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Heat shock protein 70-hom gene polymorphism and protein expression in multiple sclerosis

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ABSTRACT

Immune-mediated and neurodegenerative mechanisms are involved in multiple sclerosis (MS). Growing evidences highlight the role of HSP70 genes in the susceptibility of some neurological diseases.
In this explorative study we analyzed a polymorphism (i.e. HSP70-hom rs2227956) of the gene HSPA1L, which encodes for the protein hsp70-hom.
We sequenced the polymorphism by polymerase chain reaction (PCR), in 191 MS patients and 365 healthy controls. The hsp70-hom protein expression was quantified by western blotting.
We reported a strong association between rs2227956 polymorphism and MS risk, which is independent from the association with HSP70-2 rs1061581, and a significant link between hsp70-hom protein expression and MS severity.

1. Introduction

Multiple sclerosis (MS) is a disimmune-mediated neurodegenerative disease characterized by demyelinated lesions scattered throughout the white and the grey matter of the brain and the spinal cord (Compston and Coles, 2008). The precise aetiology of MS is actually unknown but evidences suggest that demyelinated lesions are induced by different immunological mechanisms, including components of both the adaptive and the innate immune system (Mallucci et al., 2015). Oxidative stress is also involved in MS pathogenesis and progression by direct and indirect mechanisms of action(Lassmann, 2014), which also encompass the heat shock proteins 70 (hsp70s) family(Mansilla et al., 2012).

Intracellular hsp70s act as chaperones (i.e. assistant proteins that prevent proteins mis-folding and/or aggregation) and anti-apoptotic proteins (Mayer, 2013). Extracellular hsp70s process and present antigens, promoting the activation of immune system (Li et al., 2002). Polymorphisms leading to either quantitative or qualitative change in hsp70 expression likely affect both the hsp70 cyto-protective and/or immune-modulatory effects.

Among the several proteins included in hsp70s family, the two major stress-inducible members (i.e. the Hsp70-1 and Hsp70-2) are encoded by HSPA1A and HSPA1B gene respectively, and the constitutively expressed non-inducible protein (i.e. Hsp70-hom) is encoded by HSPA1L gene (Brocchieri et al., 2008). These three genes are located on chromosome 6 (6p21.3) (Milner and Campbell, 1990), within the human leukocyte antigen (HLA) class III region. Polymorphisms of the HLA class III have not yet been included in genome wide association studies aimed at studying MS genetic susceptibility, which actually have found >110 known MS risk variants in and out HLA class I and II region (International Multiple Sclerosis Genetics Consortium, Nat Genet 2013; Moutsianas et al., 2015). The HSPA1L gene located within the HLA class III region may be a good candidate gene for the analysis of MS genetic susceptibility adding some information on MS genetic risk. Those results will be validated in bigger studies.

While polymorphisms within HSPA1A exons are silent (Milner and Campbell, 1992), we recently demonstrated that +1267 A/G HSPA1B (rs1061581) polymorphism is associated with an increased MS risk and MS patients with GG or GA genotype display a significant reduction of hsp70-2 expression compared to patients with AA genotype (Boiocchi et al., 2014). Polymorphisms in the HSPA1L gene are mainly located in the region coding for the substrate-binding domain. Among

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these, HSP70-hom rs2075800 has been associated with rheumatoid arthritis (Jenkins et al., 2000) and uveitis in patients with sarcoidosis (Spagnolo et al., 2007) and HSP70-hom rs2227956 has been linked to Loegren's syndrome and sarcoidosis (Bogunia-Kubik et al., 2006).

The aims of this study were to analyze the effect of +2437 HSP70-hom (rs2227956) polymorphism on MS risk and MS severity and its contribution to oxidative stress, in order to better define the real contribution of the gene HSPA1L as a MS onset/severity genetic susceptibility locus.

2. Material and methods

2.1. Subjects and ethics statement

We consecutively included in this study 195 Caucasian patients with a diagnosis of MS according to the 2010 revised McDonald Criteria (Polman et al., 2011), patients were recruited from the MS Centre of the National Neurological Institute "C. Mondino" (Pavia, Italy). The neurological disability of MS patients was quantified by the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) while the clinical impact of MS was calculated applying the Multiple Sclerosis Severity Score (MSSS) (Roxburgh et al., 2005) which relates scores on EDSS to disease durations. From our extended clinical experience, we arbitrary chose a cut–off value of an MSSS of 3: MS patients with a MSSS < 3 were considered affected by a mild form of disease; MSSS ≥ 3 reflected instead patients with a moderate to severe form of MS. The control population includes 439 Caucasian subjects. Controls were randomly selected from healthy individuals, as judged by regular checks, attending the National Neurological Institute C. Mondino. MS patients and controls demographical and clinical characteristics were recorded when blood samples were collected and are listed in Table 1 for the subjects with no missing information included in the analyses.

The study has been approved by local ethics committees and has been conducted in accordance with principles expressed in the Declaration of Helsinki.

2.2. Gene polymorphism analysis

Human genomic DNA was obtained from 200 μl of whole blood [collected by venipuncture in Vacutainer tubes containing ethylenedinitrilotetraacetic (EDTA, BD)], using the QIAamp DNA Blood Mini Kit (QIAGEN) following the manufacturer's instructions. The concentration and purity of DNA was determined by spectrophotometric analysis. In order to establish alleles and genotypes for the investigated polymorphism, +2437 HSP70-hom (rs2227956 C) and +1267 HSP70-2 (rs1061581 G) we used a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Amplification was performed using I-Cycler (BioRad). For the +12437 T/C HSP70-hom and +1267 A/G HSP70-2 polymorphisms, and the following amplification protocol was applied: initial incubation at 95 °C for 5 min, followed by 35 cycles each of 95 °C for 30 s, 53 °C for 1 min, 72 °C for 1 min and 30 s; final extension is carried out at 72 °C for 7 min. The PCR products were visualized by electrophoresis in 2% agarose gel (Agarose Standard, Eurobio). Genotypes were determined by digestion with an appropriate restriction enzyme. The restriction patterns were obtained by gel electrophoresis in 3% agarose gel (Table 1 supplement). To confirm the reliability of our data obtained by PCR- RFLP we performed further genotyping by RealTime PCR. Genotyping was performed with TaqMan® SNP Genotyping Assays on LightCycler 480 system (LC480) (Roche S.p.A., Milano, Italy). Assays were purchased from Applied Biosystem (C__25630755_10). The overlap of the data was 100%.

2.3. Peripheral blood mononuclear cells (PBMCs) isolation from whole blood

Five milliliter of blood were diluted 1:1 with Ficoll (Histopaque-1077, Sigma-Aldrich) and centrifuged at 450 ×*g* for 30 min. PBMCs above the Ficoll ring were harvested and washed twice with phosphate buffered saline 1× (PBS). The cellular pellets were stored at −80 °C until further analysis.

2.4. Western blotting

PBMCs from MS patients and controls were collected and homogenized in a buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 50 mM 2-mercaptoethanol, 0.32 mM sucrose, and a protease inhibitor cocktail at the dilution suggested by the manufacturer (Roche Molecular Biochemicals), by using a Teflon/glass homogenizer and sonicating twice for 10 s. The protein content was measured via Bradford's method using bovine serum albumin (Sigma Aldrich) as standard. Proteins were diluted in sodium dodecyl sulfate (SDS) protein gel loading solution 2×, boiled for 5 min, separated by 12% SDS-polyacrylamide gel electrophoresis, and then processed as previously described. The mouse anti-HSP70-hom antibody (Enzo Lifescience) was diluted at 1:1000, and the mouse anti-α-tubulin (Sigma-Aldrich) at 1:1000. All the antibodies were diluted in TBST buffer [10 mM Tris-HCl, 100 mM NaCl, 0.1% (*v*/*v*) Tween 20, pH 7.5] containing 6% (*v*/*v*) milk. The nitrocellulose membrane signals were detected by chemiluminescence. The experiments were performed in duplicate for each sample using α-tubulin to normalize the data. The analysis was performed on the densitometric values obtained using the V1.62 NIH Image software after image acquisition.

2.5. Statistical analysis

To assess the association between the +2437 T/C HSP70-hom polymorphism and MS we performed an unconditional logistic regression analysis adjusted by sex and age; adjusted Odds Ratios (OR) with 95% confidence intervals (95% CI) were derived and used as measure of effect. An additive allelic model and a genotypic model were fitted to estimate i) the C allele risk; ii) the heterozygous TC versus wild type TT risk and iii) the homozygous CC versus wild type TT genotype risk. Moreover, allele frequencies in controls were examined to detect any significant deviation from the Hardy–Weinberg Equilibrium using a

Table 1
Demographic and clinical characteristic of MS patients and controls. Data are expressed as number of subjects and percentage (%) or mean ± standard deviation. *t*-Test for age and χ^2 test for gender are calculated to compare cases and controls, $p < 0.05$ is considered statistically significant. § Comparison of age or sex among MS patients and MS patients sub-group is not significant ($p > 0.05$). † Comparison of age or sex among controls and controls sub-group is not significant ($p > 0.05$).

	MS patients	Controls		MS patients sub-group	Controls sub-group	
	($n = 191$)	($n = 365$)	*p*-Value	($n = 47$)	($n = 29$)	*p*-Value
Age (years)	44.1 ± 10.7	35.7 ± 8.6	<0.001	45.4 ± 11.33§	39.3 ± 13.1†	>0.05
Sex (F/M ratio)	1.81	1.31	<0.05	1.61§	0.70†	<0.01
MS duration (years)	12.6 ± 8.3			12.0 ± 7.5§		
EDSS	2.66 ± 2.14			2.97 ± 2.19§		
MSSS	3.0 ± 2.4			3.30 ± 2.35§		

Abbreviation: EDSS = expanded disability status scale; F/M = female to male ratio. MS = multiple sclerosis; MSSS = multiple sclerosis severity score.