

Original Article

Association of IL-13 single nucleotide polymorphisms in Iranian patients to multiple sclerosis

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Abstract: MS is an autoimmune disease and interleukin 13 (IL-13) has been proposed to be an important neuro-protective mediator in MS. Because of plausible effect of single nucleotide polymorphisms (SNPs) in expression level or biological activity of any cytokine, we sought to investigate association of IL-13 SNPs, C-1112T, A-1512C and G+2044A, with risk to MS. Sixty-eight RRMS patients and 110 healthy controls were involved in this study. After extraction of genomic DNA, frequency of genotypes and alleles were determined by PCR-RFLP and data were analyzed statistically. Results showed significant higher frequency of CC, CC, and AA genotypes and C, C, and A alleles of -1112CT, -1512AC and +2044GA SNPs respectively, in patients group. There was significant association between -1112C allele with onset age of MS. No significant association was seen between any of genotypes or alleles with expanded disability status scale (EDSS) of patients. Our findings showed significant association between three studied SNPs of IL-13 with susceptibility to MS in Iranian patients. More studies should be done on other IL-13 SNPs, and also polymorphisms of IL-13 receptor and other cytokines to determine the exact role of SNPs in protecting or predisposing of individuals for MS.

Keywords: Multiple sclerosis, interleukin 13, polymorphism, PCR, RFLP

Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS). It attacks the insulating cover, myelin sheath, of nerve cells in the CNS and damages the axons at different levels. The disease is categorized in four major groups based on the course of disease: Relapsing Remitting (RRMS), Secondary Progressive (SPMS), Primary Progressive (PPMS), and Progressive Relapsing (PRMS) [1]. Prevalence of MS is only about 0.1% in Europe and the US but it affects young individuals (typically presents in adults 20 to 45 years of age) which results a heavy medical and economic load on the society [2]. Its female to male incidence ratio is about two and Iran was at a mid-level in comparison with other countries. But, it has been shown that the incidence

rate has dramatically increased within the past five years [3].

Although immunopathologic process of MS has been elucidated in experimental autoimmune encephalomyelitis (EAE) mouse model, there are still vast number of questions should be answered. To date, it has been shown that MS develops in genetically susceptible individuals following exposure to yet unidentified environmental triggers [4]. Both anti- and pro-inflammatory cytokines secreted by different subtypes of helper T (Th) and also other immune cells play crucial roles in orchestrating harmful or protecting immune response in MS [5, 6]. IL-13 is a pleiotropic cytokine, secreted mainly by Th2 cells could exert anti-inflammatory effects [4]. In MS, it has been shown that IL-13 plays a neuroprotective role [7], and has effi-

Table 1. Primers used to amplify flanking regions of three studied SNPs

SNP	Primers	Sequence
+2044 G/A	Forward	5`-CTT CCG TGA GGA CTG AAT GAG ACG GTC-3`
	Reverse	5`-GCA AAT AAT GAT GCT TTC GAA GTT TCA GTG GA-3`
-1512 C/A	Forward	5`-CAA CCG CCG CGC CAG CGC CTT CTC-3`
	Reverse	5`-CCGCTACTTGGCCGTGTGACCGC-3`
-1112 C/T	Forward	5`-GGA ATC CAG CAT GCC TTG TGA GG-3`
	Reverse	5`-GTC GCC TTT TCC TGC TCT TCC CGC-3`

cient involvement in modulation of neuronal integrity and synaptic function [8]. Further evidence for anti-inflammatory roles of IL-13 was provided by Wilson et al. that showed protection of mice from lethal intestinal inflammation by in vitro and in vivo negative regulation of Th17 cells [9]. Recently, Th17 cells and their major cytokine IL-17 have been considered as major player in disruption of blood brain barrier (BBB) [10]. Gene coding for IL-13 is located on long arm of chromosome 5, and contains four exons which lead to 146 amino acid protein [11]. Several SNPs have been identified in coding and non-coding regions of IL-13 genes [12]. It has been shown that C-1112T may affect the expression level of IL-13 [13], and G+2044A in exon 4 could give rise to replacement of Arg by Gln and increased affinity of IL-13 to its receptor [14]. A-1512C also is located in promoter region of IL-13 gene and postulated to influence the cytokine expression level. Association of above-mentioned polymorphisms in allergic rhinitis [15], asthma [16], childhood asthma [17], glioma [18], autoimmune thyroid disease (AITD) [19], Elderly-associated Chronic Inflammatory Diseases [20], Atopic Dermatitis [21], Type 1 Diabetes (T1D) [22], Graves' disease (GD) [23], and aggressive periodontitis [24] have been also investigated.

In this study, we aimed to determine the genotype of three IL-13 SNPs (C-1112T, A-1512C, and G+2044A) in Iranian patients with MS and healthy control group, and analyze the plausible association of any genotype or allele with susceptibility to MS.

Materials and method

Patients and control subjects

Sixty eight (23 male and 45 female) Iranian patients aged between 14 and 51 years with clinically definite RRMS were recruited. Patients

according to the revised 2010 McDonald criteria [25] were diagnosed by specialists at Neurosciences Research Center of Tabriz University of Medical Sciences. Mean age of patients and controls were 33.23 and 30.77, respectively.

Control group consisted of 110 age and gender matched healthy subjects without any history of autoimmune, asthma, allergy and chronic infectious diseases. 110 individuals of control group were selected from Tabriz Blood Transfusion Center. Informed consent was obtained from all the participants or their legal guardians. A questionnaire was completed for each person and a blood sample was taken from each individual for DNA extraction. This study was approved by The Ethic Committee of Tabriz University of Medical Sciences.

Genotyping of IL-13 SNPs

Whole peripheral blood sample was collected in tubes containing Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulant. Genomic DNA was extracted by standard salting-out method and stored at -20°C until use. Genotypes of G+2044G, A-1512C and C-1112T were determined by conventional PCR method followed by Enzymatic digestion using BspLI (NlaIV), Bsh-1236I (BstUI), respectively. Primers used to amplify flanking regions of SNPs are shown in **Table 1** [26]. PCR reactions were run in final volume of 25 µl and in program consisted of initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation (50 seconds), annealing temperature (62°C, 65.5°C and 59°C, respectively , 50 seconds) and extension (72°C, 30 seconds). The reaction finished after final extension at 72°C for 5 minutes, and specific bands were electrophoresis on agarose gel and visualized by ethidium bromide staining. Determining of genotypes was performed by subjecting specific bands to digestion by above mentioned restriction enzyme for 16 hrs. Digestion products were analyzed after agarose gel electrophoresis and interpretation was made according to the pattern of digestion mentioned in result section. Genotyping by PCR-RFLP was confirmed by sequencing of sample from each genotype.

Association of IL-13 SNPs with MS

Table 2. Genotypes and allele frequencies of the G+2044A, A-1512C and C-1112T polymorphisms of IL-13 in MS patients and control groups. Values in parentheses represent percentages of the group. Odds ratio (OR) was calculated as compared to patients with Multiple Sclerosis (MS). (CI = confidential interval)

IL-13 SNP		MS patients; n = 68 (%)	Healthy control; n = 110 (%)	χ^2 P-value	Age-Adjusted OR (95% CI)	P-value	Crude OR	P-value			
G2044A	Genotype	A/A	13 (19.12)	12 (10.91)	0.019*	3.01 (1.18-7.67)	2.90	0.024*			
		G/A	33 (48.53)	39 (35.45)		2.40 (1.21-4.78)			0.012*	2.26	0.017*
		G/G	22 (32.35)	59 (53.64)							
	Allele	G	77 (56.62)	157 (71.36)	0.004*	1.96 (1.24-3.07)	1.90	0.005*			
		A	59 (43.38)	63 (28.64)							
A-1512C ^c	Genotype	A/A	39 (58.21)	79 (71.82)	0.048*	1.45 (0.73-2.90)	4.72	0.030*			
		A/C	21 (31.34)	28 (25.45)		4.75 (1.15-19.60)			0.031*	1.51	0.230
		C/C	7 (10.45)	3 (2.73)							
	Allele	A	99 (73.88)	186 (84.55)	0.014*	1.89 (1.10-3.23)	1.93	0.015*			
		C	35 (26.12)	34 (15.45)							
C-1112T ^e	Genotype	C/C	16 (27.12)	15 (13.64)	0.095	0.38 (0.15-0.99)	0.43	0.052			
		C/T	15 (25.42)	35 (31.82)		0.42 (0.18-0.99)			0.048*	0.40	0.054
		T/T	28 (47.46)	60 (54.55)							
	Allele	C	47 (39.83)	65 (29.55)	0.056	0.62 (0.39-1.00)	0.63	0.055			
		T	71 (60.17)	155 (70.45)							

*P-value < 0.05 was regarded as significant. ^cOne patient sample was considered as not defined. ^eNine patient samples were considered as not defined.

Statistical analysis

Statistical Analysis was performed by Stata, version 11 for windows. Genotype and allele frequency of IL-13 gene (C-1112T, A-1512C, G+2044A) polymorphisms were compared using Chi-squared and logistic regression in two groups. Association between onset-age and Expanded Disability Status Scale (EDSS) with genotypes and allele frequency were done by ANOVA and T-test respectively. P-value less than 0.05 was regarded as significant.

Results

Amplification of flanking regions of C-1112T, A-1512C and G+2044A SNPs resulted in 247 bp, 245 bp and 236 bp bands, respectively. For C-1112T SNP, 247, 224/23 and 247/224/23 bp bands were interpreted as CC, TT and CT genotypes. Digestion patterns for A-1512C SNP were as 214/31, 192/31/22 and 214/192/22/31 bp for AA, CC and AC genotypes, respectively. Digestion of specific band for G+2044A SNP gave rise to 210/26 bp for A/A, 178/26/32 bp for G/G and 210/178/28/32 bp bands for GA genotypes. Deviations from Hardy-Weinberg (HW) equilibrium were examined for each SNP with exact tests. The distributions of the A-1512C and G+2044A genotypes in control and MS individuals were in accordance with HW equilibrium, but genotype frequencies in

C-1112T were in HW disequilibrium, especially in patient group. Genotype and allelic distributions for the studied SNPs are shown in **Table 2**. As mentioned in more details later, comparison between case and control groups in A-1512C and G+2044A indicated statistically significant differences in genotype frequencies. Similarly, allele frequencies showed significant differences between two groups. However the differences in genotype and allele frequencies in C-1112T were statistically slight significant between patient and MS groups.

For G+2044A SNP, the data showed significant higher frequency of AA genotype (P = 0.019) and allele A (P = 0.004) in patients compared to healthy controls. On the other hand, CC genotype and allele C at A-1512C was significantly more frequent in patients (P = 0.048 and P = 0.014, respectively). Differences in frequency of genotype and alleles at C-1112T SNP between two studied group, however, were significant weakly (P = 0.095, P = 0.056).

Individuals with AA genotype (OR = 2.90) and carriers of A allele (OR = 1.90) in G+2044A SNP, and CC genotype (OR = 4.72) and carriers of C allele (OR = 1.93) in A-1512C SNP showed significant increased risk of developing multiple sclerosis. However in C-1112T SNP individuals with TT genotype (OR = 0.43) and carriers of T allele (OR = 0.63) showed a slight increased

Association of IL-13 SNPs with MS

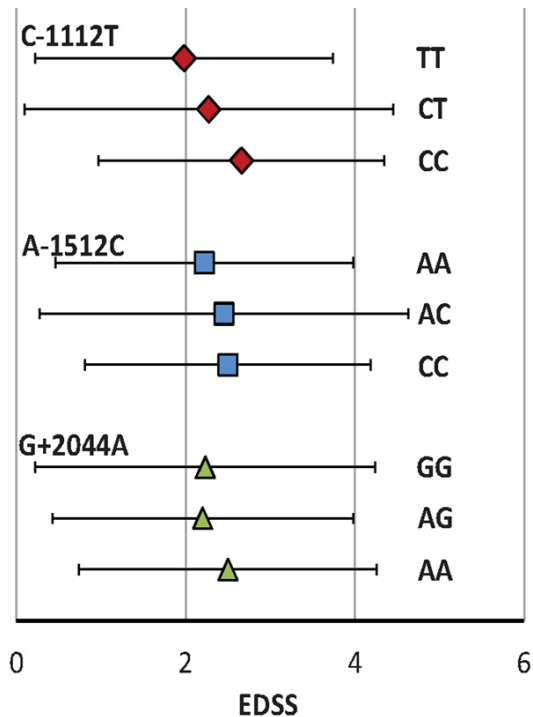


Figure 1. Association of genotypes with EDSS. Association of genotypes with EDSS in each SNP has been analyzed to determine whether investigated SNPs influence severity of disease or not. Statistical analysis does not show any significant difference between EDSS mean value among the genotypes of each SNP.

risk of developing multiple sclerosis. In our analysis, a significant association was found between -1112T ($P = 0.0211$) and onset-age but our results did not show any significant differences between allele frequencies (G+2044A $P = 0.6070$, A-1512C $P = 0.8349$) and genotype frequency (G+2044A $P = 0.5618$, A-1512C $P = 0.8752$ and- C-1112T $P = 0.1239$) in relation to onset-age without considering subjects' gender or health status. Comparing genotypes (allele) frequency at different EDSS values (that had been determined based on clinical diagnosis by neurologists) did not show any significant differences (G+2044A $P = 0.2508$ ($P = 0.4732$), A-1512C $P = 0.7480$ ($P = 0.8122$) and C-1112T $P = 0.6075$ ($P = 0.2686$)). **Figure 1** shows EDSS for various genotypes in the investigated SNPs.

Discussion

Multiple sclerosis appears to be developed as a result of a complex interaction between genetic and environmental factors. Although all specific genes controlling susceptibility to MS have not

been identified, recent genome-wide association studies have provided evidences for the linkage to loci on multiple chromosomes (e.g. [27, 28]). Multiple genetic analysis across diverse ethnic populations provided strong support for the candidacy of the IL-13 gene as a susceptibility factor in different atopic and autoimmune diseases [12, 19, 23]. In this study we investigated frequencies of IL-13 C-1112T, A-1512C, G+2044A genotypes and alleles and found higher frequency of CC, CC and AA genotypes and C, C and A alleles, respectively in MS patients compared with healthy individuals.

It has been reported previously that -1112 T allele is associated with atopic dermatitis (e.g. [21]). In agreement with this study, Nie et al. performed a meta-analysis and showed that -1112 T allele is more frequent than -1112C allele in asthmatic patients (OR = 1.25, 95% CI 1.18-1.32, $P = 2 \times 10^{-14}$) [16]. They concluded that -1112T allele may serve as a risk allele for predisposition to Th2 mediated disorders including asthma. Inversely lower frequency of -1112T allele in patients with GD has been reported by Hiromatsu et al. [23]. As there is positive correlation between inheritance of -1112T allele and higher production rate of IL-13 [13], higher frequency of -1112C allele in our patient group (**Table 2**) may be attributed to lower production of IL-13 cytokine in this group. As IL-13 has been reported to be a neuroprotective cytokine [8], lower production of IL-13 may give rise to susceptibility to MS.

Our results showed significant higher frequency of -15121C allele in MS patients (**Table 2**). This finding is consistent with Bugawan et al. [22] showed significant correlation between -1512 C allele and T1D, another Th1 mediated autoimmune disease. On the contrary, this allele has been showed as less frequent allele in asthma [29, 30]. One exception was Shazia et al. [31], reported that -1512C allele is more frequent in atopic asthma and rhinitis. They attributed such incompatible result to heterogeneity of their study population. Because of residing -1512 SNP in promoter region, it is postulated that by dampening expression level of IL-13, -1512C allele could be serve as a risk factor for susceptibility to Th1-mediated autoimmune disease including MS.

+2044 A allele, on the other hand, was significantly more frequent in our MS patients group.

Other studies also showed higher frequency of this allele in Th2-mediated allergic disease and Th1-mediated T1D. Such ambiguous association become more complex by the finding that G to A transition in +2044 nucleotide leads to non-conservative replacement of Arg 130 with Gln and enhanced activity affinity of IL-13 to its receptor [14, 32]. It is showed that such amino acid replacement could increase biological activity of IL-13, plays a pivotal role in promoting allergic conditions [14] but exert inhibitory effects on Th1-mediated immune responses [1]. So, higher frequency of +2044 A allele in our patients group is unexpected. One explanation is that alleles residing in promoter region of IL-13 gene which could decrease expression level of cytokine were significantly higher in MS patients. Additive effect of two alleles might decrease IL-13 expression level in patients in a way that presence of +2044 A allele which increases biologic activities of IL-13, could be not functional.

In conclusion, we showed significant association of IL-13 SNPs with susceptibility to MS in Iranian population. More studies are needed to be done on other IL-13 SNPs and also genes coding for IL-13 receptors in our and other populations to determine exact role of SNPs in predisposing or protecting individuals to MS. On the other hand, because a complex cytokine network determine the fate of any immune response including autoimmunity, vast of investigations should be performed to conclude how any cytokine function in immunopathogenesis of MS.

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Disclosure of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Association of IL-13 SNPs with MS

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