Neuromyelitis Optica and Nucleotide Variations of Aquaporin-4 Gene

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Abstract

***Purpose*:** Neuromyelitis opitca (NMO) is an autoimmune disease that relates to deposition of anti-Aquaporin-4 (AQP4) IgG in the central nervous system (CNS). However, called seronegative NMO when patients are negative for AQP4 antibody. Nevertheless, NMO is most probably an antibody mediated disease. The aim of this study was to assess the association between seronegative NMO disease and variations in the AQP4 gene region.

***Methods*:** A total of 24 seronegative NMO patients were enrolled in the study in 12 months. The frequency of neucleotide variations in 5' untranslated region (5'-UTR), coding, noncoding, and 3' untranslated region (3'-UTR) regions were found and compared with the corresponding rates reported previously.

***Results*:** The frequencies of the studied variations showed no statistical difference with the rates reported previously. However, a variation was found in 7% of the alleles in the intronic region between exons 1 and 2 which has not been reported so far.

***Conclusion*:** Nucleotide variation in different regions of AQP4 gene can affect antigenicity of AQP4 in different ways one of which is making a change in AQP4 isoform (M1/M23) expression ratio. While the variation found in this study in the intronic region of the gene can affect isoform expression ratio, further investigation is needed to determine its role.

***Keywords*:** Aquaporin, Neuromyelitis Optica, Devic's Disease, Single Nucleotide Polymorphisms, Sequencing, Orthogonal Arrays of Particle

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Introduction

Neuromyelitis opitca (NMO) is an autoimmune disease which predominantly affects spinal cord and optic nerves.[1] In terms of pathogenesis, IgG and complement deposits are present in spinal cord and optic nerve lesions. It is suggested that IgG against Aquaporin-4 (AQP4) molecule induces complement dependent astrocyte necrosis and death.[1,2] Meanwhile, the serum titre of AQP4 antibody is correlated with the clinical activity of the disease.[3,4] However, a number of NMO patients are negative for AQP4 antibody even when utterly sensitive assays are employed.[1] The NMO disease in those patients is considered seronegative.

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Nevertheless, NMO is most probably an antibody mediated disease because both seropositive and seronegative patients show improvement with plasma exchange and B-cell depletion.[1,4] The affinity of the antibody depends on AQP4 molecule assembly on cell membrane because some antibodies recognize the epitopes produced by supramolecular aggregates of AQP4.[3] Those supramolecular aggregates are formed as square arrangements called orthogonal arrays of particles (OAPs). AQP4 is expressed in the central nervous system (CNS) in two isoforms: long isoform or M1, and short isoform or M23. The more M23 molecules compared to M1, the larger OAP size will be. In other words, OAP size is determined by the amount of M23 isoform.[3]

The reasons for NMO seronegativity are diverse. Besides a negative indirect immunoflourecence (IF) assay due to NMO antigenic variation, other reasons for seronegativity are: 1) titer of NMO antibody is lower than detection limit of the assay which in turn can be due to treatment or natural remission of the disease. An NMO negative test due to low titer antibody can only be identified by following the patient and repeating the test during relapse period[5]; 2) a new epitope that is made by quaternary structure of supramolecular aggregation of AQP4 molecules that cannot be detected by simple molecules present in cell-based assays[5]; 3) unidentified antigen against which antibody is produced. For instance, in some seronegative NMO patients, a complement dependent immune response caused by an antibody against myelin oligodendrocyte glycoprotein has been reported.[6]

In NMO seronegative patients, no study has been conducted to date on the association of mutations or single nucleotide polymorphisms (SNP) and their role in susceptibility to the disease. The aim of this study was to assess the association between seronegative NMO disease and variations in the AQP4 gene region.

Methods

Sample collection and preparation

A total of 24 AQP-4 seronegative NMO patients were selected in 12 months for investigation in this retrospective study according to Wingerchuck clinical and radiological criteria.[7] The enrolled patients were considered having NMO disease by fulfilling both clinical and radiologic criteria. All patients were Anti-AQP4 antibody negative by cell-based IF assay as instructed by the manufacturer (Lübeck, Germany).

After obtaining written consent, 2 mL of EDTA anti-coagulated whole blood were collected from each patient. Plasma was separated from blood cells by centrifugation at 1000 g for 10 minutes and was stored in a temperature controlled refrigerator at 4 °C up to 10 days until IF assay was performed. Buffy coat was also stored in -20 °C until DNA was purified.

DNA purification

Nucleic acid was extracted using Roche High Pure Template Preparation Kit (Roche Diagnostics, Indianapolis, IN) as instructed by the manufacturer. Briefly, buffy coat was incubated with 200 µL of tissue lysis buffer and 40 µL of proteinase K in a dry block at 55 ^{o}C for 48 hours. After addition of 200 µL of binding buffer and 100 µL of isopropanol, the mixture was brought to column. Then washing was performed by inhibitor removal or wash buffers, for three times, DNA was eluted into a final volume of 200 µL and stored in -20 ^{o}C.

Polymerase chain reaction

Primer3 web-based software version 0.4.0 was employed for primer design.[8] Primers were designed for amplification of 5' untranslated region (5'-UTR), coding, noncoding, and 3' untranslated region (3'-UTR) regions of AQP4 gene. The PCR was performed in a 20 µL reaction containing 0.2 µM of each forward and reverse primer, and 10 µL of SYBR Premix Ex Taq II (Takara Bio, Ostu, Shiga, Japan). The sequence of the primers and reaction conditions are summerized in table 1.

Sequencing and alignment

PCR products with the right melting temperature were sent for purification and sequencing to Macrogen (Geum chun-gu, Seoul, Korea) where the sequencing was performed by ABI 3730 XL machine. Sequencing results were aligned by MEGA software version 4.0.2.[9] Subsequently, nucleotide variations were found and confirmed by visually inspecting the

chromatogram. The frequency of each allele in the sequenced region was recorded. Finally, each variation was searched in Ensmbl and NCBI SNP databases.[10,11] The frequency of each SNP was compared to the frequency that was reported by Sorani and co-workers[12] by means of χ^2 statistical test.

Table 1. Primers, polymerase chain reaction product length, and polymerase chain reaction conditions for amplification of different regions of Aquaporin-4 gene

Region	Primer Sequence (5'-3')	Product size (base pair)	Initial Denaturation	45 Cycles	
				Denaturation	Annealing /Extension
5'-UTR	CAGAGTGCAGCTCTCATTGC TGCCCAATTTCAACTACAACA	342	95 ºC for 30''	94 ºC for 10''	60 ºC for 30''
AQP4 exon 1	CTGGGTGTAGTGGCTTCTGA GTCCAAAGCAAAGGGAGATG	246	95 ºC for 30''	94 ºC for 10''	60 ºC for 30''
AQP4 intron	AATTTTCGGGCCACCTAGA CTCCAAGCTTCTTGCAAACTC	206	95 ºC for 30''	94 ºC for 10''	56 ºC for 30''
AQP4 exon 1	AAGTGTGGACCTTTGTGTACCAG AGGCCTCCCACCACACTG	401	95 ºC for 30''	94 ºC for 10''	63 ºC for 30''
AQP4 exons 2 & 3	ATTGTCATCGAAAATACTCTTGC GGAAGATGGGAACTATCAATATGA	611	95 ºC for 30''	94 ºC for 20''	57 ºC for 60''
AQP4 exon 4	GCCCATCATAGGAGCTGTC ATGGGTGGAAGGAAATCTGAG	330	95 ºC for 30''	94 ºC for 10''	60 ºC for 30''
3'-UTR	GGAGGAACGGAAGAAACCTA CTTGAATGTGCATGACTGTGA	293	95 ºC for 30''	94 ºC for 10''	58 ºC for 30''

AQP4: Aquaporin-4, 5'-UTR: 5' untranslated region, 3'-UTR: 3' untranslated region

Results

Five patients were male and the rest were female. The age of the patients ranged from 16 to 58 years with mean and standard deviation of 34.2 and 9.7 years, respectively. The frequencies of the studied SNPs are summarized in table 2. The frequencies of the studied SNPs showed no statistical difference with the rates reported by Sorani et al.[12] However, a variation was found in 7% of the alleles (3 out of 42) in the intronic region between exons 1 and 2 which is shown in figure 1. The aforementioned variation has not been reported so far.[12,13]

Table 2. Characteristics of different nucleotide variations in different regions of Aquaporin-4 gene

	Gene region	ID	Genomic Position (GRCh37)	Genomic position	Nucleotide change	Amino acid position	Amino acid change	Frequency	Sorani's Study[12]	p-value
1	3'-UTR	Rs3763043	24435818	22689816	G>A			25%	0.317	0.48
2	CODING		24436339	22690337	G>A	270	ALANIN=THR	2%	NA	
3	NON CODING		24442039	22696037	A>T			7%	NA	
4	NON CODING	Rs455671	24442056	22696054	T>C			28%	0.189	0.44
5	CODING	Rs72557968	24442227	22696225	G>A	122	GLN=GLN	2%	0.054	
6	CODING	Rs35248760	24442392	22696390	G>T	67	PRO=PRO	10%	0.149	
7	CODING	Rs150587304	24442537	22696535	G>T G>C	19	ARG=ISO ARG=THR	0%	NA	
8	5'-UTR	Rs162007	24445847	22699845	C>T			29%	0.183	0.33

AQP4: Aquaporin-4, NA: Not available, 5'-UTR: 5' untranslated region, 3'-UTR: 3' untranslated region

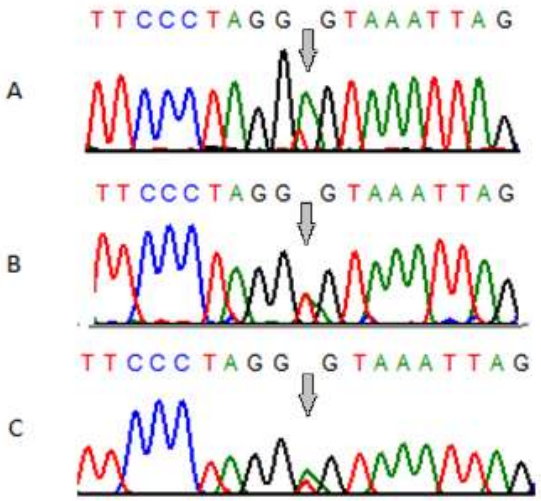

Figure 1. The site of mutation in the intronic region between exons 1 and 2 of AQP4 gene is shown by arrow. Panels A, B and C belong to patients number 8, 9 and 17 respectively who are all heterozygous (A/T).

Discussion

Nucleotide variations in different regions of AQP4 gene; promoter, introns, and exons; can affect the antigenicity of AQP4 protein in different ways: 1) direct change of antigenicity by making a change in amino acid sequence of extra-membranous domain of AQP4 protein; 2) indirect change of antigenicity by altering the OAP size on glial cell membrane which is described by Martello et al,[13] 3) altering the OAP size by changing the amount of M1 to M23 molecules expressed on the plasma membrane.[3] M23 is the major isoform of AQP4 expressed in CNS,[13] and– in contrast to M23– M1 has 22 more amino acids in the N-terminus region. Meanwhile, M1 to M23 isoform expression can be regulated by gene promoter, post-translational regulation of protein synthesis, or intron-mediated transcriptional regulation.

Each M1 or M23 isoform possesses a different transcription initiation site at the 5'-UTR region of the gene.[14] Therefore, isoform expressions can be affected by variations in 5'-UTR region of the gene, and by factors that interact with that region. No association has been found to date between NMO disease and variations of 5'-UTR region of AQP4 gene.[12,13] Meanwhile, no association has been found between the variations of this region and the disease in the current study.

Researchers have suggested that M1/M23 ratio is also regulated by post-translational mechanisms such as leaky-scanning, RNAi, palmitoylation, calcium elevation, and protein kinase C activation.[15-17] Considering that micro-RNA can complement with pre-mRNA,[14] a mutation in the intronic region of the gene can be important in gene post-transcription regulation.

In prokaryotic cells, removal of the introns often decreases mRNA expression. This phenomenon – called intron-mediated transcriptional regulation – is divided into splicing-independent and splicing-dependent categories.[18,19] In splicing-independent regulation, the intron contains binding sites for enhancers/repressors or neucleosome positioning.[18] Moreover, the mechanisms for splicing-dependent regulation, which requires 5'proximity of intron, are more diverse: snRNP interaction with promoters, mRNA stabilization, or mRNA transport.[18]

We have found a variation in NMO seronegative patient in the intronic region of M23 isoform of AQP4 gene near 5' region. A variation in that region can affect the ratio of M23 in contrast to M1, the OPA size and its antigenicity.

A limitation of the current study was small sample size, however, it should be mentioned

that seronegative NMO is a small proportion of NMO disease which itself is an infrequent disorder.[20] Another limitation was that the variation found in this study is not described before; therefore, its prevalence in Caucasians is not known. While the aforementioned variation was not present in Pubmed or Ensemble databases, it would be very unlikely that this variation is ubiquitous.

Conclusion

In conclusion, we screened multiple regions of AQP4 gene from 5'-UTR to 3'-UTR. None of the variations in this region was significantly different from its prevalence in general population. However, we found a novel variation in the intronic region of AQP4 gene in genomic position 24442039. While a variation in that region can theoretically affect the ratio of M23 in contrast to M1, further investigation with more cases and controls is needed to determine the importance and exact mechanism.

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