ORIGINAL ARTICLE

NRAMP1 Polymorphism and Viral Factors in Sardinian Multiple Sclerosis Patients

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ABSTRACT: ***Background:*** Multiple sclerosis (MS) is believed to be an autoimmune disease occurring in genetically predisposed individuals after an appropriate environmental exposure such as viral infections. Recent studies suggest a significant association between MS and the functional 5'-(GT)n polymorphism in the promoter region of the *NRAMP1* gene. In the present study we aimed to evaluate the contribution of the allelic variation in the *NRAMP1* promoter to MS susceptibility and to study the role of viral infection in relation to specific *NRAMP1* genotypes, in a Sardinian cohort. ***Methods:*** Sixty MS patients and 66 healthy individuals were genotyped, and screened for the presence of Epstein-bar virus (EBV) and JC virus (JCV) sequences. ***Results:*** Consistent with previous autoimmune disease studies, allele 3 at the functional 5'(GT)n promoter region repeat polymorphism, was significantly overrepresented among MS patients when compared to controls (p=0.02). The EBV and JCV sequences were detected in 8/60 (13.33%) and in 4/60 (6.66%) of MS patients respectively and in 5/66 (7.57%) and in 0/66 of controls. ***Conclusion:*** The allelic variation in the NRAMP1 promoter may contribute to MS susceptibility in the Sardinian population. The viral sequences were not confined to a specific NRAMP1 genotype.

RÉSUMÉ: Le polymorphisme NRAMP1 et les facteurs viraux chez les Sardes atteints de sclérose en plaques. ***Contexte :*** La sclérose en plaques (SEP) est considérée comme une maladie autoimmune qui survient chez des individus qui y sont prédisposés génétiquement, après une exposition environnementale particulière telle une infection virale. Des études récentes suggèrent qu'il existe une association significative entre la SEP et le polymorphisme fonctionnel 5'-(GT)n situé dans le promoteur du gène NRAMP1. Notre but était d'évaluer la contribution de la variation allélique dans le promoteur du gène NRAMP1 à la susceptibilité à la SEP et d'étudier le rôle de l'infection virale en relation à des génotypes NRAMP1 spécifiques dans une cohorte de Sardes. ***Méthodes :*** Soixante patients atteints de SEP et 66 volontaires sains ont été génotypés et on a également recherché la présence de séquences de l'EBV et du JCV. ***Résultats :*** Tel que démontré dans des études antérieures sur les maladies autoimmunes, l'allèle 3 du polymorphisme de répétitions situé dans la région fonctionnelle 5'(GT)n du promoteur était significativement surexprimé chez les patients atteints de SEP par rapport aux témoins (p = 0,02). Des séquences de l'EBV et du JCV ont été détectées respectivement chez 8 (13,33%) et chez 4 (6,66%) des 60 patients atteints de SEP et chez 5 (7,57%) et 0 des 66 témoins. ***Conclusion :*** La variation allélique située dans le promoteur de NRAMP1 pourrait contribuer à la prédisposition à la SEP chez les Sardes. Nous n'avons pas confirmé l'association de séquences virales à un génotype NRAMP1 spécifique.

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Multiple sclerosis (MS) is a chronic inflammatory disease of central nervous system. Although the aetiology of MS remains unclear, both a complex genetic trait with multiple susceptibility-conferring genes as well as environmental agents such as viral infections, have been identified as important risk factors.[1,2]

Concerning the genetic susceptibility to MS, the class II major histocompatibility loci and genes controlling T-cell receptors and cytokines appear to be important. Interestingly a recent study by Kotze et al[3] performed in a genetically homogenous African population of South Africa, suggest an association between MS and the gene encoding the natural resistance-associated macrophage protein 1 (*NRAMP1*). *NRAMP1* (alternate name: solute carrier 11a1; MIM*600266) is an iron transporter at the phagolysosomal membrane of macrophages and neutrophils and associated with macrophage activation.[4,5] To date, a number of polymorphisms at the

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NRAMP1 gene have previously been associated with susceptibility to both these putative infectious agents and to these autoimmune disorders.[6] Among them, a 5′-(GT)n repeat polymorphism in the promoter region of the NRAMP1 gene appear of particular interest, since it has been shown to affect levels of gene expression.[7] *In vitro* studies of this polymorphism suggested direct contribution of alleles to autoimmune (allele 3) and infectious (allele 2) disease susceptibility.[7] Nevertheless, Comabella et al[8] failed to find evidence of association between *NRAMP1* polymorphisms and MS susceptibility in the Spanish population. Since the relative studies are limited we can not exclude the role of the *NRMAP1* gene polymorphisms in MS susceptibility, given that very recently Kissler et al[9] demonstrated in mice that Nramp1 silencing using RNA interference (RNAi) reduced the frequency of type 1 diabetes, and protected against experimental autoimmune encephalomyelitis, a widely used model for multiple sclerosis, further supporting a role for *NRAMP1* in autoimmunity.

On the hand, viruses such as the Epstein-bar virus (EBV) or the JC virus (JCV), that involved in the central nervous system (CNS) infections are attractive candidates as aetiological agents in chronic neurological disorders such as MS.[10,11]

The aim of the present study was to investigate a possible association of *NRAMP1* functional 5′-(GT)n repeat polymorphism and MS risk and to screen the samples for the presence of EBV and JCV in order to assess the significance of these viruses as environmental triggers of MS in genetically predisposed individuals.

Materials and Methods

Patients and samples

Blood samples were obtained with written informed consent from 60 unrelated MS patients of Sardinian origin. All patients had clinically definitive MS according the Poser criteria. Disability was measured using the Kurtzke Expanded Disability Status Scale (EDSS) and graded as mild/moderate (EDSS ≤ 5.5) or severe (EDSS > 6). The unrelated control population consisted of 66 healthy individuals from the same age and population/ethnic group.

NRAMP1 genotyping

The DNA was isolated from blood with the NucleoSpin blood kit (Macherey-Nagel, Germany). To confirm the integrity of the DNA, initially a 430-bp sequence in the human glyceraldehyde-3-phosphatate dehydrogenase gene was amplified.

The 5'-(GT)n repeat polymorphism[12] was defined using polymerase chain reaction (PCR) and automated sequencing analysis of the PCR products using a Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Darmstadt, Germany), and an ABI 377 automated sequencer. The polymorphic region was amplified using the forward 5' GACATGAAGACTCGCATTAG 3' and reverse 5' TCAAGTC TCCACCAGCCTAGT 3' primers.

Amplification of viral sequences

For detection of EBV DNA, the primer sequences 5'-TCGCGTTGCTAGGCCACCTT-3' and 5'-CTTGGATGGC GGAGTCAGCG-3' representing nucleotide positions 1043-1062 and 1319-1338 at the BamHI-W region of the EBV genome, respectively were used. The sample reagent mixtures were preheated to 94°C in a thermal cycler for ten minutes, ran at 94°C for 20 seconds, 60°C for 20 seconds, and 72°C for 20 seconds for 35 cycles, and then 72°C for seven minutes followed by soaking at 15°C for cooling.

For detection of JCV sequence, a 277-bp fragment was amplified in a nested PCR assay. For the first round the primers JCDAL-1: 5'-TCA TGT GGA TGC TGT CAA CC-3' and JCDAL-3: 5'-CTG TCT ACA CAG GGC ACT AT-3' given a product of 369 bp were used. Reactions of 50 µl were heated at 94°C for five minutes then cycled 40 times of denaturation at 94°C for 30 seconds, annealing at 57ºC for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension step at 72ºC for five minutes. PCR products were then used as template in a second round reaction using primers JCDAL-2: 5'-TGC TAC AGT ATC AAC AGC CT-3' and JCDAL-4: 5'-TGG GTT AAA GTC ATG CTC CT-3' to produce a 277-bp fragment. Cycling in this case was: an initial denaturation step at 94ºC for five minutes, followed by 40 cycles at 94ºC for 30 seconds, 55 ºC for 30 seconds and 72ºC for 30 seconds and a final extension step at 72ºC for five minutes. In all cases negative control contained H_2O without DNA sample was used.

Statistical analysis

Statistical analysis was performed by GraphPad InStat (version 3.00, GraphPad Software, Inc., San Diego, CA, USA). Two tailed Fisher exact test and the χ^2 calculation were applied as appropriate.

Results

The MS patients had a mean age of 44.52 ± 11.23 years, mean age at disease onset 30.72 ± 10.61 and median EDSS of 3.51 (range 0-8). There were 47 women and 13 men. There were 28 relapsing-remitting MS (RRMS) patients, 18 secondary progressive MS (SPMS) patients and 14 primary progressive MS (PPMS) patients.

As shown in the Table, the homozygous genotype allele 3/allele 3 as well as the allele 3 at the functional 5'(GT)n repeat polymorphism, were associated with higher risk of MS when compared to control (OR = 2.49; $p = 0.017$ and OR = 1.80; $p = 0.02$, respectively). Alleles 4, 6, and 7 were not identified within the Sardinian population studied.

When based on EDSS score (mild/moderate and severe disability) the MS cases were divided into two groups, no differences in the genotype and allele frequencies of 5'-(GT)n polymorphism were observed between groups. Additionally, analysis of association between the 5'-(GT)n polymorphisms and the clinical form of the disease (RRMS, SPMS and PPMS) showed no differences between groups.

The EBV and JCV sequences were detected at a low frequency in controls and MS patients. Specifically, in MS patients, EBV and JCV were detected in 8/60 (13.33%) and in 4/60 (6.66%) respectively, and in controls EBV and JCV were detected in 5/66 (7.57%) and in 0/66 respectively. Viral sequences were not confined to a specific genotype for the *NRAMP1* promoter polymorphism.

Table: Genotype and allele frequencies of 5'-(GT)n *NRAMP1* gene polymorphisms in MS patients and healthy controls

	Genotype frequencies		
	Controls (n=66)	**MS patients (n=60)**	***p*; OR (95% CI)**
Allele 1/ Allele 1	1 (1.51%)	0	1; 0.36 (0.01-9.03)
Allele 1/ Allele 2	2 (3.03 %)	1 (1.67%)	1; 0.54 (0.05-6.14)
Allele 1/ Allele 3	1 (1.51%)	0	1; 0.36 (0.01-9.03)
Allele 1/ Allele 5	0	1 (1.67%)	0.47; 3.35 (0.13-83.95)
Allele 2/ Allele 2	13 (19.69%)	10 (16.67%)	0.82; 0.81 (0.33-2.03)
Allele 2/ Allele 3	14 (21.21%)	10 (16.67%)	0.65; 0.74 (0.30-1.83)
Allele 2/ Allele 5	4 (6.06%)	4 (6.67%)	1; 1.11 (0.26-4.64)
Allele 3/ Allele 3	18 (27.27%)	29 (48.33%)	0.017; 2.49 (1.18-5.24)
Allele 3/ Allele 5	9 (13.64%)	4 (6.67%)	0.25; 0.45 (0.13-1.55)
Allele 5/ Allele 5	4 (6.06%)	1 (1.67%)	0.37; 0.26 (0.03-2.42)
	Allele frequencies		
	Controls (n=66)	**MS patients (n=60)**	***p*; OR (95% CI)**
Allele 1	5 (3.78%)	2 (1.67%)	0.30; 0.43 (0.08-2.26)
Allele 2	46 (34.84%)	35 (29.16%)	0.33; 0.77 (0.45-1.31)
Allele 3	60 (45.45%)	72 (60%)	0.02; 1.80 (1.09-2.97)
Allele 5	21 (15.91%)	11 (9.16%)	0.11; 0.53 (0.24-1.16)

OR = odds ratio; CI = confidence interval

Discussion

In the present study, the 5'-(GT)n polymorphism in the promoter of *NRAMP1* gene was analyzed as a candidate polymorphism for MS susceptibility in a Sardinian population. We found a higher incidence of allele 3 of the 5'-(GT)n *NRAMP1* promoter polymorphism in MS patients. When our MS cases were analyzed based on EDSS score and clinical form of the disease, no differences in the frequencies of *NRAMP1* alleles were found in patients with severe disability and disease relapse.

Our results concerning the contribution of 5'-(GT)n polymorphism in MS susceptibility are partly in agreement with the findings of Kotze et al[3] that support the contribution of allele 5 of the 5'-(GT)n polymorphism in MS susceptibility, in a South African population. In our study, whereas we find a much higher frequency of allele 5 in our population compared to the South African study (15.91% versus 3% in MS patients)[3] we observed that the allele 3 is significant overrepresented in Sardinian MS patients. Kotze et al[3] and our results opposed in the finding of Comabella et al.[8] This discrepancy may be explained by the small sample size used, differences in environmental, genetic and ethnic background of populations. Further studies in larger populations of different races, ethnic backgrounds and environmental exposures are needed to clarify this issue. Nevertheless, our results are in agreement with previous findings suggested that allele 3 of *NRAMP1* promoter polymorphism linked to autoimmune, whereas allele 2 to infectious diseases.[13]

Concerning the viral infections in MS, EBV is one of the viruses associated with MS in adults,[14] and more recently with MS in children.[15] However, the causative role of EBV in adult onset MS is challenged by the inherent delay between early life exposure to the virus and presentation of MS. Additionally, JCV it is know that can be reactivated from its latent state at a time of immunosuppression induced by immune impairments or treatments leading to progressive multifocal leucoencephalopathy. The possibility that JCV may also replicate in the brains of other patients with demyelinative diseases of the CNS, as MS cannot be excluded.[16] Since the incidence of EBV and JCV in the sample tested was low, we can not support a causative role of these viruses for MS in the Sardinian population. Although, our samples size was too small to safely exclude the involvement of EBV and JCV in MS pathogenesis, viral sequences found did not correspond to a specific *NRAMP1* allele in MS patients. Our findings concerning the EBV implication in MS pathogenesis in relation to *NRAMP1* genotypes are in agreement with de Villiers et al.[17]

In view of the emerging role of polymorphisms in complex diseases, and the functional significance of *NRAMP1* promoter polymorphism in autoimmune and infectious disease predisposition, we conclude that the allelic variation in *NRAMP1* promoter may contribute to MS susceptibility in the Sardinian population. Since our sample size is very small, further epidemiological studies on enlarged sized samples are required to determine whether the *NRAMP1* polymorphisms are of primary importance in susceptibility to MS.

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