Investigation of CD24 and Its Expression in Iranian Relapsing-Remitting Multiple Sclerosis

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ABSTRACT

CD24 is a glycosylphosphatidylinositol (GPI)-linked cell surface glycoprotein expressed in central nervous system cells. Recent investigations have suggested that CD24 participates in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). However, a limited number of studies have been published regarding the contribution of CD24 to the risk and severity of MS in humans. We investigated the contribution of a CD24 single nucleotide polymorphism (SNP) to MS disease risk and severity. We also studied mRNA expression of the CD24 gene in Iranian MS patients using quantitative real-time polymerase chain reaction (PCR). Our findings showed that the CD24v/v genotype was significantly more frequent in MS patients compared with controls (p = .004). Moreover, a statistically significant difference in the Multiple Sclerosis Severity Score (MSSS) was found between MS patients carrying CD24a/a and CD24v/v genotypes (p = .008). The results also indicated that the expression of CD24 mRNA was 1.7 times more in MS patients compared with controls. In conclusion, our results suggest that the CD24v/v genotype influences both MS disease risk and severity in Iranian MS patients, and the high disease severity in CD24v/v patients may indicate that they require more aggressive treatment than do patients carrying CD24a/a.

KEYWORDS: CD24, CD24 mRNA expression, multiple sclerosis, Multiple Sclerosis Severity Score (MSSS), quantitative real-time PCR

INTRODUCTION

Multiple sclerosis (MS) is the most common neurological disease of the central nervous system (CNS) worldwide, affecting 1/1,000 Caucasians (Noseworthy, Gold, & Hartung, 1999). It is an autoimmune disorder mediated by autoreactive T-cell responses to myelin antigens (Agrawal & Yong, 2007). The onset of MS is commonly between 20 and 40 years of age, and its incidence among women is almost twice that of men (Dean, 1994). The etiology of MS remains elusive, but there is evidence that both genetic and environmental factors play roles in disease susceptibility (Oksenberg, Bernini, Barcellos, & Hauser, 2001). Numerous epidemiological studies have shown that there is an increased risk of MS among MS relatives (Robertson et al., 1997; Sadovnick, Ebers, Dymnt, & Risch, 1996). Moreover, twin studies in different populations have demonstrated that a monozygotic twin of a patient with MS has a higher risk (about 5–6 fold) of MS than a dizygotic twin (Ebers, Sadovnick, & Risch, 1995; Sadovnick et al., 1996). Collectively, these findings suggest that MS is a complex disease and, like other complex diseases, disease risk and possibly disease severity are affected by multiple genetic factors. The human leukocyte antigen (HLA) region is the primary region that has repeatedly shown significant association with and linkage to MS, and investigation of
this region revealed that the HLA-DRB1*1501 allele and the HLA-DRB1*1501-DQA1*0102-DQB1*0602 haplotype influence disease risk (Fogdell, Hillert, Sachs, & Olerup, 1995; Zamani et al., 1995). However, polymorphisms in the HLA region only explain approximately half of the genetic contribution to MS (McElroy & Oksenberg, 2008). Recent investigations have identified a broad spectrum of non-HLA genes that apparently influence MS risk in different populations (Alcina et al., 2009; Ramagopalan, Anderson, Sadovnick, & Ebers, 2007; Shahbazi et al., 2009, 2010).

CD24 is one of the non-HLA genes suggested to contribute to the pathogenesis of MS (Bai et al., 2004). It is a glycosylphosphatidylinositol (GPI)-linked cell surface glycoprotein expressed in a broad range of hematopoietic system cells, including T cells (Zhou, Wu, Nielsen, & Liu, 1997), B cells (Liu et al., 1992), macrophages, and neutrophils (Hernández-Campo et al., 2007). It is also known that CD24 is abundantly expressed in CNS cells such as astrocytes and microglia (Zhou et al., 2003). CD24 contributes to the pathogenesis of MS through several mechanisms, including expansion of autoreactive T cells in the target organ and regulation of homeostatic proliferation (Bai et al., 2004). Moreover, it has been shown that CD24 mediates a CD28-independent costimulatory pathway that can induce the activation of CD4 and CD8 T cells (De Bruijn, Petersen, & Jackson, 1996). CD24 is also required for the induction of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Liu et al., 2007). Human CD24 mRNA has a 0.24-kb open reading frame (ORF) and a 1.8-kb 39 untranslated region (UTR). A replacement of C with T in the coding region of CD24 at nucleotide position 170 from the translation start site (P170) results in the neoconservative substitution of alanine for valine preceding the putative cleavage site for the CD24 GPI anchor (Wang et al., 2007). Generally, it seems that single nucleotide polymorphisms (SNPs) in coding regions or regulatory sites of genes can have large impacts on gene expression and function. Therefore, they can be considered a major cause of disease outcome.

To understand the importance of the CD24 SNP in influencing the relative risk of MS, a few studies were conducted in different populations to determine the association of the CD24 SNP with MS (Liu & Zheng, 2007; Ronaghi, Vallian, & Etemadifar, 2009; Zhou et al., 2003). However, a limited number of studies have been published regarding the expression of CD24 at protein or mRNA levels in MS patients. In the present study, we used a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to investigate the contribution of the CD24 SNP to MS disease risk and severity in Iranian MS patients. In addition, we compared CD24 mRNA expression in MS patients and healthy controls using TaqMan quantitative real-time PCR.

**MATERIALS AND METHODS**

Each participant in the patient and control groups gave their informed consent before sample collection, and the study was performed according to the instructions of the local ethics committee. A total of 120 unrelated Iranian relapsing-remitting multiple sclerosis (RRMS) patients (mean age: 38.2 ± 8.5 years, range: 21–61), diagnosed according to the recommended diagnostic criteria for MS [the McDonald criteria (McDonald et al., 2001)], were studied in the Department of Neurogenetics of the Iranian Center of Neurological Research. All these patients were treated with interferon beta (β). Sixty-one patients received 30 µg of interferon β1a intramuscularly once weekly (Avonex), 38 patients received 250 µg of interferon β1b subcutaneously every other day (Betaferon), and the remaining 21 patients received 44 µg of interferon β1a subcutaneously three times weekly (Rebif). An expert neurologist clinically assessed the patients, and clinical information, including the Expanded Disability Status Scale (EDSS), a method of quantifying the degree of disability in MS (Kurtzke, 1983), and the global Multiple Sclerosis Severity Score (MSSS), a method that provides a novel approach by relating EDSS scores to the distribution of disability in patients with comparable disease durations (Roxburgh et al., 2005), was recorded. One hundred and twenty age, sex, and geographically matched healthy volunteers (mean age: 37.2 ± 7.8 years, range: 22–65) with no history of autoimmune or inflammatory disorders were randomly selected to serve as controls. Blood samples were collected in 5% ethylenediaminetetraacetic acid (EDTA) tubes from patients and controls for RNA and DNA extraction.

**DNA Extraction and CD24 SNP Genotyping**

Genomic DNA was extracted from peripheral blood samples (10 mL peripheral blood in 5% EDTA) using a modified salting out method (Miller, Dykes, & Polesky, 1988). CD24 SNP genotyping was performed using the PCR with subsequent RFLP analysis. 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. PCR reactions were performed in a thermal cycler (Eppendorf, UK) using previously described primers: forward: 5′-TTGTGTGCCACCTTTGCATTTTTGAGGC-3′ and reverse: 5′-GGATTGGGTTTAGAAGATGGGGAAA-3′ (NCBI Reference Sequence: NW_001838990.2; Zhou et al.,
2003). The PCR conditions were as follows: 5 min at 94°C followed by 94°C for 30 s, 62°C for 30 s, and 72°C for 45 s, repeated for 35 cycles, and finally 5 min at 72°C as the final extension. The predicted CD24 PCR fragment is 453-bp long. PCR products were digested with BstXI 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 using ethidium bromide.

RNA Extraction and Quantitative Reverse Transcription PCR (RT-PCR)

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) using the Trizol (Invitrogen) method. The RNA concentration was measured using a NanoDrop ND 1000 (Nanodrop Technologies Inc., Wilmington, Delaware, USA). The 260/280 ratio was above 1.75 for all samples. Total RNA (400 ng) was reverse transcribed with the QuantiTect Reverse Transcriptase kit (Qiagen, catalog no. 205311). Quantitative real-time PCR was performed using a Corbett Rotor-Gene 6000 with the QuantiFast Probe PCR kit (Qiagen, catalog no. 204254) in accordance with the manufacturer’s instructions. All primers and probes were synthesized by Metabion international AG (Germany). The following primers and probe that we designed were used to quantify CD24 mRNA expression: forward primer: 5’-ACCCACGCAGATTTATTTCCA-3’ and reverse primer: 5’-CGAAGAGACTGGCTGTGAC-3’ and probe: 5’-Fam-ATCCAACTAATGCCACCA-CGAAGAGACTGGCTGTTGAC-3’. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was amplified to serve as an endogenous reference (internal control gene) with the following primers and probe: forward primer: 5’-CCCACTTCCACACTTTG-3’ and reverse primer: 5’-TCATACCAGAAATGACGC-TTGAC-3’ and probe: 5’-Fam-CTGCGATTGCCC-TCAACGACCA-BHQ-1-3’. The gliceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was amplified to serve as an endogenous reference (internal control gene) with the following primers and probe: forward primer: 5’-CCCACCTCCACACTTTG-3’ and reverse primer: 5’-TCATACCAGAAATGAGC-TTGAC-3’ and probe: 5’-Fam-CTGCGATTGCCC-TCAACGACCA-BHQ-1-3’. Real-time PCR was performed using optimal conditions. cDNA product (2 μL) was amplified using the QuantiFast Probe PCR kit according to the manufacturer’s instructions with the following program: 3 min at 95°C as a PCR initial activation step followed by two-step cycling at 95°C for 3 s and 60°C for 30 s, repeated for 40 cycles. A 1/1,000 dilution of pooled cDNA from healthy controls was carried out using a chi-square test with Yates’ correction. 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 honestly significant difference (HSD) post hoc test were used to compare the global MSSS in each genotype in MS patients and to determine which groups differ from each other. Relative expression was determined by the comparative method (2−ΔΔCT), and an independent student t-test was used to compare ΔCT between two groups. A p value <.05 was considered statistically significant.

RESULTS

The Influence of CD24 Genotype on MS Susceptibility and Severity

The distribution of CD24 allele and genotype frequencies in MS patients and healthy controls is shown in Table 1. Three different genotypes could be detected after PCR amplification and BstXI digestion of total genomic DNA. The genotype distribution of this polymorphism did not deviate from the Hardy–Weinberg equilibrium in patient and control groups. A comparison of genotype frequencies between MS patients and controls indicates that the CD24v/v genotype was significantly more frequent in MS patients than in controls (OR = 3.500, p = .004). This result suggests that the CD24v/v genotype may play a role in MS susceptibility.

MS disease severity is usually evaluated using EDSS, but we used the global MSSS to assess disease severity because it combines disease duration with EDSS to...
provide a better measurement of MS disease severity. A comparison of the MSSS among different CD24 genotypes in MS patients is shown in Tables 2 and 3. The results showed a significant difference in the MSSS of the three different genotypes \((F = 4.824, p = .010)\). Subsequent analysis using Tukey’s HSD post hoc test (Table 3) revealed a statistically significant difference in the MSSS between the CD24\(^a\)/a and the CD24\(^v\)/v genotype \((p = .008)\). These data suggest that the CD24\(^v\)/v genotype may influence both disease susceptibility and severity in Iranian MS patients.

**Analysis of CD24 Gene Expression in MS**

We investigated CD24 gene expression in PBMC from patients with MS and controls. The amount of CD24 mRNA normalized to the endogenous reference and relative to a calibrator was expressed as follows: \[\frac{\text{Ave. CD24}_{\text{Ct} \text{Calibrator}} - \text{Ave. GAPDH}_{\text{Ct}}}{\Delta \Delta \text{Ct}}.\] Finally, average relative expression was determined by the comparative method \((2^{-\Delta \Delta \text{Ct}})\). A comparison of CD24 gene expression between MS patients and healthy controls is shown in Table 4. The results indicated a significant difference in the \(\Delta \text{Ct}\) between MS patients and controls \((p = .00001)\). As shown in Table 4, when we calculated the relative amount of CD24 mRNA, it showed a 1.7-fold upregulation of CD24 mRNA in MS patients compared with controls. To investigate the relative expression of CD24 among different CD24 genotypes in MS patients, we used the \(\Delta \Delta \text{Ct}\) (Figure 1). This result did not show any significant difference among the three CD24\(^v\)/v genotypes \((F = 0.268, p = .765)\).

**DISCUSSION**

Polymorphisms in the HLA region explain approximately half of the genetic contribution to MS (McElroy & Oksenberg, 2008). Several genome-wide association studies have shown the possibility of contributions from non-HLA genes to the pathogenesis of MS. One of these studies suggested a linkage disequilibrium in distal 6q, where an interesting candidate gene, CD24, is located (Haines et al., 1996). It is well established that SNPs in immune-related genes influence host susceptibility to autoimmune disorders such as MS (McElroy & Oksenberg, 2008; Zhou et al., 2003). Non-synonymous SNPs altering amino acid sequences are believed to be responsible for most well-known disease susceptibilities and phenotypic variations and have been emphasized as suitable targets in candidate gene screens (Tabor, Risch, & Myers, 2002). Several studies previously conducted in different populations showed an association of a CD24 SNP with MS (Liu & Zheng, 2007; Zhou et al., 2003).

### Table 1. The distribution of CD24 allele and genotype frequencies in MS patients and healthy controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>MS patients</th>
<th>Controls</th>
<th>(p) value</th>
<th>(p^c)</th>
<th>OR (95% CI) (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>(n^2 = 240)</td>
<td>(n = 240)</td>
<td>.024</td>
<td>.031</td>
<td>0.642 (0.436–0.945)</td>
</tr>
<tr>
<td>V</td>
<td>152 (63.3%)</td>
<td>175 (72.9%)</td>
<td>.024</td>
<td>.031</td>
<td>1.559 (1.058–2.291)</td>
</tr>
</tbody>
</table>

Note: \(p\) value < .05 considered statistically significant were showed in bold.

\(a\)The two-tailed \(p\) value with Yates’ correction.

\(b\)Odds ratio (OR) with 95% confidence interval (CI).

\(c\)Number of chromosomes.

\(d\)Number of patients.

### Table 2. The correlation of MSSS with CD24 genotypes in MS patients

<table>
<thead>
<tr>
<th>CD24 genotypes</th>
<th>MSSS</th>
<th>SE(a)</th>
<th>N(b)</th>
<th>(F)</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24(^a)/a</td>
<td>4.67</td>
<td>0.30</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD24(^v)/v</td>
<td>5.50</td>
<td>0.39</td>
<td>40</td>
<td>4.824</td>
<td>.010</td>
</tr>
<tr>
<td>CD24(^v)/v</td>
<td>6.46</td>
<td>0.52</td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: \(p\) value < .05 considered statistically significant were showed in bold.

\(a\)Standard error of the mean.

\(b\)Number of patients.

### Table 3. Comparison of the MSSS among different CD24 genotypes in MS patients using Tukey’s HSD test

<table>
<thead>
<tr>
<th>CD24 genotypes</th>
<th>MSSS mean difference</th>
<th>SE(b)</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24(^a)/a and CD24(^v)/v</td>
<td>-0.83</td>
<td>0.49901</td>
<td>.224</td>
</tr>
<tr>
<td>CD24(^a)/a and CD24(^v)/v</td>
<td>-1.79</td>
<td>0.58809</td>
<td>.008</td>
</tr>
<tr>
<td>CD24(^v)/v and CD24(^v)/v</td>
<td>-0.96</td>
<td>0.62238</td>
<td>.275</td>
</tr>
</tbody>
</table>

Note: \(p\) value < .05 considered statistically significant were showed in bold.

\(a\)Standard error of the mean.
TABLE 4. Comparison of CD24 gene expression between MS patients and healthy controls

<table>
<thead>
<tr>
<th>Case or control</th>
<th>N</th>
<th>Mean</th>
<th>SE</th>
<th>ΔΔCt</th>
<th>RΔ = 2⁻[^ΔΔCt]</th>
<th>R_MS/R_CONT[^e]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS patients</td>
<td>120</td>
<td>7.2551</td>
<td>0.1508</td>
<td>-3.6321</td>
<td>12.3985</td>
<td>1.7116</td>
</tr>
<tr>
<td>Controls</td>
<td>120</td>
<td>8.0888</td>
<td>0.1145</td>
<td>-2.8567</td>
<td>7.2435</td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>1 × 10⁻⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: p value < .05 considered statistically significant were showed in bold.
[^a]: Ave. CD24_Ct – Ave. GAPDH_Ct.
[^b]: Standard error of the mean.
[^c]: Ave. ΔCt – Ave. ΔCt_Calibrator (1/1,000 dilution of pooled cDNA from healthy controls).
[^d]: Relative amount of the target.
[^e]: The ratio of the relative amount of target in MS patients to the relative amount of target in healthy controls.

Ronaghi et al., 2009; Zhou et al., 2003). However, a limited number of studies have been published regarding the expression of CD24 protein and mRNA in PBMCs in MS.

In this study, we investigated both the contribution of the CD24 SNP to the relative risk of MS and also, for the first time, CD24 mRNA expression in Iranian MS patients. Our findings showed that the CD24^v/v genotype was significantly more frequent in MS patients (OR = 3.500, p = .004); these findings were consistent with the study previously conducted by Zhou et al. (2003), in which a significant association was found between MS and the CD24^v/v genotype (6.3% of the controls carried the CD24^v/v genotype vs. 13.2% in MS, p = .023; Zhou et al., 2003). Moreover, our data were also similar to those from other studies carried out in Spanish and Iranian populations (Otaegui et al., 2006; Ronaghi et al., 2009). In 2006, Goris et al. investigated the association of the CD24^v/v genotype with susceptibility and progression of MS in a relatively broad population from Belgium and the UK, and their results did not show an association of the CD24^v/v genotype with MS. Thus, our findings confirmed pervious data (Goris et al., 2006; Otaegui et al., 2006; Ronaghi et al., 2009), suggesting that CD24^v/v, but not CD24^a/a or CD24^a/v, may act as a modifier of MS risk. To investigate the correlation of CD24 genotypes and MS disease severity, we used the global MSSS instead of EDSS for a better measurement of disease severity and progression. Our findings revealed that there is a statistically significant difference in the MSSS between CD24^a/a and CD24^v/v genotypes (p = .008, Table 3). This finding indicates that the CD24^v/v genotype is associated with both disease susceptibility and severity in Iranian MS patients. Zhou et al. (2003) also reported that the severity of MS in 50% of the CD24^v/v patients reached an EDSS of 6.0 within 5 years, whereas 50% of the CD24^a/a and CD24^v/v patients reached that milestone in 13 and 16 years, respectively. Therefore, it can be concluded that MS patients with the CD24^v/v genotype have greater disease severity and more rapid progression than those with the CD24^a/a and CD24^v/v genotypes. Consequently, it can be suggested that MS patients bearing the CD24^v/v genotype need better guidelines for optimal treatment than those with the CD24^a/a and CD24^v/v genotypes.

To date, a limited number of studies have investigated CD24 expression at the protein or mRNA level in PBMCs (Wang et al., 2007; Zhou et al., 2003). We used quantitative TaqMan real-time PCR to detect CD24 mRNA expression. A comparison of CD24 gene expression between patients and controls showed that the expression of CD24 mRNA in MS patients was 1.7 times more than that in controls. Although the mRNA level of MS patients with the CD24^v/v genotype was slightly higher than that of patients with CD24^a/a and CD24^v/v genotypes, there was no significant difference.

FIGURE 1. The comparison of CD24 relative expression among different CD24 SNP genotypes in MS patients shows no statistically significant difference in CD24 gene expression among different genotypes. ΔΔCt: Ave. ΔCt – Ave. ΔCt_Calibrator.
in the mRNA level between the different CD24 genotypes among MS patients ($F = 0.268, p = .765$). Our findings were inconsistent with the study conducted by Zhou et al. (2003) regarding CD24 expression at the protein level by two-color flow cytometry. Their results indicated that regardless of genotype, CD24 is expressed on both T cells and non-T cells, but T cells from CD24/a patients expressed sixfold less cell-surface CD24 than did T cells from CD24/v patients (Zhou et al., 2003). The difference in the distribution of blood cellularities in MS patients and healthy controls and also the differences caused in this cellularity by the treatment of MS with interferon $\beta$ may explain this inconsistency. CD24 is a GPI-linked protein with 32 amino acids in the mature human protein. It has been speculated (Zhou et al., 2003) that the replacement of alanine with valine at amino acid 31, immediately preceding the putative cleavage site for the GPI anchor, may increase the efficiency of cleavage and result in an increased expression of CD24/v on the cell surface. It seems the inconsistency that exists between CD24 expression at the protein and the mRNA level could likely result from processing of the CD24 protein itself that leads to the expression of CD24 protein on T cells with different efficiencies.

In conclusion, our findings suggest that the CD24/v genotype may influence both MS disease risk and severity in Iranian MS patients. High disease severity measured by the MSSS in the CD24/v patients may suggest that they need better guidelines for optimal treatment. Moreover, our data showed that CD24 mRNA expression was 1.7 times more in MS patients compared with controls. CD24 mRNA expression in CD24/v patients was slightly more than that in CD24/a and CD24/vv MS patients, but this was not a large enough increase to be significantly different. It appears that the high disease severity provided by the CD24/v genotype may be caused by post-mRNA processing on the CD24 protein and alterations to the efficacy of T cells. As our study was limited by the small sample size, it would be great that our finding be extended and confirmed in other populations and a larger sample size.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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