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ORIGINAL ARTICLE

A single nucleotide polymorphism within *Ninjurin 2* is associated with risk of multiple sclerosis

Rezvan Noroozi[1] · Iman Azari[1] · Mohammad Taheri[2] · Mir Davood Omrani[2] · Soudeh Ghafouri-Fard[1]

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Abstract

Multiple sclerosis (MS) is a devastating inflammatory disease of the central nervous system (CNS) associated with loss of myelin sheaths. The role of Schwan cells in the remyelination of MS lesions has been documented. However, the detailed steps of this process are unknown. *Ninjurin 2* (*NINJ2*) encodes an adhesion protein with high expression in Schwann cells adjoining the distal piece of injured nerve. Based on the role of this protein in neurite outgrowth, it might participate in the process of nerve regeneration after nerve damage. In the present study, we genotyped two *NINJ2* single nucleotide polymorphisms (SNPs) namely rs11833579 and rs3809263 in a population of Iranian patients with MS as well as healthy individuals. The frequency of T allele of the rs3809263 was significantly higher in MS patients compared with healthy subjects (OR (95% CI) = 1.33 (1.08–1.63), adjusted *P* value = 0.01). TT genotype of this SNP was associated with MS risk compared with CC genotype (OR (95% CI) = 2.22 (1.37–3.57), adjusted *P* value = 0.009). Moreover, the rs3809263 was associated with MS risk in recessive model (OR (95% CI) = 2.09 (1.33–3.31), adjusted P value = 0.003). There were no significant difference in the alleles and genotypes frequencies of rs11833579 between cases and controls. The current research suggests contribution of *NINJ2* in the pathogenesis of MS and warrants further studies for elaboration of the underlying mechanism of such contribution.

Keywords *Ninjurin 2* · *NINJ2* · Multiple sclerosis

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). This disorder is associated with loss of myelin sheaths in axons as well as death of oligodendrocytes (Kocsis and Waxman 2007). The ability of several cells from oligodendrocyte lineage as well as Schwann cells in transmigration to demyelinated lesions and remyelination of them has been reported in animal studies (Radtke et al. 2007). In human subjects, although remyelination of MS lesions has been documented, this process is usually insufficient and ultimately fails in most of lesions and patients. Although the exact steps of this process are not defined, it has been revealed that the process includes primary migration oligodendrocyte progenitor cells to lesions and differentiation of these cells into myelinating oligodendrocytes (Chari 2007). Elaboration of this process would help in identification of patients' prognosis and design of novel treatment strategies.

Ninjurin 2 (*NINJ2*) encodes an adhesion protein with high expression in Schwann cells adjoining the distal piece of injured nerve. Based on the role of this protein in neurite outgrowth, it participates in the process of nerve regeneration after nerve damage (Araki and Milbrandt 2000). A paralog of this gene is *Ninjurin 1* (*NINJ1*). NINJ1 has been recognized as an over-expressed protein

Rezvan Noroozi and Iman Azari contributed equally to this work.

✉ Mohammad Taheri
mohammad_823@yahoo.com

✉ Soudeh Ghafouri-Fard
s.ghafourifard@sbmu.ac.ir

[1] Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[2] Urogenital Stem Cell Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Table 1 Demographic and clinical data of study participants

Variables	MS patients	Controls
Female/Male [no. (%)]	300 (73%)/110 (27%)	300(73%)/110 (27%)
Age (mean ± SD, Y)	35.9 ± 2.6	37.1 ± 1.3
Age range (Y)	16–65	17–66
Age at onset (mean ± SD, Y)	28.9 ± 2.5	–
Duration (mean ± SD, Y)	6.8 ± 1.5	–
EDSS score (mean ± SD)	4.3 ± 3.2	–

in the human blood brain barrier (BBB) endothelial cells (ECs) obtained from MS lesions. NINJ1 has a critical role in the transmigration of inflammatory antigen presenting cells (APCs) across the BBB (Ifergan et al. 2011). As a protein which regulates the attachment of monocytes to endothelial cells and thus controls the relocation of immune cells to the CNS, *NINJ2* modulates the onset of relapses in MS patients (Thompson et al. 2018). The rs7298096 within *NINJ2* has been shown to modulate time to first relapse after treatment with IFN-β. This single nucleotide polymorphism (SNP) is also associated with peripheral expression of *NINJ2*. Notably, based on the results of co-expression investigations, *NINJ2* participates in immune system-associated pathways (Thompson et al. 2018). Further studies have shown the regulatory role of *NINJ2* on expression of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-8 and IL-6 in human vascular endothelial cells. *NINJ2* can also modulate LPS-associated endothelial activation through NF-κB and Toll-like receptor 4 (TLR4) (Wang et al. 2017). Therefore, *NINJ2* has prominent roles in nerve regeneration, immune response regulation and development of MS. Based on these results; we hypothesized that SNPs within *NINJ2* might affect risk of MS. Consequently, we conducted the current case-control study to genotype two *NINJ2* single nucleotide polymorphisms (SNPs) namely rs11833579 and rs3809263 in a population of Iranian patients with MS and healthy individuals. These two SNPs were selected based on the previous reports demonstrating the effect of rs3809263 in modulating *NINJ2* expression (Zhang et al. 2016) and their contribution in conferring risk of a human disease namely ischemic stroke as reported by independent studies (Bis et al. 2014; Zhu et al. 2014; Ikram et al. 2009).

Material and methods

Study participants

A total of 410 relapsing-remitting MS (RRMS) patients and 410 age- and sex-matched healthy subjects were enrolled in the current study. Patients were recruited from Department of neurology, Farshchian Hospital, Hamadan Province, West of Iran. Patients were assessed and diagnosed based on the revised McDonald criteria for MS (Thompson et al. 2018). Persons recruited in the control group had no individual or family history of MS or other autoimmune disorders. The study protocol was approved by the ethical committee of Shahid Behaeshti University of Medical Sciences. All study participants signed the informed consent forms. Table 1 shows the demographic and clinical data of study participants.

Genotyping

The rs11833579 and rs3809263 SNPs within the *NINJ2* gene were selected for genotyping. Table 2 shows the characteristics of the mentioned SNPs.

Three milliliters of the peripheral blood were obtained from all study participants. DNA was extracted from all samples using salting out method. SNPs were genotyped using the tetra-primer amplification-refractory mutation system (ARMS)-PCR technique. The results were verified by sequencing of 10% of samples (ABI 3730xl DNA analyzer, Macrogen, Korea). Table 3 shows the nucleotide sequences of primers, the annealing temperatures and the predicted amplicon sizes. The PCR program consisted a primary denaturing step at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, specific annealing temperature for 30 s, 72 °C for 1 min and an ultimate extension step at 72 °C for 5 min.

Statistical analyses

Data was analyzed using SNP Analyzer 2.0 online tool (Yoo et al. 2008). Associations between disease status and each SNP or haplotype were judged using Pearson's chi-square. A haplotype-specific test with one degree-of-

Table 2 Characteristics of selected SNPs of *NINJ2* gene

SNP	Position	Minor Allele	MAF	MAC	Type
rs11833579	Chr 12:666033	A	0.30	1488	Intergenic variant
rs3809263	Chr 12:664290	T	0.32	1581	upstream variant

Table 3 Nucleotide sequences of primers used for genotyping

SNP	Primer sequence	Tm	Annealing temperature	PCR product size (bp)
rs11833579	Forward inner primer (A allele): CTTTCTGGAAAACCTTAATTCGGCTA	63 °C	59 °C	170 bp (A allele)
	Reverse inner primer (G allele): GGATAAATAGTTAATATGTTGCTTCTTGC	57 °C		236 bp (G allele)
	Forward outer primer: AATTTTTTTTTAATTGAGCTAGATGTGGC	60 °C		351 bp (two outer primers)
	Reverse outer primer: ATATTCGAGTACTGTTCTCTTTTGCATT	60 °C		
rs3809263	Forward inner primer (A allele): CTTCAAGCCCTGAATTGGATTACTGG	69 °C	62 °C	262 bp (G allele)
	Reverse inner primer (G allele): GTAGACGTGCTTGGCAGAGTGTTCAT	70 °C		227 bp (A allele)
	Forward outer primer: GACTAAAATATGGCACCCATCCTATCATC	69 °C		437 bp (two outer primers)
	Reverse outer primer: ATGGAGCATGGAGTAGTTGTACCTTCGA	69 °C		

freedom was applied for assessment of associations between expected haplotypes and MS risk. D' and r factors were measured for appraisal of linkage disequilibrium between rs11833579 and rs3809263 SNPs. Associations were weighed in allelic, co-dominant, dominant and recessive models. Judgment was based on the calculated odds ratios (OR), 95% confidence interval of OR (95% CI), *P* value and Bonferroni adjusted *P* values. P values less than 0.05 were regarded as significant.

Results

The allele and genotype frequencies of rs11833579 and rs3809263 SNPs in both cases and controls were in agreement with Hardy-Weinberg principle (Table 4).

The frequency of T allele of the rs3809263 was significantly higher in MS patients compared with healthy subjects (OR (95% CI) = 1.33 (1.08–1.63), adjusted *P* value = 0.01). TT genotype of this SNP was associated with MS risk compared with CC genotype (OR (95% CI) = 2.22 (1.37–3.57), adjusted *P* value = 0.009). Moreover, the rs3809263 was associated with MS risk in recessive model (OR (95% CI) = 2.09 (1.33–3.31), adjusted P value = 0.003). There were no significant difference in the alleles and genotypes frequencies of rs11833579 between cases and controls (Tables 5 and 6).

Table 4 Exact test for Hardy-Weinberg equilibrium

SNP	rs11833579			*P* value	rs3809263			*P* value
	GG	AG	AA		CC	CT	TT	
MS	161	196	53	0.58	168	182	60	0.35
Healthy Control	152	191	67	0.59	191	188	31	0.1

Based on the D' and r statistics (D' = 0.53, r = 0.09), the mentioned SNPS were not in strong linkage disequilibrium. We also assessed the associations between anticipated haplotypes of these SNPs and MS risk. After correction for multiple comparisons, no significant difference was detected in haplotype frequencies between cases and controls.

Discussion

In the present case-control study, we genotyped two *NINJ2* SNPs in a population of Iranian MS patients and healthy subjects. Of note, we detected significant associations between rs3809263 and MS risk in allelic, co-dominant and recessive models. This SNP has been reported as a functional SNP in this gene which is associated with risk of stroke. Zhang et al. have genotyped this SNP in 414 Chinese patients with large artery atherosclerotic stroke and 423 healthy subjects. They reported a remarkable lower risk of stroke for the rs3809263 CT and TT genotypes. Furthermore, they have shown higher *NINJ2* mRNA expression in TT genotype carriers (Zhang et al. 2016). Notably, the risk allele for MS (T allele) as reported in the current study seems to have protective effect against stroke in Chinese population. This observation might be explained by different roles of NINJ2 in the pathogenic processes of these disorders. Based on the putative role of *NINJ2* in neurite outgrowth (Araki and Milbrandt 2000), the presence of certain substances in the MS sera with inhibitory effects on neurite outgrowth and synapse formation (Chen et al. 2015), and the reported up-regulation of myelin-associated neurite outgrowth inhibitor (Nogo-A) in remaining oligodendrocytes at the border of demyelinating lesions of MS (Satoh et al. 2005),

Table 5 Genotypes and alleles frequencies in MS patients and controls

SNP	Model		Frequency in cases (%)	Frequency in controls (%)	OR (95% CI)	*P* value	Adjusted *P* value
rs11833579	Allele	A vs. G	302 (37) 518 (63)	325 (40) 495 (60)	0.89 (0.73–1.08)	0.24	0.48
	Co-dominant	AA vs. GG AG vs. GG	53 (12.9) 196 (47.8)	67 (16.3) 191 (46.6)	1.34 (0.88–2.04) 1.03 (0.77–1.39)	0.38	0.76
	Dominant	AG + AA vs. GG	249 (60.7) 161 (39.3)	258 (62.9) 152 (37.1)	0.91 (0.69–1.21)	0.52	1.00
	Recessive	AA vs. AG + GG	53 (12.9) 214 (52.2)	67 (16.3) 219 (53.4)	0.76 (0.51–1.2)	0.17	0.33
rs3809263	Allele	T vs. C	302 (37) 518 (63)	250 (30) 570 (70)	1.33 (1.08–1.63)	0.007	0.01
	Co-dominant	TT vs. CC CT vs. CC	60 (14.6) 182 (44.4)	31 (7.6) 188 (45.9)	2.22 (1.37–3.57) 1.1 (0.82–1.47)	0.004	0.009
	Dominant	CT + TT vs. CC	242 (59) 168 (41)	219 (53.4) 191 (46.6)	1.26 (0.95–1.66)	0.10	0.20
	Recessive	TT vs. CT + CC	60 (14.6) 350 (85.4)	31 (7.6) 379 (92.4)	2.09 (1.33–3.31)	0.001	0.003

we expected lower expression of *NINJ2* in MS patients. However, the TT genotype which was regarded as the risk genotype in our study, has been associated with higher expression of NINJ2 in Chinese stroke patients (Zhang et al. 2016). Future studies are needed to assess the relation between rs3809263 genotypes and NINJ2 expression in different tissues, distinct populations and diverse disease backgrounds.

Based on our results, the rs11833579 has been not associated with risk of MS in any inheritance model. This SNP is located upstream of *NINJ2*. Although some previous studies have linked this SNP with risk of stroke, a meta-analysis of the available literature failed to detect any association between rs11833579 and risk of stroke (Lian et al. 2012). This meta-analysis along with our data cause doubt about the functionality of this SNP.

To the best of our knowledge, the current study is the first study evaluating the association between *NINJ2* variants and MS risk. The main strength of our study was inclusion of an acceptable sample size of MS patients with a certain disease course (RRMS patients). We state lack of assessment of *NINJ2* expression as a limitation of our study. Moreover, lack of validation on independent sample is another limitation of our study. So, we suggest future investigation of expression levels of NINJ2 in association with its genomic variants to verify the role of each variant in the alteration of its expression levels.

Taken together, the present study introduces the rs3809263 as a marker of susceptibility to MS in Iranian population. However, the underlying cause of the observed association is not clear. Based on the multifactorial nature of MS disease, this SNP might affect neuronal growth, immune response or response to environmental risk factors. These possibilities should be assessed in functional studies.

Table 6 Haplotype frequencies in cases and controls

rs11833579	rs3809263	Frequency in cases	Frequency in controls	Total Frequency	OR (95% CI)	*P* value	Adjusted *P* value
G	C	0.33	0.35	0.34	0.89 (0.73–1.1)	0.29	1.00
A	C	0.30	0.34	0.32	0.84 (0.69–1.03)	0.09	0.39
G	T	0.30	0.25	0.28	1.28 (1.04–1.59)	0.02	0.08
A	T	0.06	0.05	0.06	1.36 (0.81–2.28)	0.24	0.96

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