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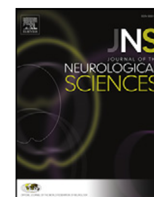

Two functional promoter polymorphisms of neuregulin 1 gene are associated with progressive forms of multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory, autoimmune, demyelinating disease of the central nervous system (CNS). Spontaneous remyelination happens in most of MS lesions but it is incomplete and inadequate and eventually fails in majority of lesions of damaged areas. According to the fact that the accuracy of this phenomenon depends on distinct temporal expression profiles of molecules, especially growth factors, we decided to study the association of two functional polymorphisms of NRG1 as a myelin-related growth factor which can promote oligodendrocyte proliferation and differentiation in CNS and PNS. SNP rs6994992 and SNP rs7014762 were genotyped by PCR-RFLP and mismatch PCR-RFLP methods, respectively, in 165 subjects with MS and 200 healthy controls. Totally, our results showed no significant difference in the allelic and genotype frequencies of these two NRG1 polymorphisms between MS patients and control group in Iranian population; but statistically significant association was found for the progressive forms of MS (secondary progressive-MS and primary progressive-MS) for functional SNP of rs6994992 polymorphism. Also, considering rs7014762 polymorphism frequencies, significant difference was observed between primary progressive MS group in comparison to the control group. Furthermore, when the samples were stratified by gender, significant association was revealed between male MS subjects and rs7014762 polymorphism. These results indicate that progression of MS disease could be influenced by functional nucleotide variations in NRG1 gene, which might have an impact on remyelination capacity in different patients.

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1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease in the central nervous system (CNS) with autoimmune and neurodegenerative properties. The most common pathologic hallmarks of the disease include variable degrees of inflammation, demyelination, axonal damage, and scar formation (gliosis) [4]. In demyelinating diseases such as MS, remyelination process is followed by pathological loss of myelin in adult CNS. This regenerative process consists of two phases; at first oligodendrocyte progenitor cells (OPCs) are recruited to demyelinated regions, then they differentiate to myelinating oligodendrocytes, and finally they contact to denuded axons for producing myelin sheaths and improving function of viable axons [19,24]. Due to inaccuracies in differentiation of OPCs into mature myelin forming cells, the remyelination and regeneration process fails in most of the lesions of MS [7]. Indeed, understanding the etiology of this failure in remyelination is essential for finding appropriate therapeutic strategies in MS disease. Some studies indicated that improvement in the ability of endogenous OP cells for efficient remyelination is obtained by meliorating the activity of growth factor signaling pathway [1].

NRG1-ErbB signaling pathway plays a critical role in migration, differentiation and survival of neural and oligodendrocyte precursors and in central nervous system development, also this signaling networks regulate myelination, neurotransmission, and synaptic plasticity [14]. Neuregulin 1 (NRG1) is a signaling protein that mediates cell–cell interactions and plays critical roles in the growth of the nervous system. This gene has 15 isoforms which are generated through alternative splicing and different promoter usage. Previous studies represented that NRG1 has pro-myelinating effect in PNS [11,26] and promotes myelination by oligodendrocyte in the CNS [30]. Taveggia et al. reported cortical hypomyelination in NRG1 type III heterozygous mice [28]. The amount of NRG1 type III expressed on myelinated axons determines myelin sheath thickness [15]. NRG1 type II can promote OPC proliferation *in vivo* and *in vitro* and is expressed in OPCs during their proliferative response to demyelination [3].

Based on genetic epidemiological studies, MS is a disease with complex genetic criteria. Since association study is an effective way to assign the degree of genetic contribution in complex diseases, this method was chosen for investigating effects of two important polymorphisms in

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NRG1 gene on MS. Rs6994992 (SNP8NRG243177) and rs7014762 are two functional promoter polymorphisms that are candidates for this study. Rs6994992 is located near to the promoter and 1.2 kb upstream of initiation transcription site of type IV NRG1. Indeed, bioinformatics analysis on rs6994992 has revealed that this polymorphism changes binding site of transcription factor implicated in NRG1 regulation.

In one study Tan et al. showed that T allele conversion to C allele in rs6994992 results in 60% decrease in the promoter activity [27]. It is known as a functional polymorphism and the TT genotype has been associated with higher levels of NRG1 type IV transcription [8]. McIntosh et al. showed that homozygotes for rs6994992 T allele was associated with decreased white matter density in the right anterior limb of the internal capsule and reduced structural connectivity [13]. Reduced white matter integrity in the left anterior thalamic radiation was shown in T allele carriers [23].

Rs7014762 is a single nucleotide polymorphism that is located in core promoter region and has 87 base pair physical distance from rs6994992. Nicodemus et al. found significant association between this SNP and NRG1 type III expression in the hippocampus of schizophrenia cases [16]. So in this study, we assessed association between SNP rs6994992 and rs7014762 in NRG1 gene and MS disease for the first time in Iranian population.

2. Material & method

2.1. Collection of MS patients and control samples

Case samples were collected from patients with MS diseases referred to Sina Hospital, and they were diagnosed by physician specialty based on diagnostic criteria by [32,33] and according to Brain magnetic resonance imaging. Control subjects were acquired from volunteers with no neurological disease history (Table 1). Totally peripheral blood samples were collected from 165 subjects with MS and 200 healthy controls and genotyped for two rs6994992 and rs7014762 polymorphisms. The control groups are coincident with case groups in sexuality, age and race. Informed consent from each case was acquired before blood sampling and the Ethics Committee of Tarbiat Modares University approved the study.

2.2. Genomic DNA extraction

DNA extraction from whole blood was done using DNG Plus™ kit (Cinnagen, Iran). According to producer instructions, blood cells are lysed by kit lysis buffer followed by DNA precipitation using isopropanol. Then extracted DNA was washed by 75% ethanol and dissolved in distilled water. Quality and concentration of extracted DNAs were examined by electrophoresis in 1% agarose gel or spectrophotometer, respectively.

2.3. Genotyping

SNP rs6994992 was genotyped by PCR-RFLP method. For each sample approximately 100 ng of extracted DNA was used to amplify 584 bp amplicon that containing rs6994992 SNP using recombinant Taq polymerase (Cinnagen, Iran). Amplification was carried out using specific primers: 5′-ACTCTATTGAAACACAACCAGC-3′ as forward primer and 5′-AGTTTGGAGGGACAGGGTCA-3′ as reverse primer. All primers were designed using PRIMER EXPRESS software (Applied Biosystems). PCR was conducted with 5 min of denaturation 94 °C, 35 cycles of 94 °C for 30 s, annealing at 61 °C for 30 s, 72 °C for 40 s and a final extension at 72 °C for 5 min. PCR products were digested by TaiI enzyme (Fermentas) at 65 °C and visualized by electrophoresis on 1% agarose gel. TaiI cannot cut the C allele, but cuts the T allele and produces two fragments with 446 bp and 138 bp in length.

Mismatch PCR-RFLP technique was used for genotyping of SNP rs7014762. The region containing rs7014762 was amplified using a mismatch reverse primer to produce a recognition site for Bam HI enzyme (Fermentas) in the presence of T allele (Fig. 1). Sequences of primers were as follows: 5′-AGTAGGATTGGATGTTTGAACC-3′ as forward primer and 5′-GGGTCATCACACTCCCTGTGTGTCTTGGCAAGGGGGG-3′ as reverse primer.

The mismatched nucleotide in reverse primer is underlined. In PCR reaction, following an initial 94 °C denaturation step (5 min), the samples were subjected to 35 cycles at 94 °C (30 s), 60 °C (30 s), 72 °C (30 s) and ending with a final extension at 72 °C (5 min). After digestion by Bam HI at 37 °C, products were subjected to electrophoresis on 12% polyacrylamide gel. The amplicon was 195 bp and Bam HI cannot cut the A allele. Genotyping of some samples from each genotype was confirmed by DNA sequencing with ABI automated DNA sequencer (Macrogen, Korea).

2.4. Statistical analysis

The calculated genotype frequencies of studied SNPs were compared with expected genotype frequencies according to the Hardy–Weinberg law. The association of SNP rs6994992 and SNP rs7014762 with MS was analyzed with Chi-square test and (version 16) SPSS software to understand difference of allele and genotype frequencies between the study groups.

A P-value of ≤0.05 was considered as significant level.

3. Results

In this study, we used PCR-RFLP and mismatch PCR-RFLP methods for genotyping SNP rs6994992 and rs7014762, respectively. By these methods, we distinguished different genotypes of individuals for rs7014762 and rs6994992 in 12% acrylamide gel (Fig. 1D) and 1% agarose gel (Fig. 2), respectively. Sequencing results were matched with genotyped results based on used methods (Fig. 3). Two mentioned SNPs in NRG1 gene were genotyped in 165 MS patients and 200 healthy controls.

Determined genotype frequencies were in Hardy–Weinberg equilibrium in the control and patient groups for both SNP. The allelic frequency of rs6994992 (C, T) was 50.3%, 49.7% in patient group *vs.* 46.6% and 53.4% in healthy group, respectively; and genotype frequency (CC, CT, TT) was 25.4%, 49.8%, 24.8% in MS group *vs.* 20.47%, 52.38%, 27.15% in healthy group, respectively. But for rs7014762 (A, T), in MS group allelic frequency was 25.8%, 74.2%, while in control group was 27.1%, 72.9%, respectively, and genotype frequency (AA, AT, TT) was 7.1%, 37.4%, 55.5% in MS group *vs.* 6.7%, 40.8%, 52.5%, in control group, respectively. According to statistical analysis and χ^2 test result, totally there was no significant difference in allelic and genotype frequency between case and control groups (Table 2).

Table 1
Demographic features of MS patient and control individuals.

	Male			Female			Frequency of MS types		
	Number	Age range	Mean onset age	Number	Age range	Mean onset age	Relapsing remitting (RR)	Secondary progressive (SP)	Primary progressive (PP)
MS	40	23–50	29.7	125	17–53	29.7	69.7%	25.3%	5%
Control	45	21–52	–	155	16–60	–	–	–	–

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