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Association of *TIM-1 5383-5397ins/del* and *TIM-3 -1541C>T polymorphisms* with multiple sclerosis in Isfahan population

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Summary

Multiple sclerosis (MS) is an organ-specific autoimmune disease in central nervous system, affecting about 2.5 million people around the world. Probable involvement of two newly identified immunoregulator molecules, TIM-1 and TIM-3, has been reported in autoimmune diseases. In this study, for the first time, the association of TIM-1 5383-5397ins/del and TIM-3 -1541C>T polymorphisms with MS in an Iranian population was considered. The results of our study showed that there is no significant association between TIM-1 5383-5397ins/del and MS (P = 0.38); however, the frequency of CT genotype of TIM-3 -1541C>T in patient group was significantly higher than the control group, and there was a significant association between CT genotype and MS (P = 0.009, OR = 4.08).

Introduction

Multiple sclerosis (MS) is an organ-specific disease and one of the most common inflammatory diseases of the central nervous system (CNS). In this disease, innate and acquired immune systems attack the myelin sheath of nerve cells (Alatab *et al.*, 2011). According to available data, about 2.5 million people around the world are suffering from this disease (Alatab *et al.*, 2011). MS is a multifactorial disease. Environmental factors. have important roles in the disease development (Lorentzen *et al.*, 2010). Also, some of single nucleotide polymorphisms (SNPs) that are associated with MS disease were identified using genomewide association studies (GWAS) such as polymorphism *in IL-7R, IL-2R, CD58, STAT3* genes (Freeman *et al.*, 2010; Sadovnick, 2012).

The T-cell/transmembrane immunoglobulin and mucin (TIM) gene is one of the genetic factors that recently demonstrated that has a critical role in various diseases like autoimmune diseases. TIM gene family, cloned in 2001 using a congenic mouse model of asthma, plays a critical role in regulating immune responses such as transplant tolerance, autoimmunity, allergy and asthma (Freeman *et al.*, 2010). The TIM gene family consists of three members, TIM-1, TIM-3 and TIM-4, on human chromosome 5q33.2, located in a chromosomal region linked with asthma, allergy and autoimmunity (Rodriguez-Manzanet *et al.*, 2009; Yeung *et al.*, 2011). This gene encodes type I cell-surface glycoprotein with common structural features (Lee *et al.*, 2011). The expression pattern of TIM1, TIM-3 and TIM4 is different in the cells; therefore, they have different effects on the regulation of immune responses (Yeung *et al.*, 2011).

In human, TIM-1 is expressed on Th2 cells and functions as a co-stimulatory signal for the T-cell activation and cytokine production. Moreover, TIM-1 is expressed on mast cells, tubular epithelial cells and a subpopulation of B cells (Xu *et al.*, 2008). TIM-3 is preferentially expressed on Th1, Tc1 and also expressed on Th17 cells, and functions as a negative regulator for immune responses of these cells (Xu *et al.*, 2008, 2011). TIM-3 is also expressed on innate immune cells like dendritic cells (DC) and can mediate phagocytosis of apoptotic cells and cross-presentation of antigen (Rodriguez-Manzanet *et al.*, 2009). Interaction of TIM-3 with galectin-9 (as a ligand of TIM-3) can lead to activation of TIM-3⁄galectin-9 pathway and downregulation of the Th1 responses (Xu *et al.*, 2011).

Identification of different polymorphisms in TIM genes, the way they affect immune responses and their association with allergic and autoimmune diseases can

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create new perspectives in diagnosis and prognosis of these diseases. In this research, we tried to investigate the frequency of two selected polymorphisms of TIM-1 and TIM-3 genes (5383-5397ins/del and -1541 C>T, respectively) in patients with MS compared with the healthy subjects in Isfahan province, Iran. Before studies have investigated the association of these two SNPs with other autoimmune diseases like RA and their findings were contradictory. As the pathogenesis of RA is like the MS disease, so we have performed this study.

Materials and methods

The study population

One hundred and thirty-eight nonrelative relapsing-remitting multiple sclerosis (RRMS) patients that their disease had been approved by a neurologist through McDonald's criteria were selected. Patients had been attended to Alzahra hospital, Isfahan, Iran and had at least two relapse and partial or complete remission with 0–10 expanded disability status scale (*EDSS)*. One hundred and thirty-eight nonpregnant healthy people with no manifestation of autoimmune and inflammatory diseases and no history of organ transplantation were randomly selected in Blood Donation Organization. Two groups were matched for age and sex.

Sample preparation and DNA extraction

Whole blood samples were taken from all subjects in EDTA test tubes (Cinnagen Co., Karaj, Iran). The genomic DNA was extracted from each sample using Genomic DNA Extraction Kit (Qiagen Inc., Gladbach, Germany) according to the manufacture's instruction. The quality and quantity of the extracted DNA were assessed by electrophoresis on 1.5% agarose gel and measuring optical density (OD) at 260 nm, respectively.

Genotyping of -1541 C>T polymorphism of TIM-3

The –1541 C>T polymorphism was genotyped by PCR-RFLP analysis. The primers used to amplify the fragments of TIM-3 promoter containing the SNP, -15 41C>T, were 5′-TCCAGCCTGAGGCTCTTGTTT-3′ and 5′-ATGCTCATTGTTGTTGGAACAG-3′. PCR was carried out in a total volume of 30 μL PCR mixture containing 150 ng of genomic DNA, 3 μL of 10× PCR buffer, 200 μM of dNTPs, 0.5 μM of each primers, 1.5 mM of MgCl2 and 1.5U of Taq DNA polymerase (Cinnagen Co.). The DNA was amplified with an initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 57° C for 30 s, 72° C for 30 s and a final extension at 72° C for 5 min using Techne thermal cycler (Techne Co., Minneapolis, MN, USA). BsaJI restriction enzyme (Fermentas Co., Wurttemberg, Germany) was used for genotyping the SNP. A total of 10 μL of the 199 base pair (bp) PCR product was incubated 2 h in 56°C with 1 μL of BsaJI enzyme. Digested products were separated on 1.5% agarose gel. Fragments 61 and 138 bp indicated the presence of homozygous CC genotype, a single 199 bp band represented the presence of homozygous TT genotype and three fragments of 61, 138 and 199 bp displayed the presence of heterozygous CT genotype (Figure 1).

Some genotypes were randomly selected and sent for sequencing to ensure the accuracy of genotyping by digestion (Bioneer Co., Daejeon, South Korea).

Genotyping of 5383-5397ins/del polymorphism of TIM-1

The 5383-5397ins/del polymorphism of TIM-1 was genotyped by PCR-PAGE analysis (SSP-PCR). PCR amplification was performed using following specific primers: 5′-TCCAGCCTGAGGCTCTTGTTT-3′ and 5′-ATGCTCATTGTTGTTGGAACAG-3′. Amplifying of DNA was carried out with the same condition for -1541C>T in the TIM-3 gene. The PCR products were separated on 8% denatured polyacrylamide gel and visualized with silver nitrate staining. A 186 bp band indicates del/del, 186 bp and 201 bp bands indicate del/ref, fragments 201 bp and 204 bp represent ref/ins, a 201 band indicates ref/ref, and a band with 204 bp represents ins/ins genotypes (Figure 2).

Some genotypes were randomly selected and sent for sequencing to ensure the accuracy of the results (Bioneer Co.).

Statistical analysis

The SPSS 16.0 software package (SPSS Company, Chicago, IL, USA) was used for data analysis. The chi-squared test was used to compare the frequency distribution of age and sex as well as genotype and allele distributions between two groups. Association between two polymorphisms with multiple sclerosis was shown as odds ratios (OR) estimates with 95% confidence intervals (95% CI). Also, logistic regression analysis was used for evaluation the effects of -1541

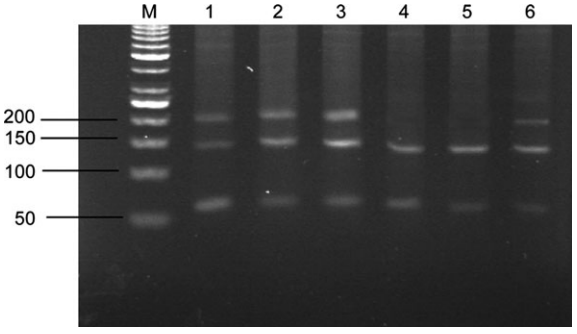

Figure 1. PCR-RFLP analysis of *TIM-3-1541 C>T polymorphism*. Lane M: 50 bp DNA lader; Lanes 1, 2, 3 and 6: heterozygous CT genotype; Lanes 4 and 5: homozygous CC genotype.

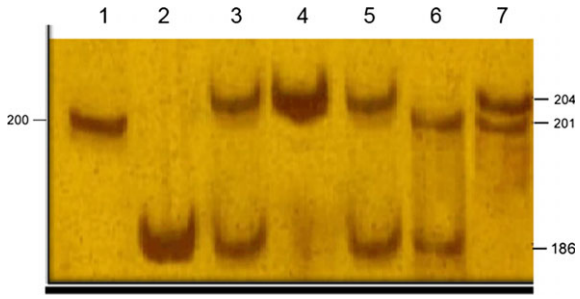

Figure 2. The results of PCR-PAGE of TIM-1 5383-5397 ins/del polymorphism. Lane 1: 200 bp Marker, Lane 2:del/del Lanes 3 and 5:ins/del, Lane 4:ins/ins, Lane 6: ref/del 7:ins/ref.

C> T SNP and 5383-5397 ins/del polymorphism on the incidence of MS. *P* value <0.05 was considered as significant in all of the tests.

Results

In this study, 138 patients with MS and 138 healthy subjects were studied as case and control groups, respectively. The characteristics of the two groups are summarized in Table 1. Both groups were matched for age and sex suitably. The genotype distributions of the TIM-3 -1541C>T and TIM-1 5383_5397ins/del polymorphisms were in agreement with Hardy–Weinberg equilibrium (P > 0.05) in both groups. The genotypes and alleles frequency of TIM-1 5383-5397ins/del are shown in Tables 2 and 3, and the frequency of the genotypes of TIM-3 -1541C>T is shown in table 4. These frequencies were compared between two groups using the Pearson chi-squared test. Frequency of *Ins* allele in patients with MS was higher than the control group, but the frequency of different genotypes of TIM-1 5383-5397ins/del in patient and control groups showed no significant differences, and therefore, there was no significant association between this polymorphism and the incidence of MS ($P = 0.38$). The frequency of CT genotype of TIM-3 -1541C>T in MS group was significantly higher than the control group, and there was a significant association between CT genotype and incidence of MS ($P = 0.009$, OR = 4.08).

Table 1. Characteristics of the studied population

Variables	Control (%)	Case	*P* value
Age (Mean± SD)	31.3 ± 6.6	32.1 ± 8.4	0.36
Sex			
Male	60 (43.47)	51 (36.95)	0.2
Female	78 (56.53)	87 (63.05)	
EDSS	n (%)		
1	91 (65.9%)		
1.5	24 (17.5%)		
2	14 (10.1%)		
3–5	9 (6.5%)		

Table 2. Comparison of allele frequency of *TIM-1 5383-5397ins/del* between patients with MS and healthy subjects (*n* = 138)

Allele Type	MS group (*n*/%)	Control group (*n*/%)	*P* value	OR (CI 95%)
ref	11 (8)	8 (5.8)	0.48	1.41 (0.55–3.61)
del	120 (87)	127 (92)	0.17	0.58 (0.26–1.27)
ins	79 (57.2)	65 (47.1)	0.046	1.54 (1.04–2.42)
Total	210 (100)	200 (100)		

Discussion

Multiple sclerosis is one of the most common inflammatory diseases of the CNS which is due to immune response to myelin sheath of nerve cells and characterized by degradation of the myelin sheath and loss of oligodendrocytes, resulting in impaired nerve conduction (Weinshenker, 1996; Alonso *et al.* 2007). The balance between Th1/Th2 cells is important in the immune responses. The studies in past years have defined the role of Th1/Th2 balance in the induction and regulation of autoimmune diseases (Charlton & Lafferty, 1995; Nicholson & Kuchroo, 1996; Kidd, 2003). TIM genes are one of the genetic factors that recently demonstrated that have critical roles in Th1/Th2 responses and autoimmune diseases like MS (Anderson & Anderson, 2006).

In this study, the frequency and the association of two polymorphisms of TIM genes, TIM-1 5383-5397ins/del and TIM-3 -1541C>T, with MS disease was investigated in an Iranian population. According to our survey in *data banks*, there was no report for the association of these polymorphisms with MS. However, findings about correlation of other

Table 3. Comparison of genotype frequency of *TIM-1 5383-5397ins/del* between patients with MS and healthy subjects (*n* = 138)

TIM-1 Genotype	MS group (n/%)	Control group (n/%)	*P* value	OR (CI 95%)
ref/ins	7 (5.1)	5 (3.6)	0.55	1.4 (0.4–4.56)
ref/del	4 (2.9)	3 (2.2)	0.7	1.3 (0.3–6.1)
ins/ins	11 (8)	6 (4.3)	0.2	1.9 (0.6–5.3)
del/del	55 (39.9)	70 (50.7)	0.09	0.7 (0.4–1.03)
ins/del	61 (44.2)	54 (39.1)	0.4	1.2 (0.7–1.9)
Ref/ref	–	–	–	–
Total	138 (100)	138 (100)		

Table 4. Comparison of genotype frequency distribution of *TIM-3* gene between patients with MS and healthy subjects (*n* = 138)

Genotype	MS group (*n*/%)	Control group (*n*/%)	*P*-value	OR (CI 95%)
CC	123 (89.1)	134 (97.1)		
CT	15 (10.9)	4 (2.9)	0.009	4.08 (1.32–12.64)
TT	–	–		
Total	138 (100)	138 (100)		

autoimmune diseases with this polymorphism promoted us to do a study on this matter.

The results of this study show higher frequency of 'Ins' allele of TIM-1 5383-5397 ins/del in MS patients compared with control group. However, no significant difference was observed in genotype frequencies between the two groups. Therefore, there is no significant association between TIM-1 5383-5397ins/del and MS disease ($P = 0.38$). A study in 2006 has been conducted for possible association between TIM-1 5383-5397ins/del and RA in Korean population, but its findings are not consistent with our results. Chae *et al.* have shown that the TIM-1 5383-5397del/del is associated with a reduced risk of developing rheumatoid arthritis (RA) in Korean population (Chae *et al.*, 2004a,b). This diversity may be due to the difference in sample size, ethnic diversity and interaction of environmental factors which may affect the impact of this polymorphism as a predisposing factor in different geographic area.

Also, the frequency and the association of TIM-3 -1541C>T was investigated with MS in this study. The results showed that frequency of CT genotype of TIM-3 -1541C>T in MS group was significantly higher than the control group, and there is a significant association between CT genotype and incidence of MS ($P = 0.009$, OR = 4.08). Based on our survey in literatures, there was no report for association of *TIM-3 -1541C>T* polymorphism with MS, but few studies have investigated for correlation of this polymorphism with other autoimmune diseases which their results were contradictory. The study on China's Hui population in 2004 showed that -1541C>T polymorphism is associated with RA which is consistent with our results. As the mechanism of pathogenesis of RA and MS diseases is somehow similar, it could be assumed that this polymorphism in TIM-3 may dampen its activity which in turn enhances Th1-mediated inflammation. However, another study on China's Han population in 2004 showed that there was no significant association between TIM-3 -1541C>T polymorphism with RA which is not consistent with our findings (Chae *et al.*, 2004a,b). These different findings may be due to different ethnicity. Also, one can conclude that the impact of this SNP is not suppressed by environmental factors in our area. So, more studies in other populations can help to clarify the role of such TIM gene polymorphisms in MS disease.

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Disclosures

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