Original Article

VLA4 Gene Polymorphism and Susceptibility to Multiple Sclerosis in Slovaks

(multiple sclerosis / VLA-4 / integrin / single-nucleotide polymorphism / association study)

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Abstract. Multiple sclerosis (MS) is an inflammatory autoimmune disease occurring in genetically sensitive individuals. As migration of immune cells into the CNS is facilitated by the Very Late Antigen 4 (VLA-4) integrin molecule, the *VLA4* gene may be considered as a plausible candidate genetic risk factor for susceptibility to MS. Therefore, the objective of our study was to investigate the association between two genetic polymorphisms located in the *VLA4* gene and the risk of multiple sclerosis. One hundred seventeen MS patients and 165 control subjects from Slovakia were genotyped for *VLA4* gene SNP polymorphisms at positions 269 (C/A) and 3061 (A/G). The same study cohorts were also genotyped for the rs3135388 polymorphism tagging the HLA-DRB1*15:01 allele, which is a known genetic factor associated with susceptibility to develop MS in many populations. Our findings show for the first time that the rs3135388 polymorphism is a strong risk factor for MS in the Slovak population. Investigation of the *VLA4* gene polymorphisms revealed a significantly higher frequency of the 3061AG genotype in MS patients compared to the controls ($P \leq 0.05$). We suggest that the 3061AG polymorphic variant is an independent genetic risk factor for MS development in our population as it was significantly associated with this disease. The association was also confirmed after applying multivariate logistic-regression analysis adjusted for gender, age and HLA-DRB1*15:01 positivity as possible influencing factors.

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Abbreviations: AP-2 – activating protein 2, CD – cluster of designation, CI – confidence interval, CNS – central nervous system, HLA – human leukocyte antigen, ITGA4 – integrin α4, MS – multiple sclerosis, OR – odds ratio, PCR – polymerase chain reaction, RFLP – restriction fragment length polymorphism, SNP – single-nucleotide polymorphism, SSP – sequence-specific primer, VCAM-1 – vascular cell adhesion molecule 1, VLA-4 – very late antigen 4.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disease of the human central nervous system. It is characterized by the presence of demyelinated plaques or multifocal inflammatory lesions caused by autoreactive immune cells. Like many other immune-mediated diseases, MS is caused by the combined action of genetic background and environmental triggers (Nicot, 2009; Sadovnick, 2012; Buc, 2013). Until now, more than 100 genetic loci have been identified as susceptibility loci to MS development, involving HLA as well as non HLA genes (Pravica et al., 2012; Lambert et al., 2013).

VLA4 gene and susceptibility to MS

An essential step in brain inflammation onset is immune cell migration through the blood-brain barrier, which is facilitated by adhesion molecules such as the Very Late Antigen 4 (VLA-4) (Berlin et al., 1995). The VLA-4 molecule is an important member of the β1 integrin family and is composed of two chains: CD49d (α4) and CD29 (β1). The involvement of VLA-4 in MS pathogenesis makes the *VLA4* gene polymorphism a candidate genetic risk factor for susceptibility to MS.

Based on the autoimmune mechanism of demyelination, genetic studies have mainly been focused on associations between MS and polymorphic variants of candidate genes that regulate the immune response. Many independent association studies have documented the crucial role of HLA genes on chromosome 6p21 on MS

development. Based on accumulative evidence obtained by genome-wide studies, the HLA association was fine-mapped to the HLA-DRB5*01:01-HLA-DRB1*15:01-HLA-DQA1*01:02-HLA-DQB1*06:02 extended haplotype (Lincoln et al., 2005; Schmidt et al., 2007; Brum et al., 2010; Sadovnick, 2012). Definitely, the HLA-DRB1*15:01 allele is the most significantly MS-associated genetic factor confirmed in a number of populations (Patrucco et al., 2009; Stankovich et al., 2009). Although the association of HLA-DRB1*15:01 with MS has been well documented in various cohorts, comprehensive genomic scans have confirmed the possible involvement of multiple other loci in MS pathogenesis, each with a minor contribution.

Genetic predisposition of *VLA4* to MS has been analysed by only a few studies so far (Andreoli et al., 2007; O'Doherty et al., 2007). The studies found only minor associations of *VLA4* with MS leading to continuation of research on this topic. Therefore, the objective of our study was to employ the candidate gene approach to evaluate the association between genetic polymorphisms located in the *VLA4* gene (*ITGA4*) on chromosome 2q31.3 and the risk of multiple sclerosis in Slovak patients. Two single-nucleotide polymorphisms (SNPs) in the α4-subunit gene were investigated: a single point mutation at position 3061 (rs1143676) causing an arginine (CGG) to glutamine (CAG) transversion at amino acid position 844 in exon 24 (Szabo and McIntyre, 1995) and a C to A transversion at position 269 (rs113276800) in the promoter region of exon 1 (Heymann et al., 2003).

Material and Methods

Study subjects

The investigated group included 117 unrelated individuals meeting criteria for multiple sclerosis according to clinical findings and imaging techniques. MS patients (37 men and 80 women) were recruited at random via several neurology clinics in Slovakia. The average age was 37.4 ± 9.7 years, the average age at disease onset was 26.1 ± 7.2 years. Clinical status was evaluated using the expanded disability status scale (EDSS, Kurtzke, 1983). Out of the MS group, 110 patients had the relapsing-remitting MS form, seven patients had secondary progressive MS, and none developed primary progressive MS.

The reference cohort in our case-control study comprised 165 unrelated age-matched volunteers (60 men and 105 women with the mean age of 35.7 ± 9.3 years). All the control subjects were without any personal or family history of MS and they were randomly recruited from a larger population sample. All patients and controls were Caucasians of Slovak descent.

Written informed consent for enrolling in the study and for personal data management was obtained both from all MS patients and the control subjects. All the investigations were carried out in accordance with the International Ethical Guidelines and the Declaration of Helsinki.

Genotyping

Both, patient and control DNA was extracted from whole blood by a modified salting out procedure (Miller et al., 1988). HLA-DRB1*15:01 genotyping was performed by determination of the rs3135388 polymorphism that tags the DRB1*15:01 allele (Goris et al., 2008). The rs3135388 polymorphism (T or C allele) was detected by sequence-specific PCR followed by restriction fragment polymorphism analysis (RFLP). Primer sequences, PCR algorithm and Bsu15I enzyme cleavage were used as described by Benesova et al. (2013).

Consequently, single-nucleotide polymorphisms in the VLA-α4-subunit gene at position 3061 (exon 24) was genotyped by PCR-RFLP as described by Andreoli et al. (2007). A 241-bp PCR product flanking the polymorphic site was amplified and afterwards digested with the MspI restrictase. The restriction products were run in 2% agarose gel for 20 min, either producing an intact PCR fragment (allele A) or two fragments of 149 bp and 92 bp (allele G). The SNP at position 269 in the promoter region of exon 1 was determined by PCR with sequence-specific primers (PCR-SSP) using the method described by Heymann et al. (2003) and Andreoli et al. (2007). Electrophoresis was performed in 1.5% agarose gel for 20 min at 10 V/cm and the gel was UV-photographed.

Statistical analysis

Allele and genotype frequencies were evaluated by direct counting. Data were tested for the goodness of fit between the observed and expected genotype frequencies and their fit to Hardy-Weinberg equilibrium. Statistical significance of differences in the allele and haplotype frequencies in MS patients and controls was evaluated by the standard χ^2 test using the InStat statistical software. The odds ratios (OR) and 95% confidence intervals (95% CI) were calculated as well and consequently estimated by multivariate logistic-regression analysis adjusted for gender, age and HLA-DRB1*15:01 positivity as possible influencing factors. Calculations were performed by the SNPstats web software available at http.//bioinfo.iconcologia.net/snpstats/start.htm. The statistical power to detect association at the 0.05 significance level was calculated by Sampsize software available at http://sampsize.sourceforge.net/iface/s3.html.

Results

Allele and genotype frequencies of the *VLA4* gene polymorphisms at positions 269 (C/A) and 3061 (A/G) observed in both, MS patients and the control group, are shown in Table 1. Genotyping of the SNP variants at 269 (C/A) revealed no statistically significant differences in either allele or genotype frequencies between the two cohorts (P = 0.20, OR = 0.72, 95% CI: 0.44–1.19). Interestingly, no homozygous AA genotype was detect-

Table 1. VLA4 allele and genotype frequencies in MS patients and healthy controls

SNP	Allele/ genotype	MS subjects (N = 117)	Controls (N = 165)	Univariate analysis		Multivariate analysis	
				P	OR (95% CI)	P	OR (95% CI)
269 C/A	C A	198 (84.62 %) 36 (15.38 %)	267 (80.91 %) 63 (19.09 %)	0.25	0.77 (0.49-1.21)	-	-
	CC CA AA	81 (69.23 %) 36 (30.77 %) 0 (00.00 %)	102 (61.82 %) 63 (38.18 %) 0 (00.00 %)	0.20	1.00 0.72 (0.44-1.19) -	0.36 -	1.00 0.78 (0.46-1.32) -
	CC CA + AA	81 (69.23 %) 36 (30.77 %)	102 (61.82 %) 63 (38.18 %)	0.20	1.00 0.72 (0.44-1.19)	0.36	1.00 0.78 (0.46-1.32)
3061 A/G	A G	159 (67.95 %) 75 (32.05 %)	231 (70.00 %) 99 (30.00 %)	0.60	1.10 (0.77-1.58)	-	-
	AA AG GG	50 (42.74 %) 59 (50.43 %) 8 (6.84 %)	85 (51.52 %) 61 (36.97 %) 19 (11.52 %)	0.07 0.61	1.00 1.64 (1.00-2.71) 0.72 (0.29-1.76)	0.09 0.60	1.00 1.60 (0.95-2.69) 0.71 (0.28-1.80)
	AA AG + GG	50 (42.74 %) 67 (57.26 %)	85 (51.52 %) 80 (48.48 %)	0.15	1.00 1.42 (0.88-2.29)	0.19	1.00 1.39 (0.85-2.28)
	AA + AG GG	109 (93.16 %) 8 (6.84 %)	146 (88.48 %) 19 (11.52 %)	0.19	1.00 0.56 (0.24-1.34)	0.20	1.00 0.57 (0.23-1.39)
	AA + GG AG	58 (49.57 %) 59 (50.43 %)	104 (63.03 %) 61 (36.97 %)	**0.0243**	1.00 1.73 (1.07-2.81)	**0.04**	1.00 1.69 (1.02-2.78)
haplotypes	CA CG AG AA	61.84 % 22.78 % 9.27 % 6.11 %	63.76 % 17.14 % 12.86 % 6.24 %	0.14 0.23 0.95	1.00 1.44 (0.89-2.33) 0.67 (0.35-1.28) 0.97 (0.41-2.32)	0.25 0.37 1.00	1.00 1.35 (0.81-2.23) 0.74 (0.38-1.44) 1.00 (0.41-2.48)

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses.
OR – odds ratio; CI – confidence interval. Univariate analysis is based on χ^2 test. Multivariate analysis is adjusted for sex, age and HLA-DRB1*15:01 positivity.

ed in both groups; however, this observation is in accordance with data obtained in other populations (Heymann et al., 2003; Andreoli et al., 2007). The absence of the AA variant caused a deviation from Hardy-Weinberg equilibrium (P = 0.047 in MS patients, P = 0.003 in controls).

Regarding the *VLA4* gene polymorphism at position 3061 (A/G), significantly higher frequencies of the AG genotypes were determined in MS patients compared to the control subjects (P = 0.0243, OR = 1.73, 95% CI: 1.07–2.81). Distribution of other genotypes, namely 3061AA and 3061GG, revealed no statistically significant differences between MS patients and controls (Table 1). Frequencies of *VLA4* genotypes at 3061 (A/G) fit the Hardy-Weinberg equilibrium (P = 0.084 in MS patients, P = 0.120 in controls).

Haplotype analysis of *VLA4* gene polymorphisms at both polymorphic positions revealed no statistically significant differences between the MS cohort and the controls (P values ranging from 0.14 to 0.95 as given in Table 1).

HLA-DRB1*15:01 confers the strongest risk for multiple sclerosis (Masterman et al., 2000; Lincoln et al., 2005; Schmidt et al., 2007). We therefore analysed the distribution of rs3135388 that tags the HLA-DRB1*15:01 allele (Hafler et al., 2007; Zivkovic et al., 2009; Benesova et al., 2013). The results obtained in our population sample confirm extremely significantly higher frequencies of the rs3135388 T allele (tagging HLA-DRB1*15:01 positivity) in MS patients compared to the control group as summarized in Table 2. Multivariate analysis of *VLA4* genotypes at 269 (C/A) and 3061 (A/G) adjusted for age, gender and HLA-DRB1*15:01 positivity revealed no significant changes in comparison with the univariate analysis (Table 1). The significantly higher prevalence

*Table 2. Allele and genotype frequencies of rs3135388 tagging HLA-DRB1*15:01*

SNP	Allele/Genotype	MS subjects (N = 117)	Controls (N = 165)	Univariate analysis	
				P	OR (95 % CI)
rs3135388	C T*	170 (72.65 %) 64 (27.35 %)	292 (88.48 %) 38 (11.52 %)	< 0.0001 < 0.0001	0.31 (0.19-0.53) 2.89 (1.86-4.51)
	CC CT* TT*	63 (53.85 %) 44 (37.61 %) 10 (8.55 %)	130 (78.79 %) 32 (19.39 %) 3 (1.82 %)	< 0.0001 0.001 0.01	0.31 (0.19-0.53) 2.51 (1.46-4.29) 5.05 (1.36-18.77)

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses.
OR – odds ratio; CI – confidence interval. Univariate analysis is based on χ^2 test.
* rs3135388 alleles and genotypes tagging HLA-DRB1*15:01 positivity

*Table 3. VLA4 allele and genotype frequencies in HLA-DRB1*15:01 positive MS patients and healthy controls*

SNP	Allele/ genotype	MS subjects (N = 54)	Controls (N = 35)	Univariate analysis		Multivariate analysis	
				P	OR (95% CI)	P	OR (95% CI)
269 C/A	C A	95 (87.96 %) 13 (12.04 %)	57 (81.43 %) 13 (18.57 %)	0.23	0.60 (0.26-1.38)	-	-
	CC CA AA	41 (75.93 %) 13 (24.07 %) 0	22 (62.86 %) 13 (37.14 %) 0	0.19 -	1.00 0.54 (0.21-1.36) -	0.20 -	1.00 0.54 (0.21-1.37) -
	CC CA + AA	41 (75.93 %) 13 (24.07 %)	22 (62.86 %) 13 (37.14 %)	0.19	1.00 0.54 (0.21-1.36)	0.20	1.00 0.54 (0.21-1.37)
3061 A/G	A G	73 (67.59 %) 35 (32.41 %)	47 (67.14 %) 23 (32.86 %)	0.95	0.98 (0.52-1.86)	-	-
	AA AG GG	22 (40.74 %) 29 (53.70 %) 3 (5.56 %)	17 (48.57 %) 13 (37.14 %) 5 (14.29 %)	0.19 0.33	1.00 1.72 (0.69-4.28) 0.46 (0.10-2.22)	0.21 0.35	1.00 1.73 (0.69-4.33) 0.48 (0.10-2.33)
	AA AG + GG	22 (40.74 %) 32 (59.26 %)	17 (48.57 %) 18 (51.43 %)	0.47	1.00 1.37 (0.58-3.24)	0.47	1.00 1.38 (0.58-3.26)
	AA + AG GG	51 (94.44 %) 3 (5.56 %)	30 (85.71 %) 5 (14.29 %)	0.17	1.00 0.35 (0.08-1.58)	0.19	1.00 0.37 (0.08-1.68)
	AA + GG AG	25 (46.30 %) 29 (53.70 %)	22 (62.86 %) 13 (37.14 %)	0.12	1.00 1.96 (0.82-4.68)	0.13	1.00 1.95 (0.81-4.70)
haplotypes	CA CG AG AA	63.37 % 24.59 % 7.81 % 4.22 %	59.78 % 21.65 % 11.21 % 7.36 %	 0.87 0.39 0.39	1.00 1.07 (0.47-2.43) 0.59 (0.18-1.95) 0.48 (0.09-2.53)	 0.90 0.44 0.35	1.00 1.05 (0.46-2.39) 0.62 (0.19-2.07) 0.45 (0.08-2.37)

Allele and genotype frequencies are presented as absolute numbers with percentage in parentheses.
OR – odds ratio; CI – confidence interval. Univariate analysis is based on χ^2 test. Multivariate analysis is adjusted for sex and age.

of 3061AG genotypes in MS patients as compared to the controls was also preserved after the adjustment (P = 0.04, OR = 1.69, 95% CI: 1.02–2.78).

In addition, we performed stratification of MS patients according to the HLA-DRB1*15:01 positivity. Genotyping in the HLA-DRB1*15:01 positive group revealed no statistically significant differences in the distribution of *VLA4* genotypes at 269 (C/A) and 3061(A/G) between the studied groups (Table 3). Similarly, no changes were determined in the haplotype analysis (P values ranging from 0.39 to 0.87), as given in detail in Table 3. After adjustment for sex and gender, there were again no significant changes in the distributions of the *VLA4* gene polymorphism at 269 (C/A) and 3061 (A/G) between MS patients and the control group (Table 3).

The statistical power to detect association at the 0.05 significance level with the given numbers of MS patients and controls was 61.1 % for *VLA4* 3061A/G. The relatively low statistical power can be due to limited sample sizes analysed in our study.

Discussion

Integrins represent important adhesive molecules that mediate migration of leukocytes across the blood brain barrier into tissues. Many studies have shown that these molecules participate in the pathogenesis of chronic inflammation diseases, including MS (reviewed by Anaya et al., 2012). The α4β1 integrin, also known as very late antigen 4 (VLA-4), belongs to the most important members of the β1 integrin subfamily. In 2004, a new drug, natalizumab, a humanized monoclonal antibody binding to the α4 subunit of VLA-4, was approved for the treatment of MS. This VLA-4 antagonist prevents interactions of VLA-4 expressed on immune cells with VCAM-1 on endothelial cells, leading to inhibition of immune cell transmigration into the brain. The drug has been found to be effective in preventing relapses, vision loss, and cognitive decline and it has been recommended as monotherapy for the treatment of highly active relapsing/remitting MS (O'Connor et al., 2005, 2011).

Genetic predisposition of VLA-4 (α4β1 integrin) to chronic inflammatory diseases of CNS, including MS, has been analysed by only a few studies so far (Andreoli et al., 2007; O'Doherty et al., 2007; Correia et al., 2009). In patients with autism, an association was found for the rs155100 SNP (A/T, P = 0.019) located in the intron 9 of the integrin α4 gene and for a number of specific marker haplotypes containing this SNP (0.00053 < P < 0.022) (Correia et al., 2009).

The study of O'Doherty et al. (2007) examined 12 SNPs located in the α4 promoter region and in the introns. Three hundred fifty two MS patients and 235 controls were enrolled in the study. The genotyping confirmed only one significant difference in the distribution of SNP rs1449263 (T/C) in intron 2 between MS patients and controls in the Basque group; other SNP distributions showed non-significant differences in the Basque group and in the Nordic group. Another study was focused on two SNPs in the α4 region of the *VLA4*

gene (269 C/A and 3061 A/G) (Andreoli et al., 2007). Genotyping of 275 MS patients and 255 controls of Italian origin showed no association of the examined *VLA4* polymorphisms to MS risk.

Our analysis of the C to A transversion at position 269 in the promoter region of exon 1 revealed no association to MS pathogenesis and confirmed the results obtained by Andreoli et al. (2007). No homozygous 269 AA genotype could be observed in either cohort, causing the deviation from Hardy-Weinberg equilibrium; however, this finding was also reported by other authors (Heymann et al., 2003; Andreoli et al., 2007). As the 269 (C/A) polymorphism is located in the α4 promoter region near the AP-2 binding sites, the AA variant may be responsible for the negative gene expression causing the functional impairment of the α4 subunit (Hilger-Eversheim et al., 2000).

A point mutation at 3061 in exon 24, which causes an arginine (CGG) to glutamine (CAG) transversion, leads to the formation of two α4 subunit variants. The G variant was named α4-mas and the A variant α4-tex (Szabo and McIntyre, 1995). Surprisingly, in healthy individuals, the frequency of α4-tex is much higher than that of α4-mas, as observed in many studies including ours (Heymann et al., 2003; Andreoli et al., 2007). Regarding the *VLA4* gene polymorphism at 3061 (A/G), in the Slovak population we determined significantly higher frequencies of 3061AG genotypes in MS patients than in the control group. The 3061G variant in MS patients could change the α4 subunit conformations leading to higher affinity binding to its ligand VCAM-1; however, this explanation needs to be proved.

Our findings do not correspond with the results of Andreoli et al. (2007) and O'Doherty et al. (2007), as they were not able to find any association of the 3061AG variant with MS**.** Similarly, the combined analysis of our data with the data published in the two other studies (Andreoli et al., 2007; O'Doherty et al., 2007) failed to confirm a genetic association of the 3061AG variant VLA-4 to MS susceptibility in the Caucasian population (P = 0.7011, OR = 0.9718, 95% CI: 0.85–1.11). This result can be explained by relatively small sample sizes analysed in our study and by genetic differences between the Slovak population and other Caucasian populations.

Our study also confirms that HLA-DRB1*15:01 confers the strongest risk of multiple sclerosis in the Slovak population, and this finding was also reported in many ethnic populations (Hafler et al., 2007; Balnyte et al 2013; Benesova et al., 2013; Isobe et al., 2013). After adjustment for sex, gender and HLA-DRB1*15:01 positivity, there was still significantly higher prevalence of 3061AG genotypes in the Slovak MS patients as compared to the controls. This finding allows us to suggest that the 3061AG variant may be independently related to the pathogenesis of multiple sclerosis. To fully assess the contribution of the *VLA4* gene to MS, further studies are needed. We plan to also examine other SNPs in the *VLA4* gene in the MS patients and investigate their possible influence on the MS development, severity of the disease and treatment.

Conclusions

In conclusion, this is the first study examining the genetic association of VLA-4 to MS susceptibility in the Slovak population. Our results confirm the rs3135388 polymorphism tagging the DRB1*15:01 allele as a strong risk factor for MS susceptibility in the Slovak population. Moreover, our findings suggest the role of the *VLA4* gene, namely the 3061AG variant, as an independent genetic risk factor for multiple sclerosis in Slovaks.

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