Iran J Public Health, Vol. 45, No.5, May 2016, pp.644-649

Original Article

TIM-3 Rs10515746 (A/C) and *Rs10053538 (C/A)* Gene Polymorphisms and Risk of Multiple Sclerosis

Esmat YAGHOOBI [1], ***Saeed ABEDIAN*** [3], ***Omid BABANI*** [4], ****Maryam IZAD*** [1, 2]

1. *Dept. of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
2. *MS Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Sina Hospital, Tehran, Iran*
3. *Dept. of Immunology, Mazandaran University Medical of Sciences, Sari, Iran*
4. *Amirkola Children's Hospital, Amirkola, Babol, Iran*

***Corresponding Author:** Email: izadm@sina.tums.ac.ir

(Received 16 May 2015; accepted 20 Nov 2015)

Abstract

Background: Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) caused by autoreactive T cells against myelin antigens. T-cell immunoglobulin mucin -3 (TIM-3) is a negative regulator glycoprotein expressed by a range of immune cells, including, Th1 cells, activated CD8+ T cells and in a lower level on Th17 cells. A defect in TIM-3 regulation has been shown in multiple sclerosis patients. In humans, several single nucleotide polymorphisms (SNPs) have been identified in the TIM-3 gene and are associated with inflammatory diseases. The aim of this study was to analyze the association between TIM-3 -574A>C and -1516 C>A SNPs in the promoter region, and susceptibility to MS.

Methods: DNA samples from 102 patients and 102 healthy controls were genotyped using RFLP-PCR method.

Results: In this case-control study, analysis of the alleles and genotypes revealed a significant higher frequency of C/C and lower frequency of A/C genotypes for -574 locus of TIM-3 gene in MS patients (P=0.0002). We also found that C/C genotype for locus of -1516 increased in MS patients, while A/C genotype decreased (P=0.012). Allele C of -574C/C and -1516 C>A SNPs were also more frequent in MS patients (P=0.036 and 0.0027 respectively).

Conclusion: -574 A>C and -1516 C>A SNPs in the promoter region of TIM3 gene may affect the disease susceptibility.

Keywords: Multiple sclerosis, TIM-3, TIM-3 polymorphism

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) caused by autoreactive T cells directed against myelin antigens. Th1 and Th17 cells are believed to contribute to the pathogenesis of MS (1).

The family of T-cell immunoglobulin and mucin domain (TIM) proteins including TIM-1, TIM-3, and TIM-4 is expressed on T cells and some other immune cells (2). TIM-3 is a negative regulator of immune responses that specially expressed on activated Th1 cells, CD8+ T cells and at a lower level on Th17 cells but not on Th2 cells (3-5). TIM-3 expressing regulatory T cells are able to do a higher level of Th1 and Th17 cells suppression compared to TIM3 negative counterparts (6). Dysregulated expression of TIM-3 on T-cells resulted in enhanced T-cell proliferation and IFN-γ secretion following T-cell stimulation (7). TIM-3 interacts with its ligand (Galectin-9) to regulate T cells responses. Blocking of Tim-3/Gal-9 interaction with specific monoclonal antibody reduced T-lymphocyte apoptosis and augmented production

of IFNγ and IL-17 in the relapsing-remitting MS (RRMS), and Healthy controls (HC), but not in the primary progressive (PPMS) group (8).
Preventing of Tim-3 signaling in CD4+ T cells influenced the localization of inflammation in the brain and spinal cord via alteration in the Th17:Th1 ratio and exacerbated experimental autoimmune encephalomyelitis (EAE) mediated by CD8+ T cells (9). Moreover, CD4+ T cell clones from the cerebrospinal fluid (CSF) of MS patients secreted significantly higher levels of IFN-γ than those from controls, but expressed lower levels of TIM3 mRNA(7). Blocking of TIM-3 during T-cell stimulation increased IFNγ- production in healthy controls but had no effect in untreated MS patients. However, treatment with glatiramer acetate or IFN-β could recover this functional defect (10). Collectively, these data indicate a dysfunction of TIM-3 immunoregulation in MS disease. These observations suggest that variation in the expression of TIM-3 may influence the susceptibility to MS and genetic polymorphism in its gene could be the reason behind such variations.
A number of single nucleotide polymorphisms (SNPs) in the promoter and coding region of human TIM-3 gene have been shown in previous studies (11). Additionally, associations of TIM-3 SNPs with disease susceptibility in different autoimmune diseases, such as rheumatoid arthritis (12), type 1 diabetes (13) and Ankylosing Spondylitis (AS) (14) have been reported.
In this study, we analyzed TIM-3 rs10053538 (C/A) and rs10515746 (A/C) SNPs located in the promoter region at positions -1516 and -574, respectively, in patients with MS.

Materials and Methods

Subjects

One hundred-two patients referred to Shariati Hospital, Tehran, Iran from March to Dec 2013 with clinically defined MS according to the Mc Donald criteria; 82 patients with relapsing-remitting MS (RRMS) or secondary progressive MS, and 20 patients with primary progressive MS (PPMS) were included in this study. The control group consisted of 102 ethnically matched, and with no past medical or family history of MS or other autoimmune diseases. All patients and controls were Iranian white origin.
Informed consent was obtained from all patients and controls. The ethical clearance was approved by clinic-based Ethic Committee of Tehran University of Medical Sciences. Demographic characteristics of the subjects are shown in Table1.

Table 1: Demographic characteristic of the patients

Group	Sex	Age (mean ± SD)	Age of onset (mean ± SD)	Duration of the disease (mean ± SD)	Median of EDSS (range)	Initial symptoms	No. relapses (mean ± SD)
Patient	Women : 68 men : 34	34.4± 9.8	27.3± 9.7	7.7 ± 5.7	5.5 (0- 9.5)	Visual: 45% Others: 55%	4.5 ± 2.8
Control	Women : 53 men : 49	31.1± 1.2	----------	----------	----------	----------	----------

SD: standard deviation
EDSS: Expanded Disability Status Scale

DNA extraction and Determination of TIM-3 gene polymorphism using RFLP method

To determine the association between the TIM-3 polymorphisms and the susceptibility to MS, we analyzed the frequency of SNPs rs10515746 (A/C) and *rs10053538 (C/A)* located in the promoter region of TIM-3 gene.
Genomic DNA was extracted from peripheral blood cells using the Sina gene DNG-Plus TM (Iran, Cat. No:DN 8118C) according to the manufacturer's instructions.

Two polymorphisms within the TIM-3 gene promoter (-574 A>C and -1516 C>A) were determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. The sequences of primers are listed in Table 2. All polymerase chain reactions were performed with 50 to 150 ng of isolated genomic DNA. PCR products containing the two polymorphic sites were then digested with the restriction enzymes Bsl I and BCC l (New England Biolabs) according to the manufacturer's instructions. Digested fragments were separated on 1% agarose gels and RFLP bands were visualized by ethidium bromide staining under UV light gel doc.

Table 2: PCR primer and restriction enzyme for SNP assays

Position	Primers	PCR Product	Restriction enzyme	Fragment sizes
-574 A>C *rs10515746 (A_C)*	F:AGTACAGATGCATCATCCATG R:GTATGCATGAGATGAAACAGG	444bp	BCC1	279,165
-1516 C>A *rs10053538 (C_A)*	F:GCCTTGACCAAGTTCATGCT R:ACCACCCCGGATAATTTTGT	404 bp	Bsl 1	66;338 ;404

F=forward; R=reverse

Statistical analysis

Genotypes and alleles frequencies were analyzed by the $\chi 2$ test or Fisher's exact test. Each polymorphism was tested in the controls and patients. Odds ratios (OR) and 95% confidence interval (CI) were calculated. *P* values <0.05 were considered statistically significant.

Result

Genotype and allele frequencies of the TIM-3 -574 A>C and -1516 C>A polymorphisms in MS patients and controls are shown in Table 3.

Table 3: Genotype and allele frequencies of two Tim-3 gene promoter SNPs in cases and controls

Position	Genotype/ Allele	Controls n (%)	MS patient n (%)	Odd Ratio[a] (95% CI)	*P*-value[b]
-574 A>C	AA	7(6.9)	6 (5.9)	6.067 (1.421-25.9)	0.0002[d]
	CC	5(4.9)	26(25.5)	1:00 (reference)[c]	
	AC	90(88.2)	70(68.6)	6.686 (2.442-18.30)	
	A	104 (50.9)	82 (40.19)	1.547 (1.045-2.290)	0.0367
	C	100 (49.01)	122 (59.8)	1:00 (reference)	
-1516 C>A	AA	84(82.4)	73(71.6)	5.753 (1.601-20.67)	0.0122
	CC	3(2.9)	15(14.7)	1:00 (reference)	
	AC	15(14.7)	14(13.7)	5.367 (1.272-22.57)	
	A	183 (89.7)	160 (78.4)	2.396 (1.367-4.202)	0.0027
	C	21 (10.29)	44 (21.56)	1:00 (reference)	

[a] Logistic regression analyses were used for calculation odds ratios with 95% confidence interval.

[b] Was determined by χ^2 test (for genotype) or Fisher exact test (for alleles) from a 2×3 and 2×2 contingency table.

[c] The first allele or genotype is considered as reference

The frequency of C allele and CC genotype in TIM-3 -574 A>C SNP is significantly increased in patients with MS compared with controls, whereas A allele and AC genotype decreased (*P*=0.03, OR=1.54, 95% CI: 1.045-2.290 and *P* =0.0002, OR=6.067, 95% CI: 1.421-25.9 respectively). There was also a significant difference in genotype and allele distributions between patients and controls in SNP -1516 C>A. Patients showed a higher frequency of C allele and C/C genotype at position -1516 C>A (*P*=0.002, OR=2.39, 95% CI: 1.036-4.2 and *P* =0.012, OR=5.75, 95% CI: 1.6-20.67 respectively).

Discussion

In the present study, we showed a significant higher frequency of C/C and lower frequency of A/C genotypes for -574 and -1516 loci of TIM-3 gene in MS patients versus controls. Moreover, allele C of -574C/C and -1516 C>A SNPs were more frequent in cases.

TIM-3 is a negative regulatory molecule expressed on activated Th1, Th17, and CD8+ T cells, regulatory T cells, monocytes, dendritic cells, mast cells, and microglia (4, 6, 15, 16). Engagement of Tim-3 with its ligand, galectin-9, down-regulate T cells responses (3, 5). Administration of galectin-9 resulted in decreased IFN-γ and IL-17 producing cells and suppression of Th1 and Th17 response (4, 17, 18). TIM-3 blockade with specific antibodies enhances secretion of IFN-γ, IL-17, IL-2, and IL-6 but not IL-10, IL-4, or TNF-α by activated CD4+ T cells (4).

Multiple sclerosis (MS) is a chronic autoimmune, inflammatory neurological disease of the central nervous system (CNS), believed to be mediated by autoreactive T cells directed against myelin antigens (1). It seems that Th1 and Th17cells secreting pro-inflammatory cytokines including IFN-γ, TNF-α, and IL-17 enhance MS pathogenicity (19). A defect in TIM-3 regulation has been shown in multiple sclerosis patients. TIM-3 blockade by anti-TIM-3 mAbs enhanced IFN-γ and IL-17 secretion from CD4+ T cells in control subjects but not in untreated patients with MS *(10)*. Mononuclear cells from CSF of MS patients secreted higher amounts of IFN-γ while expressed lower levels of TIM3 in comparison to controls (7, 20).

Several single nucleotide polymorphisms have been shown in promoter and coding region of TIM-3 gene that might control the expression level of the protein (11, 14). Associations of TIM-3 single nucleotide polymorphisms with disease susceptibility in autoimmune diseases including rheumatoid arthritis (12), type 1 diabetes (13) and Ankylosing Spondylitis (AS) (14) have been investigated. So far, there is no report investigating the role of TIM-3 polymorphisms in multiple sclerosis. However, previous studies regarding the association of TIM-3 polymorphism with other chronic inflammatory and autoimmune diseases showed inconsistent results (12, 21-25). For example, Wang et al. and other researchers showed that frequency of the TIM-3 - 574GT genotype and -574T allele were significantly increased in patients with Ankylosing spondylitis (AS), rheumatoid arthritis and HIV+ non-Hodgkin lymphoma (12, 14, 24), while, frequency of -574 GG genotype and G allele decreased in rheumatoid arthritis, asthma and allergic rhinitis patients (12, 22). Patients with AS carrying polymorphic - 574GT genotype has significantly lower TIM-3 mRNA and protein levels in CD4+ T cells, CD8+ T cells, and monocytes (14).

TIM-3 -574 A>C and -1516 C>A SNPs might not contribute to SLE, idiopathic thrombocytopenic purpura (ITP) and type-1 diabetes susceptibility (13, 23, 26).

Conclusion

Since TIM-3 has a suppressor role in T-cell responses, and genetic variations in its gene could affect the level of TIM-3 production, -574 and -1516 C>A SNPs could influence the individual susceptibility to MS.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission,

redundancy, etc.) have been completely observed by the authors.

Acknowledgements

This study was financially sponsored by Tehran University of Medical Sciences. The authors declare that there is no conflict of interests.

References

1. Nylander A, Hafler DA (2012). Multiple sclerosis. *J Clin Invest,* 122(4):1180-1188.
2. Kuchroo VK, Meyers JH, Umetsu DT, DeKruyff RH (2006). TIM family of genes in immunity and tolerance. *Adv Immunol,* 91:227-249.
3. Sabatos CA, Chakravarti S, Cha E, et al. (2003). Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nature immunol,* 4(11):1102-1110.
4. Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, et al. (2009). TIM-3 is expressed on activated human CD4+T cells and regulates Th1 and Th17 cytokines. *Eur J Immunol,* 39(9):2492-2501.
5. Gorman JV, Colgan JD (2014). Regulation of T cell responses by the receptor molecule Tim-3. *Immunol Res,* 59(1-3):56-65.
6. Gautron AS, Dominguez-Villar M, de Marcken M, Hafler DA (2014). Enhanced suppressor function of TIM-3 FoxP3 regulatory T cells. *Eur J Immunol,* 44(9):2703-2711
7. Koguchi K, Anderson DE, Yang L, O'Connor KC, Kuchroo VK, Hafler DA (2006). Dysregulated T cell expression of TIM3 in multiple sclerosis. *J Exp Med,* 203(6):1413-1418.
8. Saresella M, Piancone F, Marventano I, et al. (2014). A role for the TIM-3/GAL-9/BAT3 pathway in determining the clinical phenotype of multiple sclerosis. *FASEB J,* 28(11):5000-5009.
9. Lee SY, Goverman JM (2013). The influence of T cell Ig mucin-3 signaling on central nervous system autoimmune disease is determined by the effector function of the pathogenic T cells. *J Immunol,* 190(10):4991-4999.
10. Yang L, Anderson DE, Kuchroo J, Hafler DA (2008). Lack of TIM-3 immunoregulation in multiple sclerosis. *J Immunol,* 180(7):4409-4414.
11. Chae SC, Song JH, Pounsambath P, Yuan HY, Lee JH, Kim JJ, Lee YC, Chung HT (2004). Molecular variations in Th1-specific cell surface gene Tim-3. *Exp Mol Med,* 36(3):274-278.
12. Chae SC, Park YR, Shim SC, Yoon KS, Chung HT (2004). The polymorphisms of Th1 cell surface gene Tim-3 are associated in a Korean population with rheumatoid arthritis. *Immunol Lett,* 95(1):91-95.
13. Bruck P, Ramos-Lopez E, Bartsch W, Bohme A, Badenhoop K (2008). TIM-3 polymorphisms in type 1 diabetes families. *J Hum Genet,* 53(6):559-564.
14. Wang M, Ji B, Wang J, Cheng X, Zhou Q, Zhou J, Cao C, Guo Q (2014). Tim-3 polymorphism downregulates gene expression and is involved in the susceptibility to ankylosing spondylitis. *DNA Cell Biol,* 33(10):723-728.
15. Anderson AC, Anderson DE, Bregoli L, Hastings WD, et al. (2007). Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. *Science,* 318(5853):1141-1143.
16. Wang F, He W, Zhou H, Yuan J, Wu K, Xu L, Chen ZK (2007). The Tim-3 ligand galectin-9 negatively regulates CD8+ alloreactive T cell and prolongs survival of skin graft. *Cell Immunol,* 250(1-2):68-74.
17. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK (2005). The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol,* 6(12):1245-1252.
18. Wang F, Xu J, Liao Y, Wang Y, Liu C, Zhu X, Chen ZK, Sun Z (2011). Tim-3 ligand galectin-9 reduces IL-17 level and accelerates Klebsiella pneumoniae infection. *Cell Immunol,* 269(1):22-28.
19. Olsson T (1995). Cytokine-producing cells in experimental autoimmune encephalomyelitis and multiple sclerosis. *Neurology,* 45 (6 Suppl 6):S11-5.
20. Khademi M, Illés Z, Gielen AW, Marta M, et al. (2004). T Cell Ig-and mucin-domain-containing molecule-3 (TIM-3) and TIM-1 molecules are differentially expressed on

human Th1 and Th2 cells and in cerebrospinal fluid-derived mononuclear cells in multiple sclerosis. *J Immunol,* 172(11):7169-7176.

21. Liao J, Zhang Q, Liao Y, Cai B, Chen J, Li L, Wang L (2014). Association of T-cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) polymorphisms with susceptibility and disease progression of HBV infection. *PLoS One,* 9(5):e98280.
22. Chae SC, Park YR, Lee YC, Lee JH, Chung HT (2004). The association of TIM-3 gene polymorphism with atopic disease in Korean population. *Hum Immunol,* 65(12):1427-1431.
23. Du WT, Zhao HF, Xu JH, Gu DS, Xue F, Ge J, Dong XW, Chen ZP, Zhou ZP, Yang RC (2009). The role of T-cell immunoglobulin-and mucin-domain-containing molecule-3 polymorphisms in idiopathic thrombocytopenic purpura. *Hum Immunol,* 70(6):398-402.
24. Song H, Ma S, Cha Z, Chen L, Tong D, Ma L, Song W, Bai J (2013). T-cell immunoglobulin- and mucin-domain-containing molecule 3 genetic variants and HIV+ non-Hodgkin lymphomas. *Inflammation,* 36(4):793-799.
25. Li Z, Li N, Zhu Q, Zhang G, Han Q, Zhang P, Xun M, Wang Y, Zeng X, Yang C, Liu Z (2013). Genetic variations of PD1 and TIM3 are differentially and interactively associated with the development of cirrhosis and HCC in patients with chronic HBV infection. *Infect Genet Evol,* 14:240-246.
26. Li W-X, Chen G-M, Yuan H, Yao Y-S, Li R-J, Pan H-F, Li X-P, Xu J-H, Tao J-H, Ye D-Q (2011). Polymorphisms of the TIM-1 and TIM-3 genes are not associated with systemic lupus erythematosus in a Chinese population. *Mutagenesis,* 26(4):507-511.