

Potential protective role of a NOD2 polymorphism in the susceptibility to multiple sclerosis is not associated with interferon therapy

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Abstract. Pattern recognition receptors, such as specific nucleotide-binding oligomerization domain protein 2, and their polymorphisms may be involved in the pathogenesis of multiple sclerosis (MS). They may also play a role in the formation of neutralizing antibodies against interferon- β (INF- β), and may exhibit lowered efficacy. Identification of these polymorphisms may be useful for early identification of potential non-responders and to allow for modification of treatment regimens earlier. The differences in genotype distribution and allele frequency of the rs3135499 and rs2066842 *NOD2* polymorphisms between patients with MS and healthy controls were analysed in the present study. The group of patients were divided into responders and non-responders to INF- β therapy to evaluate the association of both polymorphisms with response to therapy. No differences in the genotype frequencies between the responder and non-responder groups were observed. However, a statistically significant difference in genotype frequencies of TT homozygotes for rs2066842 between patients with MS and healthy controls was observed ($\chi^2=11.8$; $P=0.003$). A recessive genotype model and allele distribution in rs2066842 suggest that the genotype TT and allele T itself are protective against MS. The odds ratio of 0.12

represents an 8.33x lower risk for MS if an individual has a TT genotype. The significantly lower incidence of the TT genotype of rs2066842 in patients with MS suggests that the TT genotype and T allele may be a protective genetic factor against MS.

Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory disorder of the central nervous system caused by the interplay of genetic and environmental factors. It is the most common cause of neurological disability in younger individuals with a lifetime risk of 1 in a 400 that affects women twice as frequently as men. The majority of patients present with the relapsing/remitting (RRMS) form of the disease, which normally has three phases: Relapses with complete recovery, relapses with a persistent disability, and secondary progression (1). The precise etiopathogenetic mechanisms of the disease remain unclear. Several disease-modifying drugs are already available and new promising drugs are emerging; however, interferon- β (INF- β) is still the favoured first-line treatment for RRMS (2,3). Nevertheless, a subgroup of patients do not respond to this treatment; only ~60% of patients on INF- β treatment were relapse-free for 2 years (responders), and in 30% of patients, the disease progressed over 2 years whilst the study was ongoing (4,5). The true therapeutic effects of INF- β can only be measured after several months of treatment, which makes management of MS even more challenging (3-5). Surrogate biomarkers for early identification of non-responder patients may enable timely adjustments to the MS therapeutic regimen, and consequently improved outcomes, but no such biomarkers are currently available (2,6). In addition, a considerable proportion of patients with MS (14-22%) on INF- β therapy develop neutralizing antibodies (NAb) against INF- β , which have been shown to reduce the positive effects of INF- β on disease progression (4,7,8). A previous study examined the role of the pattern recognition receptors (PRRs) in facilitating NAb formation, as PRRs participate in the immune response towards foreign substances (6).

Specific nucleotide-binding oligomerization domain protein 2 (NOD2; OMIM#, 605956) belongs to the family of PRRs, and specific *NOD2* polymorphisms have already been

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Abbreviations: MS, multiple sclerosis; RRMS, relapsing/remitting MS; NOD2, specific nucleotide-binding oligomerization domain protein 2; PRR, pattern recognition receptor; INF- β , interferon- β ; NAb, neutralizing antibody; SNP, single nucleotide polymorphism; IL, interleukin; EDSS, expanded disability status score; RAR, retinoic acid receptor; RXR, retinoid X receptor

Key words: pattern recognition receptor, INF- β , NAb, multiple sclerosis

investigated with regard to MS. One study observed an association between rs3135499 and time to relapse of MS, as well as rs2066842 and frequency of NAb development. However, these observations were not considered statistically significant after correction for multiple comparisons (6). The association of rs2066842 with the Th17 response to the myelin basic protein in patients with MS has also been suggested, but not confirmed (9). Another study tested multiple polymorphisms in genes of the innate immune system and again found no link between rs2066842 and MS diagnosis (10).

The aim of the present study was to investigate the association between *NOD2* gene polymorphisms and susceptibility to MS, as well as the association with response to IFN- β treatment in a cohort of Slovenian and Croatian patients with MS who were treated with IFN- β .

Materials and methods

Study population. A total of 267 patients with MS (203 females and 64 males; median age at onset, 28 years; age range 14-55 years) treated with IFN- β at the University Medical Centre Ljubljana (Ljubljana, Slovenia) and the Clinical Hospital Center Rijeka (Rijeka, Croatia) were included in the present study. All patients were diagnosed prior to enrolment using the McDonald diagnostic criteria (11). Patients with MS and other chronic diseases were excluded. For the evaluation of treatment response (responder/non-responder), clinical status was followed prospectively for at least 2 years following initiation of therapy (12). An exacerbation was defined as new, worsening or recurrent neurological symptoms attributable to MS. A responder was defined as a patient with no MS relapses within the first 2 years after treatment initiation, independent of the Kurtzke's Expanded Disability Status Score (EDSS) (13). After applying the above mentioned criteria, there were 175 responders and 92 non-responders in the group of patients with MS. The group of healthy controls consisted of 234 individuals (17 females and 217 males; median age at onset 40 years; age range 22-68 years) with no neurological disease in their health history. The present study was approved by the Ethical Committees of both participating centres, and all subjects provided informed consent to participate.

Sample collection and selection of single nucleotide polymorphisms (SNPs). Genomic DNA was extracted from peripheral blood leukocytes using a QIAmp blood kit (Qiagen GmbH) according to the manufacturer's protocol.

The *NOD2* polymorphisms were selected based on previous studies and data (rs3135499 and rs2066842) (6,9,10). Additional information regarding the selected SNPs was obtained using GeneCards (genecards.org), The UCSC Genome Browser, ENSEMBL and Algen Promo (14-17). Genotyping was performed using the TaqMan® Genotyping assays using allele-specific quantitative PCR and TaqMan® MGB probes, according to the manufacturer's protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.). An ABI PRISM® 7000 Sequence Detection system was used for PCR amplification and signal detection, and ABI PRISM® 7000 SDS version 1.2 with the RQ Study Results software (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for data analysis.

Statistical analysis. Genotype frequency data was acquired from 1000 Genomes, Candidate Gene SNP Selection (GenePipe) and Ensembl (accessed on May 2021) (18,19). The association between the genotypes and INF- β responder/non-responder status was calculated using a χ^2 test or a Fisher's exact test. $P < 0.05$ was considered to indicate a statistically significant difference (α). The Hardy-Weinberg equilibrium equation was used to determine if the sample was representative. Data were analysed using the Court lab-HW (20) calculator and SPSS version 22 (IBM Corp.).

Results

In the group of 267 patients, there were 204 female and 63 male patients (Table I). Both males and females were distributed evenly amongst the responder and non-responder groups, with females accounting for 76% of patients with MS, 75% of the responder group and 78% of the non-responder group. The observed genotype distribution of rs3135499 and rs2066842 showed no significant differences when compared with those predicted from the Hardy-Weinberg equilibrium for either patients or controls ($P > 0.05$).

The rs2066842 polymorphism displayed a statistically significant difference in the genotype frequencies in patients with MS, with the TT genotype being significantly less common in the group of patients with MS than expected ($\chi^2 = 11.8$; $P = 0.003$). There were no significant deviations in the rs3135499 allele distribution (Table II).

In addition, further statistical analysis for the recessive model and allele distribution of rs2066842 were calculated. The findings suggest that the TT genotype and the T allele itself are protective against MS (Table III); the TT genotype was associated with an 8.33x lower risk for MS (odds ratio = 0.12, $P = 0.0008$).

Because of the highly significant difference in genotype frequencies between MS patients and controls the genotype distribution of rs2066842 and rs3135499 were compared between the control group with the data for the European population from the 1000 Genomes database, and found that both distributions were very similar (data not shown) (15).

The comparison of rs3135499 and rs2066842 genotype frequencies between the responder and non-responder groups showed no significant deviations when all patients with MS were grouped together or when stratified by sex (Table IV).

Discussion

In the present study, two *NOD2* polymorphisms and their association with MS were studied. A statistically significant lower frequency of the TT genotype and T allele of rs2066842 in patients with MS was found. It is hypothesized that the T allele has a protective effect against MS with the carriers of TT being 8.33x less likely to develop MS than the carriers of a CT or CC genotype.

These results do not imply a direct causal relationship, nevertheless rs2066842 may have an influence on susceptibility to MS. No specific mechanism by which the TT genotype or the T allele may have a protective effect on MS has been studied yet, to the best of our knowledge. *In silico* predictions for SNP function link rs2066842 to a nonsense-mediated mRNA

Table I. Clinical characteristics of MS patients.

Characteristics	All	Women	Men
Total (%)	267	204 (76%)	63 (24%)
Age at onset ^a	28.7±8.1	28.5±8.4	29.6±7.0
Duration, years ^a	9.9±6.9	10.3±7.1	8.4±6.0
EDSS ^{a,b}	3.3±2.9	3.3±3.1	3.1±2.0
PI ^{a,c}	0.38±0.28	0.36±0.28	0.42±0.29

^aMean ± standard deviation; ^bDisability was assessed using Kurtzke's EDSS; ^cPI is expressed as ratio EDSS/disease duration in years. EDSS, Expanded Disability Status Scale; PI, progression index.

Table II. Comparison of genotype frequencies of the rs3135499 and rs206684 polymorphisms between patients with MS and the healthy controls.

Single nucleotide polymorphisms	Genotype	MS, n (%)	Control, n (%)	χ^2	P-value
rs3135499	AA	64 (24.1)	60 (26.5)	$\chi^2=1.952$	P=0.377
	AC	154 (57.9)	117 (51.8)		
	CC	48 (18.0)	49 (21.7)		
	Σ	266 (100.0)	226 (100.0)		
rs2066842	TT	2 (0.7)	14 (6.0)	$\chi^2=11.8$	P=0.003 ^a
	TC	102 (38.2)	93 (39.7)		
	CC	163 (61.1)	127 (54.3)		
	Σ	267 (100.0)	234 (100.0)		

^aP<0.01. MS, multiple sclerosis.

Table III. ORs for rs2066842 genotypes and alleles in patients with MS compared with the control subjects.

Statistics	TT vs. TC and CC	Allele T vs. Allele C
OR (95% CI)	0.12 (0.03-0.53)	0.71 (0.53-0.96)
P-value	0.0008 ^b	0.024 ^a

^aP<0.05, ^bP<0.001. CI, confidence interval; MS, multiple sclerosis; OR, odds ratio.

decay, a process that influences the amount of transcript and therefore, the amount of protein produced, and its function (14,15). In addition, both E2F-1 and retinoid X receptor (RXR)- α transcription factors can bind to allele C, but only E2F-1 can bind to the protective allele T (17).

The RXR- α has already been investigated in association with MS. Mean plasma retinol levels in untreated RRMS patients were lower compared with the IFN- β 1a treated patients, as well as compared with patients with a non-inflammatory neurological disease. In the IFN- β 1a treated patients, the expression of 5 retinoid receptor subtypes was reported (retinoic acid receptor (RAR)- α , RAR- γ , RXR- α , RXR- β and RXR- γ), whilst only the first three were expressed in untreated RRMS patients. Therefore, the disease activity in MS may be

associated with retinol levels, and activation of retinoid receptors may be a mechanism of action of IFN- β (21).

A previous study reported no relationship between rs2066842 and MS diagnosis, in contrast with the results of the present study, and this may be due to the heterogeneity of the study group recruited by Enevold *et al* (10), where all types of patients with MS were included, as opposed to the present study, which only included RRMS patients receiving immunomodulatory treatment.

In addition, the association between *NOD2* gene polymorphisms rs2066842 and rs3135499 with response to therapy with IFN- β in MS patients were analysed. To the best of our knowledge, this is the first study to do so. No significant differences in genotype frequencies for rs2066842 and rs3135499 between the responder and non-responder groups, as a whole, or when stratified by sex was observed. The highly variable course of MS significantly complicates the prediction of the outcome of IFN- β therapy and predictors of response would greatly enhance the management of patients. So far, no such surrogate endpoint exists, to the best of our knowledge. Studies have investigated the role of genetic polymorphisms in NAb formation, mostly in relation to HLA II characteristics, but the results have not been implemented in clinical practice (22-24).

Important insights into the immunopathogenetic concepts of MS have been gained. A study by Arellano *et al* (25) demonstrated a notable increase in production of Th17 and Th1 lymphocytes and associated cytokines (such as, IFN- γ

Table IV. Comparison of genotype frequency of polymorphisms rs3135499 and rs206684 based on response to treatment and sex.

Single nucleotide polymorphisms	Sex	Genotype	Responders, n (%)	Non-responders, n (%)	Statistics	P-value
rs3135499	All	AA	43 (24.7)	21 (22.8)	0.209 ^a	0.901
		AC	99 (56.9)	55 (59.8)		
		CC	32 (18.4)	16 (17.4)		
		Σ	174 (100)	92 (100)		
	Male	AA	10 (22.7)	7 (35)	1.12 ^b	0.626
		AC	25 (56.8)	10 (50)		
		CC	9 (20.5)	3 (15)		
		Σ	44 (100)	20 (100)		
	Female	AA	33 (25.4)	14 (19.4)	0.951 ^a	0.622
		AC	74 (56.9)	45 (62.5)		
		CC	23 (17.7)	13 (18.1)		
		Σ	130 (100)	72 (100)		
rs2066842	All	TT	2 (1.15)	0 (0)	0.852 ^b	0.644
		TC	65 (37.15)	37 (40.2)		
		CC	108 (61.7)	55 (59.8)		
		Σ	175 (100)	92 (100)		
	Male	TT	1 (2.3)	0 (0)	0.588 ^b	0.856
		TC	19 (43.2)	8 (40)		
		CC	24 (54.5)	12 (60)		
		Σ	44 (100)	20 (100)		
	Female	TT	1 (0.8)	0 (0)	1.011 ^b	0.706
		TC	46 (35.1)	29 (40.3)		
		CC	84 (64.1)	43 (59.7)		
		Σ	131 (100)	72 (100)		

^aAnalysed using a χ^2 test. ^bAnalysed using a Fischer's exact test.

and IL-17F) in patients with MS, which could additionally be used to differentiate between MS subtypes. Several other studies have also shown evidence of increased pro-inflammatory cytokine production, for example, the macrophage migration inhibitory factor (MIF) family of cytokines, and its homolog MIF 2 in patients with MS (26,27). On the other hand, the production of endogenous anti-inflammatory cytokines and their antagonists (IL-37, IL-1 receptor antagonist, heme oxygenase, amongst others) seems to be reduced in patients with MS (28-30). This indicates that future studies are warranted to reveal the complex immunological pathways involved in MS pathogenesis.

The role of *NOD2* polymorphisms in the pathogenesis of MS and response to IFN- β therapy is most likely only one part of the complex multidirectional system of genetic, immunological and environmental factors yet to be elucidated. Considering the major role PRRs serve in our immune system, a connection between *NOD2* and MS seemed promising and worth exploring, since little research has been published on this topic so far (9,10). Since PRRs are directly involved in our immune response to foreign molecules, it seemed prudent to explore the potential association between the *NOD2* and IFN- β treatment response in MS.

The limitation of the relatively small sample size in the present study should be mentioned. Yet, this is a representative sample from the local region, and thus represents a true *NOD2* rs2066842 polymorphism association with susceptibility to MS. Furthermore, given that no studies have performed a detailed haplotype-based analysis of *NOD2* in patients with MS, to the best of our knowledge, the possibility of allelic heterogeneity cannot be ruled out. A possible MS risk gene could exist and be in linkage disequilibrium with the *NOD2* rs2066842 allele.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AZ, AM, SR, NSC, BP and LL prepared the materials, and collected and analysed the data. AZ and LL wrote the manuscript, and all authors revised the manuscript. All authors have read and approved the final manuscript. AZ and LL confirm the authenticity of the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethical Committees of both participating centres and all subjects provided written informed consent to participate.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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