Association study of four polymorphisms in the interleukin-7 receptor alpha gene with multiple sclerosis in Eastern Iran

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ARTICLE INFO

ABSTRACT

Objective(s): Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) with unknown etiology. Various genetics and environmental factors contribute to the pathogenesis of the disease. The interleukin-7 receptor alpha chain (IL-7Ra) was identified as the first non-major histocompatibility complex (non-MHC) MS susceptibility locus. In this study we are trying to find the association of IL-7Ra gene polymorphisms with MS susceptibility in Eastern Iran.
Materials and Methods: A case-control study was performed in two provinces Sistan & Baluchistan and Khorasan with 219 patients and 258 unrelated matched healthy controls, using PCR-RFLP method for four single nucleotide polymorphisms (SNPs) rs7718919, rs11567685, rs11567686 and rs6897932 of IL-7Ra gene.
Results: We found a tendency toward association with genotyping analyses in SNP rs7718919 (*P*=0.048, OR=4.344, and 95% CI=0.892-21.146); also genotype and allele frequency in gender and MS subtype stratification were shown to have significant association with MS. Analysis of two provinces separately showed a significant difference in results of the allele and genotype frequencies. Moreover, haplotyping analysis showed that (GTGC) has an association only in the male secondary-progressive multiple sclerosis (SPMS) patients in comparison to the healthy controls (*P*=0.043, OR=0.413, and 95% CI=0.179–0.955).
Conclusion: IL7-Ra could be a susceptible gene to MS within the Eastern Iran population especially after MS and gender stratification.

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Introduction

Multiple sclerosis (MS) is a neurodegenerative disease in the central nervous system (CNS) that usually occurs in young adults (1-3). The etiology of MS is unknown but numerous families and twin studies have shown that there is a strong genetic component underlying the etiology of MS (4-7). It has been confirmed that moderate contributions of more than 50 non-HLA risk loci underlie the development and progression of the disease (8). Many different approaches including genetic linkage, candidate gene association, and gene expression studies have been used to identify the genetic basis of MS (9). Association study is a useful strategy for the identification of genetic risk loci using genetic markers such as single nucleotide polymorphisms (SNPs). So far, it has been achieved that a lot of SNPs located in different chromosomes are associated with MS.

The well-established human leukocyte antigen (HLA) association does not completely explain the genetic impact on disease susceptibility. Therefore many studies are performed for analysis of other genes involved in the susceptibility of MS in different geographical regions. More than three decades after the apparition of susceptibility effect of HLA genes, the interleukin-7 receptor alpha chain (IL-7Ra) or (CD127) located on chromosome 5P13 was identified as the first non-major histocompatibility complex (non-MHC) MS susceptibility locus (10). Promising results soon led researchers to propose IL-7Ra as a susceptible gene for MS (11-14). IL-7Ra gene polymorphisms analyses were performed on an Iranian population in 2011 (15). The discovery and

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validation of genetic risk factors in more ethnic populations may help toward the understanding of MS pathogenesis by providing valuable information on biological pathways that needs to be investigated. Most of the MS risks SNPs are involved in T cell homeostasis and differentiation (3).

IL-7Ra gene has crucial roles in some processes in the immune system such as development, maturation, and homeostasis of T and B cells (16). This gene is influential in VDJ recombination during lymphocyte development, and also regulates the access of signal transducers and activators of transcription 5 (STAT5) to the T cell receptor gamma (TCR γ) locus (17). Also the potential causative role of this gene in MS has been revealed in some functional studies (18). We analyzed four SNPs in IL-7Ra gene which have been confirmed as risk factors for susceptibility to MS mainly in European and Western countries (13, 19, 20). The frequency of susceptible allele might be different between population based on their genetics background (10, 21, 22). One of the most interesting risk SNPs identified was rs6897932, located in IL-7Ra, also known as CD127, which is involved in homeostasis and longevity of T lymphocytes (23). Because autoreactive T cells are thought to play an important role in MS, a genetic variation within this important survival factor can be important for the disease (24).

The SNP rs6897932 affects alternative splicing of exon 6, leading to the increase of exon 6 skipping, and finally the increase in production of soluble form of IL-7Ra for individuals carrying the risk allele (25). Three other SNPs located in promoter region are rs7718919, rs11567685, and rs11567686 that might influence the expression of IL-7Rα.

Here we hypothesized that these four SNPs may have important effects in susceptibility to MS in Eastern Iran. This area bordered by Turkmenistan in the North, Pakistan and Afghanistan in the East and the Oman Sea in the South has a specific environment and genetic background which likely varies from other Iranian ethnic groups as we saw in β-thalassemia mutation spectrum of this region and newly reported epidemiological study of MS in Iranian population (26, 27). We investigated this hypothesis by allelic, genotypic, and haplotypic analysis of these four risk SNPs. We did not find any strong association in total MS patients except a tendency toward association in genotyping analyses in SNP rs7718919 (*P*=0.048); although in gender and MS subtype stratification, we found some genotypes that were significantly associated with MS; interestingly in total male patients of SNP rs7718919, *P*-value was 0.031, OR=0.951, and 95% CI=0.898-1.007. Also analyses of two provinces separately showed different results which interestingly were different from previous results that were reported in the patients of capital of Iran,

Tehran. We think that these information warrant further investigation of these SNPs in other ethnic groups and populations.

Materials and Methods
MS patients and control subjects
During 2 years (2010 to 2012) we conducted this study on 477 individuals from Eastern Iran with Mean±SD age of 30.39±0.54 consisting of 219 patients and 258 age and gender matched controls. The patients were diagnosed by neurologist according to diagnostic criteria that described by McDonald *et al* (28). This group has at least one episode of MS such as optic neuritis, sensory sings, and weakness. All patients and healthy controls were from Sistan & Baluchistan (S&B) and Khorasan (KH) provinces of Iran and informed consent was obtained from all of them before blood sampling. This work was approved by the Ethics Committee of University of Zabol.

DNA extraction and genotyping
Peripheral blood samples (5 ml) were collected in EDTA tubes and DNA was extracted from whole blood using boiling method. Quantity and quality controls were performed by spectrophotometer and visualized by electrophoresis on 1% agarose gel. The DNA samples which have proper characteristics were stored in -20 ℃ for future analysis. Genotyping was performed via polymerase chain reaction (PCR) with subsequent restriction fragment length polymorphism (RFLP) analysis. To determine the genotype of sample, approximately 100 ng of genomic DNA was amplified using recombinant Taq polymerase (Cinnagen, Iran) and 100 nmol/l of promoter SNPs forward:
(5'- GGCATTCAGGTTTGGGGGAGTC -3') and reverse:
(5'- AAGGACATGAAGAGACAGAGCC -3') and also exon 6 SNP forward:
(5'- CCAGGGGAGATGGATCCTATCTTACGAA- 3') and reverse:
(5'-GATAGAGATACCGATACTGGGCAC-3'). All of primers were designed by CLC Main Work bench software (Version 5) and checked with BLAST software in NCBI. The primer SNPs amplified a fragment with 1186 bp length from genomic DNA in which cover of promoter SNPs. Promoter SNPs amplification was performed in a 25 μl reaction volume and PCR condition was initial denaturation at 94oC/ 5 min, followed by 35 cycles at 94 ℃ for 30 sec, 66 ℃ for 30 sec, and 72 ℃ for 60 sec. Termination of cycle sequencing was performed at 72 ℃ for 5 min as final extension. The mismatch primer SNP amplified a fragment with 217 bp in size. SNP rs6897932 PCR was performed under condition of an initial denaturation at 94 ℃/ 5 min, followed by 35 cycles at 94 ℃ for 30 sec, 62 ℃ for 30 sec, 72 ℃ for 30 sec, and final extension at 72 ℃/5 min. DNA genotyping for SNP rs6897932 was performed by a designed mismatch PCR-RFLP method, using a forward mismatch primer to create a new HinfI (Takara Bio Inc,

Japan) restriction site in the mutant allele site for overnight and then was subjected to electrophoresis on 12% polyacrylamide gel. The purified PCR products were digested for SNPs rs7718919 and rs11567686 using HphI enzyme (Fermentas, Cinnagene, Iran) and SNP rs11567685 was genotyped using PstI enzyme (Takara Bio Inc., Japan) for overnight and subjected to electrophoresis on 1% agarose gel.

Statistical analysis

The frequency of alleles and genotypes for case and control groups were identified and the association analysis of 4 SNPs with MS was performed using Chi-square test and Fisher's exact test. Hardy-Weinberg equilibrium (HWE) was used to check allelic equilibrium between samples. Odd`s ratio (OR) and 95% interval confidence (CI) were applied to estimate the contribution of the risk factors. All statistical analyses were performed using SPSS version 19 and frequencies of haplotypes (Hap) were estimated using the PHASE software (V. 2.1, USA). The conventional *P*-value of ≤ 0.05 was considered as overall significant level.

Results

The characteristics of the MS patients and healthy controls are presented in Table 1 and the genotypic analysis of total MS patients versus healthy controls is given in Table 2.

Allele and genotype frequency for the four SNPs of IL7Ra gene in stratification
Based on subtype

Significant association was gained while the subtype stratification was applied on genotype level: relapsing-remitting (RRMS) in SNP rs7718919 (*P*=0.03) and for secondary-progressive multiple sclerosis (SPMS) in SNP rs11567685 (*P*=0.009).   Also allelic frequency of rs11567685 SNP showed a significant association for SP MS patients (*P*=0.032, OR= 0.474, and 95% CI= 0.236-0.949) (data is not shown).

Based on gender

Allelic frequency of SNP rs7718919 in the males which are affected by MS showed a significant association to disease (*P*=0.000, OR= 0.030, and 95% CI= 0.009–0.099). Also genotype frequency of different subtypes was applied in this SNP for males; RRMS and SPMS showed a significant association with the disease (*P*=0.026, OR=0.947, and 95% CI=0.879-1.021 and *P*=0.005, OR=0.917, and 95% CI=0.773-1.087), respectively. Interestingly, genotyping of SNP rs6897932 in SP MS of the male group demonstrated a significant statistical difference between cases and Allelic analysis in the female group showed significant results for SP MS in SNP rs11567685 (*P*=0.040; OR=0.419, 95% CI=0.179-0.978) also this association was gained in the genotypic level of RR MS patients in the case of SNP

Table 1. Demographic characteristics of patients and controls

Individual (n=477)	MS (n=219)	HCs (n=256)
Mean±SDE age	31.77±0.863	29.40 ± 0.687
Male	65 (35.48± 1.750)	97 (28.82±1.213)
Females	153 (30.20±0.941)	159 (29.54 ± 0.826)
RR/SP/PP	154/38/25	-
S&B Males	34 (34.52±2.135)	54 (27.95±1.528)
S&B Females	78 (29.51 ± 1.101)	76 (27.51±0.953)
KH Males	31	43
KH Females	76	83

HCs, Healthy controls; RR, relapsing-remitting; SP, secondary progressive; PP, primary progressive; S&B= Sistan &Baluchistan, KH= Khorasan

rs11567685 (*P*=0.048; OR=0.369, 95% CI= 0.133-1.027) (data is not shown). Controls (*P*=0.004, OR=0.917, and 95%CI=0.773–1.087) but not in the females (data is not shown)

Based on province

We stratified the dataset by province so as to evaluate any difference in the results. The significant results are presented in Table 3.

Haplotype analysis

The results of total MS patients revealed that Hap GTGC is associated with MS susceptibility in the male SPMS patients (*P*=0.043, OR=0.413, and 95% CI=0.179–0.955), but in the total patients we did not yield significant association of any other Hap (results are not shown).

Discussion

In this study, we found that rs7718919 located in the promoter region of IL-7Ra gene was slightly associated with total (*P*=0.048) and also with male (*P*= 0.031) MS patients that were not previously reported in Iranian (15) and Australian population (29). However they showed that the presence of minor T allele in rs7718919 SNP's potential to facilitate the binding of different transcription factors, including nuclear factor to gene promoter in T cells in silico (29). When gender, subtype, and residence stratification were applied, SNP rs11567686 and SNP rs11567685 which are located respectively in 449 bp and 504 bp distances from start codon on the promoter of IL7Ra gene showed positive association with MS which has already been reported. The studies clarified that 3 SNPs in promoter region of IL-7Ra gene can influence the expression of this gene (25). The association of the high risk T allele of rs11567685 with MS has been reported by two previous studies (21, 13), but such linkage failed to be confirmed by a third one (7); however, the functional effects of these SNPs need to be further investigated.

Table 2. Allele and genotype frequencies of IL7Ra SNPs in MS patients versus healthy controls. Threshold of significance was considered in $P \leq 0.05$ Significant values are indicated in bold letters

SNP/Risk allele		MS n (%)	HC n (%)	MS vs. HCs P-values	Odd`s Ratio (95% CI)
rs7718919/ G	GG	187 (92.6%)	225 (92.2%)	(GG+GT) vs. TT	4.344 (0.892 – 21.146)
	GT	8 (4.0%)	17 (7.0%)	0.048	
	TT	7 (3.5%)	2 (0.8%)		
	G	191 (94.55%)	233.5 (95.70%)	G vs. T	0.781 (0.328 – 1.858)
	T	11 (5.45%)	10.5 (4.30%)	0.575	
rs11567685/ T	TT	120 (54.8%)	143 (55.4%)	(TT+TC) vs. CC	0.780 (0.423 – 1.440)
	TC	80 (36.5%)	87 (33.7%)	0.427	
	CC	19 (8.7%)	28 (10.9%)		
	T	160 (73.06%)	186.5 (72.29%)	T vs. C	1.040 (0.694 – 1.558)
	C	59 (26.94%)	71.5 (27.71%)	0.850	
rs11567686/A	AA	54 (26.7%)	84 (34.4%)	(AA+AG) vs. GG	1.027 (0.664 – 1.589)
	AG	99 (49.0%)	102 (41.8%)	0.905	
	GG	49 (24.3%)	58 (23.8%)		
	A	103.50 (51.24%)	135 (55.33%)	A vs. G	0.848 (0.584 – 1.233)
	G	98.50 (48.76%)	109 (44.67%)	0.389	
rs6897932/C	CC	129 (61.4%)	159 (64.6%)	(CC+CT) vs. TT	2.356 (0.212 – 26.165)
	CT	79 (37.6%)	86 (35.0%)	0.427	
	TT	2 (1.0%)	1 (0.4%)		
	C	168.5 (80.24%)	202 (82.11%)	C vs. T	0.884 (0.552 – 1.416)
	T	41.5 (19.76%)	44 (17.89%)	0.609	

HCs: Healthy Controls; MS: Multiple sclerosis; n: number

Table 3. Stratification of gender and subtype frequency of IL7Ra SNPs in MS patients versus healthy controls in two province of Eastern Iran; only significant values are shown

SNPs	MS Allele analyses gender /subtype/P-value		Odd`s Ratio (95% CI)		MS Genotype analyses gender/subtype/P-value		Odd`s Ratio (95% CI)	
	S&B	KH	S&B	KH	S&B	KH	S&B	KH
	NS in Females	NS in Females	NS in Females	NS in Females	NS in Females	NS in Females	NS in Females	NS in Females
rs7718919	Males/SP/0.029	NS in Males	0.292 0.091 – 0.933	NS in Males	Males/SP/0.0001	Males/RR/0.028	0.001 0.0001 – 229864.900	0.002 0.0001- 665394.727
					Male/RR/0.017		0.002 0.0001 – 563205.349	
	Females /SP/0.000	Females /PP/0.12	0.189 0.101 – 0.355	2.345 1.196 – 4.597	Females/SP/0.0001	Females/PP/0.0001	0.211 0.090 – 0.494	1690.268 0.0001 – 5.544E11
rs11567685	Males/SP/0.001	Males/PP/0.027	0.367 0.203 – 0.663	0.527 0.298 – 0.931	Males/SP/0.011	Males/PP/0.0001	0.375 0.174 – 0.810	0.211 0.096 – 0.463
	Males/PP/0.011		2.569 1.227 – 5.382		Males/PP/0.001	Males/SP/0.002	1249.739 0.0001 – 4.107E11	105.233 0.206 - 53801.407
rs11567686	NS in Females	Females /PP/0.0001	NS in Females	130307.310 0.0001 – 1.079E32	Females/SP,PP/0.04	Females/PP/0.0001	2.138 1.065 –4.292	0.0001 0.0001 – 10158.321
	NS in Males	Males/SP/0.014	NS in Males	2.017 1.147 – 3.549	NS in Males	NS in Males	NS in Males	NS in Males
rs6897932	NS in Females NS in Males	NS in Females NS in Males	NS in Females NS in Males	NS in Females NS in Males	NS in Females Males/SP/0.0001	NS in Females NS in Males	NS in Females 0.007 0.0001 – 3.552	NS in Females NS in Males
					Males/RR/0.016		0.016 0.0001 –8.349	

S&B= Sistan & Baluchistan province, KH= Khorasan province, NS= not seen

SNP rs6897932 located on exon 6 is very important for transmembrane and soluble form of IL-7Ra protein and represents the most consistently replicated susceptibility gene to MS besides the HLA class II region to date (23). A recent study showed that the soluble form of the IL-7Rα potentiates the biological availability and activity of IL-7 providing basis for autoimmune susceptibility (30). We found the association of this SNP only in males with increased relapse rate in Eastern Iran while after stratification based on province; the stronger result was gained again in the same sex. A number of studies have illustrated an association between MS and the high risk allele of rs6987932. These studies covered different ethnic groups (9, 18, 20, 31, 32). Akkad *et al* confirmed the association of SNP rs6897932 with susceptibility to MS, although he suggested that the SNP rs6897932 may not be the disease causing variation in this gene (7).

The comparison analysis of Hap in patients and controls presented a trend towards association in Hap 1 (GTGC) only in the males which have been repeated in this gender while the genotype frequencies were analyzed. Other previous studies have not analyzed the gender stratification but they examined haplotype of total MS population and found the relationship of Hap 2 (GCAC) in primary progressive (PP) subtype and Hap 3 (GTAT) in SP subtype with the disease (13).

Based on subtype and province analysis, we found that the males have superior association to MS in some SNPs and haplotype than the females. The effect of gender on MS disease severity has been evaluated but did not yield any significant difference between the males and females (33) while natural history of MS studies have shown that females are quicker to diagnose MS signals (34). The epidemiology study of MS has shown diverse results in province stratification, also the prevalence of MS between males and females were opposite in different subareas. The influence of sex on the disease still needs to be further investigated (35).

Iran is a big country laid on many different latitudes and is inhabited by populations with distinct ethnical backgrounds, preferentially concentrated in distinct geographic areas (36). Although Khorasan and Sistan & Baluchistan provinces are neighbors, they showed slightly different association results when stratification was applied. The reason of this variation might depend on difference of life styles and the difference in habitat (27). The number of patients registered in the medical network of S & B and KH that have our inclusion criteria is restricted.

Conclusion

This study has utilized a simple PCR-RFLP method to investigate genetic polymorphism of IL-7Rα in MS. However these data need further study on other and larger Iranian populations, especially on RNA and protein levels to confirm the role of these variations in expression of IL-7Ra gene and their pathological effects on MS susceptibility. Ethnic background accompanying the disease subtype and specific environment triggers might determine the MS susceptibility, so more investigations on IL-7Ra polymorphism in other Iranian ethnics are still needed.

Conflicts of interest

The authors have no conflicting financial interests.

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