IL-7Ra Association and Genotype-Dependent Severity and Response to IFN-β Therapy in Multiple Sclerosis

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Abstract- Multiple sclerosis (MS) is a neurodegenerative disease arising from interactions of both environmental and genetic factors. The SNP rs6897932 is located in *IL-7Ra* gene associated with MS susceptibility in some population. In this study, we investigated the possible association of SNP rs6897932 with MS susceptibility in 157 Iranian MS patients and 152 healthy controls. We also studied genotype-dependent severity and response to IFN-β in MS. Unlike some previous studies, our results clearly demonstrate that there are no significant differences in distribution of the SNP rs6897932 in our chosen Iranian MS patients and controls. Furthermore our results show, Interferon beta (IFN-β) therapy over a period of two years demonstrated an *IL-7Ra* genotype-dependent therapeutic effect in MS population. Patients carrying TT or TC genotypes gave a better response to IFN-β treatment, whereas patients carrying the homozygous CC genotype were the worst responders to IFN-β treatment. In other words, despite the lack of linkage the therapeutic response to the severity and progression of MS was related to the genotype of SNP rs6897932 presented in our patient. Therefore, in light of our findings reported here, to reach higher certainties, further studies are needed to associate *IL-7Ra* gene with the pathogenesis of MS.

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Introduction

Multiple sclerosis (MS) is the most common chronic demyelinating disease of the central nervous system (CNS) in adolescents, affecting about 2.1 million people worldwide (1). Although MS is idiopathic, many factors are considered to play a role in the pathogenesis of the disease (2,3). In general, 85% of the patients demonstrate relapsing-remitting multiple sclerosis disease course (RRMS), 10-15% of the older patients exhibit primary progressive multiple sclerosis course (PPMS) and the rest present the secondary progressive multiple sclerosis phase (SPMS) (4). Abundant studies among families and twins have demonstrated a clear correlation between genetic variants and MS disease (5). More than 50 non-HLA risk loci are estimated to be influential in the development and advancement of the disease (6). In this regard, the genetic background of Multiple Sclerosis has been investigated by searching for candidate gene associations, genetic linkage, and gene expression (7). Although genetic predisposition of the disease is not yet totally confirmed, the data obtained from the human leukocyte antigen (HLA) association studies revealed new candidate genes associated with the pathogenesis of MS. Among these candidate genes, we can mention interleukin 7 receptor (IL7R), interleukin 2 receptors A (IL2RA), CD58 and C-type lectin domain family 16 (CLEC16A) (8). And yet, many studies surveyed other genes engaged in the genetic predisposition of MS in different geographical regions (9). For a quarter century, the relevant studies on HLA, as major histocompatibility complex (MHC) genes, have strongly implicated the potential association of this family of genes with the pathogenesis of MS. Likewise, interleukin-7 receptor alpha chain (*IL-7Rα* or a CD127) positioned on chromosome 5p13, has been identified as the first non-MHC gene strongly associated with the genetic predisposition of MS (10). While four common haplotypes for *IL-7Ra* gene have previously been described, the studies of functional relevance or effect of

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these haplotypes on the pathogenesis of MS remain challenging. Haplotype 2, rs6897932, is known as protection against the disease and is tagged by a single base T/C allele. This single nucleotide polymorphism (SNP) rs6897932 is situated in exon 6 of *IL-7Ra* gene. *IL-7Ra* is expressed as a heterodimer on the surface of T cells, dendritic cells (DCs) and other myeloid cells and serves as receptors for hematopoietic growth factor Interleukin 7 (IL-7) which is produced and secreted by the stromal cells in the bone marrow (2). IL-7 growth factor plays a critical role in proper functioning of immune system, particularly, in lymphocyte differentiation, survival, proliferation, and reactivation through V(D)J recombination, signaling cascade for lymphopoiesis of T and B lymphocytes and control of accessibility of T lymphocyte γ receptor locus by STAT5 (18,19,26). Impaired function of *IL-7Ra* has strongly been implicated in development of MS disease (4). Consistent with the effect of *IL7*, studies performed by Europeans and others suggest that there is an association between SNP (rs6897932), a splicing determinant polymorphism in exon 6 of *IL7Ra* gene, and MS disease (11-13). In carriers of rs6897932 SNP, the production of soluble IL7Rα is elevated. This effect is due to rs6897932 SNP induced miss-splicing (deactivation of exon 6 splice silencer) and increased exon 6 skipping (14). In other words, presence of exon 6 in the message leads to the production of full functioning membrane-bound *IL7Rα*receptor (rIL7Rα), whereas the absence of exon 6 creates a soluble dysfunctional form of the protein (sIL7Rα). In this regard, allele C of rs6897932 has been identified as the SNP causing exon 6 skipping and truncation of *IL7Rα* protein, whereas allele T leads to synthesis of the normal coding message (retaining exon 6) for production of the full functional membrane-bound receptor (15,16). Therefore in line with these findings, allelic distribution of rs6897932 SNP have been reported to be associated with variable manifestations of the MS.*IL-7Ra* heterogeneity survey among Iranian and German populations was reported (17). In their studies, the authors have reported that regardless of the MS type there were no significant association between SNP rs6897932 and MS while with regard to disease progression, a significant association of the C allele of the SNP and chronic state of the SPMS was observed. Hoe *et al*., (2010), and McKay *et al*., (2013), have also discussed the effect of IFN-β (the most commonly used immunomodulatory drug in MS) on the promoter region of IL-R7a, indicating that *IL-R7a* production is upregulated in normal haplotype 1 more than the affected haplotype 2 and haplotype 4, the two haplotypes associated with the MS disease. Homozygous haplotype 1 (exon 6 of *IL-7Ra* spliced correctly) give rise to production of full functioning *IL7-Ra* receptor while heterozygous haplotype 2 in addition to normal functioning receptor also produces the nonfunctioning soluble form of the receptor (exon 6 spliced out). IFN-β being involved in viral protection and immune modulation, processes that could be pathogenically significant in MS, Hoe *et al*., argue further that IFN-β may contribute to the haplotype-dependent genetic association of *IL-7Ra* signaling with MS (29-30).

This study was an attempt to replicate the potential association of *IL-7Rα* with pathogenesis of MS by investigating the allelic distribution of rs6897932 SNP and its response to IFN-β in a selected Iranian population of normal individuals versus MS patients. These studies might also provide some insights on whether the frequency of genetic susceptibility of alleles could be different among populations with different genetic backgrounds.

Materials and Methods

Sample collection

During 2014-2015, a total of 157 MS patients from Imam Khomeini and Shariati Hospital of Tehran, Iran (113 women and 44 men, with mean age of 38.41±0.63 years, and the age of disease onset of 31.69±0.57 years) were chosen for this study. The diagnosis of MS was made according to the McDonald criteria (20). Average duration of disease in these patients was 6.85 years. As controls, we included 152 healthy individuals for this study (95 women and 54 men, with average age of 36.85±0.42). Informed consent was obtained from each participant before the study. Age, sex, different types of MS, expanded disability status scale (EDSS), age of onset, interval of the first relapse from the first attack, the number of relapses in relapsing forms, family history of genetic diseases such as autoimmune diseases, history of interferon beta (IFNβ) treatment and other types of drugs were recorded for each patient. Blood sampling and fillings were performed according to patient's consent.

DNA extraction

Peripheral blood from patients and controls was collected in EDTA tube and genomic DNA was extracted by salting out method (21). Demographic and clinical profiles of MS patients and controls are shown

in table 1.

Table 1. Demographic and clinical information of MS patients

Characteristics	Patients	Controls
Age (years) means	38.41±0.63	-
Female / male (n)	113/44 (71.79% / 28.21%)	95/57(62.5% / 37.5%)
Age of onset (years) mean	31.69±0.57	36.83±0.42
Disease duration (years) mean	6.85±0.51	-
Subgroup (n)		-
RRMS	120	-
SPMS	18	-
CIS	8	-
PPMS	11	-
EDSS mean	4.16	-
MSSS mean	5.13	-
Positive history of autoimmune	0%	-

Abbreviations:
EDSS: Expanded disability status scale,
MSSS: Multiple sclerosis severity score
RRMS: Relapsing Remitting Multiple sclerosis
SPMS: Secondary Progressive Multiple sclerosis
CIS: clinically isolated Syndrome
PPMS: Primary Progressive Multiple sclerosis

Genotyping

IL-7Rα SNP (rs6897932 polymorphism) was determined by sequence-specific primer- Polymerase chain reaction (SSP-PCR). For exon 6 polymorphism, the sequence of primers include a specific forward primer for T allele; 1= 5´-GGGGAGATGGATCCTATCTTACTTA**T**-3′ and forward primer for C allele; 2= 5´-GGGGAGATGGATCCTATCTTACTTA**C**-3′ that were used with a common reverse primer; C= 5´-CTGGGCACTAAATTCGTGAAATGCCA-3´. The SSP-PCR reactions were set up in a total volume of 12 µl with 1 µl of specific A GeneAmp PCR System 9700 (Life Technologies Europe BV, Naerum, Denmark) and Mastercycler Eppendorf (AH diagnostics AS, Aarhus, Denmark) was used. The PCR samples were loaded onto 6% non-denaturing polyacrylamide gels and run for 35 min at 156V. Subsequently, the gels were stained with ethidium bromide (EtBr) (Sigma-Aldrich, Denmark) and genotypes of samples were determined.

Disease severity and statistical analysis

For the measurements of cross-sectional disease severity in response to β-Interferon (IFN-β), Expanded Disability Status Scale (EDSS) methodology was employed. The global Multiple Sclerosis Severity Score (MSSS), progression of the severity of disease, was calculated employing the severity measurements (EDSS) of the MS disease at the time of blood sampling (MSSS1) and two years later (MSSS2). All Genotyping data were entered into a database and analyzed with SPSS, version 15 for Windows (Chicago, IL, USA). Pearson`s$\chi 2$ (Chi-square) test were performed and Fisher-extract tests were utilized for comparisons of frequencies of the two alleles, for rs6897932 SNP and its genotypes between MS and controls. The *Bonferroni correction* was used for multiple testing. After this correction, the corrected *P* (*Pc*) of <0.05 was assumed to be statistically significant.

Results

Demographic and clinical distribution of patients

For this study, we collected blood samples from 157 randomly chosen individuals with phenotypic presentation of the MS disease. The average age of these patients was estimated to be at 38.41±0.63 years (Table 1). Of this random population of MS patients, 71% (113 individuals) were women whereas 28% (44) constituted the male patients. The average age of onset of MS disease among these patients was 31.69±0.57 years, with the average duration of disease in these patients estimated to be 6.85±0.51 years. The control population in this study included 152 normal individuals, of which 62.5% (95 individuals) were men and 37% (57 individuals) were women, maintaining the ratio of male and female as in our patient group. The average age for the normal individuals in this study was 36.83±0.42

years. The majority of MS patients (120 individuals) were with RRMS condition, 18 with SPMS, 11 with PPMS and 8 patients exhibited clinically isolated syndrome (CIS). As shown in Table 1. The Expanded Disability Score mean (EDSS mean) of 4.16 with the Multiple Sclerosis Severity Score mean (MSSS mean) of 5.13is reported for the MS patients in this study. There was no history of autoimmune disorder reported for the patients in this study (Table 1).

Frequency of *IL-7Ra* polymorphism

In this study, employing SSP-PCR methodology, different alleles of SNP rs6897932 in the MS patients and control group were compared to determine whether there is an association of the previously described diseased allele (diseased allele C vs the normal allele T; 15,16) with that of our patient group as well. Among our 157 patients studied, there were 95 patients with genotype of CC, 57 patients with TC and 5 patients with TT, which correspond to frequencies of 60.5%, 36.3% and 3.1% respectively (Table 2). Among the members of our control group (152 individuals), 12 exhibited genotype of TT, 48 TC and 92 CC, which correspond to similar frequencies as in patients, 60.5%, 31.5%, and 7.9%, respectively. Naturally, with respect to the observed genotypes, the allele distribution among our patient group, with total of 314 alleles, was 67 allele T (21.3%) vs 247 allele C (78.7%). Similar to the allele distribution among the normal individuals, with total of 304 alleles, was 72 allele T (23.7%) vs 232 allele C (76.3%) (Table 2). As shown in table 2 and chart 2, similarities of the genotypes and the distribution of alleles in patients and the control members were estimated with high degrees of confidence. The statistical analysis of Fisher-exact test also indicated that there is no association between the *IL-7Ra* SNP rs6897932 and our Iranian MS patients (Table 2).

Table 2. Allele frequencies and SNP rs6897932 genotypes in IL7Ra gene in patient vs. control groups

IL7R C>T	Patients	Frequency	Control	Frequency	*P*	OR	CI (95%)
Genotypes	(n=157)		(n = 152)				
TT	5	0.031(3.1%)	12	0.079(7.9%)	0.057	2.61	0.895-7.583
TC	57	0.363(36.3%)	48	0.315(31.5%)	0.224	0.81	0.505-1.298
CC	95	0.605(60.5%)	92	0.605(60.5%)	0.545	1.00	0.634-1.579
Allele		(n = 314)			(n = 304)		
T	67	0.213(21.3%)	72	0.237(23.7%)	0.273	1.144	0.784-1.669
C	247	0.787(78.7%)	232	0.763(7.63)	0.273	0.874	0.599-1.275

Gender distribution of *IL-7Ra* polymorphism

Polymorphism of *IL-7Ra* with respect to the SNP rs6897932 genotypes were compared in both female and male patients. As can be followed in Table 3, although, there were almost three times more women than men in our patient group the distribution of polymorphism of the SNP rs6897932 in men and women were not that different. The distribution of CC alleles in male and female patients, were 54.5% and 62.8%, respectively. The distribution of TC alleles was 34.5% in female vs 40.9% in male, and that of TT was 2.7% in female and 4.5% in male. The control group consisted of the same number of male and female individual participants to maintain the same Gender distribution as that of the patient group (Chart 3).

Table 3. Frequency of IL7Ra polymorphism in both genders

Genotype	Female (N=113)	Male (N=44)	*P*
TT	3	2	NS
TC	39	18	NS
CC	71	24	NS

NS=Not Significant

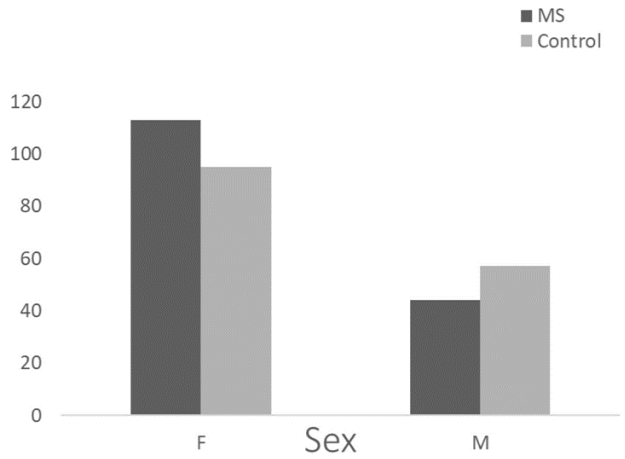

Chart 1. Sex distribution of MS patients and healthy controls

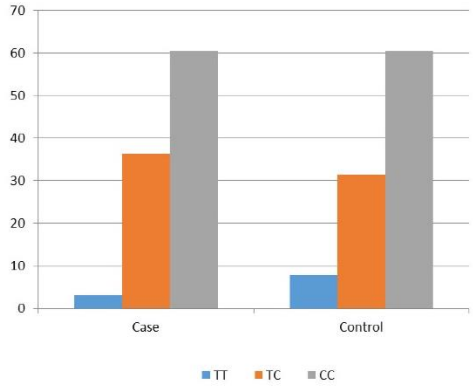

Chart 2. Distribution of *IL-7Ra* genotypes in case and control groups.

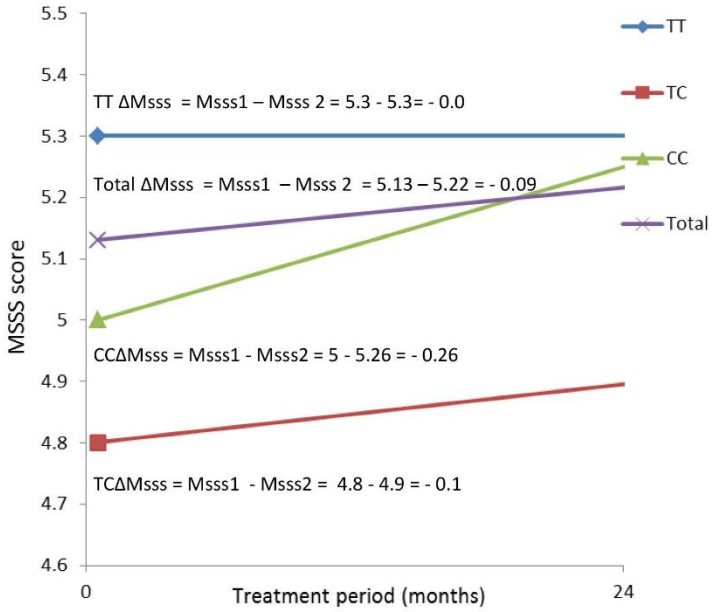

Chart 3. *IL-7Ra* genotype-related therapeutic response to IFN-β in 157 MS patients after 24 months

***IL-7Ra* genotype-based response to β-interferon therapy**

To measure the progression and severity of the disease in our patient group (only 40 patients were available for this analysis) in response to IFN-β the two methodology of EDSS and MSSS were employed. As shown in chart 3, the severity of disease in response to IFN-β at initial time of treatment is a higher for patients with TT genotype of SNP rs6897932 (MSSS 1=5.3) than those of CC (MSSS 1=5.0) or TC (MSSS 1=4.8) and the total (MSSS 1=5.13) genotypes. The treatment of IFN-β two years later also induced different responses in patients with different genotypes. Progression of MS disease in patients with TT (ΔMSSS=0.0) or TC (ΔMSSS=0.1) genotype with repeat of IFN-β treatment two years after the initial treatment was either non or low respectively (good responders for IFB-β), while the progression of severity of the MS disease in patients with CC genotype was higher than the other genotypes (ΔMSSS=0.26; bad responders to IFN-β).

Discussion

In this study, like the study by Alsahebfosuol *et al*., (2016), on RRMS patients (22), we report no association between rs6897932 and MS disease in this Iranian patient population. On the other hand, there are number of similar studies done in Iran or other countries that contradict our findings. MS distributions vary considerably based on geographic location and ethnic origins of different populations (23). Recent studies have revealed a high prevalence of MS in the Iranian population with no apparent etiology (24). Studies of the past quarter century on the disorder of MS is suggestive of multiple gene loci rather than a single susceptibility locus involvement in such complex phenotype, linkage to various chromosomal loci for MS have been identified (25). To this end, MS disorder could be due to impairment of multiple gene interactions (26,27). The association between SNP rs6897932 and multiple sclerosis in the Olmsted County collection appeared to be much stronger than predicted on the basis of the recent studies (13,27). In a study in Sweden, rs6897932 and rs2303137 are identified as the most relevant SNP polymorphisms in their MS population. This study provides the evidence for the presence of heterogeneity in *IL7Rα* gene that codes for the T lymphocyte surface receptor of interleukin 7 alpha chain (IL7-Ra), a non-HLA genetic risk in multiple sclerosis. This finding suggest a distinct place for lymphocyte signaling pathways and immune cell maturation apart from the HLA role in immunity for pathophysiology of MS (13). Gregory *et al*., have shown an over transmission of C-C-C-A haplotype (haplotype-specific P ¼ 0.006) to offspring affected with MS and under transmission of T-T-C-T haplotype (haplotype-specific P ¼ 0.00005). Common risk allele (C) at the coding SNP rs6897932 (single-locus P ¼ 0.0006) determines these two haplotypes, the C allele at rs1494555 (single-locus P ¼ 0.03) and the T allele at rs987106 (single-locus P ¼ 0.01) (15). The common 'C' allele of the non-synonymous coding SNP rs6897932 (T244I) in exon 6 of *IL7R* gene is strongly associated with increased multiple sclerosis risk, which was in accordance with reports in four independent data categories; 2 case-control studies and 2 family-based studies. Although our sequencing of *IL7Ra* did not identify any new variants, it remains a formal possibility that a rare allele in linkage disequilibrium (LD) with rs6897932 is the susceptibility allele. Interestingly, a scarce allele in a small number of individuals with the rs6897932 risk allele is identified which apparently has an extremely high impact (15). Additionally, Traboulsee *et al*., (2004), have shown a significant association between progressive MS and rs6897932 suggesting that *IL7Rα* may not be disease-causing but a determinant of disease course (28). Some of the previous findings suggest that the genetic variations are different between sporadic and familial MS, onset of sporadic disease have been determined by common variants of small effects, whereas familial MS is created by deleterious variants (14,16). Moreover, in a Dutch population, homozygosity for the *IL-7Rα* exon 6 rs6897932 C risk allele is found to be associated with MS, while no association between high risk genotype of rs6897932 (i.e., CC or CT) and mRNA levels for *IL-7Rα* or its imbedded levels on the surface of T cells was identified (12). Additionally, study done by hoe *et al*., (2010), suggests that MS patients with different IL-7 receptor alpha chain haplotypes differ in their response to IFN-β (29). In another study, it has been shown that T cells with *IL7Rα* haplotypes of 1 and 2 (haplotypes associated with reduced cytoplasmic *ILR-7a* receptor, reduced Exon 6 spliced out), but not that of 4 (haplotype with increase cytoplasmic levels of the receptor: increased exon 6 spliced out), produce better response to IFN-β, the most commonly used immunomodulatory drug in MS (30). Therefore, the haplotype-dependent variation in the regulation of *IL-7Rα* production by IFN-β may contribute to the genetic association of *IL-7Rα* with MS

which was also part of our observations (29). Interestingly, in response to IFN-β, we have also found that the severity of disease in our patient population with TT genotype was higher than those of TC or CC genotypes, patients carrying TT genotype being more responsive to IFN-β treatment. On the other hand, in response to the therapeutic effect of IFN-β over time, unlike the *IL-7Ra* genotypes of TT and CT, the patients with homozygous CC genotype demonstrated bad responders to the IFN-β treatment (shown in Chart 3). This finding is in line with the studies done by Hoe *et. al*., (2010), and McKay *et al*., (2010) suggesting the haplotype dependent effect of IFN-β on expression of *IL-7Ra* in MS patients (20-30). Components of the *IL-7Ra* signaling pathway(s) could very well be targeted as candidate genes involve in the pathogenesis of MS.

Although, a number of studies have illustrated an association between MS and the high-risk allele of rs6987932, such association was not seen in our population. Despite this lack of association, we observed that patients with T/T and C/T genotypes were better responders to IFN-β treatment while those patients carrying CC genotype were bad responders to IFN-β therapy. It seems the rs6897932 SNP plays an important role in the effect of IFN-β in the treatment of the MS disease rather than causing the development of MS in the Iranian population studied here. Therefore, considering our findings in this study, further Pharmacogenetic based studies are required for understanding the nature of this differential response of IFN-β treatment on the MS disease.

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