Received: 4 August 2021 | Revised: 7 September 2021 | Accepted: 22 September 2021

DOI: 10.1111/cns.13738

ORIGINAL ARTICLE

CNS Neuroscience & Therapeutics Open Access WILEY

BLK polymorphisms and expression level in neuromyelitis optica spectrum disorder

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Funding information
This study was supported by the National Natural Science Foundation of China (No. 81873759)

Abstract

Aim: This study aimed to determine the correlation between B-lymphoid tyrosine kinase (BLK) polymorphism, mRNA gene expression of BLK, and NMOSD in a Chinese Han population.

Background: B-lymphoid tyrosine kinase gene expressed mainly in B cells plays a key role in various autoimmune disorders. However, no studies have investigated the association of BLK polymorphisms with neuromyelitis optica spectrum disorder (NMOSD).

Methods: Han Chinese population of 310 subjects were recruited to analyze three single nucleotide polymorphisms (rs13277113, rs4840568, and rs2248932) under allele, genotype, and haplotype frequencies, followed by clinical characteristics stratified analysis. Real-time PCR was used to analyze mRNA expression levels of BLK in the peripheral blood mononuclear cells of 64 subjects.

Results: Patients with NMOSD showed lower frequencies of the minor allele G of rs2248932 than healthy controls (odds ratio (OR) =0.57, 95% confidence intervals (CI) 0.39–0.83, $p = 0.003$). The association between minor allele G of rs2248932 and reduced NMOSD susceptibility was found by applying genetic models of inheritance (codominant, dominant, and recessive) and haplotypes analysis. Subsequently, by stratification analysis for AQP4-positivity, the minor allele G frequencies of rs2248932 in AQP4-positive subgroup were significantly lower than in the healthy controls (OR =0.46, 95% CI 0.30–0.72, $p = 0.001$). Notably, the genotype GG of rs2248932 was more frequent in AQP4-negative subgroup ($n = 14$) than in AQP4-positive subgroup ($n = 93$) ($p = 0.003$, OR =0.05, 95% CI =0.01–0.57). BLK mRNA expression levels in the NMOSD patients ($n = 36$) were lower than in healthy controls ($n = 28$) ($p < 0.05$). However, the acute non-treatment ($n = 7$), who were untreated patients in the acute phase from the NMOSD group, showed BLK mRNA expression levels 1.8-fold higher than healthy controls ($n = 8$) ($p < 0.05$).

Conclusion: This study evaluated that the minor allele G of rs2248932 in BLK is associated with reduced susceptibility to NMOSD and protected the risk of AQP4-positive. BLK mRNA expression in NMOSD was lower as compared to healthy controls while significantly increased in acute-untreated patients.

KEYWORDS
B-lymphoid tyrosine kinase, mRNA, neuromyelitis optica spectrum disorder, rs2248932, single nucleotide polymorphisms

1 | INTRODUCTION

NMOSD is a relatively rare inflammatory, autoimmune, and demyelinating disorder of the central nervous system (CNS), predominantly affecting the optic nerve and the spinal cord. The international panel of experts published that the concept of NMOSD is defined as an astrocytopathy mediated by aquaporin-4 immunoglobulin G (AQP4-IgG), which is produced by differentiation of B cells to plasma cells.[1] Up to date, a study reported that serum AQP4-IgG could be detected in 60%–80% of patients with NMOSD, while the diagnostic criteria differentiate patients with positive or negative/unknown AQP4-IgG status.[2] Intriguingly, some epidemiological surveys revealed that women are more often affected by NMOSD than men, along with the sex ratio being about 9:1. In the Asian populations, the prevalence of NMOSD is higher than in white populations.[3,4] The definite etiology and pathogenesis of NMOSD have not been completely elucidated so far, but multiple genetic and environmental risk factors that contribute to the susceptibility of NMOSD have been established in recent years. In previous studies, some polymorphisms in several immunomodulatory genes have shown an association with an increased risk of NMOSD, including IRAK1,[5] CD58,[6,7] AQP4,[8,9] HLA,[10,11] and IL17.[12] In contrast, compared to other autoimmune diseases, such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), and autoimmune thyroid diseases, there are still only a few studies regarding the genetics of NMOSD. All the genes/loci that have been identified represent a small proportion of the NMOSD-heritability only, partly due to many other genetic loci that remain unknown.

The BLK gene located on chromosome 8p23.1 encodes a non-receptor tyrosine kinase of the Src family of proto-oncogenes. It is typically involved in B cell proliferation as well as differentiation. The protein plays a crucial role in B cell receptor signaling and might be significant for the development of the B cell repertoire as well as mature B cell function.[13,14] Furthermore, BLK is involved in the immune tolerance of B lymphocytes, affecting the function of B cells, which may lead to autoreactive or regulatory cellular responses, increasing the risk of autoimmune diseases. Most studies have provided reliable evidence that the single nucleotide polymorphisms (SNPs) of BLK exert a key role in susceptibility to various autoimmune disorders, such as SLE,[15] rheumatoid arthritis (RA),[16] SS,[17] thyroid disorders,[18] and systemic sclerosis.[19] Similarly, NMOSD is also mainly a B cell-mediated autoimmune disease.[20] The clinical and laboratory studies of NMOSD have demonstrated the pivotal role of B cells in pathogenesis.[21] As a result, the BLK variants may also confer susceptibility to NMOSD. However, it may add to our knowledge that no research has investigated the correlation between BLK gene SNPs and mRNA expression with NMOSD. Therefore, this study detected whether BLK SNPs at these loci predispose individuals from a Han Chinese population to NMOSD. This study analyzed the relationship between BLK alleles, genetic models, linkage disequilibrium (LD), and haplotypes with NMOSD. Meanwhile, we conducted a stratified analysis of genotypes based on AQP4-IgG status and clinical symptoms-onset. In addition, we also evaluated the BLK mRNA expression levels in peripheral blood mononuclear cells (PBMC) from NMOSD patients and healthy people in Han Chinese.

Highlights

- This study demonstrated that the minor allele G of rs2248932 in BLK is associated with reduced susceptibility to NMOSD.
- The minor allele G and GG genotype frequencies of rs2248932 were significantly lower in the AQP4-positive subgroup compared with the HCs.
- The minor alleles G of rs2248932 reduced the risk of AQP4-positive compared with AQP4-negative.
- The NMOSD patients showed significantly reduced BLK mRNA expressions levels compared with HCs.
- However, the acute-untreated NMOSD patients showed significantly higher BLK mRNA expressions levels compared with HCs.

Significance statement

- NMOSD is a relatively rare inflammatory, autoimmune, and demyelinating disorder of the CNS, which predominantly affects the optic nerve and spinal cord. BLK is involved in the immune tolerance of B lymphocytes, affecting the function of B cells, increasing the risk of autoimmune diseases. BLK variants may also confer susceptibility to NMOSD. However, to our knowledge, the correlation between BLK gene SNPs and mRNA expression with NMOSD has not been investigated. Our findings increase genetic insights into the role of BLK in NMOSD pathogenesis and promote the development of screening strategies targeting the alleles susceptible to NMOSD for early control.

2 | MATERIALS AND METHODS

2.1 | Participants

The present study included 310 Han Chinese individuals from Northern China, 121 with NMOSD, and 189 healthy controls (HCs). All participants were consecutively recruited from the

Neurology Department of the Second Hospital of Hebei Medical University between April 2019 and June 2021. All patients met the revised criteria for the diagnosis of NMOSD.[22] No patients were combined with other systemic, autoimmune, neurological, or infectious diseases. None of the HCs suffered from autoimmune diseases, nervous system diseases, tumors, or other common systemic diseases.

Subsequently, we performed allele-specific expression analysis for BLK in PBMC from the NMOSD patients and HCs, with sex and age being matched. Demographics and clinical characteristics were recorded for each subject, including gender, age, age at onset, onset symptoms, core clinical syndromes, disease duration, serum AQP4-IgG status (cell-based assay),[23] and MRI lesions. This study was authorized by the Ethics Committee of The Second Hospital of Hebei Medical University. Before this study, all participants gave informed written consent.

2.2 | DNA extraction and single nucleotide polymorphisms genotyping

The peripheral blood samples (1–3 ml) were collected EDTA anticoagulated venous blood samples from all subjects. Genomic DNA samples were extracted and purified from each specimen using the Blood Genome DNA Extraction Kit provided by Shanghai Generay Biotech Co., Ltd and were stored at −80°C until genotyping. SNPs selection was preceded by research in PubMed. BLK SNPs with minor allele frequency (MAF) below 5% (< 0.05) were excluded from the study. We selected three BLK genetic variants based on previous studies of other autoimmune disorders (rs13277113, rs4840568, and rs2248932). Sequences of the three SNPs from the BLK gene and primer information are described (Table 1). SNP genotyping was performed using the SNaPshot technique (Thermo Fisher Inc. Shanghai, China).

2.3 | RNA extraction and real-time PCR analysis of the mRNA

The PBMC was isolated from the fresh peripheral blood (3–4 ml) of the patients and controls to extract the mRNA. Afterward, mRNA was isolated by TRIzol Reagent (Tiangen Biochemical Technology (Beijing) Co., Ltd., DP421) according to the manufacturer's instructions. For the reverse transcriptase reaction into cDNA, we used the PrimeScript RT reagent kit (Takara Bio Inc., Japan). PCR amplification was performed by using QuantStudio™ 7 Flex Real-Time PCR System with the conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and a final extension of 72°C for 10 min. The mRNA relative expression levels were computed using the $2^{-\Delta\Delta Ct}$ method.

2.3.1 | BLK primers

Forward 5′ AGGTCACTCGTCACAGGAAGA.
Reverse 5′ GCCTTGTTGATTGGAGCAAGA.

2.4 | Statistical analysis

The clinical data, demographic, disease activity, and laboratory variables in relation to BLK were described as mean ± standard deviation (SD), and frequencies were presented as numbers and percentages. The Hardy-Weinberg equilibrium (HWE) test was performed for genotypes distribution of each polymorphism, with $p > 0.05$ indicated no significant deviation in allele distribution among subjects. Differences in gender and age between patients with NMOSD and controls were analyzed using the Chi-square test and Student's *t*-test (normality), respectively.

Subsequently, logistic regression analysis was applied to assess the association with NMOSD susceptibility under allelic, codominant, dominant, recessive, and overdominant models after adjusting for sex as well as age. Moreover, stratified analysis was applied for clinical characteristics with BLK variants. BLK LD patterns haplotype analysis was conducted with SHEsis software ($r^2 > 0.8$ means strong LD) (http://analysis.bio-x.cn/myAnalysis.php).[24] The nonparametric Mann-Whitney U test (non-normality) was used to compare the mRNA expression of BLK between the two groups and assess differences in the mRNA expression in genotypes of rs13277113, rs4840568 as well as rs2248932 in the NMOSD patients. Kruskal-Wallis (non-normality) with Dunn's test was used to compare the mRNA expression of BLK among three groups. Other statistical analyses were performed by using SPSS 19.0 (IBM Corp). The *p*-value lower than 0.05 was regarded as statistically significant.

TABLE 1 SNPs in BLK and primer sequences used for PCR amplification

Rs_num	SNP substitution	Chr	Chr_Position	Primer sequence
rs13277113	G > A	8	NC_000008.11:g.11491677	F: GCAAGATGTCCGTAGACTCA R: GATTCACCTCAGAAGGGTCTA
rs4840568	G > A	8	NC_000008.11:g.11493510	F: GAAATGAACTCCTTGAGAAAG R: AGTGTCTTGATATTGGCTTAT
rs2248932	A > G	8	NC_000008.11:g.11534141	F: AATGCAGATACCAGCACT R: CCAATGGAGTCAGTTATGATT

3 | RESULTS

3.1 | Clinical characteristics

Demographics and clinical characteristics of all participants were collected at the time of blood sampling (Table 2). These include gender, mean age, mean age at onset, AQP4-IgG status, core clinical syndromes. A total of 121 patients with NMOSD (109 females,90.08%) and 189 HCs (170 females,89.94%) were recruited (sex, $p = 0.969$, $p > 0.05$). The average ages were 44.64 ± 16.10 years (NMOSD) and 44.92 ± 10.07 years (HCs, $p = 0.851$, $p > 0.05$). A significant difference was not indicated in gender or age between patients and controls ($p > 0.05$) (Table 2). The mean onset age of NMOSD was 42.93 ± 16.79 years. Furthermore, 107 (88.4%) patients with NMOSD tested serum AQP4-IgG. In the NMOSD patients, the AQP4-Ab status data were available, 86.9% (93/107) were AQP4-positive, and 13.1% (14/107) were AQP4-negative.

TABLE 2 Demographics and clinical characteristics of participants

	NMOSD	HCs	*p*-values
Gender, female/male (*F*%)	109:12 (90.08 %)	170:19 (89.94%)	0.969
Age, year (mean ± SD)	44.64 ± 16.10	44.92 ± 10.07	0.851
Age at onset, year (mean ± SD)	42.93 ± 16.790	NA	NA
AQP4-IgG[a], no. (%) of patients	107/121 (88.4)		
Seropositive	93/107 (86.9)	NA	NA
Seronegative	14/107 (13.1)	NA	NA
Onset syndromes[b], no. (%) of patients			
Optic neuritis	22/121 (18.2)	NA	NA
Acute myelitis	41/121 (33.9)	NA	NA
Brain attacks	5/121 (4.1)	NA	NA
Mix attacks	53/121 (43.8)	NA	NA

Abbreviations: NA, not applicable; SD, standard deviation.
[a]Data on AQP4-IgG was available for 107 patients.
[b]Data on clinical syndromes were available for 121 patients; brain attacks include brainstem and brain attacks.

3.2 | Analysis of alleles and genotypes frequencies

The distribution of BLK allele frequencies in both NMOSD patients and controls was consistent with HWE ($p > 0.05$). Moreover, we took into account the MAF of the three BLK SNPs in the present study. All of the MAF was greater than 5%. Evaluation of genotyping quality has been presented (Table 3).

Furthermore, the distributions of alleles and genotypes of BLK rs13277113, rs4840568, and rs2248932 polymorphisms in patients and controls are shown (Table 4). Nonsignificant evidence was found for the association of NMOSD with neither BLK rs13277113 polymorphism nor rs4840568 polymorphism ($p > 0.05$). However, we detected a significant difference in genotype distribution of the BLK rs2248932 polymorphisms between NMOSD patients and HCs. The frequencies of the minor allele G of rs2248932 were lower in the patient group (0.202) compared with the control group (0.310) and were significantly associated with decreased risk of NMOSD (OR =0.57, 95% CI 0.39–0.83, $p = 0.003$).

We also analyzed the distribution of the three SNPs polymorphisms by using four genetic models of inheritance (codominant, dominant, recessive, and overdominant).

The results showed that the GG genotype of rs2248932 was significantly associated with reduced susceptibility to NMOSD in the codominant model (G/G compared with A/A, OR: 0.29, 95% CI: 0.10–0.80, $p = 0.014$), dominant model (A/G+G/G compared with A/A, OR: 0.55, 95% CI: 0.35–0.88, $p = 0.012$), and recessive model (G/G compared with A/G+A/A, OR: 0.34, 95% CI: 0.13–0.94, $p = 0.023$), while indicating that the minor allele G of rs2248932 was a protective allele for NMOSD. Detailed values are presented (Table 4).

3.3 | Analysis of linkage disequilibrium and haplotypes

In three SNPs, LD was calculated according to the D' and r^2 values. The rs13277113 and rs4840568 variants exhibited a strong LD (D' =0.97, $r^2 = 0.95$, both D' and $r^2 > 0.9$) (Figure 1). Rs2248932 exhibited moderate LD with rs13277113 and rs4840568, respectively (all D' =0.75, r^2 = 0.50). This study analyzed the BLK haplotypes based on the LD between the three SNPs. Haplotypes were constructed following the order of rs13277113, rs4840568, rs2248932,

TABLE 3 Data of quality evaluation for genotyping

SNP	Groups	Test of HWE (*p*-value)	MAF (present-study data) Allele	Frequencies	MAF (CHB)
rs13277113	NMOSD	0.250	G	0.290	0.252
	Control	0.538	G		
rs4840568	NMOSD	0.299	G	0.290	0.252
	Control	0.971	G		
rs2248932	NMOSD	0.982	G	0.267	0.223
	Control	0.325	G		

Abbreviations: CHB, Han Chinese in Beijing; MAF, minor allele frequency.

TABLE 4 Allelic and genotypic frequencies of the BLK three-SNP association analysis in NMOSD and controls

Gene SNP	Model	Genotype	HCs n (%)	NMOSD n (%)	OR (95% CI)	p-values
rs13277113 A/G	Alleles	A	262 (0.693)	178 (0.736)	1	0.26
		G	116 (0.307)	64 (0.264)	O.81 (0.57~1.16)	
	Codominant	A/A	89 (0.471)	63 (0.521)	1	0.41
		A/G	84 (0.444)	52 (0.430)	0.88 (0.55-1.42)	
		G/G	16 (0.085)	6 (0.050)	0.52 (0.19-1.42)	
	Dominant	A/A	89 (0.471)	63 (0.521)	1	0.40
		A/G-G/G	100 (0.529)	58 (0.479)	0.82 (0.52-1.30)	
	Recessive	A/A-A/G	173 (0.915)	115 (0.95)	1	0.22
		G/G	16 (0.085)	6 (0.05)	0.55 (0.21-1.47)	
	Overdominant	A/A-G/G	105 (0.556)	69 (0.570)	1	0.82
		A/G	84 (0.444)	52 (0.430)	0.95 (0.60-1.51)	
rs4840568 A/G	Alleles	A	261 (0.690)	179 (0.740)	1	0.19
		G	117 (0.310)	63 (0.260)	0.79 (0.55-1.13)	
	Codominant	A/A	90 (0.476)	64 (0.529)	1	0.27
		A/G	81 (0.429)	51 (0.421)	0.89 (0.55-1.44)	
		G/G	18 (0.095)	6 (0.050)	0.46 (0.17-1.23)	
	Dominant	A/A	90 (0.476)	64 (0.529)	1	0.37
		A/G-G/G	99 (0.524)	57 (0.471)	0.81 (0.51-1.28)	
	Recessive	A/A-A/G	171 (0.905)	115 (0.950)	1	0.12
		G/G	18 (0.095)	6 (0.050)	0.49 (0.19-1.27)	
	Overdominant	A/A-G/G	108 (0.571)	70 (0.579)	1	0.92
		A/G	81 (0.429)	51 (0.421)	0.98 (0.61-1.56)	
rs2248932	Alleles	A	261 (0.690)	193 (0.798)	1	0.003
		G	117 (0.310)	49 (0.202)	0.57 (0.39-0.83)	
	Codominant	A/A	93 (0.492)	77 (0.636)	1	0.014
		A/G	75 (0.397)	39 (0.322)	0.63 (0.38-1.03)	
		G/G	21 (0.111)	5 (0.041)	0.29 (0.10-0.80)	
	Dominant	A/A	93 (0.492)	77 (0.636)	1	0.012
		A/G-G/G	96 (0.508)	44 (0.364)	0.55 (0.35-0.88)	
	Recessive	A/A-A/G	168 (0.889)	116 (0.959)	1	0.023
		G/G	21 (0.111)	5 (0.041)	0.34 (0.13-0.94)	
	Overdominant	A/A-G/G	114 (0.603)	82 (0.678)	1	0.18
		A/G	75 (0.397)	39 (0.322)	0.72 (0.45-1.17)	

The results of genotypic frequencies using logistic regression analyses were adjusted by age and sex.
Abbreviations: CI, confidence intervals; HCs, healthy controls; NMOSD, neuromyelitis optica spectrum disorders; OR, odds ratios.

and the haplotypes (AGA, AGG, and GAA) with a frequency <3.0% in both patients and controls were excluded. However, the remaining 4 haplotypes are described (Table 5). Three haplotypes, including AAA, AAG, and GGG, displayed a statistically significant difference between the patients and controls. In particular, the haplotype AAA carrying the major alleles A of the three SNPs rendered an increased risk of NMOSD (p = 0.032, OR =1.467, 95% CI =1.033–2.085), while the two haplotypes (AAG and GGG) carrying the minor alleles G of rs2248932 might be associated with protection against NMOSD (p = 0.034, OR =0.373, 95% CI =0.144–0.962; p = 0.038, OR =0.652, 95% CI =0.435–0.979, respectively). In this regard, the minor alleles G of rs2248932 were also shown to be protective for NMOSD susceptibility.

3.4 | Stratification analysis of BLK polymorphisms according to clinical characteristics of patients with NMOSD

To investigate BLK polymorphisms were associated with the clinical characteristics of NMOSD patients, we further analyzed AQP4-IgG status and onset symptoms based on genotypes of rs13277113,

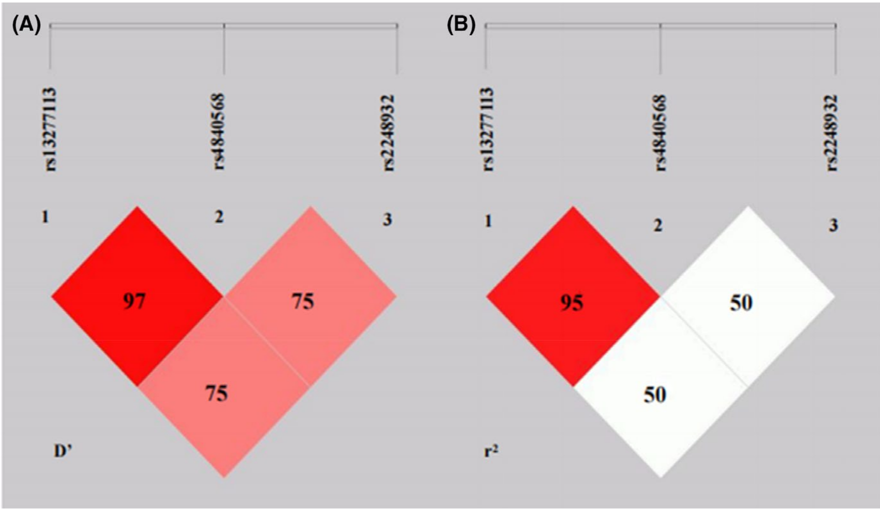

FIGURE 1 Linkage disequilibrium (LD) patterns in BLK. D' (A) and r^2 (B) mean LD coefficients of the three SNPs. Each block represents the LD relationship between two SNPs. The rs13277113 and rs4840568 variants exhibited strong LD for both D' and $r^2 > 0.9$. Rs2248932 indicated moderate LD with rs13277113 and rs4840568, respectively (D' =0.75, $r^2 = 0.50$)

TABLE 5 Haplotype analysis of BLK polymorphisms in NMOSD and controls

Haplotype	Case (%)	HCs (%)	Chi-square	*p*-value	OR (95% CI)
A A A	0.709	0.629	4.596	0.032	1.467 (1.033-2.085)
A A G	0.023	0.059	4.477	0.034	0.373 (0.144-0.962)
G G A	0.080	0.057	1.338	0.247	1.452 (0.769-2.742)
G G G	0.176	0.247	4.288	0.038	0.652 (0.435-0.979)

Logistic chosen for haplotype analysis: rs13277113, rs4840568, and rs2248932.

rs4840568, and rs2248932. The NMOSD genotyping samples can be divided into the AQP4-positive (n = 93) and AQP4-negative (n = 14) subgroups. First, we further assessed the stratification analysis for AQP4-positivity. Particularly, it was found that the minor allele G frequencies of rs2248932 were significantly lower in the AQP4-positive subgroup compared with the HCs (OR =0.46, 95% CI 0.30–0.72, p = 0.001) (Table 6). Subsequently, genotypes of BLK SNPs were analyzed using four genetic models, and the genotype GG of rs2248932 was found to reduce NMOSD risk in all three genetic models (codominant, dominant, and recessive), which was significantly much more different from before the stratification analysis for AQP4-positivity. In this respect, a significant association was not observed between the other two SNPs (rs13277113, rs4840568) and the risk of NMOSD. Second, the frequencies of the genotypes of rs2248932 displayed a statistical difference between the AQP4-positive subgroup and the AQP4-negative subgroup. The genotype GG of rs2248932 was more frequent in the AQP4-negative subgroup than in the AQP4-positive subgroup (p = 0.003, OR =0.05, 95% CI =0.01–0.57). It is suggested that the minor alleles G of rs2248932 reduced the risk of AQP4-positive. Finally, the stratification analysis according to onset symptoms indicated no significant difference (Table 7).

3.5 | Analysis of BLK mRNA expression between NMOSD patients and healthy controls

In the NMOSD group, the number was only 36, while mRNA expression levels of BLK in PBMC could be measured (3 males and 33 females, mean age 46.72 ± 15.324 years) and randomly recruited 28 HCs (5 males and 23 females, mean age 41.93 ± 11.981 years), there was no significant difference in gender or age between patients and controls (gender: p = 0.282; age: p = 0.178). In the NMOSD group, 33 patients were AQP4-positive, and 3 patients were AQP4-negative. We found that the NMOSD patients significantly reduced BLK mRNA expression levels than HCs (p = 0.025) (Figure 2A). Furthermore, the 36 NMOSD patients were divided into 3 groups are following: (1) Acute non-treatment group (ANT): 7 patients in the acute phase were admitted without any medication before the time of blood collecting, and no patients took any drugs for 1 month prior to its onset (all patients tested positive for serum AQP4-IgG); (2) Acute long-term oral glucocorticoids group (AOGC): 20 patients in acute phase had routinely used long-term oral glucocorticoids before this acute onset (3 patients were AQP4-negative, and 17 patients in the NMOSD group were AQP4-positive); (3) remission group: 9 patients in the stable phase were taking long-term oral drugs (including glucocorticoids, azathioprine, or mycophenolate mofetil, etc.). The 9 patients tested positive for serum AQP4-IgG. In a comparison of the ANT group with age- and gender-matched HCs, the BLK mRNA expression in the ANT group was 1.8-fold higher than in the controls (n = 8) (p = 0.040) (Figure 2B). After age and sex correction in the three groups among the NMOSD patient cohort, we found the BLK expression levels were ranked in the three groups in order from high to low: ANT >AOGC > remission group. According to the comparison results, the BLK mRNA expression levels in the ANT group were higher than the other two groups (p = 0.0135). And there were statistical differences in the ANT

TABLE 6 Stratification analysis for AQP4-positivity: allelic and genotypic frequencies of the BLK three-SNP association analysis in AQP4-positive subgroup and controls

Gene SNP	Model	Genotype	HCs *n* = 189 (%)	AQP4-positive subgroup *n* = 93 (%)	OR (95% CI)	*p*-values
rs13277113 A/G	Alleles	A	262 (0.693)	140 (0.753)	1	0.14
		G	116 (0.307)	46 (0.247)	0.74 (0.50~1.11)	
	Codominant	A/A	89 (0.471)	50 (0.538)	1	0.15
		A/G	84 (0.444)	40 (0.430)	0.86 (0.51-1.44)	
		G/G	16 (0.085)	3 (0.032)	0.32 (0.09-1.15)	
	Dominant	A/A	89 (0.471)	50 (0.538)	1	0.30
		A/G-G/G	100 (0.529)	43 (0.462)	0.77 (0.46-1.26)	
	Recessive	A/A-A/G	173 (0.915)	90 (0.968)	1	0.07
		G/G	16 (0.085)	3 (0.032)	0.34 (0.10-1.21)	
	Overdominant	A/A-G/G	105 (0.556)	53 (0.570)	1	0.87
		A/G	84 (0.444)	40 (0.430)	0.96 (0.58-1.59)	
rs4840568 A/G	Alleles	A	261 (0.690)	141 (0.758)	1	0.10
		G	117 (0.310)	45 (0.242)	0.71 (0.48-1.06)	
	Codominant	A/A	90 (0.476)	51 (0.548)	1	0.09
		A/G	81 (0.429)	39 (0.419)	0.86 (0.5-1.45)	
		G/G	18 (0.095)	3 (0.032)	0.28 (0.08-1.00)	
	Dominant	A/A	90 (0.476)	51 (0.548)	1	0.26
		A/G-G/G	99 (0.524)	42 (0.452)	0.75 (0.45-1.24)	
	Recessive	A/A-A/G	171 (0.905)	90 (0.968)	1	0.03
		G/G	18 (0.095)	3 (0.032)	0.30 (0.09-1.05)	
	Overdominant	A/A-G/G	108 (0.571)	54 (0.581)	1	0.94
		A/G	81 (0.429)	39 (0.419)	0.98 (0.61-1.56)	
rs2248932	Alleles	A	261 (0.690)	154 (0.828)	1	0.001
		G	117 (0.310)	32 (0.172)	0.46 (0.30-0.72)	
	Codominant	A/A	93 (0.492)	62 (0.667)	1	5×10^{-4}
		A/G	75 (0.397)	30 (0.323)	0.60 (0.35-1.02)	
		G/G	21 (0.111)	1 (0.011)	0.07 (0.01-0.54)	
	Dominant	A/A	93 (0.492)	62 (0.667)	1	0.005
		A/G-G/G	96 (0.508)	31 (0.333)	0.48 (0.29-0.81)	
	Recessive	A/A-A/G	168 (0.889)	92 (0.989)	1	7×10^{-4}
		G/G	21 (0.111)	1 (0.011)	0.09 (0.01-0.66)	
	Overdominant	A/A-G/G	114 (0.603)	63 (0.667)	1	0.22
		A/G	75 (0.397)	30 (0.323)	0.72 (0.43-1.22)	

group compared with the remission group ($p = 0.0146$). Although it was not statistically different in the AOGC group compared to the remission group, the trend is higher in the AOGC group ($p = 0.0755$) (Figure 3). In addition, we did not find any significant difference in the BLK mRNA expression levels according to the different genotypes in the NMOSD patients (n = 31). However, NMOSD patients carrying the AA genotype tended to have higher levels of BLK than carriers of the AG genotype ($p > 0.05$) (Figure 4).

4 | DISCUSSION

In the present study, the genetic relationship of BLK variants with NMOSD susceptibility was explored in the Chinese Han population. Predominantly, BLK rs2248932 was associated with a lower risk of NMOSD. Specifically, the minor allele G of rs2248932 conferred protective effects against NMOSD and protected the risk of AQP4-positive. Additionally, the haplotype AAA of rs2248932 is genetically

TABLE 7 Association of BLK rs13277113, rs4840568, and rs2248932 with clinical characteristics of patients with NMOSD

	rs13277113				rs4840568				rs2248932			
Clinical characteristics	**AA**	**AG**	**GG**	**P**	**AA**	**AG**	**GG**	**P**	**AA**	**AG**	**GG**	**P**
AQP4-IgG positive *n* (%)	50	40	3	0.066	51	39	3	0.068	62	30	1	0.003
	(53.8)	(0.43)	(3.2)		(54.8)	(41.9)	(3.2)		(66.7)	(32.3)	(1.1)	
AQP4-IgG negative *n* (%)	7	4	3		7	4	3		10	1	3	
	(50.0)	(28.6)	(21.4)		(50)	(28.6)	(21.4)		(71.4)	(7.1)	(21.4)	
OR (95% CI)	1	1.4	0.14		1	1.34	0.14		1	4.84	0.05	
		(0.38-5.12)	(0.02-0.83)			(0.37-4.90)	(0.02-0.82)			(0.59-39.57)	(0.01-0.57)	
Onset syndromes, *n* (%)												
Optic neuritis	12	10	0	0.893	12	10	0	0.911	16	6	0	0.363
	(54.5)	(45.5)	(0.0)		(54.5)	(45.5)	(0.0)		(72.7)	(27.3)	(0.0)	
Acute myelitis	20	18	3		22	16	3		23	17	1	
	(48.8)	(43.9)	(7.3)		(53.7)	(39)	(7.3)		(56.1)	(41.5)	(2.4)	
Brain attacks	2	3	0		2	3	0		5	0	0	
	(40.0)	(60.0)	(0.0)		(40.0)	(60.0)	(0.0)		(100.0)	(0.0)	(0.0)	
Mix attacks	29	21	3		28	22	3		33	16	4	
	(54.7)	(39.6)	(5.7)		(52.8)	(41.5)	(5.7)		(62.3)	(30.2)	(7.5)	

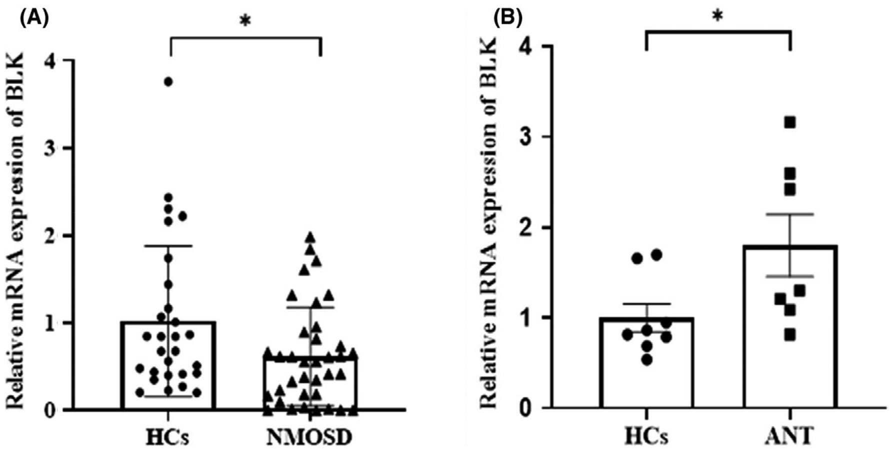

FIGURE 2 BLK mRNA levels in peripheral blood mononuclear cells (PBMCs) were determined by the $2^{-\Delta\Delta Ct}$ method using GAPDH as a reference gene. *P*-value was calculated by comparisons of individuals' relative expression using the $2^{-\Delta\Delta Ct}$ method. RT-PCR determined the relative quantification (RQ) of the BLK mRNA expression level in PBMCs of HCs and the NMOSD patients. (A) BLK mRNA expressions were decreased in the total NMOSD patients (n = 36) compared with healthy controls ($n = 28$) ($p = 0.025$). (B) BLK mRNA expressions were 1.8-fold higher in the acute untreated group (n = 7) than in healthy controls ($n = 8$) ($p = 0.040$). *$p < 0.05$ by nonparametric Mann-Whitney U test (non-normality). HCs, healthy controls; ANT, acute non-treatment; Each *p*-value was corrected for age and sex

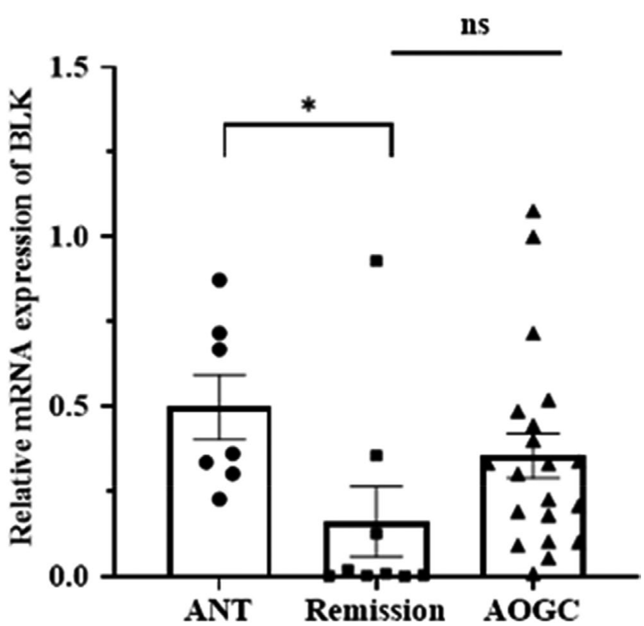

FIGURE 3 There were 3 groups divided in the 36 NMOSD patients: ANT, acute non-treatment ($n = 7$); AOGC, acute long-term oral glucocorticoids group ($n = 20$); and remission group ($n = 9$). The BLK expression levels were ranked in the 3 groups in order from high to low: ANT >AOGC > remission group. The BLK mRNA expression levels in ANT were higher compared with the other two groups ($p = 0.0135$). It was statistically different in ANT compared with the remission group ($p = 0.0146$). Although, there was no statistical difference in the AOGC compared to the remission group, and the trend is higher in the AOGC group ($p = 0.0755$). *$p < 0.05$ and ns (not significant) using Kruskal-Wallis test with Dunn's correction (non-normality) for three groups comparison test. Each *p*-value was corrected for age and sex

associated with an elevated risk of NMOSD. Two haplotypes (AAG and GGG) that carry the minor allele G of rs2248932 may be protective factors for NMOSD susceptibility.

Moreover, previous studies indicated that BLK was linked to numerous autoimmune diseases,[15-19] the relationship of BLK polymorphism with NMOSD predisposition has not been reported. To the best of our knowledge, this study is the first to explore the effect of genetic polymorphisms in BLK variants on NMOSD occurrence among the Chinese Han population. This result may provide a theoretical basis for gene modification therapy of NMOSD in the future.

BLK expressed mainly in B cells is a member of the Src family of tyrosine kinases mediating the signal transduction from the B cell receptor. It might be important for the development of the B cell development and mature B cell function.[25] It is demonstrated that the main mechanisms of BLK-mediated autoimmune systemic diseases are as follows: BLK is activated during B cell receptor (BCR) signaling and plays a key role in the phosphorylation of downstream BCR signaling pathways. BLK gene polymorphisms regulate B cells functions through BCR signaling.[26] Another study has shown that BLK risk alleles may influence early B cell development by affecting BLK expression.[27] Furthermore, B cells are regarded as a necessary part of NMOSD immunopathology, whereas dysregulation of B cells is considered to be implicated in the pathogenic mechanisms of NMOSD.[21] Consequently, it is conceivable that BLK gene polymorphisms can be involved in the pathogenesis of NMOSD through alterations in B cell functions and early development.

Considering NMOSD is a relatively rare autoimmune disease of the CNS with complex genetic and pathogenic modes of inheritance. We tested multiple genetic models to explore the association between BLK gene polymorphisms and NMOSD to gain further insight into the feasible genetic pattern of the SNP loci of the BLK gene in NMOSD. Our study showed that the homozygous GG genotype of rs2248932 was associated with a reduced risk of NMOSD compared with the wild AA genotype under the codominant, dominant, and recessive inheritance models. This finding is similar to that of Zhang et al., who did a large study that genotyped SNP rs2248932 in 1,396

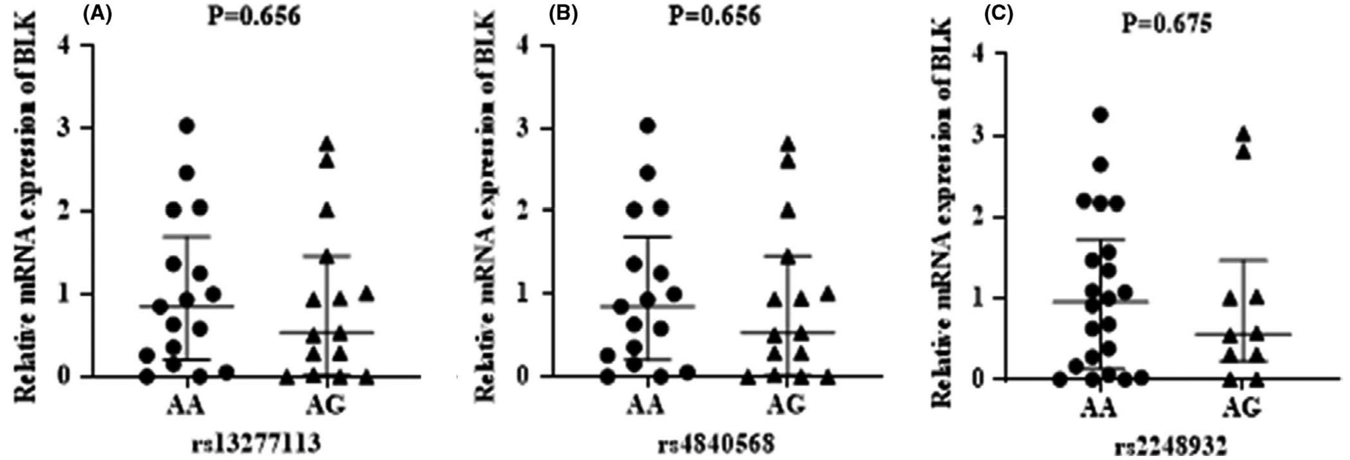

FIGURE 4 The BLK mRNA expression levels in different genotypes of rs13277113, rs4840568, and rs2248932 in the NMOSD patients. (A), (B), (C) The 31 subjects from the cases group were analyzed, including rs13277113 (AA 17, AG 15, GG 0), rs4840568 (AA 17, AG 15, GG 0), and rs2248932 (AA 22, AG 10, GG 0). Mann-Whitney U test (non-normality) was applied for the statistical analysis. Significant differences were not observed in BLK mRNA expression levels in the NMOSD patients with different genotypes ($p > 0.05$). Each p-value was corrected for age and sex

SLE patients of Chinese Han and 4,362 ethnically matched control subjects using the Sequenom MassArray system. Furthermore, it is confirmed that SNP rs2248932 in the BLK gene was significantly associated with SLE. This risk allele was the major allele in the Chinese Han. In genotypic analysis, the minor allele of rs2248932 was lower in patients than controls and was compatible with a dominant model, which was similar to our findings.[28] A meta-analysis further confirmed the strong association between Caucasians and Asians.[29] Conversely, the frequencies of alleles, genotypes, and haplotypes rs2248932 of FAM167A-BLK were not significantly different between the primary Sjogren's syndrome patients and controls.[30]

In contrast, Zhang H et al. suggested that the functional SNP BLK rs2248932 variant allele was associated with RA development.[31] These findings indicate that the BLK gene is involved in various autoimmune diseases. Multiple autoimmune diseases have different pathogenesis and variable clinical subphenotypes. The high heterogeneity of manifestations may result in a different pathogenic mode of inheritance. In addition, analysis of the three tag-SNPs identifies three haplotypes greatly associated with NMOSD: the AAA serving as a risk factor, the AAG, and GGG as protective factors. These results identified that BLK polymorphisms are associated with NMOSD in the Han Chinese population. The previous study showed that the function of the BLK risk haplotype in RA is associated with enhanced activation of BCR-stimulated B cells with an increase in T cell and B cell collaboration.[26]

Subsequently, we further assessed a stratified analysis of AQP4-IgG status and onset symptoms for the genotypes. Stratification for AQP4-IgG positivity, a significant association with susceptibility to NMOSD, persisted in rs2248932. Intriguingly, the results suggested that the GG-genotype of rs2248932 may be a protective factor for AQP4-positive NMOSD susceptibility. Nowadays, a study conducted that AQP4-positive patients are more likely to relapse compared to AQP4-negative counterparts. In clinical manifestations, the two sub-types also demonstrate distinct differences and treatment responses.[32] In this regard, a previous study provided evidence that distinct genetic profiles characterize AQP4-negative and AQP4-positive NMOSD while also sharing common genetic determinants.[33] Our reports support the stated conclusion. Afterward, we further analyzed onset symptoms based on the genotypes of rs13277113, rs4840568, and rs2248932. Unfortunately, no correlation was observed between any of the three SNPs and onset symptoms of NMOSD. These data will provide new clues for subsequent investigation on the relationship between BLK polymorphisms and NMOSD.

SNP rs2248932 was located in the first intron region of the BLK gene. Accumulating evidence has indicated that introns play a vital role in regulating gene expression.[34] Furthermore, intronic variation may exert its phenotypic effects by changing gene expression levels.[35] We evaluated the potential relationship of the three BLK SNPs and mRNA expression levels in this regard. However, there were no significant differences in BLK mRNA expression levels in NMOSD patients with different genotypes. This result is probably also due to a small number of case samples.

We further examined the mRNA expression of BLK in 36 NMOSD patients and 28 HCs, because the BLK SNPs involved in SLE susceptibility may affect the levels of mRNA expression of genes in SLE.[36] In NMOSD patients, BLK mRNA levels were downregulated as compared with HCs ($p = 0.025$). Similarly, Dang et al. found a significantly lower BLK mRNA expression in SLE patients than HCs.[37] Interestingly, one finding was that acute un-treatment NMOSD patients showed significantly higher BLK mRNA expression than HCs (1.8-fold, $p = 0.040$). Meanwhile, the BLK mRNA expression in the acute non-treatment patients was the highest in the NMOSD patients ($p = 0.0135$). Statistical differences were indicated in the acute non-treatment patients compared with patients in the remission period ($p = 0.0146$). The BLK mRNA expression levels in the acute patients with long-term oral glucocorticoids were lower than in the acute non-treatment patients and higher than in the remission group, although there was no statistical difference. Perhaps it was because of the small sample size. These results suggest that BLK mRNA expression levels in NMOSD patients without treatment may be altered significantly during the acute phase of the disease. Perhaps BLK expression levels can be used as a predictor of disease activity in the future. In this respect, we first reported the BLK expression in PBMC of NMOSD patients and compared BLK mRNA expression levels between the acute non-treatment group and HCs for the first time. We need to further confirm in large sample size, and more mechanistic research is needed to support this finding. Consequently, it is an important mechanism and must be taken into consideration for potential treatment.

5 | LIMITATIONS

This study has several potential limitations which should be acknowledged here. First, all enrolled participants were only Chinese; thus, the association between BLK variants and susceptibility of NMOSD should be further conducted with a large and different study population. Second, the sample size of our study is not large enough to get the optimal statistical power to detect a correlation with the size of the weak effect; thus, the results of our research should be further verified in a larger cohort. Last, we have not yet examined more closely the functional roles of rs2248932 in the pathogenesis of NMOSD, but we will continue to explore that in future studies.

6 | FUTURE PERSPECTIVE

In the present study, the genetic relationship of BLK variants with NMOSD susceptibility was explored in the Chinese Han population. Overall, BLK rs2248932 was associated with a lower risk of NMOSD. To the best of our knowledge, this study is the first to explore the effect of genetic polymorphisms in BLK variants on NMOSD occurrence among the Chinese Han population. This result may provide a theoretical basis for gene modification therapy of NMOSD in the future. BLK mRNA levels in NMOSD patients were downregulated as compared with healthy controls. However, one interesting finding was that acute un-treatment NMOSD patients showed significantly higher BLK mRNA expression than healthy controls (1.8-fold, $p = 0.040$). It could indicate that BLK mRNA expression levels in NMOSD patients without treatment may be altered considerably during the acute phase of the disease. Perhaps BLK expression levels may be used as a predictor of disease activity in the future. In this respect, we first reported the BLK expression in PBMC of NMOSD patients and compared BLK mRNA expression levels between the acute-untreated group and healthy controls for the first time. We need to further confirm in large sample size, and more mechanistic research is needed to support this finding. Our findings increase genetic insights into the role of BLK in NMOSD pathogenesis and promote the development of screening strategies targeting the alleles susceptible to NMOSD for early control.

7 | CONCLUSION

This result showed that the minor allele G of BLK rs2248932 conferred protective effects against NMOSD and AQP4-positive NMOSD. Subsequently, BLK mRNA expression was lower in the total NMOSD group but significantly higher in acute untreated patients when compared with healthy controls. Our findings increase genetic insights into the role of BLK in NMOSD pathogenesis and promote the development of screening strategies targeting the alleles susceptible to NMOSD for early control.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Arshad Mehmood from The Second Hospital of Hebei Medical University for assisting with professional editing and proofreading for this manuscript. Also, the authors are grateful to all the healthy volunteers and patients for their participation.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

ETHICAL APPROVAL

The experiment was approved by the Institutional Animal Care and Use Committee of Hebei Medical University and the Experimental Ethics Committee of the Second Hospital of Hebei Medical University.

CONSENT TO PARTICIPATE

Before this study, all participants gave informed written consent.

DATA AVAILABILITY STATEMENT

The supplementary material for this article can be found online. All processed data used in this study can be obtained from the corresponding author on reasonable request.

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How to cite this article: Yin B-W, Li B, Mehmood A, et al. BLK polymorphisms and expression level in neuromyelitis optica spectrum disorder. *CNS Neurosci Ther*. 2021;27:1549–1560. https:doi/org/10.1111/cns.13738