

Association of *Mycobacterium avium* subsp. *paratuberculosis* and *SLC11A1* polymorphisms in Sardinian multiple sclerosis patients

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Abstract

Introduction: Recent findings propose that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection could act as risk factor in favoring multiple sclerosis (MS) progression. *SLC11A1* is a gene associated with mycobacterial survival in the host and it may be involved in the induction and maintenance of autoimmune disease.

Methodology: In this preliminary study, 100 MS patients and 100 healthy controls (HCs) from Sardinia were enrolled. Eight single nucleotide polymorphisms (SNPs) in the *SLC11A1* gene were searched by PCR RFLP-genotyping. IS900 specie specific PCR was undertaken to search for MAP presence. Indirect ELISA was performed to assess if MS patients displayed a stronger humoral response against MAP2694 protein compared to the HCs.

Results: Only rs2276631 SNP was associated with MS. MAP DNA was detected in 23 out of 100 MS patients (23%) and in 7 out of 100 HCs (7%). A strong humoral response against MAP2694 protein was detected in 36% of MS patients and only in 3% of HCs. A correlation between ELISA sero-positivity and the rs2276631 SNP was also found.

Conclusion: Our preliminary results suggest that the Sardinian population might be prone to develop autoimmune disease due to polymorphisms in immunomodulating the *SLC11A1* gene, which is important in the immune response against intracellular bacteria such as MAP.

Key words: *Mycobacterium avium* subsp. *paratuberculosis*; SLC11A1; multiple sclerosis; Sardinia

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Introduction

Multiple sclerosis (MS) is a complex multifactorial autoimmune disease with unknown etiology not imputable to a single genetic or environmental factor. We recently demonstrated that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is an environmental agent associated with MS in the Sardinian population [1,2]. The rate of MS in Sardinia is one of the highest worldwide [3], probably due to the particular genetic structure of Sardinian people. *SLC11A1* is located in chromosome 2q35 and encodes the natural resistance associated macrophage protein 1 (NRAMP1). This protein is a divalent transition metal transporter that regulates macrophage activation in infectious and autoimmune diseases; it also exerts a role in the resistance to intracellular pathogens including mycobacteria [4]. Recently it has been associated with human *Mycobacterium avium* complex infection [5].

We conducted an ongoing study aiming to understand in which way genes and environment

interact with each other. We have genotyped 8 *SLC11A1* polymorphisms, aiming to establish if they could be associated with MAP presence. We are currently searching for MAP DNA within peripheral blood mononuclear cells (PBMCs) of MS patients and healthy controls (HCs). MAP specific antibody (Abs) detection directed against MAP2694 recombinant antigen was also confirmed. Logistic regression analysis revealed an interesting correlation between Abs presence and the genotype AA at rs2276631 SNP. These are preliminary findings that need to be confirmed in a larger study.

Methodology

Study population

This opening study included 100 MS patients and 100 HCs. Blood samples were collected as described elsewhere [1] and all of them were evaluated blindly.

MS patients were diagnosed according to the 2010 Revised McDonald criteria [6]. All of them were

Sardinian. Seventy were female and 30 were male with an F/M ratio of 2.3/1. Age at inclusion = 40 (SD ± 10.8), age at onset = 31 (SD ± 10.8), and disease duration from onset = 12 (SD ± 9.7). The clinical course was relapsing remitting in 82%, secondary progressive in 12.5%, and primary progressive in 5.5%. The neurological disability evaluated with the EDSS score was a mean of 2 (SD ± 2) and ranged from 0 to 6.5.

HCs were all ethnically Sardinian for at least three generations; 65 were females and 35 were males (F/M: 1.9/1). The mean of actual age was 46 years. Subjects with no personal or familial (first-degree relatives) history of autoimmune diseases were included in the study.

We consider MAP infected individual the one that manifest a strong humoral response against MAP specific protein and/or the one that are positive to IS900 PCR. In fact, MAP infection is mainly asymptomatic, and the clinical symptoms (such as general malaise, chronic weight, abdominal pain and diarrhea) may manifest only after several years.

SNP selection and genotyping

Genomic DNA was purified from whole blood according to standard procedures [7]. Eight polymorphisms (rs2276631, rs3731865, rs3731864, rs17221959, rs2695342, rs2279015, rs17235409, rs17235416) were genotyped by PCR restriction fragment length polymorphism (RFLP) analysis, and the set of specific primers were designed based on the GenBank reference sequence (accession no. NT_005403). In detail, the following primer sequences were used: rs2276631 forward (5'-TGCCACCATCCCTATACCCAG-3') and rs2276631 reverse (3'-TCTCGAAAGTGTCCCACTCAG-5'); rs3731865 forward (5'-TCTCTGGCTGAAGGCTCTCC-3') and rs3731865 reverse (3'-TGTGCTATCAGTTGAGCCTC-5'); rs3731864 forward (5'-CTGGACCAGGCTGGGCTGAC-3') and rs3731864 reverse (3'-CCACCACTCCCCTATGACGTG-5'); rs17221959 forward (5'-CTTGTCTGACCAGGCTCCT-3') and rs17221959 reverse (3'-CATGGCTCCGACTGAGTGAG-5'); rs2695342 forward (5'-TCCTTGATCTTCGTAGTCTC-3') and rs2695342 reverse (3'-GGCTTACAGGACATGAGTAC-5'); rs2279015 forward (5'-GCAAGTTGAGGAGCCAAGAC-3') and rs2279015 reverse (3'-ACCTGCATCAACTCCTCTTC-5'); rs17235409/rs17235416 forward (5'-

GCATCTCCCCAATTCATGGT-3') and rs17235409/rs17235416 reverse (3'-AACTGTCCCACTCTATCCTG-5') [8].

IS900 Map PCR

Detection of MAP DNA in 100 MS patients and 100 HCs was performed as previously described [2]. Summing up, DNA was extracted from the PBMCs of both MS patients and HCs according to CTAB methodology. The presence of MAP specific DNA was detected by PCR amplification of IS900 sequences. Two rounds of amplifications were undertaken. First, primers LIZ 1 and LIZ 2, and secondly, primers AV1 and AV 2, were used to amplify a 298-bp fragment specific for MAP.

MAP2694 cloning and expression

MAP2694 was cloned and expressed as previously published [1].

ELISA

ELISA was performed as previously reported [2]. Briefly, ELISA plates were coated with 5 µg/ml of MAP2694 protein in carbonate bicarbonate buffer overnight at 4°C. The following day, the plates were blocked with 5% non-fat dried milk for one hour at room temperature. After that, 1:100 diluted sera samples were added for two hours. Finally, an alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich) diluted 1:1000 was added for one hour and then the substrate was added. Plates were read at 405 nm on a VERSA Tunable Max Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Plates were washed between each incubation with 0.1% Tween-20 in PBS.

Results

Eight *SLC11A1* SNPs were typed by PCR (RFLP) in 100 MS patients and 100 HCs. Only rs2276631 SNP was associated with MS. The genotype frequency for the allele AA was 40% in MS patients and 7% in the HCs ($\chi^2 = 28.5$, $p < 0.0001$). The frequency of A allele was 60% in MS patients and 38.5% in HCs ($\chi^2 = 17.6$, $OR = 1.95$, $p < 0.0001$). Both results were statistically significant (Table).

MAP DNA presence was detected by IS900 PCR in 23 out of 100 MS patients (23%) and in 7 out of 100 HCs (7%); the association was considered to be very statistically significant ($\chi^2 = 8$, $p = 0.0023$, $OR = 3.9$).

Table. Allelic and genotypic analyses of the associations between SLC11A1 polymorphisms and multiple sclerosis

SNP Identity	Location	Allele frequency		OR (CI 95%)	p	Genotype frequency			p
		A (%)	a (%)			AA (%)	Aa (%)	aa (%)	
rs2276631	Exon 3								
MS (n=100)		120 (60%)	80 (40%)	1.95 (1.31-2.91)	<0.0001	40 (40%)	40 (40%)	20 (20%)	<0.0001
HCs (n=100)		77 (38.5%)	123 (61.5%)			7 (7%)	63 (63%)	30 (30%)	
rs3731865	Intron 4								
MS (n=100)		135 (67.5%)	65 (32.5%)	NS	NS	46 (46%)	43 (43%)	11 (11%)	NS
HCs (n=100)		133 (66.5%)	67 (33.5%)			42 (42%)	49 (49%)	9 (9%)	
rs3731864	Intron 5								
MS (n=100)		5 (2.5%)	195 (97.5%)	NS	NS	0 (0%)	5 (5%)	95 (95%)	NS
HCs (n=100)		6 (3%)	194 (97%)			0 (0%)	6 (6%)	94 (94%)	
rs17221959	Exon 8								
MS (n=100)		97 (48.5%)	103 (51.5%)	NS	NS	19 (19%)	59 (59%)	22 (22%)	NS
HCs (n=100)		86 (43%)	114 (57%)			10 (10%)	66 (66%)	24 (24%)	
rs2695342	Exon 9								
MS (n=100)		122 (61%)	78 (39%)	NS	NS	22 (22%)	78 (78%)	0 (0%)	NS
HCs (n=100)		125 (62.5%)	75 (37.5%)			25 (25%)	75 (75%)	0 (0%)	
rs2279015	Intron 13								
MS (n=100)		68 (34%)	132 (66%)	NS	NS	1 (1%)	66 (66%)	33 (33%)	NS
HCs (n=100)		71 (35.5%)	129 (64.5%)			0 (0%)	71 (71%)	29 (29%)	
rs17235409	Exon 15								
MS (n=100)		13 (6.5%)	187 (93.5%)	NS	NS	4 (4%)	5 (5%)	91 (91%)	NS
HCs (n=100)		10 (5%)	190 (95%)			3 (3%)	4 (4%)	93 (93%)	
rs17235416	3'UTR								
MS (n=100)		5 (5%)	195 (97.5%)	NS	NS	0 (0%)	5 (5%)	95 (95%)	NS
HCs (n=100)		3 (3%)	197 (98.5%)			0 (0%)	3 (3%)	97 (97%)	

Single Nucleotide Polymorphism (SNP). Odds ratio (OR) was calculated at 95% confidence intervals (CI); p value was calculated by Chi-square with Yates' correction and Fisher's exact test (the results was the same)

In the same population study, antibodies against MAP2694 were searched. A strong humoral response was detected in 36% of MS patients and only in 3% of HCs, showing an association extremely statistically significant ($\chi^2 = 32$, $p < 0.0001$, OR = 18). Receiver operator characteristic (ROC) curves analysis (AUC = 0.76) is shown in the Figure.

Moreover, binary logistic regression allowed us to observe a correlation between rs2276631 and MAP6994 sero-positivity ($\chi^2 = 39$; MAP2694 OR = 6, $p < 0.0001$; rs2276631 OR = 3, $p = 0.008$).

Discussion

The etiology of MS is thought to be caused by the joint action of genetic factors in the presence of other unidentified environmental agents. Several studies have suggested that different pathogens could trigger autoimmunity by molecular mimicry [9]. Counting one of the highest MS prevalence worldwide, Sardinia is an ideal setting for studying MS [3]. Genetic predisposition alone cannot account for such a high rate of MS, justifying the search for environmental agents capable of triggering the disease.

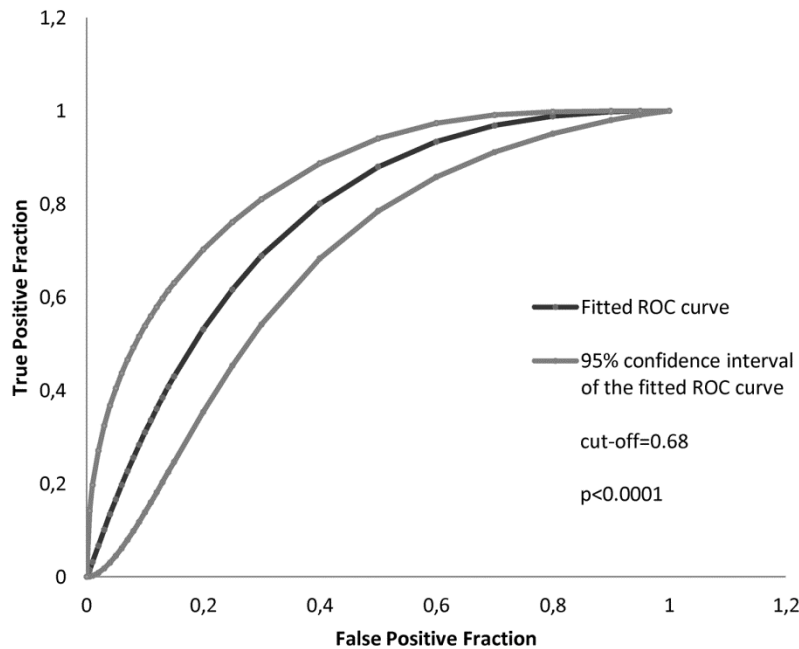
MAP is an obligate pathogenic bacterium, which was proven to play a role in several autoimmune diseases. Recently our group reported MAP involvement in MS [1,2]. The association was

postulated in light of the strong humoral response directed against two MAP specific proteins (MAP2694, MAPFprB), and the detection of MAP’s DNA in the peripheral blood of MS patients. To note, *SLC11A1* polymorphisms have been previously linked with mycobacterial infections and autoimmune disease [10]. This gene exerts pleiotropic effects on macrophage function, regulating both cytokine production and antigen presentation. All of these activities are not only essential for protection against mycobacterial infection, but also critically involved in the induction and progression of autoimmune diseases.

In view of this observation, we wanted to investigate whether the *SLC11A1* polymorphisms were associated with an altered susceptibility to MS; in particular we aimed to elucidate if there was an interaction between predisposing genes and MAP presence. The association of the HLA class II locus with MS and several novel susceptibility loci with modest effects have been recently identified [11]. The role of *SLC11A1* in MS is controversial: on one hand, some studies deny an association with this pathology [12], while on the other hand, a significant association was reported between MS and the *SLC11A1* gene [11].

At present we have PCR-RFLP typed 100 MS patients and 100 HCs for eight *SLC11A1*-SNPs. A larger sample size study has been initiated, which will

Figure. Receiver operator characteristic (ROC) curves analysis of anti-MAP2694 antibodies (Abs) in 100 MS patients and 100 healthy controls



The cut-off was set at > 95% specificity calculated accordingly. Cut-off, area under ROC curve (AUC), and *p* value, are given in the bottom right corner.

look at more SNPs within this locus. The methodology will be improved as well, undertaking sequence analyses. To note, polymorphisms at the functional 5'(GT) n promoter region will be studied. The 5'(GT)n repeat polymorphism (rs34448891) has been previously associated with MS [13].

Presently we report a novel association between MS and the rs2276631 SNP within the *SLC11A1* gene. The polymorphism rs2276631 is a nucleotide substitution that occurs in exon 3 (codon 66 for Phe). It consists of a C/T substitution at residue 274, which could impair iron transport through the channel. This in turn could avoid fagosome acidification and allow the mycobacterial survival inside the macrophage.

The presence of MAP DNA and a strong humoral response against MAP2694 antigen was also detected in MS Sardinian patients. Our preliminary results suggest that rs2276631 SNP might contribute to increased susceptibility to MS. Furthermore, a correlation between ELISA sero-positivity and SNP presence in 20% of the cases was observed. The association between rs2276631 SNP and Type 1 diabetes was recently reported [7], suggesting that the same polymorphism may be common to the major autoimmune diseases widespread in Sardinia.

Conclusion

Although this is an ongoing study, and it can be misleading to make premature conclusions, this first round of data suggests that the Sardinian population might be prone to develop autoimmune diseases due to polymorphisms in the *SLC11A1* gene, which is important in the immune response against intracellular bacteria. Further studies are needed now to validate this report. MAP or another infectious agent could act as a trigger in igniting MS in genetically prone individuals.

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