Journal of Neuroimmunology 210 (2009) 100–103

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

CASP-9: A susceptibility locus for multiple sclerosis in Italy

V. Andreoli [a,*], F. Trecroci [a], A. La Russa [a], P. Valentino [b], F. Condino [a], V. Latorre [a], R. Nisticò [b], D. Pirritano [b], F. Del Giudice [b], M. Canino [b], R. Cittadella [a], A. Quattrone [a,b]

[a] Institute of Neurological Sciences, National Research Council, Pianolago di Mangone, Cosenza, Italy
[b] Institute of Neurology, Campus di Germaneto, University "Magna Graecia", Catanzaro, Italy

ARTICLE INFO

Article history:
Received 10 December 2008
Received in revised form 24 February 2009
Accepted 5 March 2009

Keywords:
Multiple sclerosis
CASP-9
Polymorphism
Association study
Genetic susceptibility

ABSTRACT

Caspase-9 is a primary effector *CASP* that executes programmed cell death, which plays an important role in the development of multiple sclerosis (MS). Polymorphisms in the *CASP-9* gene may influence its activity, thereby modulating the susceptibility to MS. To test this hypothesis, we evaluated a SNP in the *CASP-9* gene in a set of Italian patients from Southern Italy and healthy control subjects. Our results suggest that the presence of the G/G genotype represents a higher risk factor in our MS population and a differential production of *CASP-9* might be a contributory factor in determining the severity of MS.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of undetermined aetiology, affecting mainly the white matter of the central nervous system (CNS) and is clinically characterised by progressive disability (Steinman, 1996). In recent years, several data have confirmed the presence of axonal damage in MS and have shown that the disability caused by the disease better correlates with axonal loss than it does with extensive demyelination (Trapp et al., 1998; Noseworthy et al., 2000). Although the initiating event is a matter of debate, epidemiological and genetic findings suggest that MS is an acquired autoimmune and inflammatory disease, triggered by unknown environmental factors in genetically susceptible individuals (Compston and Coles, 2002). These results demonstrate that individuals might be predisposed to MS as a result of the inheritance of many genetic factors of modest contribution that, if revealed, may present important targets for new therapies (Stewart, 1997; Oksenberg et al., 2001). What seems certain is that MS is a disease with heterogeneous pathogenic mechanisms, and several studies support the argument that MS is a primary disease of either axons, neurons or oligodendrocytes and that immune response is secondary to neurodegeneration (Trapp et al., 1998; Bö et al., 2003). Recently, neuronal apoptosis has been described in cortical MS lesions (Peterson et al., 2001) and in experimental autoimmune encephalomyelitis (EAE), a rat model of MS (Meyer et al., 2001). The apoptotic programme is executed by a family of essential proteases known as caspases (Nicholson and Thornberry, 1997). So far, at least two main caspase-activating cascades have been characterised: the mitochondria-mediated caspase-3 activation by caspase-9 (intrinsic pathway) and death-receptor-induced caspase-3 activation by caspase-8 (extrinsic pathway) (Zheng and Flavell, 2000). Particularly, caspase-9 (*CASP-9*) plays a crucial role in the initiation phase of the intrinsic pathway for apoptosis. In fact, many proapoptotic stimuli engage the apoptotic machinery in the cells by causing the release of cytochrome-*c* from mitochondria, which then induces oligomerisation of a protein called Apoptotic protease activating factor-1 (Apaf-1) and recruitment of *CASP-9* into a large complex known as the apoptosome. Apoptosome then activates the *CASP-9* cascade downstream with effector caspases, leading to apoptosis (Srinivasula, 1998; Li et al., 1997). The mechanism is evolutionarily conserved and may play an important role in mediating neuronal death; dysregulation of this normal control mechanism then could be a contributor to various diseases characterised by excessive or inadequate cell death. These findings raise the intriguing possibility that genetic variations in the *CASP-9* gene could influence susceptibility to the disease. *CASP-9*, mapped to the short arm of chromosome 1p36 (Hadano et al., 1999) in humans, is composed of nine exons; some polymorphisms have also been described within this gene. However, the potential role of the single nucleotide polymorphism (SNP) of the *CASP-9* gene in establishing susceptibility to MS has never been clearly defined. In particular, a SNP in the coding region *CASP-9* Ex5+32G>A causes a conservative change of a glutamine with an arginine (Q221R) (Hirano et al., 2001) and, thus, may have functional significance. In order to shed light on a

* Corresponding author. Institute of Neurological Sciences, National Research Council, Pianolago di Mangone, 87050, Cosenza, Italy. Tel.: +39 09849801300; fax: +39 0984969306.
E-mail address: v.andreoli@isn.cnr.it (V. Andreoli).

0165-5728/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2009.03.013

biologically potential role of the *CASP-9* gene in susceptibility to MS, we have genotyped this SNP in a collection of MS patients and healthy control subjects from Southern Italy. This is the first study to investigate possible relationships between MS and *CASP-9* gene polymorphisms in Italian patients.

2. Materials and methods

2.1. Patients and healthy individuals

Our study sample consisted of 295 unrelated cases with clinically defined MS (Poser et al., 1983; McDonald et al., 2001), all Caucasians from the south of Italy (Calabria), and followed at the Institute of Neurology, University "Magna Graecia" of Catanzaro. Patients whose disease course was classified (Lublin and Reingold, 1996) as Relapsing–Remitting MS (RRMS, $n=215$, 73.0%), Secondary Progressive MS (SPMS, $n=62$, 21.0%) or Primary Progressive MS (PPMS, $n=18$, 6.0%) were enrolled in this study. One hundred eighty-five patients were women and 110 were men. The following clinical and genetic variables were recorded for each patient: age, sex, disease duration, age at disease onset and level of disability according to the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). Clinical characteristics of the MS patients are summarised in Table 1. The control population included 295 unrelated healthy subjects (130 women and 165 men; mean age ± SD: 35.3 ± 8.2 years), enrolled during a previous study without history of inflammatory and/or degenerative neurological diseases. To avoid any bias attributed to the ethnic origin of the study population, only Caucasian MS patients and controls whose grandparents were all born in Calabria were included in the analysis. The differences in the sex ratio and ages between the patients and the controls were not significant ($p>0.05$). Patients and controls gave their written informed consent before the examination and blood testing, and the study was approved by the local ethical committee.

2.2. CASP9 Ex5 +32G>A genotyping

Blood samples for the genomic DNA studies were obtained from peripheral blood leukocytes and DNA was extracted according to standard procedure. In principle, DNA was amplified using PCR in a total volume of 50 μl containing 15 pmol of each primer, designed according to the published sequence (Hirano et al., 2001), 200 ng genomic DNA and AmpliTaq Gold (Applied Biosystems), and using standard conditions on a PTC-100™ Programmable Thermal Controller (MJ Res. Inc. Genenco). Lastly, a 185 bp fragment containing the A→G transversion in exon 5 was amplified using the primers F:5′-CGGTCCAGTCTGCATCTAGAC-3′ and R:5′-ATGCCTGCCCAGGG AACAGT-3′ (annealing temperature 59 °C). Ten microlitres of the PCR product were incubated with 10 U BstUI (New England Biolabs, Beverly, MA, USA) in a total volume of 25 μl for 3 h at 60 °C. This gave products that either remained intact (allele A) or were cut into two fragments of 94 and 91 bp (allele G). Both digested and undigested DNA fragments were visible for the respective restriction site in heterozygous samples. Amplified DNA fragments and digestion products were separated on 3% agarose gels and visualised by ethidium bromide.

Table 1
Demographic and clinical variables of MS subjects analysed.

Variable	Patients (*n*: 295)
Male sex: no. (%)	110 (37.3)
Age (yr): mean (SD)	39.8 ± 12.0
Age at onset (yr): mean (SD)	28.1 ± 9.1
Disease duration (yr): mean (SD)	13.5 ± 9.4
Disease course: no (%)	215 (73.0)
RR	62 (21.0)
SP	18 (6.0)
PP	
EDSS score: median (range)	3.3 (0–8.5)

Data are given as means ± SD and percentage.
EDSS: Expanded Disability Status Scale.

Table 2
CASP9 Ex5 + 32G>A genotypes and alleles of cases and controls and their association with the risk of MS.

Ex5 + 32G>A (Q221R)	Controls ($n=295$)	Patients ($n=295$)	OR (95%CI)	*p* value
Genotype	No. (%)	No. (%)		
A/A	116 (39.3)	65 (22.0)	1.0	
A/G	134 (45.4)	154 (52.2)	2.03 (1.34–3.07)	
G/G	45 (15.3)	76 (25.8)	2.73 (1.63–4.55)	<0.001
Allele				
A	366 (62.0)	284 (48.1)	1.0	
G	224 (38.0)	306 (51.9)	1.68 (1.31–2.16)	<0.001

Categorical variables are expressed as frequency and percentage.
The differences among groups distribution were assessed using χ^2 test.
Odds ratios and 95% confidence intervals were calculated according to a multivariate logistic-regression model, adjusted for age and sex.

2.3. Data analysis

The Hardy–Weinberg equilibrium was tested using Pearson's χ^2 goodness of fit test for *CASP-9* Ex5 + 32G>A polymorphism. Using univariate analyses for this bi-allelic marker individual *p*-values were calculated using standard 3×2 and 2×2 χ^2 contingency tables comparing genotype and allele counts in MS cases against controls. Categorical variables were expressed as counts and percentages, continuous variables were shown by mean and standard deviation and discrete variables by median and range. Relative risks for MS, estimated as the odds ratios [ORs] and 95% confidence intervals (95% CI), were calculated by multivariate logistic-regression analysis, adjusting for sex and age. To evaluate possible differences in clinical features among each + 32G>A polymorphism variant in the patients group, one-way analysis of variance (ANOVA test) or Kruskal–Wallis test was used. Differences in sex distributions among groups were evaluated with the χ^2 test. In all tests, a *p*-value less than 0.05 denoted the presence of a statistically significant difference. Statistical analyses were performed with Statistical Package for Social Sciences software (SPSS version, 12.0, Chicago, IL, USA) for Windows.

3. Results

3.1. CASP9 Ex5 +32G>A genotypes and susceptibility to MS

In our study, control and patient sample genotype frequencies were distributed according to Hardy–Weinberg equilibrium (controls, $p=0.54$; MS, $p=0.48$). Table 2 depicts the allele and genotype frequency distribution and association of the studied polymorphism in MS individuals. We found that the frequency of the G/G homozygote in MS patients (25.8%) was significantly higher than in the controls (15.3%), and the subjects who carried this genotype had a 2.73-fold increased risk for developing MS ($p<0.001$, OR = 2.73, 95% CI = 1.63–4.55). In the public databases the A allele is reported to be the largest allele of this SNP (rs1052576) in the European population. Here, *CASP-9* A allele was observed to be significantly high in our controls (62.0%), providing a reduced risk; the G allele was also over-represented in MS patients (51.9%) in comparison with the controls (38.0%, $p<0.001$, OR = 1.68, 95% CI = 1.31–2.16). Our results show that the *CASP-9*-G allele was associated with increased predisposition to MS. These data emphasize that the striking associations observed for this genetic variation in *CASP-9* genes may play an important role in the aetiology of MS.