The Influence of Combined Genotypes of the HLADRB1*1501 and CD24 Single Nucleotide Polymorphism on Disease Severity of Iranian Multiple Sclerosis Patients

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Abstract- Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. It is a clinically heterogeneous disorder especially in terms of disease severity. Current investigations suggest that genes and gene-gene interactions not only influence on susceptibility to MS but also affect the disease severity. In this study, we investigated the contribution of the HLADRB1*1501 allele and single nucleotide polymorphism (SNP) in CD24 gene and also combined genotypes of the HLADRB1*1501 and CD24 SNP to disease severity in Iranian MS patients. We have reported previously that the HLA- DRB1*1501 allele and the CD24v/v genotype associated with disease susceptibility and some other studies proposed that HLA-DRB1*1501 allele be associated with MS severity. In this study, the results showed a significant difference in the Multiple Sclerosis Severity Score (MSSS) of the nine different genotypes (F=2.838, *P*=0.007). Subsequent analysis revealed a statistically significant difference in the MSSS between the MS patients who were carriers of HLA-DRB1*1501/1501 and those who were not carriers of HLA-DRB1*1501/1501 genotypes (*P*=0.04). Moreover, the MS patients carrying combined genotypes of the HLA- DRB1*1501/x-CD24 v/v had statistically severe disease than the patients who did not carry the HLA- DRB1*1501- CD24 v/v (*P*=0.047). In conclusion, our findings suggest that, HLA-DRB1*1501/1501 and bigenic genotypes of the HLA- DRB1*1501/x- CD24 v/v may influence on disease severity in Iranian MS patients.

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Introduction

Multiple Sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system which is characterized by blood-brain barrier (BBB) disruption, demyelination and hard axonal damage (1). It primarily affects young adults, with an age of onset typically between 20 and 40 years old, and its incidence among women is almost twice that of men (2). The course of MS is difficult to predict, and the disease may lie dormant or progress steadily. Current evidence showed that inflammation and neurodegeneration are two overlapping and connected effector arms in pathogenesis of MS. Bursts of focal inflammation are responsible for the episodic, relapsing- remitting phase of MS, whereas neurodegeneration and axonal loss are thought to have an important role in the occurrence of progressive symptoms, which are the leading cause of disability (3). Accumulating evidence from epidemiological and genetic studies indicates that, as with many common diseases, genetic and environmental factors may play an important role in the pathogenesis of disease. (4,5). The human leukocyte antigen (HLA) region is the primary region that has repeatedly shown a significant association with and linkage to MS, and investigation of this region revealed that the HLADRB1*1501 allele and the HLA-DRB1*1501-DQA1*0102-DQB1*0602 haplotype influence disease risk (6,7). However,

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polymorphisms in the HLA region do not confer all genetic component of MS pathophysiology (8) and only explain approximately half of the genetic contribution to MS (9).

Several studies have identified a broad spectrum of non-HLA genes influence MS risk in different populations (10,11). CD24 is a glycosylphosphatidylinositol (GPI) linked cell surface glycoprotein expressed in a variety of hematopoietic system cells, including T cells (12), B cells (13), and also central nervous system cells such as astrocytes and microglia (14). It is one of the non-HLA genes required for the induction of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, and suggested contributing to the pathogenesis of MS through several mechanisms, including expansion of autoreactive T cells in the target organ and regulation of homeostatic proliferation (15).

Several line of evidence have indicated that genes may not only play a role in MS susceptibility but also influence the phenotypic expression of the disease (16,17). The classical twin studies provided evidence that monozygotic twins are much more concordant for disease course than dizygotic twins or siblings (18). As MS is clinically heterogeneous in terms of duration of disease, age of onset, disease progression, it seems gene and gene –gene interaction (epistatic effect) may affect clinical severity of disease. Recent studies have shown that allelic variations at the HLA-DRB1 locus may influence MS disease severity. Although some studies suggested that HLA-DRB1*1501 allele be associated with disease severity (16,17), other could not find this association (19). The role of genetic factors in determining disease severity becomes even more complicated, as disease heterogeneity, ethnic variations and environmental factors may also affect the natural progression of MS disease. Based on mentioned evidence, in the present study, we aimed to investigate the contribution of the HLADRB1*1501 allele and also combined genotype of the HLADRB1*1501 and CD24 SNP to MS disease severity in Iranian MS patients.

Materials and Methods

Patients

A total number of 120 unrelated Iranian relapsing-remitting multiple sclerosis (RRMS) patients (mean age: 38.2±8.5years, range: 21-61), diagnosed based on McDonald criteria (20), were studied in the Department of Neurogenetics of the Iranian Center of Neurological Research. Clinical information, such as gender, age, age at disease onset, expanded disability status scale (EDSS) (21), and global Multiple Sclerosis Severity Score (MSSS) (22) was recorded at time of blood sampling. All cases suffered at least one attack and had no other autoimmune disorders. Clinical severity was measured by the MSSS. The MSSS provides a better measurement of MS disease severity than the EDSS score because it includes disease duration with the EDSS value. One hundred twenty ages- and sex-matched healthy controls (mean age: 36.8±7.9 years, range: 22-63), with no history of autoimmune or inflammatory disorders, were enrolled in this study from the same geographic regions as the patients. Our study was performed according to the instructions of the local Ethics Committee and each participant in the patient and control groups gave their informed consent before sample collection.

Genotyping

Total genomic DNA was extracted from peripheral blood cells (10 mL peripheral blood in 5% EDTA) according to the standard salting out method. The HLA-DRB1 genotyping was performed by Inno lipa DRB kit (Innogenetics NV). Briefly, first the highly polymorphic second exon of HLA-DRB genes was amplified by PCR and then the high resolution genotyping of HLA-DRB1 gene was performed using Inno lipa DRB kits according to the manufacturer's recommendations. Finally Positive signals were detected by a non-radioactive colorimetric method (Innogenetics NV). High-resolution (4-6-digit) HLA-DRB1 *15 was performed using the HISTO TYPE SSP High Resolution Kit according to the manufacturer's recommendations (BAG HEALTH CARE, Germany).

The polymerase chain reaction (PCR) with subsequent restriction fragment length polymorphism (RFLP) was used to genotype CD24 SNP. A C to T substitution in the coding region of CD24 at nucleotide position 170 from the translation start site (P170) creates a BstX1 restriction site that can be used to differentiate alleles in RFLP analysis (23).. PCR reactions were performed in a Thermal Cycler (Eppendorf, UK) using previously described primers: forward: 5′-TTGTTGCCACTTGGCATTTTTGAGGC-3′ and reverse: 5′- GGATTGGGTTTAGAAGATGGGGAAA -3′ (14). The PCR conditions were as follows: 5 min at 94°C followed by 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 45 seconds, repeated for 35 cycles, and finally 5 min at 72 °C as final extension. The predicted CD24 PCR fragment is 453 bp long. PCR products were digested with BstX1 restriction endonuclease under optimal conditions. PCR products of

allele T were cut into two small fragments (317 and 136 bp), whereas allele C remained undigested (453 bp). The products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide.

Statistical analysis

All information was entered into a database and data analysis was performed by the Statistical Package for Social Sciences software (SPSS 16). To assess disease severity in MS patients, the global MSSS was calculated, and normal distribution was checked using the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) and Tukey's HSD (Honestly Significant Difference) Post-hoc test were used both to compare the global MSSS in each genotype in MS patients and to determine which groups differ from each other.

Results

Demographic and clinical features

The clinical data of MS patients are summarized in table 1. One hundred and twenty patients with MS (79 females and 41 males) were included in the study. The mean age of MS patients was 38.2±8.5years.The female-to-male ratio was 1.9:1 for the patient group. The mean EDSS and global MSSS were calculated at the time of blood collection. The mean EDSS was 4.2±2.0, and the mean MSSS was 5.3±2.4. The mean disease duration and disease onset were 8.5±4.3and 29.7±7.2years, respectively. No significant association was found among HLA-DRB1*1500 and CD24 alleles and combined genotypes with clinical features, EDSS, MSSS, in Iranian MS patients.

Table 1. Demographic and clinical characteristics of 120 Iranian MS patients

Clinical information	MS patients
Age (years) mean±SD	38.2±8.5
female/male (n)	79/41
age of onset (years) mean±SD	29.7±7.2
duration of disease (years) mean±SD	8.5±4.3
EDSSa mean±SD	4.2±2.0
MSSS b mean±SD	5.3±2.4
Positive family history	12
Negative family history	108
Positive history of autoimmune disease	36
Negative history of autoimmune disease	84

EDSS: Expanded disability Status Scale
MSSS: Multiple Sclerosis severity Score

The influence of HLADRB1*1501 and bigenic HLA-DRB1*1501-CD24 genotypes on MS severity

The global MSSS was used to assess disease severity in this study. To investigate whether or not the MS patients who were carriers of HLA-DRB1*1501/x (x: Refers to any alleles of HLA-DRB1) genotypes and those who were carriers of combined or bigenic genotypes of HLA-DRB1*1501/x-CD24 influence disease severity, we analyzed the correlation of MSSS with HLA-DRB1*1501/x and combined genotypes of HLA-DRB1*1501/x-CD24 in MS patients (Tables 3,4).

Comparison of the MSSS among different HLA-DRB1*1501 genotypes in MS patients is shown in table 2. The results showed a significant difference in the MSSS of the nine different genotypes (F=2.838, *P*=0.007).

Subsequent analysis using Tukey's HSD Post-hoc test (Table 3) revealed a borderline significant difference in the MSSS was found between the patients with HLA-DRB1*1501/1501 and the patients who were not carriers of HLA-DRB1*1501/1501. This statistically significant difference in the MSSS increased between the patients carrying HLA-DRB1*1501/1501 and DRB1*1501/0701 (*P*=0.024) and even more between HLA-DRB1*1501/1501 and DRB1*1501/0101 (*P*=0.004) genotypes (Table 3). The Comparison of MSSS with combined genotypes of HLA-DRB1*1501/x-CD24 in MS patients is shown in Table 4 and 5. The results showed a significant difference in the MSSS of the different combined genotypes (F=4.824, *P*=0.031). Subsequent analysis using Tukey's HSD Post-hoc test (Table 5) revealed a statistically significant difference in the MSSS between the DRB1*1501-CD24 v/v and DRB1*xx-CD24 xx (*P*=0.047).

Table 2. The correlation of MSSS with HLA-DRB1*1501 genotypes in MS patients

HLA-DRB1*1501/x genotypes	Mean MSSS	S. E	F	*P*-value
***1501/1501**	9.09	0.01		
***1501/0101**	1.45	0.01	F(8,111) =2.838	**0.007**
***1501/0701**	3.09	2.15		
***1501/0401**	8.82	1.17		
***1501/0301**	4.60	1.07		
***1501/1101**	6.91	0.77		
***1501/1401**	8.54	0.01		
***1501/x**	7.08	0.46		
***xx/xx**	5.24	0.29		

MSSS: Multiple Sclerosis severity Score, S.E: Standard error of the mean x: Refers to any alleles of HLADRB1
xx: Refers to any genotypes of HLADRB1 other than HLADRB1*1501/x

Discussion

Immunogenetic studies, which focused on high-risk populations such as Northern European descendants, indicated that the HLA-DRB1*1501 allele and the HLA-DRB1*1501-DQA1*0102-DQB1*0602 haplotype is associated with MS (6,7). A few studies in other populations have failed to show this association (24, 25). Recent investigations have identified a group of non-HLA genes that influence MS risk in different populations (10,11).Recently, association of some other genes particularly in interaction with DRB1*1501 allele have been reported in Iranian MS patients (26). There is several line of evidence to suggest that genetic factors not only influence on susceptibility to MS but also affect the disease severity (16,17). Gene-gene interactions (epistatic effects) are also considered to be a prominent factor that influences the clinical outcome of MS patients (27).

For the first time we studied the influence of HLADRB1 and CD24 genes and also their combined genotypes on clinical severity of MS patients in Iran. The result showed that the MS patients who were carriers of combined genotypes of the HLA-DRB1*1501/x-CD24 v/v had statistically severe disease than those who were only carriers for one of the HLA-DRB1*1501/x or -CD24 v/v. (Table 3, 5).

Table 3. Comparison of the MSSS among different HLA-DRB1 genotypes in MS patients using Tukey's HSDc test

	HLA-DRB1 genotypes	MSSS Mean Difference	S.E	*P*-value
***1501/1501 VS**	*1501/0101	7.64	2.62	0.004
	*1501/0701	6.00	2.62	0.024
	*1501/0401	0.26	2.62	0.920
	*1501/0301	4.48	2.10	0.035
	*1501/1101	2.18	2.10	0.302
	*1501/1401	0.55	2.62	0.834
	*1501/xx	2.00	2.07	0.335
	*xx/xx	3.84	1.87	0.043
***1501/x VS**	*1501/1501	-2.00	2.07	0.335
	*1501/0101	5.63	2.07	0.008
	*1501/0701	3.99	2.07	0.057
	*1501/0401	-1.74	2.07	0.402
	*1501/0301	2.47	1.35	0.071
	*1501/1101	0.17	1.35	0.900
	*1501/1401	-1.45	2.07	0.483
	*xx/xx	1.83	0.96	0.060

S.E: standard error of the mean. x: Refers to any alleles of HLADRB1
xx: Refers to any genotypes of HLADRB1 other than HLADRB1*1501/x,
MSSS: Multiple Sclerosis severity Score

Table 4. The correlation of MSSS with combined genotypes of HLA-DRB1*1501-CD24 in MS patients

DRB1*1501-CD24	Mean MSSS	S. E	N	F	*P*-value
DRB1*1501/x-CD24 a/a	4.40	0.64	14		
DRB1*1501/x-CD24 a/v	6.75	0.24	9		
DRB1*1501/x-CD24 v/v	6.85	0.67	9	F(3,116)=4.824	0.031
DRB1*x/x-CD24 xx	5.14	0.27	88		
Total	5.30	0.22	120		

S.E: standard error of the mean
xx: Refers to any genotypes of HLADRB1 other than HLADRB1*1501/x
MSSS: Multiple Sclerosis severity Score

Although our patients who were carriers for DRB1*1501 (*1501/x vs xx/xx) had a higher mean MSSS (7.08 vs. 5.24), the presence of this allele was not associated with their disease severity (*P*=0.060). This finding is consistent with the results from a study conducted by Barcellos *et al*., (19), but they contradict the results from another study published by Okuda *et al*., (16). Differences in ethnic background and also heterogeneity of MS disease may partly explain inconsistency between our results and the above mentioned study. As shown in Table 3, only the patients who were carriers for HLA-DRB1*0101 allele had a better outcome than the patients who were homozygous or heterozygous for HLA-DRB1*1501. Recently, several epistatic interactions were uncovered between HLA-DRB1 allele and susceptibility and severity to MS.

It seems HLA-DRB1*0101 may have a dominant negative epistasis in reducing the severity of MS. According to Table 5, the MS patients who were carriers of combined genotypes of the HLA- DRB1*1501/x-CD24 v/v had statistically higher MSSS than the patients who were only carriers for one of the HLA-DRB1*1501/x or -CD24 v/v. It seems-CD24 v/v in conjunction with HLA- DRB1*1501 have synergistic epistasis in increasing clinical severity of MS patients.

In conclusion, our findings suggest that the MS patients are carrying combined genotypes of the HLA-DRB1*1501/x-CD24 v/v had clinically sever disease than the patients who were only carriers for one of the HLA- DRB1*1501/x or -CD24 v/v. High disease severity measured by MSSS in the MS patients carrying combined genotypes of the HLA- DRB1*1501-CD24 v/v may suggest that they need more aggressive treatment.

Table 5. Comparison of the MSSS among combined genotypes of HLA-DRB1*1501-CD24 in MS patients using Tukey's HSD[c] test

DRB1*1501/x-CD24	DRB1*1501/x-CD24	Mean MSSS	S. E	*P*-value
DRB1*1501/x-CD24 a/a VS	DRB1*1501/x-CD24 a/v	-2.35	1.04	0.025
	DRB1*1501/x-CD24 v/v	-2.44	1.04	0.020
	DRB1*xx-CD24 xx	-0.73	0.70	0.294
DRB1*1501/x-CD24 a/v VS	DRB1*1501/x-CD24 a/a	2.35	1.04	0.025
	DRB1*1501/x-CD24 v/v	-0.09	1.14	0.937
	DRB1*xx-CD24 xx	1.61	0.85	0.060
DRB1*1501/x-CD24 v/v VS	DRB1*1501/x-CD24 a/a	2.44	1.04	0.020
	DRB1*1501/x-CD24 a/v	0.09	1.14	0.937
	DRB1*xx-CD24 xx	1.71	0.85	0.047

S.E: standard error of the mean
xx: Refers to any genotypes of HLADRB1 other than HLADRB1*1501/x
MSSS: Multiple Sclerosis severity Score

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