis (AS) is a chronic inflammatory disease, belonging to a family of rheumatological diseases called the spondyloarthropathies, which includes psoriatic arthritis [1]. The spondyloarthropathies also include reactive arthritis, juvenile-onset SpA and undifferentiated Spa, and are associated with inflammatory bowel disease [2]. Males are more often affected than females, with a ratio of approximately 3.78:1 [3]. In 80% of patients, the first symptoms of disease become apparent before the age of 30 years [4]. The main clinical manifestations of AS include: inflammatory back pain and stiffness, peripheral oligoarthritis, enthesitis, and anterior uveitis [5].The exact etiology of AS is not well understood, but genetic and environmental factors play important roles in its pathogenesis [6]. AS is highly heritable; ∼90% of the risk of disease progression is genetically determined [7]. *HLA-B27* is the most important genetic factor involved in the pathogenesis of AS [8]. The roles of other genes have been recently shown to be involved in the pathogenesis of AS [9-12]. With the advent of high-throughput Genome-wide Association Studies (GWAS), the associations between AS and two other important genes, *ERAP1* and *IL-23R*, has been determined [13-15]. These two genes are responsible for 26% and 9% of the risk of disease, respectively [7]. *IL-23R* gene is located on chromosome 1 (1p31.3) and encodes the IL-23 receptor, which, in combination with IL-12 receptor B1, makes the heterodimeric IL-23 receptor. IL-23 is a key cytokine for maintaining and amplifying Th17 cells that are engaged in several inflammatory conditions such as inflammatory arthritis [16]. Studies of common genetic variants show that individuals who carry a particular SNP allele at one site often predictably carry specific alleles at other, nearby variant sites. This correlation is known as linkage disequilibrium (LD); a particular combination of alleles along a chromosome is termed a haplotype [17]. Diplotype is defined as a specific combination of two haplotypes [18]. In this study, we evaluated the possible association of five SNPs, haplotypes and diplotypes of the *IL-23R* gene, with disease susceptibility and severity in Iranian patients with ankylosing spondylitis.

MATERIALS AND METHODS

The study population consisted of 294 AS patients (232 males and 62 females) with an average age of 38.17 years,

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and 352 age-, sex-, and ethnicity-matched healthy controls (256 males and 96 females) with an average age of 37.42 years. All of the patients recruited from the outpatient clinic of the Rheumatology Research Center (RRC) and the Iranian AS association were examined by rheumatologists and were found to meet modified New York Criteria for the disease. For evaluation of disease severity and functional capacities, a protocol based on the Assessment of SpondyloArthritis International Society (ASAS) core set [19] was used. This consisted of: 1. Disease activity by Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [20]; 2. Function by Bath Ankylosing Spondylitis Functional Index (BASFI) [21]; and 3. Damage or deformity of the spine by Bath Ankylosing Spondylitis Metrology Index (BASMI) [22]. BASDAI and BASFI questionnaires had been translated into Persian in our previous study [23].

Healthy control subjects and their family members had no history of rheumatological or autoimmune diseases. The local ethics committee of the Tehran University of Medical Science approved the study. All patients gave their informed consent prior to the study.

DNA analysis

Genomic DNA was extracted from peripheral blood samples using the standard phenol-chloroform technique [24]. The concentration and purity of the DNA collected were evaluated using the Thermo Scientific NanoDrop 2000c instrument. Five SNPs (rs11209026, rs11209032, rs1495965, rs11465804, and rs1004819) of the *IL-23R* gene were genotyped. SNP genotyping reactions were performed using the Real-Time PCR TaqMan genotyping technique according to manufacturer's instructions (Applied Biosystems, Foster City, USA). The characteristics of five selected SNPs are shown in *table 1*. Amplification reactions were performed in the Applied Biosystems StepOnePlus Real-Time PCR system, and allele calling by the analysis of allelic discrimination plots with AB SDS 2.2 software.

Statistical analysis

Data analysis was performed using IBM SPSS Software (Version 20) and R software revision (version 2.15) [25]. The odds ratio and P-value were calculated for each allele and genotype in the case and control groups. Any deviation from Hardy–Weinberg equilibrium in the control group was tested (see *table 2*) for all five SNPs. Haplotype frequencies in both case and control groups were calculated, and the most frequent haplotypes in each group were estimated. Statistical analysis of diplotypes were performed using IBM SPSS software (Version 20) and Microsoft Office Excel 2007. Detecting diplotypes is a dilemma for both geneticists and statisticians. However, we used a simple, innovative method by using Microsoft Office Excel formulae for this purpose. Firstly, the SNP genotype codes of all individuals were entered into an Excel datasheet. Then, the CONCATENATE formula was used to link the genotype codes of all SNPs together as a 5-letter (numbers) text. After that, we explored the frequencies of all these diplotypes obtained and omitted those with frequencies lower than 0.02 in the sample population. We then used the IF logical operator of Excel to label individuals for certain diplotypes. Finally, we imported these data into SPSS for the rest of the statistical tests such as Chi-square to obtain odds ratios and confidence intervals for all diplotypes. For investigating the associations between genetic findings (alleles, genotypes, haplotypes, and diplotypes) and clinical status (AS *versus* healthy controls), the chi-square test was used.

RESULTS

The Iranian ankylosing spondylitis cohort population consisted of 232 males (78.9%) and 62 females (21.1%) with an average age of 38.17 (± 10.7 SD) years (range 18-66 years) and disease duration of 15.75 (± 9.4 SD) years (range 1-59 years), and of which 73.8% of patients were HLA-B27 positive. Also, the healthy control group consisted of 256 males (72.7%) and 96 females (27.3%) with

Table 1
IL23R genetic variants analyzed in ankylosing spondylitis (AS) patients and controls. Positions are given as per dbSNP build 37.3.

Gene	Chromosome	Position	Gene location	TaqMan assay ID	NCBI SNP reference	Variation
IL-23R	1	67705958	Exon	C_1272298_10	rs11209026	A/G
IL-23R	1	67740092	Intergenic	C_2720238_10	rs11209032	A/G
IL-23R	1	67753508	Intergenic	C_8361864_10	rs1495965	A/G
IL-23R	1	67702526	Intron	C_31222838_10	rs11465804	G/T
IL-23R	1	67670213	Intron	C_1272321_10	rs1004819	C/T

Table 2
Characteristics of Iranian ankylosing spondylitis (AS) patients and healthy controls.

	AS patients (n = 294)	Controls (n = 352)
Male/Female	232 (78.9%)/62 (21.1%)	256 (72.7%)/96 (27.3%)
HLA-B27 positive	73.8%	3.4%
Age, years	38.17 ± 10.7	37.42 ± 10.1
Disease duration, years	15.75 ± 9.4	
BASDAI	4.0 ± 1.9	
BASFI	4.7 ± 2.5	
BASMI	3.8 ± 2.6	

an average age of 37.42 (± 10.1 SD) years (range 19-76 years), and of which 3.4% were HLA-B27 positive. The mean of BASDAI, BASFI, and BASMI were 4.0 ± 1.9, 4.7 ± 2.5, and 3.8 ± 2.6 respectively. Epidemiological data from the cases and controls are summarized in *table 2*.

All the SNPs studied were in Hardy-Weinberg equilibrium in the control group. Five single-nucleotide polymorphisms (SNP), rs11209026, rs11209032, rs1495965, rs11465804, and rs1004819 were genotyped, and major allele frequencies (referent allele) between cases and controls were assessed as 0.976 *versus* 0.996, 0.527 *versus* 0.545, 0.556 *versus* 0.558, 0.973 *versus* 0.962, and 0.485 *versus* 0.552 respectively. We observed that only rs1004819 had a significant (p = 0.016; allele A, OR [95% CI]: 1.31 [1.05-1.6]) association with AS (*table 3*). Furthermore, there was no association between these SNPs and HLA-B27 and its subtypes, in both case and control groups. There was no association between these SNPs and BASMI, BASFI and BASDAI (data not shown).

The most frequent haplotype in both case and control groups based on haplotype frequency was (rs11209026, rs11209032, rs1495965, rs11465804, rs1004819) ACGAT, this haplotype showed significant differences between case and control groups (p = 0.036; OR [95% CI]: 1.621 [1.172-2.243]). Moreover, ACGAG was a significant, protective haplotype (p = 0.036; OR [95% CI]: 0.031 [0.002–0.416]) for AS (*table 4*). Diplotype analysis (*table 5*) revealed two protective diplotypes: D8, $\frac{GCGAG}{GTGGG}$; and D9, $\frac{ACGAG}{GCGAG}$. D1 showed a significant protection against AS (p = 0.003; OR [95% CI]: 0.313 [0.145–0.676]), and D9 was also protective for AS (p = 0.029; OR [95% CI]: 0.416 [0.194-0.891]).

DISCUSSION

AS is a complex disease that does not follow Mendelian genetic patterns. Several environmental and genetic factors are involved in pathogenesis of the disease. HLA-B27 is

Table 3
Allele and genotype distribution of IL23R in ankylosing spondylitis (AS) patients and healthy controls

dbSNP	Alleles/genotypes	AS (n = 294) N (%)	Control (n = 352) N (%)	p-value†	OR (95% CI)
	G	308 (52.7)	388 (54.5)	-	1 (Reference)
	A	276 (47.3)	324 (45.5)	0.528	1.07 (0.85-1.3)
rs11209032	GG	81 (27.7)	106(29.8)	-	1 (Reference)
	AG	146 (50.0)	176 (49.4)	0.887	1.09 (0.74-1.6)
	AA	65 (22.3)	74 (20.8)	0.649	1.15 (0.72-1.8)
	G	570 (97.6)	688 (96.6)	-	1 (Reference)
	A	14 (2.4)	24 (3.4)	0.301	0.7 (0.33-1.4)
rs11209026	GG	279 (95.5)	335(94.1)	-	1 (Reference)
	AG	12 (4.1)	18 (5.1)	0.568	0.8 (0.3-1.8)
	AA	1 (0.3)	3 (0.8)	0.299	0.4 (0-5)
	C	326 (55.6)	397 (55.8)	-	1 (Reference)
	T	260 (44.4)	315 (44.2)	0.963	1.01(0.83-1.26)
rs1495965	CC	94 (32.1)	114 (32)	-	1 (Reference)
	TC	138 (47.1)	169 (47.5)	0.925	1.02 (0.66-1.56)
	TT	61(20.8)	73 (20.5)	0.922	1.01 (0.62-1.67)
	G	568 (97.3)	683 (96.2)	-	1 (Reference)
	T	16 (2.7)	27 (3.8)	0.288	0.71 (0.35-1.4)
rs11465804	GG	277 (94.9)	332 (93.5)	-	1 (Reference)
	TG	14 (4.8)	19 (5.4)	0.748	0.88 (0.4-1.9)
	TT	1 (0.3)	4 (1.1)	0.204	0.3 (0-3)
	G	284 (48.5)	393 (55.2)	-	1 (Reference)
	A	302 (51.5)	319 (44.8)	**0.016**	**1.31 (1.05-1.6)**
rs1004819	GG	69 (23.5)	110 (30.9)	-	1 (Reference)
	AG	146 (49.8)	173 (48.6)	0.754	1.3 (0.91-2)
	AA	78 (26.6)	73 (20.5)	0.067	**1.7 (1.07-2.7)**

AS: ankylosing spondylitis; †chi-square test; OR: odds ratio; CI: confidence interval

Table 4
Overall haplotype associations of the single-nucleotide polymorphisms of IL-23R with ankylosing spondylitis (AS)

Block 1 Haplotypes						Frequencies			
Row	**rs1004819**	**rs1495965**	**rs11209026**	**rs11209032**	**rs11465804**	**Hap.freq (AS)**	**Hap.freq (Control)**	**OR (95% CI*)**	**p-value†**
1	A	C	G	A	T	0.41	0.3	**1.621 (1.172-2.243)**	**0.036**
2	G	T	G	G	T	0.33	0.28	1.267 (0.906-1.772)	0.592
3	A	T	G	G	T	0.09	0.05	1.879 (1.009-3.499)	0.255
4	G	C	G	G	T	0.07	0.08	0.866 (0.48-1.561)	0.972
5	G	C	G	A	T	0.05	0.07	0.699 (0.36-1.358)	0.771
6	A	C	G	A	G	0.002	0.06	**0.031 (0.002-0.416)**	**0.0009**

AS: ankylosing spondylitis; * 95% confidence interval for odds ratio; †chi-square test

Table 5
Overall diplotype associations of the single-nucleotide polymorphisms of IL-23R with ankylosing spondylitis

Diplotype arrays						Frequencies			
Row	**rs1004819**	**rs1495965**	**rs11209026**	**rs11209032**	**rs11465804**	**Dip. Freq (AS)**	**Dip. Freq (Control)**	**OR (95% CI*)**	**p-value†**
D1	AG	CT	GG	AG	GG	80 (10.2)	81 (10.3)	0.920 (0.651-1.301)	0.659
D2	AA	CC	GG	AA	GG	53 (6.8)	51 (6.5)	0.983 (0.650-1.485)	1
D3	GG	TT	GG	GG	GG	31 (4)	42 (5.4)	0.674 (0.414-1.097)	0.14
D4	AG	CC	GG	AG	GG	20 (2.6)	29 (3.7)	0.635 (0.353-1.144)	0.141
D5	AG	TT	GG	GG	GG	20 (2.6)	19(2.4)	0.998 (0.524-1.900)	1
D6	GG	CT	GG	GG	GG	16(2.00)	23 (2.9)	0.645 (0.335-1.241)	0.193
D7	AA	CT	GG	AG	GG	19 (2.4)	19 (2.4)	0.945 (0.492-1.814)	0.87
D8	**GG**	**CT**	**GG**	**AG**	**GG**	**9 (1.1)**	**26 (3.3)**	**0.313 (0.145-0.676)**	**0.003**
D9	**AG**	**CC**	**GG**	**AA**	**GG**	**10 (1.3)**	**22 (2.8)**	**0.416 (0.194-0.891)**	**0.029**
D10	AG	CT	AG	AG	GT	6 (0.8)	9 (1.1)	0.626 (0.221-1.777)	0.44
D11	GG	CC	GG	AG	GG	6 (0.8)	6 (0.8)	0.947 (0.303-2.962)	1
D12	AG	CT	GG	GG	GG	4 (0.5)	6 (0.8)	0.628 (0.176-2.243)	0.537

AS: ankylosing spondylitis; * 95% confidence interval for odds ratio; †chi-square test

the most common genetic factor involved in the initiation and pathology of this disease. It is estimated that up to 90% of patients with AS could be HLA-B27-positive in most ethnic populations throughout the world; however, only 2% of HLA-B27-positive individuals in the general populations could be affected by AS, which might point to the role of other genetic/environmental factors in the susceptibility and pathogenesis of the disease [26-28]. In Iranian population, about 70% of AS patients are HLA-B27 positive; therefore, the prevalence of HLA-B27 is lower than other parts of the world [29]. However, in the Iranian population, the effect of other non-MHC and environmental factors may be greater than for other ethnic populations.

We performed this case–control genotyping study to investigate the association between AS and *IL23R* gene polymorphisms in Iranian population. IL-23 is an inflammatory cytokine that binds to its receptor on several cells, including memory T cells, effector T cell, natural killer cells, dendritic cells and macrophages [30]. It also causes Th17 cells expansion and stabilization. IL-6 and TGF-β are required for the commitment of naïve CD4+ T cell to a Th17 cell lineage, but subsequent exposure to IL-23 and IL-1 is important for complete differentiation and effector function of these cells. It seems that Th17 cells clear extracellular pathogens, including bacterial, viral, fungal and parasitic pathogens [31]. However, they can be potential inducers of autoimmunity and tissue inflammation. IL-17A is a prototypic cytokine of the IL-17 family (IL-17A, B, C, D, E and F) produced by Th17 cells, that induce the production of pro-inflammatory cytokines, chemokines and metalloproteinases from different tissues and cell types. Neutrophilia is the most typical feature of this inflammatory condition [16]. Dysregulation of the IL-23/IL-17 immune axis is essential for the pathogenesis of autoimmune diseases, including collagen-induced arthritis (CIA), inflammatory colitis and autoimmune uveitis [32]. Certain SNP alleles in the IL-23R gene can up-regulate its expression on several immune cells, and amplify the inflammatory condition. Based on a literature review, we analyzed five important SNPs of the IL-23R gene in Iranian ankylosing spondylitis patients. Previous studies in western countries revealed the strong association between these five SNPs and AS [33, 34]. The rs11209026 and rs11209032 SNPs showed the strongest associations with AS susceptibility with a *P*-value less than 10^{-9} [33]. rs11209026 G/A produced a strong, protective effect on susceptibility to AS by changing the highly conserved Arg381 to Gln381, which subsequently modifies the interaction between IL23R and JAK2 and results in a reduction in cellular signaling in response to IL-23 [35]. rs1004819 is an intronic SNP, and could perhaps regulate the splicing of IL-23R mRNA [36]. The biological effect of other SNPs studied on the function of IL-23R is unclear. We observed that only rs1004819 has a significant association with AS, while the remaining four SNP alleles do not seem to be associated with AS in our population. In the other words, the mechanism of AS pathogenesis might be different between Iranians and other ethnic groups. Surprisingly, our data concerning rs11209026 in AS patients are similar to those of a Chinese study that confirms the difference between disease mechanisms in an Asian population compared to other ethnic populations [37]. Interestingly, our study showed that rs11209026 was not associated with AS, while this causative SNP is not polymorphic in the Han Chinese population; this SNP does not seem to play any role in AS in the Chinese population. The minor frequency of this SNP in patients and healthy controls in our study was similar to frequencies found in the 1000 Genomes Project. However, interestingly, we found two significant protective diplotypes. We also found ACGAT to be a susceptible haplotype, and ACGAG to be a protective haplotype for AS. It is also important to note that in our Iranian population, the aforementioned rs11209026 G/A *IL-23R* SNP is not highly varied and most of the extracted haplotypes and diplotypes are G/G homozygotes for this SNP. Thus, we believe the protective role of genetic variations of *IL-23R* in this Iranian population depends upon the other, closely linked SNPs in the *IL-23R* gene, which was discovered through diplotype analysis.

Geographic factors result in much separation of the population and subsequently, genetic diversity. The alleles of some SNPs are found with significantly different frequencies among different geographic populations. The results from this study go some way to explain the genetic diversity among different ethnic groups: *IL-23R* gene polymorphisms alone were not shown to be associated with AS in

Iranian population. Other studies should be carried out on other genes associated with the IL-23R signaling pathway in this population.

Our results suggest that there are significant differences between Iranian, Caucasian and Chinese populations, and that comprehensive gene mapping studies of Iranian cohorts are thus likely to be very valuable in unravelling the genetic causes of AS and other rheumatic diseases.

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