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NO ASSOCIATION OF AQP4 POLYMORPHISMS WITH NEUROMYELITIS OPTICA AND MULTIPLE SCLEROSIS

Abstract

Multiple sclerosis (MS) and neuromyelitis optica (NMO) are inflammatory demyelinating disorders of the central nervous system (CNS). Various genetic and environmental factors have been identified to contribute to etiology of MS and NMO. Aquaporin 4 (AQP4), is the most abundant water channel in CNS. AQP4 is expressed in astrocytes of the brain, spinal cord, optic nerve and supportive cells in sensory organs. In contrast to MS, immunoreactivity of AQP4 is abolished in NMO lesions. However, conflicting results have been reported regarding the association between AQP4 polymorphisms and demyelinating disorders. Considering the ethnic differences of genetic variations, replications in other cohorts are required. In this study, single nucleotide polymorphisms (SNPs) of AQP4 gene in patients with NMO/neuromyelitis optica spectrum disorders (NMOSD), and MS in the Northern Han Chinese population were examined. Six selected AQP4 SNPs were genotyped by high-resolution melting (HRM) method. Compared with healthy control (HC), there was no significant difference of AQP4 allele and genotype frequency in MS or NMO/NMOSD group. This study showed no significant association of common AQP4 SNPs with MS or NMO/NMOSD, strongly suggesting that polymorphisms of AQP4 gene are unlikely to confer MS or NMO/NMOSD susceptibility, at least in Northern Han Chinese population.

Keywords

• Aquaporin 4 (AQP4) gene • Multiple sclerosis • Neuromyelitis optica • Neuromyelitis optica spectrum disorders • Single nucleotide polymorphisms (SNPs)

1. Introduction

Inflammatory demyelinating diseases (IDDs) consist of a heterogeneous group of autoimmune disorders of the central nervous system (CNS), including multiple sclerosis (MS), neuromyelitis optica (NMO), acute disseminated encephalomyelitis, transverse myelitis, and optic neuritis [1]. NMO, also known as Devic's disease, is rare in Caucasian individuals but relatively prevalent in West Indian and Asian populations [2, 3]. NMO is characterized by simultaneous bilateral optic neuritis and transverse myelitis [4]. Serum NMO immunoglobulin G (IgG), an autoantibody against aquaporin 4 (AQP4) is a highly specific marker for NMO [5]. In addition, NMO is further characterized by attack-associated longitudinally extensive transverse myelitis (LETM) on spinal magnetic resonance imaging (MRI), lack of brain lesions at disease onset, and absence of cerebrospinal fluid oligoclonal bands [2, 6, 7].

Accumulating evidence demonstrated that anti-AQP4 antibody (Ab) was detectable not


only in typical NMO patients but also in those who do not fulfill the diagnostic criteria for NMO, including those with myelitis, optic neuritis, and brainstem/hypothalamic syndromes associated with abnormal imaging [2]. Based on new clinical and neuroimaging findings, the international panel for NMO spectrum disorders (NMOSD) proposed a change in diagnostic criteria of NMOSD to include AQP4-Ab-seropositive and -seronegative patients [8].

Unlike NMO, various genetic and environmental factors contribute to etiology of MS [9, 10]. Among the genetic factors, genome-wide studies and meta-analyses have revealed strong associations of the major histocompatibility complex (MHC) region on chromosome 6 with the risk of developing MS [11, 12]. The HLA-DRB1*1501 allele confers MS risk in Caucasians [13] and Japanese individuals [14, 15]. As for NMO, the DPB1*0501 allele was associated with NMO in Japanese and Southern Han Chinese populations [16, 17], while the DRB1*03 allele was associated with NMO in

Caucasians [18]. Of note, a very recent study showed no association between non-MHC MS risk loci with NMO in Chinese cohort [19]. Taken together, all these findings indicate that the genetic susceptibility of NMO is markedly distinct from MS.

AQP4, expressed in astrocytes in the brain, spinal cord, optic nerve and supportive cells in sensory organs, is the most abundantly distributed water channel in CNS [20]. NMO-IgG binds to sites of CNS pia, subpia and Virchow-Robin spaces, identical to the NMO lesions. In contrast to MS, expression or immunoreactivity of AQP4 is abolished in NMO lesions [21, 22]. These findings raise the likelihood of AQP4 as a NMO-associated susceptibility gene. However, conflicting results have been reported regarding the association between AQP4 polymorphisms and NMO. Several studies showed no association of AQP4 variants with NMO in Caucasian and Korean populations [23, 24]. However, single nucleotide polymorphism (SNP) rs151244 in

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the *AQP4* promoter region was associated with NMO susceptibility in the Southern Han Chinese population [25]. In general, the Han Chinese population is divided into two major groups, namely Northern Han and Southern Han, in terms of the clustering analysis and frequency distribution of HLA polymorphisms [26]. For instance, it is well known that A*30-B*13-DRB1*07 is the most common haplotype in the Northern Han Chinese, while A*02-B*46-DRB1*09 is predominant in the Southern Han Chinese [27-32]. On the basis of such distinctive genetic characteristics, we speculate that Northern Han Chinese could be genetically different from Southern Han Chinese and even the Japanese race, based on the association of the DPB1*0501 haplotype and NMO in the latter two populations [16, 17]. Nevertheless, considering the ethnic differences of genetic variations, replications in other cohorts are required. In this study, we examined the associations of common *AQP4* SNPs with NMO/NMOSD and MS in Northern Han Chinese population.

2. Materials and methods

2.1 Patients and controls

Sixty-two patients with NMOSDs (23 men and 39 women) were enrolled in the study. All cases undertook detection of serum NMO-IgG using an anti-*AQP4* Ab assay on an anti-*AQP4*-transfected cell line from a commercial BIOCHIP kit (Euroimmun, Lübeck, Germany). The diagnosis was based on the criteria for NMO [33] or NMOSD [8]. Eighty-seven patients with MS (32 men and 55 women) were included and all of them met the revised McDonald criteria of 2005 [34]. Patients with primary progressive MS and clinically isolated syndrome (CIS) were

excluded from this study. Disability status for both MS and NMO/NMOSD were evaluated using the expanded disability status scale [35]. One hundred and nine healthy volunteers were included as age- and sex-matched normal controls (43 men and 66 women). All participating subjects were ethnic Han Chinese and given informed consent before the start of the study, which was approved by the Ethical Committee of Peking University People's Hospital (PKUPH).

2.2 SNPs selection and genotyping

SNPs of *AQP4* were considered from the database of Han Chinese in Beijing (CHB) in the HapMap Genome Browser release#27 (<http://hapmap.ncbi.nlm.nih.gov/>) and as a result, six common SNPs were selected on the criteria of minor allele frequency (MAF) ≥ 0.15 (Fig. 1). Six SNPs were genotyped using high resolution melting (HRM) assay. Briefly, genomic DNA was extracted and purified from whole blood using chloroform/p-chlorophenol extraction. Primers

of SNPs were designed using LightScanner Primer Design Software (Idaho Technology Inc., Salt Lake City, UT, USA) and checked for specificity by BLAST (The Basic Local Alignment Search Tool) on National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>), in combination with polymerase chain reaction (PCR) for further confirmation (Table 1). PCR was performed in a total reaction volume of 10 μ l, including 1 μ l DNA (20 ng), 5 μ l 2 \times Taq DNA polymerase Mix (Tiangen Biotech, Beijing, P. R. China), 1 μ l of 300 nM forward and reverse primers (polyacrylamide gel electrophoresis-purified), 1 μ l of 30 μ M each high and low internal controls and 1 μ l LC Green HRM dye (Idaho Technology Inc., Salt Lake City, UT, USA). The touchdown PCR was carried out in 96-well plates on LightScanner 96 (Idaho Technology Inc., Salt Lake City, UT, USA). Each program had an initial denaturation at 94°C for 10 min; 94°C for 30 s, annealing temperature 45 s and extension at 72°C for 45 s, and 35 cycles of 10 min at 72°C

Table 1. Primer sequence of SNPs.

	Mutation	Primer	Size (bp)	Annealing temperature (°C)
rs16942851	G/T	5'-CATTGAGCCTACATTATCCAT-3' 5'-AAACAACCTGCGACTGGTATT-3'	92	61
rs1058424	A/T	5'-TGGTATTCTCTCTCAATGC-3' 5'-TCAAATTTCTGGGCTTTAGTCC-3'	59	59
rs335929	A/C	5'-GGCTTTAGTCCCACATTACCT-3' 5'-GCTCATCAGTTACTTCCTTCC-3'	112	60
rs335931	A/G	5'-ACAAGCTAAGAATGTTC-3' 5'-AGCACTTGAAAATGTAGATG-3'	99	57
rs162007	A/G	5'-TGTGGCAATCGTAAGTTAT-3' 5'-AAATGTTTTGCATTCTGA-3'	93	60
rs3763043	C/T	5'-AGTCTTGCTGACAGAACTCA-3' 5'-TGTGCATGACTGTGACATACTG-3'	146	61



Figure 1. Physical map of the six SNPs selected for this study.

and 1 min at 4°C. The samples in 96-well plates were then loaded in the LightScanner 96 System (Idaho Technology Inc., Salt Lake City, UT, USA) with a temperature rise from 65°C to 95°C at the speed of 0.1°C/s. The sample assay was performed by analyzing data with the LightScanner software. Finally, normalized and temperature-adjusted melting curves of case and controls subjects were produced for SNPs genotyping. For quality control, genotyping results (each type ≥ 2) were compared to sequencing results (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in patients with NMO or HC (Fig. 2).

2.3 Statistical analysis

Hardy-Weinberg equilibrium (HWE) of six SNPs was calculated for MS, NMO/NMOSDs and HC group. Comparisons of genotype distributions between NMOSD patients, MS patients and normal subjects were performed using

logistic regression analysis, with age and sex as covariates, by using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). Significant associations were shown in boldface (P value ≤ 0.05). The common alleles were used as the referent genotype to the heterozygote and homozygote of the minor allele in referent analysis. By using the Haploview v4.2 software from the Broad Institute (<http://www.broadinstitute.org/mpg/haploview>), linkage disequilibrium (LD) among the SNPs was evaluated by examining Lewontin's D' ($|D'|$) and the LD coefficient (r^2) between all pairs of biallelic loci.

3. Results

3.1. Basic characteristics

The basic characteristics of patients and controls, including sex, age and clinical data are shown in Table 2; the expanded disability

status scale (EDSS) was significantly higher in the NMO/NMOSD patients compared with MS patients.

3.2 SNP association analysis

The SNPs were in Hardy-Weinberg equilibrium (HWE) in the control subjects ($p > 0.05$), except for rs1058424, which is located in the 3'UTR region. Therefore, we excluded this SNP from the logistic analysis.

Near the 5' end of the gene, the genotype distribution of rs162007 was significantly different under recessive model and co-dominant model. The p value of the association test by logistic regression models with age and sex adjustment was 0.006 in the recessive model and 0.009 in the co-dominant model. The frequency of the homozygous (A/A) was lower in NMO/NMOSD cases than in controls (6.56% vs. 25.24%, $p_{\text{adjusted}} = 0.009$) with odds ratio (OR) of 0.21 (95% CI 0.06 - 0.67) relative

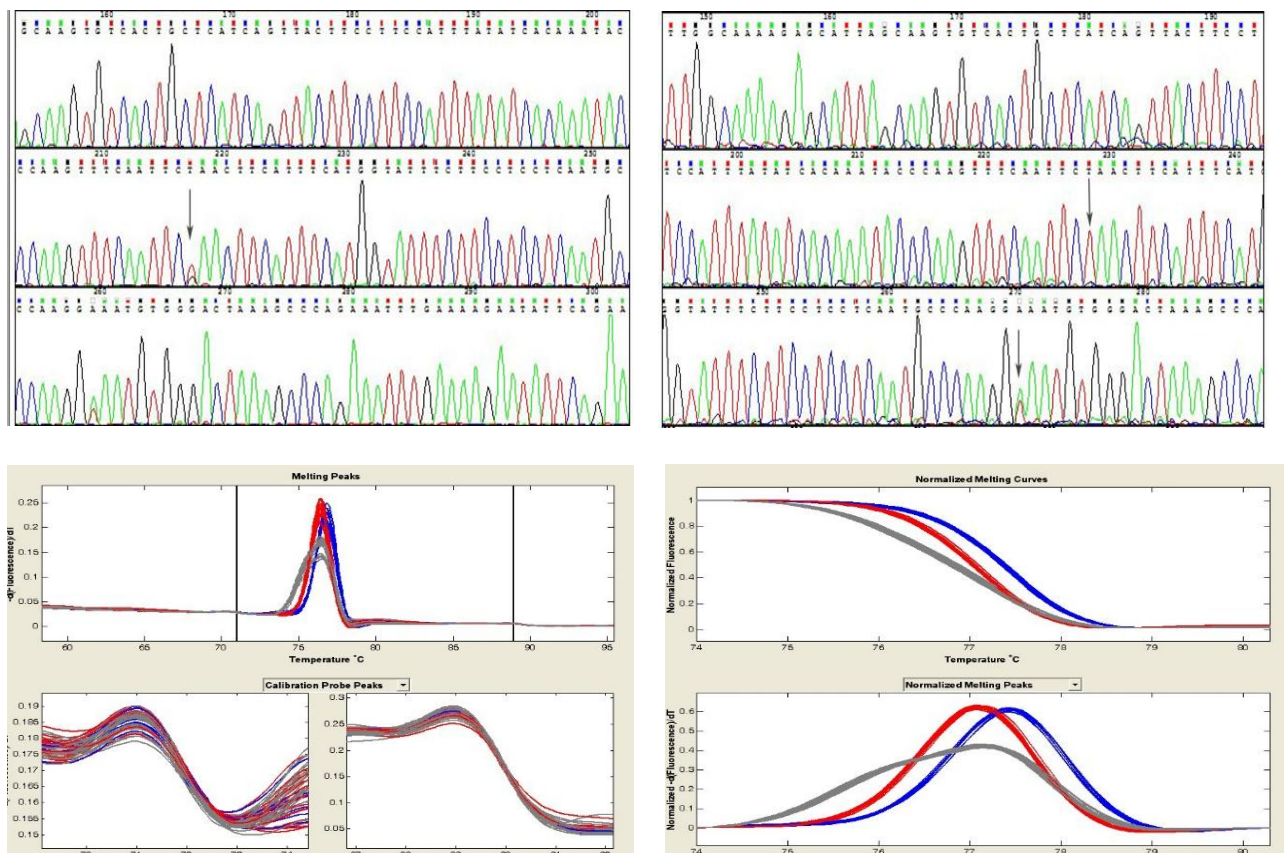


Figure 2. Sequencing (upper panel) simultaneously performed in comparison with genotyping results (each type ≥ 2) by high resolution melting method (lower panel) in patients with neuromyelitis optica.

to that in wild-type homozygotes (G/G) (Tables 3 and 4). In the stepwise logistic regression to screen variables, age and rs162007 were different in NMO/NMOSDs and HC, and these differences were statistically significant. But in the single factor regression model, age was not significantly different in the two groups. In the *post hoc* analysis, interactive analysis between age and rs162007 showed that age, rs162007 and interaction of age and rs162007 had no effect on NMO/NMOSDs. So we concluded that age, as a confounding factor, may interfere with the effect of rs162007 on disease.

A linkage disequilibrium plot is shown in Fig. 3. One linkage disequilibrium block was constructed for the *AQP4* gene. The block consisted of three SNPs: rs335931 in the intron, rs16942851 near 3' end of the gene, and rs335929 in 3' untranslated region (UTR). No significant difference in the block was found between the cases and the controls ($p > 0.05$, Fig. 3). Three haplotypes, ht1 to ht3, showed relatively high MAF of over 0.1 (Fig. 4), but non-significant difference in the frequency was found between the cases and the controls (Table 5).

4. Discussion

The human *AQP4* gene (18q11.2–q12.1) consists of five exons and four introns. For the encoded *AQP4* protein, two membrane-bound isoforms, M1 and M23, exist due to alternative splicing. The coding region of *AQP4* gene is highly conserved, while there is considerable variation in the non-coding regions of this gene among different ethnic groups [36]. In general, nucleotide variants in the coding

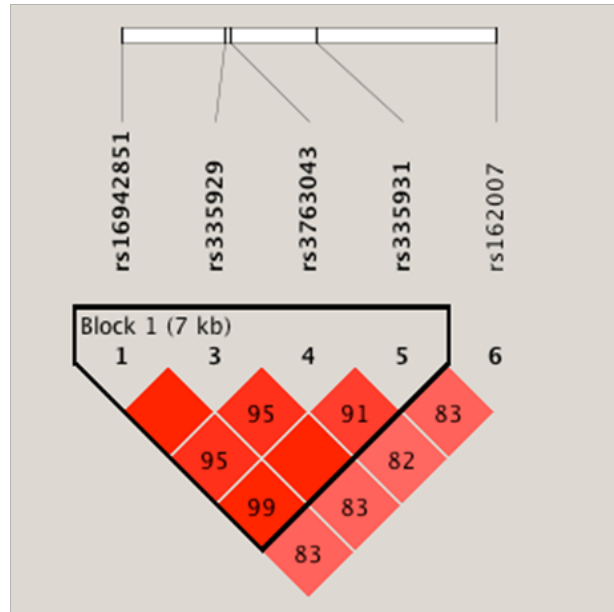


Figure 3. Linkage disequilibrium plot for five SNPs. The pairwise D' values indicating linkage disequilibrium coefficients between two SNPs are described in pink or red boxes (brighter colors indicate a higher D' value and red indicates D' = 1). The number below each SNP represents its order.

Haplotype	rs16942851	rs335929	rs3763043	rs335931	Freq.
ht1	G	C	C	G	0.411
ht2	T	A	T	A	0.364
ht3	T	A	C	A	0.207
others	0.018

Figure 4. Haplotypes of *AQP4* SNPs in all subjects.

Table 2. Basic characteristics of neuromyelitis optica (NMO) / neuromyelitis optica spectrum disorders (NMOSD), multiple sclerosis (MS) and healthy controls (HC).

	MS	NMO/NMOSD	HC	P value
M/F	32/55	23/39	43/66	-
Age (years)	33 [6-79]	36 [8-85]	30 [17-70]	*0.7891
Duration (months)	12.0 [0.1-156.0]	13.5 [0.25-336.0]	-	*0.7976
EDSS	3.0 [1.0-7.5]	5.0 [1.0-9.5]	-	*< 0.0001
Relapse times	2.0 [1.0-8.0]	3.0 [1.0-10.0]	-	*0.2795
Anti-AQP4 antibody n (%)	0(0)	45 (72.6%)	-	

EDSS: expanded disability status scale.
*calculated by using the Kruskal-Wallis test

Table 3. Genotype frequency of *AQP4* as well as logistic regression analysis in neuromyelitis optica (NMO) / neuromyelitis optica spectrum disorders (NMOSD), multiple sclerosis (MS) and healthy controls (HC).

		HC n (%)	MS n (%)	NMO/ NMOSD n (%)	P value	HC vs MS OR (95% CI)	P*	NMO/NMOSD vs HC P value	OR (95% CI)	p*
rs335931	AA	29 (27.88)	33 (37.93)	20 (32.26)	1.00		0.211	1.00		0.566
	AG	52 (50.00)	42 (48.28)	32 (51.61)	0.311	0.72 (0.38-1.37)		0.684	0.86 (0.42-1.78)	
	GG	23 (22.12)	12 (13.79)	10 (16.13)	0.081	0.47 (0.20-1.10)		0.289	0.60 (0.23-1.54)	
Dominant AA vs AG+GG					0.150	0.64 (0.35-1.18)		0.478	0.78 (0.39-1.55)	
Recessive AA+AG vs GG					0.149	0.57 (0.26-1.22)		0.324	0.66 (0.29-1.51)	
rs335929	AA	30 (27.78)	33 (37.93)	21 (33.87)	1.00		0.252	1.00		0.520
	AC	56 (51.85)	42 (48.28)	32 (51.61)	0.239	0.68 (0.36-1.29)		0.558	0.81 (0.40-1.65)	
	CC	22 (20.37)	12 (13.79)	9 (14.52)	0.115	0.50 (0.21-1.19)		0.254	0.57 (0.22-1.50)	
Dominant AA vs AC+CC					0.136	0.63 (0.34-1.16)		0.384	0.74 (0.38-1.46)	
Recessive AA+AC vs CC					0.241	0.63 (0.29-1.36)		0.327	0.65 (0.28-1.54)	
rs16942851	TT	31 (28.70)	33 (37.50)	21 (33.87)	1.00		0.305	1.00		0.667
	TG	55 (50.93)	42 (47.73)	31 (50.00)	0.311	0.72 (0.38-1.36)		0.583	0.82 (0.40-1.67)	
	GG	22 (20.37)	12 (13.64)	10 (16.13)	0.136	0.52 (0.22-1.23)		0.376	0.65 (0.26-1.67)	
Dominant TT vs TG+GG					0.181	0.66 (0.36-1.21)		0.451	0.77 (0.39-1.52)	
Recessive TT+TG vs GG					0.246	0.63 (0.29-1.37)		0.478	0.74 (0.32-1.70)	
rs162007	GG	31 (30.10)	38 (43.68)	23 (37.70)	1.00		0.152	1.00		0.023
	AG	46 (44.66)	33 (37.93)	34 (55.74)	0.111	0.59 (0.31-1.13)		0.917	0.96 (0.47-1.96)	
	AA	26 (25.24)	16 (18.39)	4 (6.56)	0.090	0.51 (0.23-1.11)		0.009	0.21 (0.06-0.67)	
Dominant GG vs AG+AA					0.056	0.56 (0.31-1.02)		0.260	0.68 (0.34-1.33)	
Recessive GG+AG vs AA					0.266	0.67 (0.33-1.36)		0.006	0.21 (0.07-0.64)	
rs3763043	CC	39 (39.00)	29 (33.72)	27 (45.00)	1.00		0.683	1.00		0.445
	TC	48 (48.00)	47 (54.65)	23 (38.33)	0.410	1.30 (0.70-2.44)		0.307	0.69 (0.34-1.40)	
	TT	13 (13.00)	10 (11.63)	10 (16.67)	0.959	1.03 (0.39-2.67)		0.732	1.19 (0.45-3.13)	
Dominant CC vs TC+TT					0.479	1.24 (0.68-2.27)		0.487	0.79 (0.41-1.52)	
Recessive CC+TC vs TT					0.773	0.88 (0.36-2.12)		0.442	1.43 (0.58-3.53)	

CI, confidence interval; OR, odds ratio; *Overall P value for that category

Table 4. Allele frequency of *AQP4* as well as logistic regression analysis in neuromyelitis optica (NMO) / neuromyelitis optica spectrum disorders (NMOSD), multiple sclerosis (MS) and healthy controls (HC).

		HC n (%)	MS n (%)	NMO/NMOSD n (%)	HC vs MS P value	OR (95% CI)	NMO/NMOSD vs HC P value	OR (95% CI)
rs335931	A	110 (52.88)	108 (62.07)	72 (58.06)	0.078	1.17 (0.99-1.40)	0.365	1.10 (0.90-1.33)
	G	98 (47.12)	66 (37.93)	52 (41.94)				
rs335929	A	116 (53.70)	108 (62.07)	74 (59.68)	0.101	1.16 (0.98-1.37)	0.308	1.11 (0.92-1.34)
	C	100 (46.30)	66 (37.93)	50 (40.32)				
rs16942851	T	117 (54.17)	108 (62.07)	73 (58.87)	0.123	1.15 (0.97-1.36)	0.428	1.09 (0.90-1.32)
	G	99 (45.83)	66 (37.93)	51 (41.13)				
rs162007	G	108 (52.43)	109 (62.64)	80 (65.57)	0.049	0.79 (0.62-1.00)	0.021	0.72 (0.54-0.96)
	A	98 (47.57)	65 (37.36)	42 (34.43)				
rs3763043	C	126 (63.00)	105 (61.05)	77 (64.17)	0.748	1.05 (0.81-1.37)	0.905	0.97 (0.72-1.31)
	T	74 (37.00)	67 (38.95)	43 (35.83)				

CI, confidence interval; OR, odds ratio

Table 5. Comparison of haplotypes frequency of AQP4 between neuromyelitis optica (NMO) / neuromyelitis optica spectrum disorders (NMOSD), multiple sclerosis (MS) and healthy controls (HC).

haplotype	NMOSD	MS	HC	NMOSD vs HC		MS vs HC	
				χ^2	P value	χ^2	P value
ht1	0.393	0.378	0.447	0.935	0.3337	1.957	0.1619
ht2	0.344	0.387	0.357	0.055	0.8149	0.425	0.5146
ht3	0.228	0.233	0.175	1.456	0.2275	1.933	0.1645

region of AQP4 may produce conformational changes in the encoded protein and modify its major functions of water transportation and coupling of potassium and glutamate channels. These functions have been implicated in NMO pathogenesis [37]. Nucleotide variability in the promoter, or in the 5' or 3' UTR, may influence AQP4 levels by modifying the affinity for transcriptional or post-transcriptional factors.

In this study, six common SNPs were selected on the basis of the CHB database and genotyped in 87 MS, 62 NMO/NMOSD patients and 109 healthy individuals. However, logistic analysis showed no significant associations of AQP4 polymorphisms with MS or NMO. These findings, together with other similar reports in Korean and Caucasian cohorts [23, 24], strongly suggest that polymorphisms of AQP4 gene may not confer a risk of NMO. Alternatively, AQP4 polymorphisms may indirectly affect disease development via the interaction with non-AQP4 susceptibility gene, such as HLA-DPB1*0501 allele. This allele is associated with NMO seropositive for anti-AQP4 Ab in Japanese and Southern Han Chinese populations [16, 17].

The distribution of allele frequencies influences disease susceptibility and varies

among different ethnic groups [38, 39]. In a study reported by the Mayo Group, one uncommon SNP, D184E (rs61731038), located at coding area of AQP4 gene, was nominally associated with NMO ($p = 0.026$, odds ratio = 13.09) in an African American population. In addition, two different missense allelic mutations at Arg19 (R19I and R19T) (rs150587304) in coding area were detected in NMO patients of African American ancestry or Jewish ancestry, in comparison to normal controls [40]. However, this finding of NMO-associated mutations R19I and R19T in AQP4 was not confirmed by a subsequent functional study [41]. In this study, these two SNPs of rs61731038 and rs150587304 were not included as common SNPs for genotyping due to their very low MAF in the Han Chinese population (less than 0.05). Of note, a recent study reported that heterozygous genotype of two 3' UTR SNPs was significantly higher in NMO cases than controls in a Southern Han Chinese population, including the A/T genotype of SNP rs1058424 ($p = 0.024$, OR = 1.670) and the C/T genotype of SNP rs3763043 ($p = 0.028$, OR = 1.638) [42]. In contrast to this, our study revealed no remarkable distinction in genotypes of the two 3' UTR SNPs between the cases and the

controls. Nevertheless, further replication in a larger cohort is needed to verify this issue.

In conclusion, no significant associations of common AQP4 SNPs with MS and NMO/NMOSD were observed, strongly suggesting that polymorphisms of AQP4 gene are unlikely to confer NMO/NMOSD or MS susceptibility, at least in Northern Han Chinese population. On the other hand, this study has limitations. An obvious limitation of this study has been its lack of statistical power due to the small number of MS and NMO/NMOSD subjects. Therefore, future investigations using fine mapping with dense SNPs and a larger sample size will help to gain more insight into the involvement of AQP4 SNPs in NMO/NMOSD susceptibilities.

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