NRAMP1 Polymorphism and Viral Factors in Sardinian Multiple Sclerosis Patients

Maria Gazouli, Leonardo Sechi, Daniela Paccagnini, Stefano Sotgiu, Giannina Arru, George Nasioulas, Dimitrios Vassilopoulos

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.


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. To date, a number of polymorphisms at the

From the Department of Neurology, School of Medicine, University of Athens (MG, DV); Molecular Biology Research Center HYGEIA “Antonis Papayiannis” (GN), Athens, Greece; Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica, Università degli Studi di Sassari (LS, DP); Institute of Clinical Neurology, University of Sassari (SS, GA), Sassari, Italy. Received January 31, 2008. Final revisions submitted April 16, 2008.

Correspondence to: Maria Gazouli, School of Medicine, University of Athens, Michalakopoulou 176, 11527 Goudi, Athens, Greece.

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. 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 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).

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NRAMP1 gene have previously been associated with susceptibility to both these putative infectious agents and to these autoimmune disorders. 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. In vitro studies of this polymorphism suggested direct contribution of alleles to autoimmune (allele 3) and infectious (allele 2) disease susceptibility. Nevertheless, Comabella et al. 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 NRAMP1 gene polymorphisms in MS susceptibility, given that very recently Kissler et al 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 aetiologiag agents in chronic neurological disorders such as MS.

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-phosphate dehydrogenase gene was amplified.

The 5′-(GT)n repeat polymorphism 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′-TCAAGTCT-3′ primers.

Amplification of viral sequences

For detection of EBV DNA, the primer sequences 5′-TCCGCTTGTCTAGGCCACCTT-3′ and 5′-CTTTGATGGCGGGAGTCAGCG-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 TGG TGT CAA CC-3′ and JCDAL-3: 5′-CTG TCT 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 57°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension step at 72°C for five minutes. PCR products were then used as template in a second round reaction using primers JCDAL-2: 5′-CTG 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 94°C for five minutes, followed by 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds and a final extension step at 72°C for five minutes. In all cases negative control contained H2O 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 χ² 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

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>Controls (n=66)</th>
<th>MS patients (n=60)</th>
<th>p; OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele 1/Allele 1</td>
<td>1 (1.51%)</td>
<td>0</td>
<td>1; 0.36 (0.01-9.03)</td>
</tr>
<tr>
<td>Allele 1/Allele 2</td>
<td>2 (3.03 %)</td>
<td>1 (1.67%)</td>
<td>1; 0.54 (0.05-16.4)</td>
</tr>
<tr>
<td>Allele 1/Allele 3</td>
<td>1 (1.51%)</td>
<td>1 (1.67%)</td>
<td>1; 0.36 (0.01-9.03)</td>
</tr>
<tr>
<td>Allele 1/Allele 5</td>
<td>0</td>
<td>1 (1.67%)</td>
<td>0.47; 3.35 (0.13-83.95)</td>
</tr>
<tr>
<td>Allele 2/Allele 2</td>
<td>13 (19.69%)</td>
<td>10 (16.67%)</td>
<td>0.82; 0.81 (0.33-2.03)</td>
</tr>
<tr>
<td>Allele 2/Allele 3</td>
<td>14 (21.21%)</td>
<td>10 (16.67%)</td>
<td>0.65; 0.74 (0.30-1.83)</td>
</tr>
<tr>
<td>Allele 2/Allele 5</td>
<td>4 (6.06%)</td>
<td>4 (6.67%)</td>
<td>1; 1.11 (0.26-4.64)</td>
</tr>
<tr>
<td>Allele 3/Allele 2</td>
<td>18 (27.27%)</td>
<td>29 (48.33%)</td>
<td>0.017; 2.49 (1.18-5.24)</td>
</tr>
<tr>
<td>Allele 3/Allele 3</td>
<td>9 (13.64%)</td>
<td>6 (10.00%)</td>
<td>0.25; 0.45 (0.13-1.55)</td>
</tr>
<tr>
<td>Allele 3/Allele 5</td>
<td>4 (6.06%)</td>
<td>1 (1.67%)</td>
<td>0.37; 0.26 (0.03-2.42)</td>
</tr>
</tbody>
</table>

Allele 1/Allele 1  5 (3.78%)  2 (1.67%)  0.30; 0.43 (0.08-2.26)  
Allele 2/Allele 2  46 (34.84%)  35 (29.16%)  0.33; 0.77 (0.45-1.31)  
Allele 3/Allele 3  60 (45.45%)  72 (60%)  0.02; 1.80 (0.99-3.97)  
Allele 5/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 autoimmunity, 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 is known that can be reactivated from its latent state at a time of immunosuppression induced by immune impairments or treatments leading to progressive multifocal leukoencephalopathy. The possibility that JCV may also replicate in the brains of other patients with demyelinating diseases of the CNS, as MS cannot be excluded.16 Since the incidence of EBV and JCV in the sample tested was low, we cannot support a causative role of these viruses for MS in the Sardinian population. Although, our sample 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.

REFERENCES


