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Neurofilament light chain gene polymorphism and risk of multiple sclerosis in Iranian patients

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Abstract

Background: Multiple sclerosis (MS) is a chronic disease characterized by inflammation and degeneration of the central nervous system (CNS). High levels of neurofilament light chain (NFL) and neurofilament heavy chain (NFH) in cerebrospinal fluid (CSF) have been associated with a wide range of neurological diseases including MS.

Subjects and Methods: Peripheral blood samples were collected from 40 relapsing remitting multiple sclerosis (RRMS) patients and 40 healthy control subjects to extract genomic DNA. Genotyping was performed by polymerase chain reaction (PCR) and sequencing technique. Genotypic and allelic distributions were compared between cases and controls. Logistic regression was used to estimate the risk of MS associated with selected SNP.

Results: The results of the present study revealed that there were significant differences in the distribution of neurofilament light gene (NEFL) genotypes and allele frequencies between Iranian RRMS patients and controls. In Iranian RRMS patients, there was a significant association between NEFL gene polymorphism rs2979687 and the risk of MS.

Conclusion: Our data indicate that there was a significant association between -374A>G NEFL gene polymorphism and risk of MS in Iranian patients. Probably, we can use it as a potential prognostic genetic marker. Further large prospective studies are required to confirm these findings.

Keywords: Multiple Sclerosis, NEFL, Polymorphism, Iranian

1. Introduction

Multiple sclerosis (MS) is a chronic disease which leads to damage and ultimately loss of myelin within the central nervous system (CNS) and neurological disability (1). There are an estimated 2.5 million patients suffering from MS. It is more common in women and in younger ages between 20 and 40 years of age (2). MS classification is as follows: Progressive relapsing MS (PRMS), Secondary progressive MS (SPMS), Primary progressive MS (PPMS) and Relapsing/Remitting MS (RRMS) (3). MS leads to symptoms such as bladder and bowel dysfunction, depression, fatigue, chronic aching pain, tremor, dizziness/vertigo, weakness, visual disorders, sexual dysfunction, spasticity (leg stiffness), swallowing disorders, and mild cognitive and memory difficulties (4).

Genetic susceptibility, infections (rubella, mumps, coronavirus, parainfluenza, *Herpes simplex*, Epstein-Barr), human T-cell lymphotropic virus type I viruses,

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imbalance of steroid hormones, geographical-latitude (ground, water properties), vitamin D deficiency (high MS frequency occurs in areas with low sunlight exposure), diet (Westernized food), stress, occupation, cigarette smoking, body mass index, and age are risk factors of MS(5, 6).

Axonal loss and neurodegeneration are main elements of MS pathology, so an objective biomarker to detect, therapeutic responses and quantify them should be of great value (7). An established biomarker for neurodegeneration and early stages of MS is body fluid neurofilament (NF) levels which is specific cytoskeletal proteins and major structural elements of neurons (8, 9). NF subunit genes, neurofilament light (NFL) chain (68 kDa), neurofilament intermediate (NFM) chain (160 kDa) and neurofilament heavy (NFH) chain (205 kDa), that are known to be involved in neurodegeneration or neuroprotection (10). Several studies have shown NF-L released from damaged axons and levels are increased in the cerebrospinal fluid (CSF) of MS patients, especially early stage of relapsing remitting form and associate with disability and radiological markers of inflammation (8, 11).

In early acute axonal damage, NFL has been mainly detected (12). A previous study showed that both CSF NF and inflammatory markers decrease in MS patients with age (13). Blood and CSF neurofilament levels could potentially be useful for both predicting and monitoring disease progression and for assessing the efficacy or toxicity of future neuroprotective treatment strategies such as MS (14, 15). Due to damage to axons of the CNS or peripheral nervous system, NFs would then occur in the CSF and the bloodstream, and NFL move from the extracellular fluid (ECF) into the CSF, hence quantification of NF from either body fluid led to estimate the amount of neuroaxonal damage caused (15, 16).

The *NEFL* gene is located on 8p21 and *NEFH* gene located on 22q12. These genes respectively codifies the light chain and heavy chain of NF protein (17). The *NEFL* gene is 5.66 kb in length and consists of four exons. This is one of the gene in the incidence of MS. Rs2979687 polymorphism is located at position A/G in the promoter region of *NEFL* gene (18).

In the present study, a single nucleotide polymorphisms (SNP) in the light subunits of NF gene was selected to be studied (rs2979687). The aim of the present research was investigating the association of *NEFL* gene -374A>*G* polymorphism with risk of MS in the Iranian patients.

2. Materials and Methods

For the present case-control study, 40 patients with the relapsing remitting type of MS (RRMS) and 40 healthy individuals were selected. The patients were between 20-47 years old and do not suffer from other neurological disorders such as Alzheimer's disease. We analyzed all the factors (smoking, family history and other environmental factors) that could affect the outcome through a questionnaire. The subjects were included under the study with their written informed consent. All the patients and controls were Iranian. Whole blood was collected by venipuncture in tubes containing EDTA. Genomic DNA was extracted from peripheral blood using the IBRC kit (IBRC, Iran). The selected gene polymorphisms were analyzed by polymerase chain reaction (PCR) and direct sequencing technique. The forward and reverse primers were designed using Perlprimer software (Table 1).

Table 1. Sequence of primers used for the investigation of the selected gene polymorphisms

Polymorphisms	Primers	PCR product size
NEFL - 374A>*G*	Forward: AAATTCCCTAGCCGCACCATC	415 bp
	Reverse: CAGCCTGCGATCGATCACG	

The thermal gradient for amplification of *NEFL* was as follows: Initial melting step of 3 minutes at 95˚C, followed by 30 cycles of 30 second denaturation at 95˚C, 30 second annealing at 56˚C, 1 minute extension at 72˚C, and a final elongation step of 5 minutes at 72˚C. The amplified PCR products of *NEFL* gene was visualized on 0.8% agarose gel electrophoresis and is shown in Figure 1.

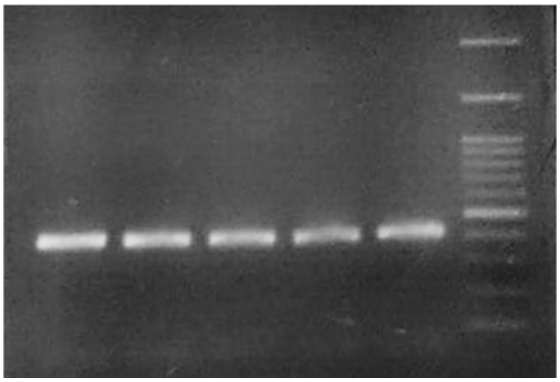

Figure 1. Agarose gel electrophoresis for PCR amplification of NEFL gene. DNA ladder (100bp); lane P1, P2 and P3 represents case; lane C1 and C2 represents healthy control individuals.

PCR products were sequenced at Pishgam Company (Tehran, Iran) according to the Sanger protocol. The resulting sequences were then analyzed for genotypes using the FinchTV software. The allele and genotype frequencies amongst cases and controls were compared by Chi-square test and p values using SPSS software. P-value less than 5% was considered as significant. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated to evaluate the

associations. The results of the sequencing are shown in Figure

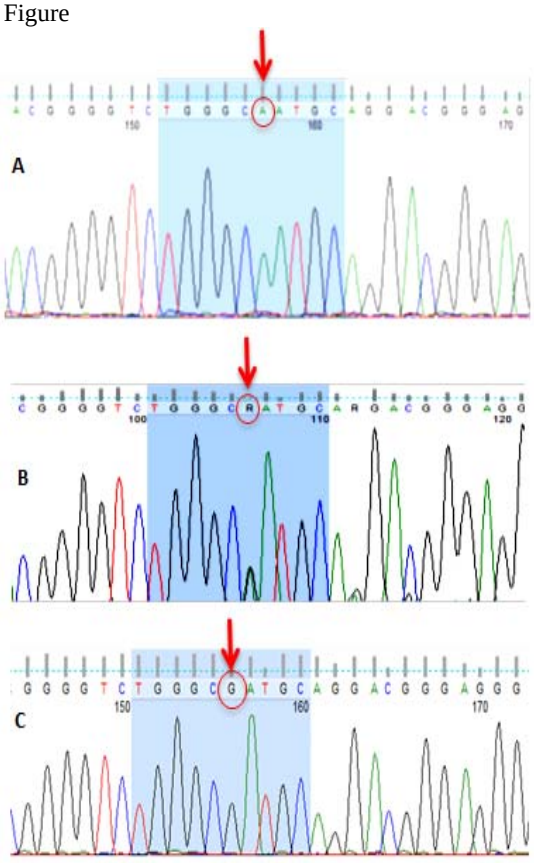

Figure 2. Results of *NEFL g*ene direct sequencing, A: Homozygote healthy mode, B: Heterozygote state, C: Patients homozygote

The direction of the regression coefficient represented the effect of the risk genotype (GG or GA) versus the reference genotype (AA) for light chain NF gene polymorphism. Sex and age were included as covariates.

Rs2979687 SNP located in the promoter region of the *NEFL* gene causes a mutation, which adenine has been converted to guanine.

3. Results

-374A>G (rs2979687) polymorphism in RRMS subjects (n = 40) and healthy controls (n = 40) were genotyped and being confirmed about the quality of DNAs by Nanodrop.

The mean age at the time of collection of patients was 32.65 years and in the control group was 36.68 years. The age and sex distribution of study subjects are shown in Table 2.

In the present study, we examined the selected polymorphism with MS by PCR and sequencing methods.

The genotypes and allele frequencies of *NEFL* gene polymorphism were shown in Table 2. The analysis pointed out that the most common genotype between case and control groups of *NEFL* was GA genotype.

The frequencies of the AA, AG and GG genotypes of *NEFL* rs2979687 were 10%, 70%, 20%, respectively, among the cases and 35%, 55%, 10%, respectively, among the controls. Subjects carrying GG homozygote (OR=7.860, 95% CI = 1.460-42.306, P = 0.016) GA heterozygote (OR = 4.931, 95% CI = 1.364-17.820, P = 0.015) and AA homozygote (P = 0.027) had a genetic association with RRMS (Tables 2 and 3). The logistic regression analysis showed that there was a significant association between relapsing remitting MS and -374A>G polymorphism of *NEFL* gene in the Iranian patients (P =0.022, X^2=7.609) (Tables 2 and 3).

Sex and age as the measure of effect were analyzed and results illustrated that there was no significant association between the age and sex of cases and controls and examined gene polymorphism (Table 2).

The results illustrated that having GG and GA genotype of -374A>*G NEFL* gene polymorphism compared to AA genotype was a significant risk factor for MS and there was a correlation between GG genotype and G allele and risk of MS.

In general, there was a statistically significant association between the *NEFL* -374A>G gene polymorphism and RRMS in the Iranian patients, and there was a significant difference between cases and controls for rs2979687 in the *NEFL* gene ($p < 0.05$).

Table 2. Allele and genotype frequency and odds ratio of -374A>*G* NEFL gene polymorphism among RRMS patients versus control group. OR, odds ratio; CI, confidence interval. Bold values indicate the significance level of p<0.05.

-374A>*G*		Group		P value	P value adjusted	OR	95% CI for OR	
		MS patient	Control				Lower	Upper
Genotype	AA	4 (10%)	14 (35%)	0.022*	0.027	Reference	-	-
	GA	28 (70%)	22 (55%)		0.015	4.931	1.364	17.820
	GG	8 (20%)	4 (10%)		0.016	7.860	1.460	42.306
Allele	A	36(45%)	50(62.5%)					
	G	44(55%)	30(37.5%)					
Age	<35	26 (65%)	25 (62.5%)		0.055	0.948	0.999	1.113
	>35	14 (35%)	15 (37.5%)					
Gender	Male	11 (27.5%)	16 (40%)		0.165	0.470	0.734	6.159
	Female	29 (72.5%)	24 (60%)					

Table 3. Chi-Square analysis

	Value	Df	P value
Pearson Chi-Square	7.609[a]	2	0.022
Likelihood ratio	7.965	2	.019
Number of valid cases	80		

4. Discussion

The search for valid biomarkers and prognostic parameters for patients with MS, especially at the beginning of the disease, could facilitate therapy decisions and help in timely diagnosis and reliable and sensitive biomarkers are therefore needed. Serum level of NF is considered as an important measurable biomarker for several neurological diseases such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and head and spinal cord trauma (19, 20). NF levels in paired CSF and serum samples in patients with MS have been shown to have increased compared with controls and indicating continuous axonal damage during the entire course of the disease (21). Several previous studies suggested increased levels of NF (which is specific cytoskeletal proteins) in blood as prognostic and predictive biomarkers in RRMS (15, 22). The strict neuronal specificity of neurofilament has hindered the mechanistic studies of recessive NEFL mutations (23).

According to this information in the present study, we examined the association between a subunit of NF (*NEFL*) gene polymorphism and the risk of relapsing remitting MS in the Iranian patients.

An association between high NFL levels and relapse in RRMS patients has been reported previously in several studies. The increase in NFL levels shows that the axonal damage is more prominent during relapse phase of the MS and suggests an association with the inflammatory process (24).

To our knowledge, this is the first study to examine associations between genetic variations in *NEFL* gene and the risk of MS, so there are not many similar studies the results of which might be useful for comparison and the discussion of the results of the present study.

Several previous studies indicate that mutation in *NEFL* causes Charcot-Marie-Tooth disease and statistically significant association between *NEFL* gene polymorphism with Charcot-Marie-Tooth disease, type 1F and type 2E (25-27). In addition, another study reported a genetic association between *NEFL* with autism in Caucasian and Japanese (28). Recently, there are some reports regarding the association between *NEFL* gene variants and human diseases such as neuroblastoma (29). Moreover, effect of *NEFL* mRNA expression level in breast cancer (30), glioblastoma multiform (GBM) (31) and ALS (32) have been shown. As well, a range of studies have shown that neurofilament light protein could be considered to be a biological marker of disease activity and axonal damage in MS patients (33).

In our case-control study, we have identified that -374A>*G NEFL* gene polymorphism (rs2979687) was associated with susceptibility to RRMS in Iranian MS patients. The results of genetic analysis suggested that GG and AG genotype and G allele of *NEFL* -374A>*G* gene polymorphism have an association with RRMS and might increase MS risk. We hope that it can be used as a diagnostic biomarker for MS. However, further large prospective studies are required to confirm the findings of our study that is a preliminary study and is presenting data for future comprehensive study for making a clinical conclusion. In addition, analysis of other SNPs in the NF gene would be needed in future studies.

Conclusion

This study confirmed for the first time that there was a significant difference between the Iranian control and patient groups regarding frequency of GG genotype and G allele of -374A>*G* polymorphism of *NEFL* gene. The findings of the present case-control study suggest that rs2979687 polymorphism in -374A>*G* position of *NEFL* gene has a significant association with the development of RRMS in the Iranian patients and this could be a risk factor for MS.

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