Association of *CD24V/V* genotype with susceptibility and progression of multiple sclerosis in Iranian population

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Abstract
A single nucleotide polymorphism (SNP) in *CD24* has been associated with multiple sclerosis (MS) in a population based study. This SNP results in the replacement of alanine (CD24A) by valine (CD24V*)* at amino acid 57 in the resulting polypeptide chain. In the current study, the genotyping of this SNP and its contribution to MS in 217 patients and 200 healthy individuals of an Iranian population was investigated. The correlation of the SNP alleles with the progression of the disease was determined using the expanded disability status scale (EDSS) and progression index (PI). The data revealed that individuals with the *CD24V/V* genotype showed a 2-fold increase in the relative risk of MS compared to patients with the *CD24A/V* (0.27) and *CD24A/A* (0.25) genotypes (P = 0.0193, Odds Ratio 2.4882, 95% CI: 1.416-4.3722). Moreover, the progression of the disease in patients with CD24V/V was much faster than other patients that were examined by ANOVA and the least significant difference (LSD) test. However, in the CD24V/V patients LSD analysis was statistically significant ($p<0.05$ and $p<0.01$). These results support the hypothesis that *CD24* may function as a genetic modifier for susceptibility and progression of MS through the *CD24V/V* genotype.
Keywords: Multiple Sclerosis; CD24; Single nucleotide polymorphism; Iranian population.

INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that seems to result from the interaction of genetic (hereditary) and non-genetic (environmental) factors. Therefore, MS could be considered a multifactorial disease, where more than one gene could be implicated in the risk and progression of the disease (Combella and Martin, 2007; Harbo and Spurkland, 2007; Miller and Leay, 2007). Recent genetic studies on MS have mainly focused on the search for susceptibility genes. Among the candidate loci, the human locus antigen (HLA) located on chromosome 6, seems to be the most important genomic region related to MS (Haines *et al*., 2002; Kalanie *et al*., 2000; Amirzargar *et al*., 1998). In addition to HLA, several genes have been shown to be associated with the risk and progression of MS including genes involved in antigen presentation, cytokine genes and T-cell receptor genes such as *CD24* (Jevsek *et al*., 2006; Bai *et al*., 2004).

CD24 is a sialoglycoprotein expressed on mature granulocytes in many B cells. The protein is a glycosylphosphatidylinositol (GPI)-anchored protein, that has been shown to be expressed by a variety of cells, most of which are involved in the immune system including neutrophils, B cells and T cells. Recently it was reported that CD24 is expressed by myofiber synaptic nuclei in both embryonic and adult mice. The *CD24* gene, which is located in the 6q21 region, might therefore be a good candidate as a marker of MS susceptibility among the genes that have been studied previously (Niino *et al*., 2007; Bai *et al*., 2004; Duperray *et al*., 1990). The *CD24* gene harbors a single-nucleotide polymorphism (SNP), which has been reported to be associated with the relative risk and progression of MS in different populations (Liu and Zheng, 2007; Zhou *et al*., 2003). This region has

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been described to be in linkage disequilibrium with MS in the population based cohort of central Ohio, USA (Liu and Zheng, 2007; Wang *et al*., 2007). Moreover, the protein encoded by the *CD24* gene has been shown to be an important factor in the induction of experimental autoimmune encephalomyelitis (EAE) in mice.

Previuosly, a population based study, preformed on the prevalence of MS in the central province of Iran, Isfahan, suggested this geographical region to be at medium to high risk for MS (Etemadifar *et al*., 2006; Ale-Yasin *et al*., 2002). In the present study, the association of the different genotypes of *CD24* SNP with the MS disease in patients residing in the province of Isfahan, Iran was investigated. The results showed that the *CD24 V/V* genotype may confer susceptibility to risk and development of MS.

MATERIALS AND METHODS

Human subjects, Expanded Disability Status Scale (EDSS) and Progression index (PI): 217 MS patients and 200 healthy individuals (controls) were selected for this study. The patients residing in Isfahan, Iran were referred from the Isfahan MS association; MS disease in all patients was confirmed according to the recommended diagnostic criteria for MS by the international panel on the diagnosis of MS (the McDonald criteria) (McDonald *et al*., 2001). All the patients were considered as "definite MS" according to the McDonald criteria. Patient's informed consent was obtained from all participants prior to sample collection. Blood samples from patients and healthy controls were collected in EDTA tubes for eventual DNA extraction and genomic analysis.

The EDSS, a method of determining degree of disability in MS, was used to quantify disability in eight functional systems (FS). EDSS steps 1.0 to 4.5 refer to people with MS who are able to ambulate. EDSS steps 5.0 to 9.5 are defined by the impairment of ambulation. EDSS 10.0 refers to the cases of death due to MS. the PI was measured by dividing the EDSS score by the number of years lapsed since the first symptoms of MS.

Genotyping of the *CD24* single nucleotide SNP: The *CD24* SNP results in the replacement of C by T at nucleotide 226 in the coding region of exon 2, (Gene Bank accession no. NM-013230). The SNP with the T nucleotide creates a *Bst*XI restriction enzyme site which makes it possible to differentiate the *CD24A* allele from the *CD24V* allele by restriction fragment length polymorphism (RFLP) analysis. For this purpose total human genomic DNA from was isolated from nucleated blood cells using standard salting out precipitation method. Allele-specific polymerase chain reaction (PCR) was performed to amplify the DNA region containing the *CD24* SNP using specific primers. The forward primer was (5′-TTG TTG CCA CTT GGC ATT TTT GAG GC-3′), and the reverse primer was (5′-GGA TTG GGT TTA GAA GAT GGG GAA A-3′). The PCR condition was as follows: denaturation at 94ºC for 3 minutes, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 56.5ºC for 1 min, and extension at 72ºC for 1 min, with a final extension at 72ºC for 3 min. The predicted PCR product was 453 bp in length. Following PCR amplification, the PCR products were digested with *Bst*XI (Fermentas, Germany) for 4h at 55ºC. The digested PCR products were resolved on a 1.5% (w/v) agarose gel. PCR products of the *CD24V* allele were cut into two small fragments (325 and 129 bp), whereas those of the *CD24A* were completely resistant to digestion. In heterozygous individuals with two types of alleles A and V (*CD24A/V* genotype), three fragments of 453, 325 and 129 were expected.

Statistical analysis: Patients and normal controls were examined for any significant difference in their genotype (allele) distribution with respect to *CD24* polymorphism at the population level. Pearson's chi square (χ^2) test was used to perform homogeneity assessments for distribution of the genotypes between patients and the control population. The number of individuals falling into each of the three genotypes in the patients and controls were quite high; therefore χ^2 test was also used to determine a valid P value.

To assess whether the MS progression was different among patients with different genotypes, the progression index (PI) score was calculated. One-way analysis of variance (ANOVA) was used to compare the PI value of the disease in each genotype between the groups. The difference in progression of the disease between the three genotypes was investigated using the least significant difference (LSD) test.

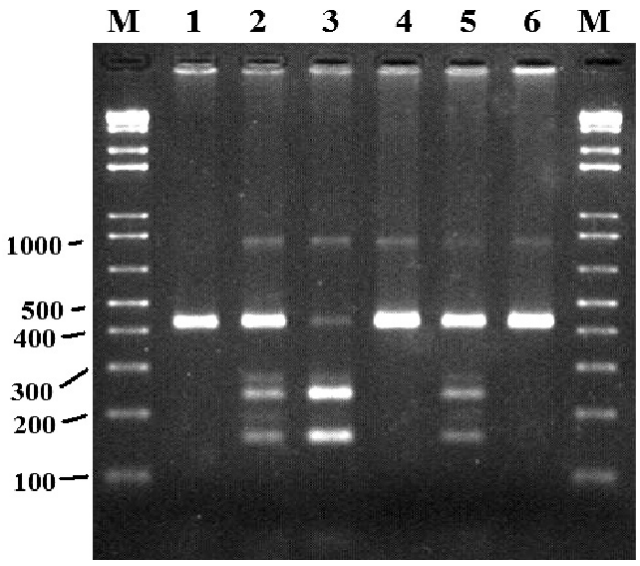

Figure 1. Restriction analysis of *CD24* SNP. DNA samples were amplified by PCR using primers specific to the *CD24* region containing an SNP corresponding to amino acid 57. The PCR products were digested with *Bst*XI restriction enzyme, and analyzed on 1.5% (w/v) agarose gel. In this representative gel, samples 1, 4 and 6 contain *CD24a/a,* samples 2 and 5, *CD24a/v* and sample 3 the *CD24v/v* genotypes, respectively. M represents a 100 bp ladder (Fermentas, Germany).

RESULTS

Genotyping of *CD24* SNP in MS patients and controls: Genotyping of *CD24* SNP was performed on 217 DNA samples from MS patients, and 200 healthy individuals. As shown in Figure 1, following PCR amplification and *Bst*XI digestion (PCR/RFLP) of total genomic DNA, three different genotypes could be detected, which were *CD24A/A, CD24A/V* and *CD24 V/V*. Figure 1 represents a typical PCR/RFLP analysis of samples from six MS patients. Lanes 1, 4 and 6 represent patients with the *CD24A/A* genotype having the 453 bp fragment, whereas lanes 2 and 5 represent heterozygous samples with the *CD24A/V* genotype consisting of the 543, 325 and 129 bp fragments. Lane 3 shows a DNA sample with the *CD24V/V* genotype displaying two bands of 325 and 129 bp.

Upon genotyping of all the patients and controls, the distribution of different genotypes among the population was examined. As represented in Table 1, while there is no significant difference in the frequency of the *CD24A/A* and *CD24A/V* genotypes between MS patients and control individuals, the *CD24V/V* genotype varies significantly between the two populations (P = 0.0193, Odds Ratio 2.4882, 95% confidence interval (CI): 1.416-4.3722). The significant increase in the *CD24V/V* genotype in individuals with the MS disease may suggest the importance of association of this genotype with the risk and susceptibility of the disease.

The *CD24V/V* genotype is associated with the progression of MS: To investigate the correlation of the *CD24* genotypes and progression of MS, the EDSS and PI of the MS patients with different genotypes were analyzed. The EDSS score and PI were determined for patients for whom more than 3 years had lapsed since the first symptom of MS. Data summarized in Table 2, show that among patients examined, 52 had *CD24A/A*, 23 had *CD24A/V* and 29 had the *CD24V/V* genotypes.

Subsequently, the possibility of differing *CD24* genotypes affecting the progression index of the disease was investigated. Table 2 shows the analysis of the progression index for different genotypes. Comparison of genotypes in each group by one-way ANOVA was performed. The results showed a large difference in the progression index of the three different genotypes (F=13.14, P=0.0001).

To investigate the difference in progression between the three genotypes, the LSD test was applied. As shown in Table 3, no significant difference between the average of the progression index of the *CD24A/A* and *CD24A/V* genotypes was observed. However, the average progression index in the *CD24V/V* patients was markedly different from that of the *CD24A/V* and *CD24A/A* patients. It should be noted that for the *CD24A/A* and *CD24A/V* genotypes, results of the LSD

Table 1. The distribution of *CD24* SNP genotypes in healthy individuals (control) and MS patients.

	CD24a/a (%)	*CD24a/v*(%)	*CD24v/v*(%)
Controls	114 (57%)	66(33%)	20(10%)
MS Patients	102(47%)	68(31%)	47(22%)
P value	NS	NS	Significant*

*P = 0.0193

NS: Not significant; MS; Multiple sclerosis; SNP: single nucleotide polymorphism.

Table 2. Analysis of the correlation of mean of the progression index (PI) with *CD24* genotypes in MS patients. *Patients for whom more than 3 years have lapsed since the first symptoms of MS. F = 13.14, P = 0.0001

Genotype	Mean of PI	Sum of PI	Number of patients*
A/A	0.25	12.92	52
A/V	0.27	6.22	23
V/V	0.51	14.80	29

PI: Progression index.

Table 3. Comparison of the progression index between different genotypes using the LSD test.

Mean Comparison	LSD	Subtraction of Means	
A/A and A/V	0.110	0.020	NS
A/A and V/V	0.126	0.240	Significant
A/V and V/V	0.104	0.260	Significant

NS: Not significant.
LSD: least significant difference.

test were not statistically significant. However, in *CD24V/V* patients, LSD analysis was statistically significant ($p < 0.05$ and $p < 0.01$). (see Table 3). Together these data suggest that the progression of MS in patients with the *CD24V/V* genotype is higher than patients with the *CD24A/A* and *CD24A/V* genotypes. Moreover, the progression of the disease is not significantly different in patients with the CD24A/A and /or CD24A/V genotypes.

DISCUSSION

Iran is considered as a medium to high risk area for MS (Etemadifar *et al*., 2006). Our previous population based study indicated the prevalence of MS in the central province of Isfahan, Iran, for the period of 2004-2005 as 35.5 per 100,000 [95% CI: 33.6-37.3] in a population of 3,923,255 (Etemadifar *et al*., 2006). A higher rate in women (54.5) than men (14.9) was detected which is much higher than previously believed (Etemadifar *et al*., 2006; Carton *et al*., 1997; Ebers *et al*., 1986). To date, few studies have examined the molecular pathogenesis of MS in Iranian patients. Those studies carried out have mainly investigated epidemiological and immunological factors related to MS such as HLA antigenic polymorphisms (Kalanie *et al*., 2000; Haines *et al*., 1996).

The presence of an SNP in the *CD24* gene, which involves a non-conservative amino acid at position 57, has been shown to mediate the association of the CD24 molecule with MS. This SNP involves a replacement of C by T at nucleotide 226, thus leading to the production of two polypeptide chains with different GPI-anchored cell surface efficiencies. Our results support previous reports on the association of the *CD24V/V* (C/C allele) genotype with risk and development of MS (Otaegui *et al*., 2006; Zhou *et al*., 2003). Analysis of the distribution frequencies of different *CD24* genotypes among the control healthy individuals and MS patients, indicates a significant difference in the *CD24V/V* homozygous genotype, but not in the *CD24A/A* and *CD24A/V* heterozygous genotypes. This may suggest a recessive role for the *CD24* SNP C allele as compared to the T allele, regarding association with MS association. Therefore, in *CD24V/V* homozygous patients, the CD24 polypeptide chain with amino acid valine at position 57 may function as a risk factor in the development of MS (see Table 1).

Our findings are similar to those reported earlier by Zhou *et al*. (2003), where a significant difference in the distribution of the *CD24V/V* genotype, but not the *CD24A/A* and *CD24A/V* genotypes was found (P = 0.0193, Odds Ratio 2.4882, 95% CI: 1.416-4.3722). Interestingly, *in vivo* and *in vitro* expression analysis of the *CD24* alleles by this group indicated a significantly higher expression for the *CD24A* allele as compared to the *CD24V* allele. Moreover, peripheral blood lymphocytes (PBLs) with *CD24V/V* genotypes were found to express higher levels of the CD24 protein compared to the *CD24A/V* and *CD24A/A* geno-

types. Further investigations on the association of the *CD24* SNP genotypes in a Spanish population has also revealed that the *CD24V/V* genotype is associated with increased risk of developing MS (Otaegui *et al*., 2006, Kamali-Sarvestani *et al*., 2006). These data support a significant role for the involvement of the *CD24V* allele in the molecular pathogenesis of MS, which warrants further investigation. Intriguingly, in a relatively broad population from Belgium and the UK, the association of *CD24V/A* with susceptibility and progression of MS has not been observed (Goris *et al*., 2006; Liu *et al*., 2007). This could further signify the role of the *CD24V/V* genotype, but not those of the CD24V/A or CD24A/A genotypes in risk and development of MS.

The severity of MS is usually measured according to the EDSS score (McDonald *et al*., 2001). Analysis of EDSS scores, indicated that the MS patients with *CD24V/V* genotypes exhibited increased risk of developing MS symptoms as compared to patients with the *CD24V/A* and *CD24A/A* genotypes. Moreover, our studies on a limited number of MS patients with family histories show that in comparison with other unaffected siblings, the *CD24V* allele seems to be preferentially transmitted to the MS offspring (unpublished data). Interestingly, population based studies of MS in relatives of patients and twins suggest a likely concordance pattern of inheritance for the possible MS genes.

Finally, examination of the progression index (PI) of MS patients shows that patients with the *CD24V/V* genotype display a more rapid progression of the disease (See Table 2). Since the PI was calculated by dividing the EDSS score by the number of years lapsed since the first symptoms of MS, it is feasible that the *CD24V/V* allele has a positive effect on the progression of the disease in *CD24V/V* patients. Moreover, the progression of the disease in patients with *CD24V/V* is much faster than other patients, when examined by ANOVA and the LSD test. However, in *CD24V/V* patients, LSD analysis was statistically significant ($p < 0.05$ and $p < 0.01$).

In summary, these data suggest that patients with the *CD24V/V* genotype are more prone to MS symptoms, and therefore may require more thorough treatment compared to patients with the *CD24V/A* and *CD24A/A* genotypes. However, further molecular verifications are required to fully justify this genotype as an important genetic modifier in the risk and progression of the MS disease.

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References

Aleyasin H, Sarai A., Alaeddin F, Ansarian E, Lotfi J, Sanati MH (2002). Multiple sclerosis: a study of 318 cases. *Arch Iran Med*. 5: 24-27.

Amirzargar A, Mytilineos J, Yousefipour A, Farjadian S, Scherer S, Opelz G, Ghaderi A (1998). HLA class II (DRB1, DQA1 and DQB1) associated genetic susceptibility in Iranian multiple sclerosis (MS) patients. *Eur J Immunogenet*. 25: 297-301.

Bai XF, Li O, Zhou Q, Zhang H, Joshi PS, Zheng X, Liu Y, Wang Y, Zheng P, Liu Y (2004). CD24 controls expansion and persistence of autoreactive T cells in the central nervous system during experimental autoimmune encephalomyelitis. *J Exp Med*. 200: 447-58.

Carton H, Vlietinck R, Debruyne J, De Keyser J, D'Hooghe MB, Loos R, Medaer R, Truyen L, Yee IM, Sadovnick AD (1997). Risks of multiple sclerosis in relatives of patients in Flanders, Belgium. *J Neurol Neurosurg Psychiatry*. 62: 329-33.

Comabella M, Martin R (2007). Genomics in multiple sclerosis-current state and future directions. *J Neuroimmunol*. 187: 1-8.

Duperray C, Boiron JM, Boucheix C, Cantaloube JF, Lavabre-Bertrand T, Attal M, Brochier J, Maraninchi D, Bataille R, Klein B (1990). The CD24 antigen discriminates between pre-B and B cells in human bone marrow. *J Immunol*. 145: 3678-83.

Ebers GC, Bulman DE, Sadovnick AD, Paty DW, Warren S, Hader W, Murray TJ, Seland TP, Duquette P, Grey T, *et al*. (1986). A population-based study of multiple sclerosis in twins. *N Engl J Med*. 315: 1638-42.

Etemadifar M, Janghorbani M, Shaygannejad V, Ashtari F (2006). Prevalence of multiple sclerosis in Isfahan, Iran. *Neuroepidemiology* 27: 39-44.

Goris A, Maranian M, Walton A, Yeo TW, Ban M, Gray J, Dubois B, Compston A, Sawcer S (2006). CD24 Ala/Val polymorphism and multiple sclerosis. *J Neuroimmunol*. 175: 200-2.

Haines JL, Bradford Y, Garcia ME, Reed AD, Neumeister E, Pericak-Vance MA, Rimmler JB, Menold MM, Martin ER, Oksenberg JR, Barcellos LF, Lincoln R, Hauser SL (2002). Multiple susceptibility loci for multiple sclerosis. *Hum Mol Genet*. 11: 2251-6.

Haines JL, Ter-Minassian M, Bazyk A, Gusella JF, Kim DJ, Terwedow H, Pericak-Vance MA, Rimmler JB, Haynes CS, Roses AD, Lee A, *et al*. (1996). A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. The Multiple Sclerosis Genetics Group. *Nat Genet*. 13: 469-71.

Harbo HF, Spurkland A (2007). Genetics in multiple sclerosis: past

and future perspectives. *Acta Neurol Scand Suppl*. 187: 34-8.

Jevsek M, Jaworski A, Polo-Parada L, Kim N, Fan J, Landmesser LT, Burden SJ (2006). CD24 is expressed by myofiber synaptic nuclei and regulates synaptic transmission. *Proc Natl Acad Sci USA*. 103: 6374-9.

Kalanie H, Kamgooyan M, Sadeghian H, Kalanie AR (2000). Histocompatibility antigen (HLA) associations with multiple sclerosis in Iran. *Mult Scler*. 6: 317-9.

Kamali-Sarvestani E, Nikseresht AR, Aliparasti MR, Vessal M (2006). IL-8 (-251 A/T) and CXCR2 (+1208 C/T) gene polymorphisms and risk of multiple sclerosis in Iranian patients. *Neurosci Lett*. 404: 159-62.

Liu JQ, Carl JW, Jr, Joshi PS, RayChaudhury A, Pu XA, Shi FD, Bai XF (2007). CD24 on the resident cells of the central nervous system enhances experimental autoimmune encephalomyelitis. *J Immunol*. 178: 6227-35.

Liu Y, Zheng P (2007). CD24: a genetic checkpoint in T cell homeostasis and autoimmune diseases. *Trends Immunol*. 28: 315-20.

Marrosu MG (2007). Susceptibility to multiple sclerosis: the role of interleukin genes. *Lancet Neurol*. 6: 846-7.

McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, *et al*. (2001). Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann Neurol*. 50: 121-7.

Miller DH, Leary, SM (2007). Primary-progressive multiple sclerosis. *Lancet Neurol*. 6: 903-12.

Niino M, Fukazawa T, Kikuchi S, Sasaki H (2007). Recent advances in genetic analysis of multiple sclerosis: genetic associations and therapeutic implications. *Expert Rev Neurother*. 7: 1175-88.

Otaegui D, Saenz A, Camano P, Blazquez L, Goicoechea M, Ruiz-Martinez J, Olaskoaga, J, Emparanza JA, Lopez de Munain A (2006). CD24 V/V is an allele associated with the risk of developing multiple sclerosis in the Spanish population. *Mult Scler*. 12: 511-4.

Wang L, Lin S, Rammohan KW, Liu Z, Liu JQ, Liu RH, Guinther N, Lima J, Zhou Q, Wang T, Zheng X, Birmingham DJ, Rovin BH, Hebert LA, Wu Y, Lynn DJ, Cooke G, Yu CY, Zheng P, Liu Y (2007). A dinucleotide deletion in CD24 confers protection against autoimmune diseases. *PLoS Genet*. 3: e49.

Zhou Q, Rammohan K, Lin S, Robinson N, Li O, Liu X, Bai XF, Yin L, Scarberry B, Du P, You M, Guan K, Zheng P, Liu Y (2003). CD24 is a genetic modifier for risk and progression of multiple sclerosis. *Proc Natl Acad Sci USA*. 100: 15041-6.