Association of HLA-A*03/A*31 and HLA-A*24/A*31 Haplotypes with Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system with unknown etiology. Recent evidences suggest the HLA contribution to Multiple sclerosis (MS) pathogenicity as they may present neuropeptides to cytotoxic lymphocytes. We aimed to investigate the association of some related HLA-A alleles and haplotypes with MS and compare the results with other Universal reports to shed light on some aspects of this universally expanded disease. In this investigation, alleles were genotyped by polymerase chain reaction with sequence-specific primers (PCR-SSP) in 50 MS patients, and 50 unrelated healthy individuals. The analysis was carried out using SPSS V.19 statistical software. The results of this study showed a significant association of HLA-A*03 and HLA-A*24 alleles with MS (P<0.0001), but HLA-A*02 and other alleles did not show any significant association (P>0.05). However, other alleles were not significantly associated (P>0.05). Interestingly, in our study, the HLA-A*31 allele was often in combination with HLA-A*03 and HLA-A*24 as risk haplotypes in MS patients. In the present study, not only HLA-A*03 and HLA-A*24 were highly associated with the risk of MS susceptibility, but also their combinations with HLA-A*31 allele were more frequent in patients. Therefore, HLA-A*31 may be introduced as a new complementary risk factor in MS pathogenesis.

Introduction

Multiple sclerosis (MS), a chronic inflammatory condition of the central nervous system (CNS), leads to demyelination and dysfunction of neurons. Further disseminated and focal damage of myelin and axons that ends up to movement disability (Weissert, 2013). The prevalence of MS has been reported from 2 up to 160 per 100,000 in different populations worldwide.

In 2013, the world health organization (WHO) reported that 2.3 million people suffer from MS worldwide (Browne et al. 2014). The prevalence of this disease has been increasing in Iran, especially since a decade ago. The prevalence of MS varies from 5.3 to 74.28 per 100,000 in various regions of Iran (Etemadifar et al. 2006, Tolou-Ghamari, 2015). Particularly, it has been shown that there has been an increase in the prevalence of MS between 2008 and 2013 (Radmehr et al, 2015). Isfahan, in central part of Iran, is one of the largest and most populous provinces. The incidence rate of 9.1 per 100,000 in 2009 was reported for this city, standardized prevalence 71.6 per 100,000 which was about two times of 2007 (Etemadifar and Maghzi, 2011). This dramatic increase in the prevalence of MS puts Isfahan amongst the regions with the highest prevalence of MS in Asia and Oceania (Etemadifar et al. 2006).

Epidemiologists have reported that MS pathogenesis is the result of genetic and environmental factors interplay (Weiner, 2008). Genetic evidences show associated loci in the human Major Histocompatibility complex (MHC) or human leukocyte antigen (HLA) region on chromosome 6p21.3 with MS. The MHC region spans 3.5 Mb, including the class I, II, and III subregions (Horton et al. 2004). The MHC region encompasses about 7.6 Mb and consists of at least 252 expressed genes, which the main proportion of which are related to different immune mechanisms (Ramagopalan et al. 2009). The severity of this disease is affected by

genes in the MHC region, but the MHC association with MS seems not as direct as scientists thought before (Natio et al. 1972). The best-established associated MHC class II alleles with MS are HLA-DRB1*15:01, DRB5*01:01, DQA1*01:02, and DQB1*06:02 (Amirzargar et al. 1998). Also, there are evidences for independent effects of markers, form MHC class I, close to HLA-A and HLA-B/HLA-C (Rioux et al. 2009). Associations between HLA class I subtypes and MS has been reported by a number of studies. For example, among Swedish patients, the HLA-A*03 allele increased the risk of MS independently of the HLA-DR15 haplotype, whereas the HLA-A*02 allele was found to decrease the risk of MS (Fogdell-Hahn et al. 2000, Brynedal et al. 2007, Link et al. 2012). Among Italian patients, HLA-A*02 is reported as a protective allele with MS (Bergamaschi et al. 2010, Bergamaschi et al. 2011). In Norwegian sporadic MS cases and Nordic and British affected sibling pairs, HLA-A*03 in contribution with HLA class II alleles was found as associated haplotypes with MS (Harbo et al. 2004). Among Iranian MS patients, the HLA-A*03 (Lotfi et al. 1978, Galehdari et al. 2018) and HLA-A*24 (Kalanie et al. 2000) alleles were found to increase the risk of MS independent from the HLA-DR15 allele, whereas the HLA-A*02 (Amirzargar et al. 2005, Ghanavati et al. 2018) and HLA-A*11 (Amirzargar et al. 2005) alleles decreased the risk of MS. Also, in silico studies showed HLA-A*31 allele as a potential important allele in MS susceptibility (Mohammadi-Milasi et al. 2020).

As mentioned above, this disease has been associated with significant rise over the past decade in Isfahan of Iran, as well as other regions worldwide. Accordingly, this study examined the frequency of some important HLA-A alleles with MS patients in Isfahan province and compared the results with other regional or Universal reports to shed light on some aspects of this universally expanded and complex disease.

Materials and Methods

MS Patients and Control Subjects

During one year (2017 to 2018), we conducted this study on 50 patients from Isfahan MS center with Mean SD age of 39.04±0.54 and 50 healthy individuals referred to the al-Zahra hospital, which did not show any autoimmune disease, no history of MS in their close relations, and with mean age of 38.5±10.6 years, with normal laboratory analysis were selected as control group. The patients were diagnosed by a neurologist according to diagnostic criteria described by Mc Donald and a primary progressive (PP), secondary progressive (SP), or relapsing-remitting (RR) disease course (McDonald et al. 2001). To be eligible, patients with RRMS had to havetwo or more attacks in the previous two years. Fifty Controls were originally from the same geographical region and were matched with cases in ethnicity. All the participants were informed about our study and completed a consent form.

DNA Extraction and Genotyping

Peripheral blood samples (5 ml) were collected in EDTA tubes, and DNAs were extracted from whole blood using S3P PCR MASTER MIX (RNA Biotech Co, Isfahan, Iran) according to the manufacturer's manual.

HLA Typing was achieved by Nested PCR and Sequence-Specific PCR (SSP-PCR) methods. Nested PCR involves the use of two primer sets and two successive PCR reactions (Haff et al. 1994) to improve sensitivity and specificity of the test. On the other hand, in PCR-SSP method, specificity is determined by the use of sequence specific primers in which a 3' single-base mismatch inhibits the priming of unspecific reactions. In this research, a combination of both methods has been employed. In the beginning, a long PCR product was amplified by using a primer pair for HLA-A gene in general with a fragment of about 2478 bp. Sequences of primers were as follow: Forward 5'-GATCATTCAGGGGTTACC-3'; Reverse 5'-CATGGCAGGTGTATCTCT-3'. The PCR amplification program was 4 min for initial denaturation at 95ºC, followed by 35 cycles of melting at 94ºC for 30s, annealing at 59ºC for 30s, and elongation at 72ºC for 30s; followed by 7 min of final elongation at 72ºC. Then with the specific designed primers for the alleles of HLA-A*02, HLA-A*03, HLA-A*11 and HLA-A*24 each allele amplified by the PCR-SSP method. A pair of control primers were designed to be present in all HLA-A alleles. Then using the products of this PCR reaction PCR-SSP, the considered alleles were genotyped. Their sequence, length, melting temperature (Tm) and position of the primers are given in Table 1.

Table 1. Oligonucleotide primer properties

Gene	Primer	Primer sequence 5 to 3	Length (mer)	Tm	Location (Exon)
HLA-A02	Sense	GACGGGGAGACACGGAAA	18	58	2
	Antisense	CGTCCAGAGGATGTATGG	18	58	3
HLA-A03	Sense	GAGACACGGAATGTGAAGGCCCAG	24	58	2
	Antisense	CTTGGTGATCTGAGCCGC	18	58	3
HLA-A11	Sense	GAGACACGGAATGTGAAGGCCCAG	24	62	2
	Antisense	GGGCCGGTGCGTGGAGTGG	19	62	3
HLA-A24	Sense	GCGGCTCAGATCACCAAG	18	59	3
	Antisense	GGCAGCTGTGGTGGTACCTTCTG	19	59	4
HLA-A31	Sense	GCCTTGAACGAGGACCTG	19	54	3
	Antisense	CATATGCGTCTTGGGGGGG	18	54	4
Internal control	Sense	CTGGAGAACGGGAAGGAG	19	58	3
	Antisense	CATGGCAGGTGTATCTCT	18	58	4

In summary, 35 cycles carried out comprising: initial denaturation at 95°C for 4 min, denaturation at 94°C for 30 sec, the SSPs was melting temperatures in the range of 58°C to 64"C for 50 sec, and extension at 72°C for 50 sec, with a final 7-min extension at 72°C. The IMGT/HLA database (http://www.ebi.ac.uk/) was used for the design of all primers and then were evaluated in ncbi/primer blast (www.ncbi.nlm.nih.gov). Finally, the results were validated by sequencing of several random samples.

Statistical Analysis

HLA analysis was performed by comparing the dominant allele frequencies between the MS patients and control subjects using Statistical Package for Social Sciences (SPSS / version 19) software. The high-frequency alleles were analyzed for association with clinical features, by calculating odds ratios (ORs) with 95% confidence intervals (CIs). The relationships between the MS cases and controls were calculated with Pearson's chi-square test. Statistical significance was considered for $p < 0.05$. These values were then corrected by Bonferroni method according to the number of analyzed alleles.

Results

Demographic Data

Summarized characteristics of patients and controls are shown in Table 2. As shown in Table 3, there was a significant increase in the frequency of HLA-A*03 and HLA-A*24 in patients with MS in comparison with those of the healthy controls; and a positive association between HLA-A*03 and MS ($P < 0.0001$, odds ratio (OR)=23.059) and HLA-A*24 ($P < 0.00001$, odds ratio (OR)=17.379) was also observed. The results showed an increase in the risk of MS and HLA-A*03 and HLA-A*24 frequency in Isfahan province. The results of our study did not replicate the negative association of HLA alleles with MS, but a protective role of HLA-A*02 for some of the clinical symptoms was observed. All four patients who were positive for the HLA-A*02 subtype had no sphincter problems and spasticity. Figure 1 presents the more frequent HLA-A alleles in MS and control subjects.

Table 2. Demographics of the MS patients and control subjects

Variable	Age (year)		Gender		EDSS 1-5	Disease duration (year)		Disease course			Familial history
	Rang	Mean ± SD	Females	Males	Median	Rang	Mean ± SD	RRMS	SPMS	PRMS	
MS patients N=50	14-70	39.04± 0.54	21	29	1.98±1.79	1-20	4.46±4	48 (96 %)	1 (2%)	1 (2%)	10 (20%)
Controls N=50	19-62	385±10.6	23	27	NA	NA	NA	NA	NA	NA	NA

MS: multiple sclerosis; EDSS: expanded disability status scale; RR: relapse remitting; PP: primary progressive; SP: secondary progressive; SD: standard deviation; NA: not applicable.

Table 3. Frequencies of HLA-A alleles in MS patients and control subjects.

HLA-A alleles	MS group frequency, %, n=50	Control group frequency, %, n=50	P-value	Odds ratio	95% confidence interval	P^c
*A02	4	2	0.6777	2.087	0.307-17.361	NS
*A03	16	1	0.0001	23.059	2.957-488.362	0.01
*A11	5	5	1	1	0.230-4352	NS
*A24	21	2	0.00001	17.379	3.522-186.087	0.001
*A31	19	10	0.0769	2.452	0.917-6.650	NS
Others	35	80	-	-	-	-

MS: multiple sclerosis; pc: probability after Bonferroni correction for p < 0.05. NS: Not Significant.
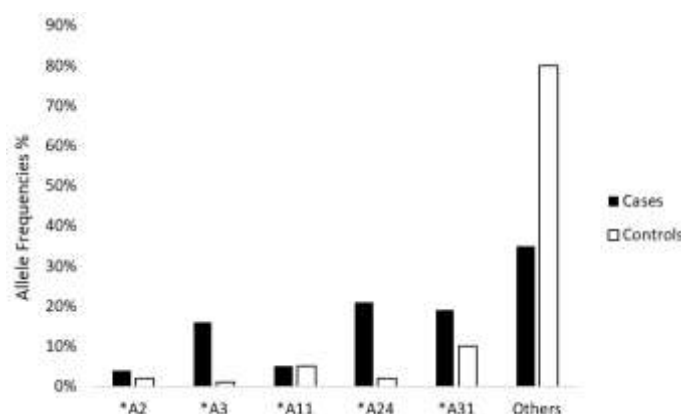

Figure 1. Frequency of HLA-A alleles in MS patients and control subjects. The results revealed that HLA-A24, A3 and A31 were the highest frequency alleles in MS patients, and HLA-A31 the highest frequency allele in control subjects. HLA, human leukocyte antigen.

Discussion

In this study, some HLA-A alleles and haplotypes were found highly associated with multiple sclerosis disease. The genetic associations of variations in MHC genes and MS have been robustly reported in different populations. Some case–control studies have proposed independent associations of HLA class I with MS (Fogdell-Hahn et al. 2000, Harbo et al. 2004, Silva et al. 2009). By using microsatellite markers in the MHC locus, a study on Sardinian families with MS disease found a telomeric region of HLA class I associated with MS but independent of HLA class II variations (Yeo et al. 2007). Parallel findings were reported by a Tasmanian study, which used microsatellite markers to study the class I region believed to play an independent role in susceptibility to and protection from MS (Rubio et al. 2002). In the recent two decades, susceptibility loci for MS, through the HLA region, were replicated in numerous populations, and HLA-A*03 has been described for increasing the risk of MS.

On the one hand, some studies have suggested independent **HLA class I** association with MS (Brynedal et al. 2007, Silva et al. 2009), and on the other hand, there are other studies that have suggested modifying effects of HLA class I alleles beside HLA-DRB1 allele as different haplotypes (Brynedal et al. 2007, Harbo et al. 2004).

Our results revealed an increased susceptibility to MS for people carrying **HLA-A*03** (P<0.0001, OR=23.059) in Isfahan province. This result is consistent with a previous study on MS patients of Tehran (Iran) (P<0.001) (Lotfi et al. 1978), Khuzistan (Iran) (P < 0.001) (Galehdari et al. 2018), Sweden (P=0.0008) (Fogdell-Hahn et al. 2000) and (2.7*10^{-3}) (Brynedal et al. 2007) but not with the result of a study in Tehran on samples referred from different regions of Iran (P>0.05) (Kalanie et al. 2000), Bahrain (Al-Nashmi et al. 2018), Norway (Harbo et al. 2004) and Brazil (Werneck et al. 2016), despite a positive trend in some studies.

Our results also revealed an increased susceptibility to MS for people carrying **HLA-A*24** (P<0.0001, OR=17.379) in Isfahan province. This result is consistent with a previous study on MS samples referred from different regions of Iran (P<0.05)

(Kalanie et al. 2000) and Kuwait MS patients who carrying A9 (P=0.031) (Al-Shammri et al. 2004), but not with the results of MS patients from Mumbai (India) (P=0.032) (Kankonkar et al. 2012), Sweden (Brynedal et al. 2007), Tehran who carrying A9 (HLA-A*23 and A*24) (Lotfi et al. 1978), and Bahraini MS patients carrying A9 (Al-Nashmi et al. 2018).

Previous studies in different populations have suggested the protective effect of **HLA-A*02** allele for MS. The HLA-A*02 associations among Portuguese (OR=0.54, p=0.001) (Silva et al. 2009), Italian (OR=0.61 P=5.28*10^{-9}) (Bergamaschi et al. 2010), Swedish (OR=0.52, P=0.0015) (Fogdell-Hahn et al. 2000) and (2007) (4.4*10-11) (Brynedal et al. 2007), African American (OR=0.72, p=0.013) (Isobe et al. 2013) and (in the absence of Cw*05 and B*44/12) in Italian (OR=0.63 P=0.0007), UK (OR=0.8 P=0.509), and UK combined case-control and family trios (OR=0.65 P=0.0099) (Bergamaschi et al. 2011), showed different levels of associations, but this allele, in all of these studies showed a protective influence. However, in our study, only a low number of MS patients and controls (2 out of 50 patients and 4 out of 50 controls) carrying this allele and maybe is the reason for not significant association of this allele with MS risk and opposite OR. This issue is consistent with the lack of significant association of HLA-A*02 and MS in Iran (Lotfi et al. 1978) and Bahrain (Al-Nashmi et al. 2018).

HLA-A*11 was not significant in our study (P>0.05), similar to Sweden (Brynedal et al. 2007), but, its association with MS is reported in Tehran (P<0.001) (Lotfi et al. 1978) and Mumbai (India) (P=0.032) (Kankonkar et al. 2012).

Interestingly as **HLA-A*31** allele was reported in an *in silico* study as a potential important allele in MS susceptibility (Mohammadi-Milasi et al. 2020), it was found more frequently in combination with highly associated HLA-A*03 and HLA-A*24 alleles in MS patients than controls (Table 4). This issue may reflect a role for this allele when combined with other risk alleles. However, further studies with a higher number of cases are required to show its effect more precisely.

Table 4. Observed HLA-A haplotypes in MS patients and controls and their attribution to EDSS.

	Other HLA-A allele	MS group frequency, %, n=50	Control group frequency, %, n=50	EDSS Mean
A*31	A*24	6	1	3.2
	A*03	5	1	2.7
	A*11	1	0	1
	others	7	8	1.5
A*24	A*03	3	0	2
	A*02	1	0	3
	A*11	1	0	1
	others	10	2	2
A*03	A*11	1	0	1
	A*02	1	0	1
	others	6	1	2

Recently, has been reported that deeper genotypes, 4-digit genotypes of HLA alleles, may show different behavior. For example, Kloverpris et al. (2011) showed that in HIV C-clade-infected subjects, HLA-*5703 was associated with a lower viral-load set point than HLAB*5702 (Kloverpriset al. 2011). Therefore, according to the controversial association of MS with different HLA-A alelles in different studies, one may suggest that this level of genotyping (2-digit resolution) may not be enough for association study of HLA alleles with MS or other related diseases, especially in heterogeneous populations. Therefore, as antigen presentation is carried out by different MHC class I and MHC class II alleles, comprehensive studies of 4-digit HLA genotyping seem necessary in diseases of interest.

To sum up, in this study HLA-A*03 and HLA – A*24 were highly associated alleles with MS, especially when combined with HLA-A*31 allele. Therefore, combinatory effects of different HLA alleles as HLA haplotypes may be considered as an informative strategy in HLA genotyping studies, either for MHC class I or MHC class II alleles.

Conflict of interest
Authors declare no conflict of interest.

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