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Investigation Of An Inducible Nitric Oxide Synthase Gene (NOS2A) Polymorphism In A Multiple Sclerosis Population

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) affecting most commonly the Caucasian population. Nitric oxide (NO) is a biological signaling and effector molecule and is especially important during inflammation. Inducible nitric oxide synthase (iNOS) is one of the three enzymes responsible for generating NO. It has been reported that there is an excessive production of NO in MS concordant with an increased expression of iNOS in MS lesions. This study investigated the role of a bi-allelic tetranucleotide polymorphism located in the promoter region of the human iNOS (NOS2A) gene in MS susceptibility. A group of MS patients (n = 101) were genotyped and compared to an age- and sex-matched group of healthy controls (n = 101). The MS group was subdivided into three subtypes, namely relapsing-remitting MS (RR-MS), secondary-progressive MS (SP-MS) and primary-progressive MS (PP-MS). Results of a chi-squared analysis and a Fisher's exact test revealed that allele and genotype distributions between cases and controls were not significantly different for the total population (χ^2 = 3.4, $P_{genotype}$ = 0.15; χ^2 = 3.4, P_{allele} = 0.082) and for each subtype of MS ($P > 0.05$). This suggests that there is no direct association of this iNOS gene variant with MS susceptibility.

Author Keywords: Multiple sclerosis; Gene association; iNOS; Polymorphism

1. Introduction

Multiple sclerosis (MS) is a serious disabling neurological disorder affecting young Caucasian individuals, with an age of onset typically ranging from 18 to 40 years. Females account for approximately 60% of MS cases [27.] with the incidence of MS in Northern Europe, Canada, and the Northern United States being approximately 10 new cases per year per 100,000 persons (between the ages of 20 and 50 years). With the exception of trauma, MS remains the most frequent cause of neurological disability for young adults. The central nervous system (CNS) in MS is affected with patches of myelin degeneration produced by multifocal inflammatory events. The disease is disseminated in time and space, with MS classified into types, based on the clinical course namely relapsing-remitting MS (RR-MS), secondary-progressive MS (SP-MS), and primary-progressive MS (PP-MS) [25.]. Studies indicate that MS has a genetic component with 30% of monozygotic twins developing the

disease compared to only 4.7% in dizygotic twins [24.]. The HLA gene complex has been shown to be associated with MS [9.], but the HLA contribution does not appear to explain all the susceptibility. Other genes have been found to be related with disease severity including the *interleukin-1 receptor antagonist* (*IL-1ra*) gene [7.], and Fc-receptor (*FcγR*) genes [19.].

Nitric oxide (NO) is a free radical agent with wide biological functions. It is produced by a group of enzymes called nitric oxide synthase (NOS). NOS catalyses the production of NO and L-citrulline from L-arginine, O_2 and NADPH. There are three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The last isoform is a calcium independent enzyme responsible for producing high output and long-lasting release of NO, exceeding the NO production level of nNOS and eNOS isoforms. Expression of iNOS is under the regulation of several cytokines such as interferon gamma (IFN-γ), tumor necrosis factor (TNF-α), and interleukin 1 beta (IL-1β) [10.] and its function is part of the macrophage-mediated response to infectious agents. Activated macrophages residing within MS brain lesions have been demonstrated [1.]. Within the cerebrospinal fluid (CSF), NO products have been found at a higher concentration in MS patients compared to healthy control subjects [20.]. Furthermore, increased iNOS activity has been detected in the CSF of MS patients with high NO concentration levels correlating with disease activity [6. and 8.]. In animals with experimental allergic encephalomyelitis (EAE), a model of MS, increased CNS mRNA levels of iNOS have been demonstrated along with high levels of cytokines, particularly involved in iNOS activation [23.]. Interestingly, this up-regulation of iNOS in EAE is concomitant with a decrease in Ca^{2+}-dependent NOS activity of eNOS and nNOS [26.]. In a human MS study, iNOS mRNA and iNOS enzymatic activity have been detected in MS plaque lesions [15.]. Inducible NOS activity resides in activated and differentiated monocytes in MS patients suggesting a role for monocyte iNOS in the autoimmune response underlying disease pathogenesis [16.]. The activity of iNOS has detrimental effects on oligodendrocytes, cells responsible for the myelination of neurons in the CNS [17.].

Nitric oxide might play a critical role in the pathogenesis of MS, and alterations of the iNOS gene could possibly have an impact on the disease. The human iNOS (NOS2A) gene has been localized to chromosome 17q11-2-q12, a locus close to a region linked with MS on chromosome 17q21-q22 [11.]. The gene consists of 27 exons and is about 40 kb in length [28.]. To determine the possible involvement of polymorphisms of iNOS in the pathogenesis of MS, a bi-allelic tetranucleotide polymorphism in the promoter region of the human iNOS gene has been examined by employing an association study approach in a group of MS patients and age/sex-matched controls.

2. Materials and methods

2.1. Subjects

The study protocol was approved by Griffith University's Ethics Committee for experimentation on humans. The case-control sample consisted of 101 MS patients and 101 healthy controls, matched for ethnicity (Caucasian), sex and age (±5 years). The MS patients were recruited from the Multiple Sclerosis Clinic at the Royal Brisbane and Women's Hospital, all from the South East Queensland region. The MS group consisted of 74% females and 26% males and was subdivided into three clinical subtypes: RR-MS, SP-MS, and PP-MS with frequencies of 42, 35, and 24%, respectively. The control group was also obtained from the South East Queensland region through the Genomics Research Centre, Southport, with each control age (±5 years), sex, and ethnicity matched to the affected population. All individuals gave informed consent before participating in the research. Genomic DNA was extracted from peripheral blood using a standard salting-out protocol.

2.2. Genotyping

DNA was extracted from frozen whole blood by a standard salting-out procedure and used as a template to generate polymerase chain reaction (PCR) products for genotyping. Within the 5′-flanking DNA of the iNOS gene there is a AAAT/AAAAAT repeat sequence extending from −756 to −716 bp 5′ to the main TATA-directed transcription initiation site [22.]. Primers specific for this bi-allelic tetranucleotide repeat polymorphism located between −891 to −575 bp of the iNOS gene were used to perform PCR (5′-TGG TGC ATG CCT GTA GTC C-3′ for the forward primer and 5-GAG GCC TCT GAG ATG TTG GTC-3′ for the reverse primer) [3.]. They were purchased from GeneWorks. The forward primer was fluorescently labeled with 5-carboxyfluorescein (FAM) dye. The PCR was performed with a PCR thermocycler machine (Gene Amp PCR System 9700; Applied Biosystems) with the following singleplex reaction: 1 unit of Taq polymerase, 0.2 μM of each primers, 7.5 μl of buffer (MasterAmp™ 2X PCR PreMix K), 25 ng of genomic DNA made to a final volume of 15 μl with sterile distilled water. The cycle parameters were as follows: 1 cycle at 94 °C for 4 min, followed by 40 cycles for 1 min at 94 °C and 1 min at 60 °C, and 1 cycle for final extension for 2 min at 72 °C.

All PCR products were then electrophoresed in ethidium-bromide-stained 2% agarose gels. Capillary electrophoresis was performed on an ABI 310 Genetic Analyser. Results were analyzed using GENOTYPER® software (Version 2.1; PE Biosystems). The accuracy and reproducibility of automated sizing of the fragments were confirmed by randomly chosen repeated analyses of identical samples.

2.3. Statistical analysis

Allele and genotype frequencies were compared using standard chi-square (independence) analysis and the Fisher's exact test, implemented in the SPSS program. Power estimates indicated that if the iNOS tetranucleotide polymorphism were to directly confer a two-fold increase in relative risk of MS, the case and control groups used in this study were of sufficient size to have >80% power to detect a significant association at the 0.05 level.

3. Results

The size of the DNA fragments amplified for the iNOS variant were 313 and 317 bp corresponding to the two alleles: no insertion (n) and insertion (i). Results for genotype and allele frequencies are provided in Table 1 and Table 2. Genotype frequencies for the control and the MS groups conformed to Hardy Weinberg equilibrium expectations reducing the risk of error during genotyping. The proportion of the alleles for the control population was similar to others found in independent studies [13. and 18.].

Table 1. Genotype frequencies of iNOS insert in Australian multiple sclerosis patients and controls

Genotype	Control ($n = 101$)	Percentage	MS ($n = 101$)	Percentage	P-value[a]
n/n	84	83.2	75	74.3	0.15
n/i	17	16.8	24	23.8	
i/I	0	0	2	2	

Allele: n, no AAAT insert; i, presence of the AAAT insert.
[a] Fisher's exact test.

Table 2. Allele frequencies of iNOS insert in Australian multiple sclerosis patients and controls

Allele	Control ($n = 202$)	Percentage	MS ($n = 202$)	Percentage	Statistic
n	185	91.6	174	86.1	$\chi^2 = 3.03$
i	17	8.4	28	13.9	$P = 0.0819$

Allele: n, no AAAT insert; i, presence of the AAAT insert;
Results for the Fisher's exact test and the chi squared analysis indicate that there is no significant difference between MS patients and the control population, either for genotype frequencies ($P = 0.15$) or for allele frequencies ($\chi^2 = 3.03$, $P = 0.0819$).

Table 3. Genotype frequencies of iNOS insert for different subtypes of MS in Australian multiple sclerosis patients and controls

Genotype	Control ($n = 42$)	RR-MS* ($n = 42$)	Control ($n = 35$)	SP-MS** ($n = 35$)	Control ($n = 24$)	PP-MS*** ($n = 24$)
n/n	34	30	29	27	21	18
n/i	8	11	6	7	3	6
i/i	0	1	0	1	0	0

Fisher's exact test.
*P=0.44
**P=0.77
***P=0.46

Table 4. Allele frequencies of iNOS insert for different subtypes of MS in Australian multiple sclerosis patients and controls

Allele	Control ($n = 84$)	RR-MS* ($n = 84$)	Control ($n = 70$)	SP-MS** ($n = 70$)	Control ($n = 48$)	PP-MS*** ($n = 48$)
n	76	71	64	61	45	42
i	8	13	6	9	3	6

Fisher's exact test.
*P=0.35
**P=0.59
***P=0.49

Results for the Fisher's exact test showed no significant differences between the subtypes of MS and the control population for both genotype frequencies or for allele frequencies (Tables 3 and 4).

4. Discussion

MS is a complex neurological disease, with genetic factors believed to play a significant role in susceptibility. Several genes have been found to be associated with the disease including the *Human Leukocyte Antigen* (*HLA*) haplotypes, *HLA DRB1*1501, DQA1*0102, DQB1 0602* [9.], the *Cytotoxic T lymphocyte-associated antigen-4* (*CTLA-4*) gene [14.], and the *osteopontin* (*OPN*) gene [21.]. Infiltrating macrophages within MS plaque lesions produce NO products generated by iNOS gene activity. In the present study, we investigated a tetranucleotide repeat polymorphism in the iNOS gene promoter in patients with different subgroups of MS and compared it with the genotypes of healthy controls with no history of MS. This study indicated no association of the tested iNOS polymorphism in an Australian MS population. Our findings support those obtained in studies on an ethnically-different Swedish population [18.] and Spanish population [4.], which showed no linkage or association of the same iNOS promoter marker polymorphism with MS. Despite these findings, it is still possible that NOS is implicated in MS susceptibility. NO is a potent mediator of inflammatory responses and can lead to the formation of new epitopes by *S*-

nitrosylation of cysteine residues of myelin proteins in MS plaques. Antibodies for such epitopes were found in different clinical forms of MS [5.]. Furthermore, IFN-β, a drug used for MS therapy, is thought to be a selective inhibitor of human glial iNOS [12.]. Although the iNOS tetranucleotide promoter polymorphism did not show association with MS in the present study, it is possible that an alteration of iNOS gene activity through mutation or polymorphism may still contribute to MS susceptibility. Interestingly, very recent single nucleotide polymorphism studies in MS using pedigree disequilibrium test data have provided some evidence for NOS2A (iNOS) involvement, and linkage between a different NOS2A promoter polymorphism marker (CCTTT)n and MS DR2 positive families was also demonstrated [2.].

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