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Chemokine receptor V Δ32 deletion in multiple sclerosis patients in Csongrád County in Hungary and the North-Bácska region in Serbia

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Key words: multiple sclerosis, genetics, CCR5 Δ32 allele

Abbreviated title: CCR5 Δ32 deletion in MS

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The roles of chemokine receptor V (CCR5) and its polymorphism, rs333 in multiple sclerosis (MS) are controversial. We investigated the receptor and its deletion in a large MS (428) and a numerous control (831) population in Csongrád County (Hungary) and North-Bácska (Serbia). Taqman probes firstly were used for the allele discrimination. There was no significant difference in genotype (OR=1.092, 95% CI=0.807-1.478, p=0.568 for wt/wt (wt=wild type allele) vs wt/Δ32, Δ32/Δ32 (Δ32=Δ32 base pair deletion allele)) or allele frequency (OR=0.914, 95% CI=0.692-1.207, p=0,525). Neither the deletion nor the wt allele affected the Expanded Disability Status Scale score or the age at onset. Our results indicate no association between the CCR5 Δ32 allele and MS.

Key words: multiple sclerosis, genetics, CCR5 Δ32 allele

Abbreviated title: CCR5 Δ32 deletion in MS

Abbreviations:

CCR5: chemokine receptor V
MS: multiple sclerosis
CNS: central nervous system
MHC: major histocompatibility complex
NK cell: natural killer cell
SNP: single nucleotide polymorphism
HIV: human immunodeficiency virus
RR: relapsing/remitting
SP: secunder progressive
EDSS: expanded disability status scale HWE: Hardy-Weinberg equilibrium BMI: body mass index

Multiple sclerosis (MS) is an autoimmune neurodegenerative disease of the central nervous system (CNS), which appears mostly in the young adult population. The disease affects 2.5 million people worldwide. The lifetime risk is 1 in 400 [1], which makes MS potentially the most common cause of neurological disability in young adults. The aetiology of the disease is only partially known; a genetic predisposition plays a clear role, acting together with unidentified environmental factors and autoimmune inflammatory mechanisms.

Despite the evidence of a genetic background [2-4], only the major histocompatibility complex (MHC) has been identified as a definite susceptibility factor both in case-control and in family-based studies [5-7]. However, the MHC locus is associated not only with MS, but also with other autoimmune diseases. Identification of the risk and protective factors has therefore been at the focus of MS research.

It is well known that the CNS inflammation in MS is characterized by myelin loss, axonal damage and gliosis, and results in progressive neurological dysfunctions. The chemokines (chemoattractant cytokines) and chemokine receptors play key roles in inflammatory processes, because they control the migration of the immune cells and can direct the migration of T-cells across the blood-brain barrier, which is probably the first step towards the development of MS.

The chemokine receptor V (CCR5), a member of the chemokine receptor family and a receptor for MIP-1α/CCL3, MIP-1β/CCL4 and RANTES/CCL5, is at the focus of genetic investigations in MS. The results of a number of studies have indicated that the receptor is upregulated within inflamed brain regions in samples from human MS patients [8-10] and in animal models of experimental autoimmune encephalomyelitis [11, 12]. The CCR5 gene (also known as CKR5, CCR-5, CD195, CKR-5, CCCKR5, CMKBR5, IDDM22 and CC-CKR-5),

located in the 3p21.31 chromosome region [13], consists of 4 exons and 2 introns. It encodes a 352 amino acid protein with a calculated molecular mass of a 40.6 Da. Two different promoters have been found upstream and downstream of exon 1 [14]. Two transcript variants encoding the same protein have been identified. The structure of the protein consists of 7 transmembrane hydrophobic α-helices, an intracellular carboxyl terminus and an extracellular amino terminus with a potential N-linked glycosylation site [15] and because of this conformation it belongs in the G-protein coupled receptor family. The CCR5 is mainly expressed in memory and effector T-lymphocytes, monocytes, macrophages, immature dendritic cells and NK cells, in which it controls cell activation and chemotaxis [16, 17]. In the CNS, the receptor is found on neurones, astrocytes and microglia [15, 18]. The possible interactions and functions of the protein in the CNS were recently reviewed [19].

A single nucleotide polymorphism (SNP) can influence the protein function. The most widely investigated polymorphism of the CCR5 gene is a 32 base pair (bp) deletion, which causes a frame shift mutation in exon 1, thereby affording protection against HIV infection in homozygotic form and resulting in a slower progression and decreased viral load in heterozygotic form [20-22]. This deletion results in a truncated protein (215 vs 352 amino acids), which excludes membrane insertion of the malfunctioining protein [13, 19, 23]. The HIV virus therefore cannot penetrate into the host cells, with the consequence that this deletion provides protection for homozygous individuals.

Investigations of the Δ32 bp deletion in MS samples and controls have revealed conflicting results. In some studies, the allele was concluded to be a risk factor for MS [24-27], whereas in others it seemed to be a protective factor [28-31], and there are results indicating that this mutation is neither a risk nor a protective factor in the case of MS [32-36] (reviewed in Table 4). There have been numerous investigations of the roles of the CCR5 gene and the Δ32

mutation in MS, but only a few which involved large MS and control samples, so our aim was to check their role in a larger MS and almost the biggest control population.

In view of the increasing incidence of MS in Europe and the lack of data regarding the genetic background of the Δ32 allele in the populations of Csongrád County (Hungary) and North-Bácska (Serbia), this study was conducted to determine the influence of the Δ32 deletion in the CCR5 gene in MS patients and healthy controls in these regions.

2.1. MS Patients and Controls

All study participants gave their written informed consent. 428 unrelated clinically definitive MS patients with the relapsing/remitting (RR) or secondary progressive (SP) form of the disease and 831 healthy controls from Csongrád County, Hungary, and the North-Bácska region in Serbia were enrolled to investigate the possible effects of the CCR5 Δ32 deletion. The patients and volunteer controls were enrolled at the Department of Neurology, Faculty of Medicine, University of Szeged, Hungary, the Institute of Preventive Medicine, Faculty of Public Health, University of Debrecen, Hungary, and the Department of Neurology, Subotica Hospital, Subotica, Serbia. Demographic data were collected by means of a questionnaire. All the MS patients in this study met the McDonald criteria and had clinically definitive MS with the RR or SP form [37]. The patient and control groups did not differ from each other in sex ratio ($p=0.942$) or mean age ($p=0.414$). The MS patient group comprised 106 males and 322 females; their average age was 43.74±11.97 years, their average age at the onset of the disease was 32.17±9.80 years and the average Expanded Disability Status Scale (EDSS) score was 2.54±1.92. The MS group consisted of 377 RR patients (91 males and 286 females, average age 42.56±11.70 years) and 51 SP patients (15 males and 36 females, average age 52.49±10.35 years). Of the 831 controls, 204 were males and 626 were females, with an average age of 44.34±13.20 years. The general sociodemographic data are to be seen in Tables 1 and 2. The study protocol was approved by the Medical Research Committee on Science and Research Ethics (No, 35764/2012/EKU (566/PI12)) and was in accordance with the Helsinki Declaration.

In the cases of 3 control samples from Debrecen, there was insufficient DNA sample for the allele discrimination protocol, and for 1 sample the gender information was missing.

2.2.1. DNA isolation

Peripheral blood was used to isolate genomic DNA by a standard desalting method [38]. The purified DNA was stored at -20 °C until further use, while the remaining of the whole blood was stored at -80 °C at the biobank of the Department of Neurology, Faculty of Medicine, University of Szeged (biobank licence: 135/2008). Some of the control samples (DNA) originated from the biobank in Debrecen, where samples are collected from throughout Hungary. Patients and control samples from Subotica, Serbia, were included within the Hungarian-Serbian cooperation programme (HUSRB/1002/214/082/01), which is supported by the EU.

2.2.2. Polymerase chain reaction (PCR) with Taqman probes

For the first time fluorescently labelled Taqman probes were used for the allele discrimination; not only is this technique faster than the standard PCR and gel electrophoresis methods, but less hazardous waste is generated and it is safer, because it does not require ethidium bromide or other intercalating dye. As compared with DNA chip experiments, it is much cheaper, but only one SNP can be investigated, in contrast with DNA chips.

For the amplification of the DNA region near the rs333 SNP, the following primers were used: forward primer: 5'-AAG AAG GTC TTC ATT ACA CC-3', reverse primer: 5'-AGC AGA GTT TTT AGG ATT CC-3'. For the allele discrimination, the following probes were applied: the wild-type allele: 5'-Fam-CAT ACA GTC AGT ATC AAT TCT GGA A-BHQ-1-3', and the deletion allele: 5'-Hex-CTC TCA TTT TCC ATA CAT TAA AGA TAG-BHQ-1-3'.

The PCR amplification was carried out with the following parameters: 95 °C for 3 min, followed by 49 cycles of 95 °C for 10 s, and then 59 °C for 50 s. A genotyping specific master mix was used (PCR Biosystem 2x PCR Bio Gentyping mix Lo-ROX). The PCR experiments were performed with a BioRad CFX96 C1000 real-time thermal cycler machine, and the genotype analysis was carried out with BioRad software (BioRad CFX Manager version 1.6). The results were checked by agarose gel electrophoresis, with exactly the same outcome (bands at 154 bp for the wild types, at 154 and 122 bp for the heterozygotes, and at 122 bp for the homozygotes).

2.2.3. Statistical methods

For evaluation of the data, SPSS software version 20.0 was used. The Chi-square test was utilized for the comparison of the distribution of genotypes and alleles, the t-test for the comparison of the averages of two groups, variance analysis in the event of more than two groups, and two-way variance analysis when there were more than two grouping criteria.

The observed genotype frequencies were in accordance with the Hardy-Weinberg equilibrium (HWE) in both the MS and the control groups.

Of the 428 MS patients, 352 were wild type, 71 were heterozygous and 5 were homozygous for the Δ32 deletion. The Δ32 allele frequency was 9.46%. Among the control samples, 670 were wild type, 146 were heterozygous and 12 were homozygous for the deletion, and the minor allele frequency for this group was 10.26%. The distributions of the genotype and allele frequencies of the CCR5 rs333 polymorphism in the MS patients and the controls are given in Table 3. There was no significant difference between the MS patients and the healthy controls in either the genotype (OR=1.092, 95% CI=0.807-1.478, p=0.568 for wt/wt vs wt/Δ32, Δ32/Δ32) or the allele frequencies (OR=0.914, 95% CI=0.692-1.207, p=0.525). No significant association was found between the rs333 polymorphism and the EDSS score (F=0.282; p=0.755) or the age at onset (F=0.416; p=0.660). Neither the deletion nor the wild- type allele affected the EDSS score (wt allele: F=0.032, p=0.858; Δ32 allele: F=0.564; p=0.453) or the age at onset of the disease (wt allele: F=0.010, p=0.921; Δ32 allele: F=0.821, p=0.365). Not only the genetic predisposition, but also the environmental factors can have a crucial impact on the development of MS. To investigate these effects jointly, the combined influence of the wild type or the deletion allele with alcohol consumption, smoking and body mass index (BMI) was examined in the MS patients, but none of these combinations proved to be significant in relation to the EDSS score or the age at onset. Our results indicate a lack of an association between the CCR5 Δ32 allele and the risk of MS in the Csongrád County (Hungary) and North-Bácska (Serbia) population.

This study investigated the role of the CCR5 Δ32 deletion in a large number of MS patients and the second biggest control population in Csongrád County (Hungary) and North-Bácska (Serbia). This population has not been involved in CCR5 trials previously. The results indicate the lack of an association between the CCR5 Δ32 allele and the risk of MS. These findings are consistent with data from several other studies [32-36, 39-41]. In contrast, a number of previous investigations have concluded that this mutation is a protective factor [28, 30, 31, 42-47] (Table 4). These studies include only one case in which the deletion allele was more common in the control population, as a strong indication of the protection [43]. In the vast majority of the articles, the protection was reflected by the higher age at the onset of the disease [28, 42, 45, 46], by the lower severity [29, 47] or by the better MRI results [30, 31, 44]. The lack or weakness of protection associated with the CCR5 Δ32 deletion allele suggests that the CCR5 receptor does not play an important role in the context of migration of the immune cells into the CNS in MS. However, it is important that the CCR5 Δ32 allele has been suggested to be a risk factor in MS [24-27]. The contradictory results may be due to the inadequate selection of the control or patient groups [26] or both [27]. In those studies, the genotype distribution did not correspond with the HWE. In an investigation of 89 MS patients and 119 controls [26], 71 of the MS patients were wild-type homozygotes, 12 were heterozygotes and 6 were homozygotes for the deletion, while 88 of the controls were wild-type homozygotes, 30 were heterozygotes and 1 was homozygous for the deletion. The result of the HWE test for the patient group was χ^2=15.85, p=0.000068; as the p value was <0.05, the group was not consistent with the HWE, the selected group did not adequately represent the population. In the control group, there was one category (homozygous deletion) with less than 5 members, which means that the value is not accurate (χ^2=0.822, p=0.364). In the Iranian population 254 MS patients and 380 controls were analysed [27]. The genotype

distribution in the MS group was 201 wild-type homozygotes, 16 heterozygotes and 37 homozygous for the deletion. In the control group, the corresponding numbers were 323, 49 and 8, respectively. The result of the HWE test for the MS group was χ^2=156.100, p<0.000001 and that for the control group was χ^2=11.724, p<0.001, so neither the MS nor the control group corresponded with the HWE. In another study, no significant difference in allele frequency or genotype prevalence was observed between the patient and control groups and the allele did not influence the age at onset of the disease [24]. Further investigations revealed that MS patients who carried the deletion died 8.44 years earlier on average. These observations were more significant in females, and the authors concluded that the deletion might serve as a prognostic marker for MS. However, the control group was not studied, and the possibility remains that the allele has a similar effect in healthy controls too. Different results emerged from two studies by one group of authors [42, 48]. In the earlier paper, they concluded that the allele is a protective factor because (CCR5Δ32, DR4) positive phenotype was negatively associated with an early MS onset [42], whereas they later identified a two-allelic combination which is MS associated [48]. The explanation of these different results on the overlapping populations may lie in the difference in the statistical methods used or in the studied MS subtypes. There has been only one article in which the allele was associated with the PP MS subtype, but the number of PP MS patients was only 30, and this outcome should therefore be checked on larger groups. The PP MS subtype is a different entity from the RR/SP MS subtypes, and it is perhaps best to disregard the PP MS results in a consideration of the RR/SP MS subtypes.

Overall, it appears likely that this receptor does not influence the migration of the immune cells into the CNS in MS in general or other receptors can take over this function. It has only a small effect; if the receptor is not functioning (homozygous Δ32 bp deletion), the results indicate only a slight protection or ineffectiveness.

Identification of the main receptor that controls the entry of the immune cells into the CNS in MS is an important question for the pathomechanism and for the pharmaceutical industry. Revealing the main mechanism and the details of the direction of the migration of the immune cells into the CNS are undoubtedly important in the future MS research.

To summarize the results, it seems very likely that the CCR5 receptor has only a weak role in MS. This study has not identified any association of the Δ32 deletion of the CCR5 gene and MS. Our results with a large number of MS patients and the second biggest control population confirm a recently published study which examined the largest MS and control population (Song and Lee 2013).

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Table 1

Sociodemographic data in the two examined groups

Group (n)	Males (%)	Females (%)	Mean age (SD) (years)	EDSS score (SD)	Age at onset (SD) (years)
MS patients (428)	106 (24.8)	322 (75.2)	43.74±11.97	2.54±1.92	32.17±9.80
Controls (831)	204 (24.6)	626 (75.4)	44.34±13.20	-	-

Abbreviations: EDSS = Expanded Disability Status Scale, SD = standard deviation

Sociodemographic data in the two examined MS subgroups

Group (n)	Males (%)	Females (%)	Mean age (SD) (years)	EDSS score (SD) (years)	Age at onset (SD) (years)
RR (377)	91 (24.1)	286 (75.09)	42.56±11.70	2.09±1.52	32.07±9.58
SP (51)	15 (29.4)	36 (70.6)	52.49±10.35	5.87±1.20	32.92±11.32

Abbreviations: EDSS = Expanded Disability Status Scale, SD = standard deviation, RR = relapsing/remitting multiple sclerosis, SP = secondary progressive multiple sclerosis

Genotype and allele frequencies of CCR5 rs333 polymorphism

Group (n)	+/+ (%)	+/Δ32 (%)	Δ32/Δ32 (%)	+ (%)	Δ32 (%)
MS patients (428)	352 (82.2)	71 (16.6)	5 (1.2)	775 (90.53)	81 (9.46)
Controls (828)	670 (81.4)	146 (17.6)	12 (1.4)	1486 (89.73)	170 (10.26)

Abbreviations: +/+ = wild type, +/Δ32 = heterozygote, Δ32/Δ32 = homozygote, + = wild-type allele, Δ32 = deletion allele

Table 4

Summary of the most important results about CCR5 and MS

Population	MS patients (% women)	Controls (% women)		Results	Reference
USA (II), Americans	sporadic 299 (~71%)			Age at onset approximately 3 years higher in patients carrying the	[28]
USA (I), Americans	index cases 120 (~75%)			Age at onset approximately 3 years higher in patients carrying the	[28]
Australian	120			No significant difference in	[39]
Italian (Sicilian)	180 (73%)		Presence of the		[29]
Russian	219 (57%)		(CCR5Δ32, DR4)		[42]
Russian overlaps with Favorova et al, 2002	286 (61.5%)	362 (43.9%)		Two-allelic combination	[48]
USA, Americans	132 (64%)			Associated with early death	[24]
Finnish	116 (57.7%)		CCR5 deletion		[25]
USA, overlaps with Barcellos et al, 2000, Americans	421	96		No significant difference in	[33]
Spanish (other origins)	102		Deletion more		[43]
Spanish (Basque)	62		Deletion		[43]

Population	MS patients (% women)	Controls (% women)	Results	Reference
Finnish, overlaps with Luomala et al, 2003	89	119	Increased frequency of Δ32/Δ32 among all MS patients; the Δ32/Δ32 genotype increased in the PP MS group	[26]
Croatian and Slovenian	325 (71%)	356	No significant difference in allele or genotype frequencies	[34]
Danish, overlaps with Sellebjerg et al, 2007	70	0	CCR5Δ32 shows a trend towards a smaller lesion burden	[44]
Danish	148 (73%)	151	Age at onset lower in patients (who have intrathecal synthesis of IgG oligoclonal band) carrying the deletions	[45]
Danish	109 (75%)	105 (70%)	CCR5Δ32 allele not associated with attack risk in patients treated with IFN-β; patients had higher percentage of CCR5-positive monocytes than controls	[49]
Irish	439	230	The population-based RR/SP MS group was associated with lower age at disease onset	[46]
Dutch, The Netherlands	637 (66%)	177 (54%)	CCR5+303*G and Δ32 low T1 lesion volume, CCR5Δ32 low black hole ratio (T1/T2 lesion volumes), CCR5Δ32 high percentage remyelinating lesions	[30]
USA, African Americans	442	293	No significant difference in allele or genotype frequencies	[35]
Iranian	254 (83.46%)	380	Δ32 allele more frequent among patients, Δ32/Δ32 genotype higher in patients than in controls; might be a risk factor	[27]
Brazilian	124 (68.5%)	127 (44.1%)	MS patients with Δ32 allele gave a lower positive gadolinium-enhancing imaging	[31]
Israelian	256	0	Progression to disability prolonged in CCR5Δ32 homozygotes and heterozygotes compared with MS patients with the wild-type genotype	[47]
German	253	0	No difference in age, sex, EDSS score, disease duration between patients carrying or non-carring the allele; no correlation between CCR5Δ32 and G-protein β3 subunit 825T polymorphism	[40]
German	221 (71.04%)	0	No association with disease severity, age at onset, gender or disease course	[41]

	MS patients		Controls			
	Population (% women)		(% women)		Results	Reference
	94 brain biopsy patients (83 German MS)				0	[41]
	Europeans	1666			No association with disease or disease	[36]
	Iranian	100			No association	[32]