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RESEARCH ARTICLE

HLA DRB1*/DQA1* Alleles and TNF-alpha G308A Polymorphism Protect against Neuromyelitis Optica in the Cuban Population

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Abstract

Background: Neuromyelitis optica (NMO) is a complex immune-mediated disease whose prevalence differs among ethnic groups, most likely due to genetic factors. The presence of the Human Leucocyte Antigens (HLA) extended haplotype is a risk for NMO. The tumor necrosis factor-alpha (TNF-a) is believed to play a role in NMO pathogenesis. Although single nucleotide polymorphisms (SNPs) in the TNF-a promoter region (pTNF-a) has been shown to influence levels of TNF-a production, such an association is not evident in the Cuban population. The aim of this study was to examine the association between the HLA alleles, pTNF-a SNPs, the amount of the TNF-a protein, and the clinical parameters of a sample of NMO patients from the Cuban population.

Methods: 20 patients diagnosed with relapsing NMO (R-NMO), and 100 unrelated healthy controls, were evaluated. Ancestry was determined and an HLA typing case-control association study was carried out. Genomic DNA was extracted from peripheral blood leucocytes. HLA DRB1 and DQ alleles typing were determined by SSP-PCR. The DNA sequence approach was used to evaluate pTNF-a SNPs. The TNF-a protein expression was measured by ELISA.

Results: Genetic ancestry estimates showed that in NMO patients the European contribution prevailed. No association of HLA alleles to NMO susceptibility was observed, although there was a slight protective effect of HLA DQA*03, DRB1*10 followed by DRB1*11 alleles. An association was found between the pTNF-a - 308 G/A and a possible protective role against NMO (OR = 0.37, p values $p < 0.001$). The TNF-a protein did not differ between NMO patients and controls. Moreover, the association of HLA alleles and SNPs was not statistically significant when the clinical parameter were evaluated.

Conclusion: Our results showed that in this sample of Cuban NMO patients HLA alleles as well as pTNF-a SNPs differ from other populations. There was no association between HLA alleles, pTNF-a SNPs and clinical variables.

Keywords

Neuromyelitis optica, Multiple sclerosis, HLA DRB, HLA DQ, Tumor necrosis factor-alpha

Abbreviations

HLA: Human Leucocyte Antigens; NMO: Neuromyelitis Optica; TNF-a: Tumor Necrosis Factor-alpha (TNF-a); pTNF-a: TNF-a promoter region

Introduction

Neuromyelitis optica (NMO) is an autoimmune, inflammatory, demyelinating disease of the central nervous system that predominantly affects the optic nerves and spinal cord. Although there are very few epidemiological studies made on NMO, its prevalence varies geographically from 0.52/100000 in Cuba to 4.4/100000 in Denmark [1,2].

There is a clear distinction between NMO and multiple sclerosis (MS) which is achieved by the detection of specific autoantibody against aquaporin-4 (anti-AQP4), the main water channel of the central nervous system.

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This has been confirmed as a biomarker distinguishing them, while the results from clinical, immunological, radiological and pathological studies have reinforced the characteristics of the disease [3-5]. The fact that some NMO patients lack anti-AQP4 seropositivity has suggested that there may be other mechanism involved. This means that NMO is considered a disease with a heterogeneous pathogenic etiology [3,6,7].

The worldwide presence of NMO cases with ethnic variations, prove the relevance of genetic factors. It has been considered a rare disorder in Europe compared with MS. A substantial proportion of inflammatory demyelinating disorders of the central nervous system (CNS) appear in non-Caucasian populations such as Afro-Brazilians, East Asians and Indians [8-11]. Nonetheless, in French and Danish studies it was mainly observed in Caucasians, suggesting that the disease is more common in Caucasians than what was previously believed [12,13].

Association studies of human leukocyte antigens (HLA) in NMO patients are few and based on small cohorts. Although certain HLA molecules seem to be related to a higher risk of NMO, Brum, et al. [14] showed that DRB1*01 was found in NMO patients of mixed (black and white) ancestry compared to MS patients of mixed ancestry where this allele is associated with a benign outcome [15]. Furthermore, Deschamps, et al., [16] observed that the distribution of DRB1 and DQB1 in NMO and MS within the African-Caribbean population shows important differences in the association of HLA with NMO and MS where DRB1*15 had no significant effect on NMO risk while it was associated with an increased susceptibility to MS. Alvarenga, et al., [17], showed that DRB1*0301 was associated with afro Brazilian and white Brazilians NMO patients. Also, DPB1*0501 has been associated to higher NMO risk in Asians [18].

The location of the TNF gene locus within the Major Histocompatibility Complex on chromosome 6 (6p21.31) suggested that the polymorphism within this locus may contribute to the pathogenesis of a wide range of autoimmune and infectious diseases [19,20].

The purpose of this study is to assess the association of DRB1*, DQA1*, and DQB1* alleles and the TNF alpha promoter gene polymorphism with the risk of NMO. All the results will reinforcement the knowledge of genes association with the disease in our sample. Furthermore, a panel of 17 Ancestral informative markers (AIMs) was genotyped in the sample, to control any possible confounding traits due to population stratification.

Methods

Patients and controls

Samples from 20 NMO patients with relapsing NMO (R-NMO) diagnosed according to Wingerchuk, et al., [5] were analyzed. Unrelated healthy controls (n = 100) were matched in terms of age, gender and ethnicity with the group of patients enrolled in the study. A written informed consent of each subject was obtained before sample collection. The protocol for the study was approved by the Ethical Research Board of the International Center of Neurological Restoration (Havana, Cuba) according to the national legislation guidelines and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association.

DNA isolation and primers

Genomic DNA was isolated from whole blood using a Wizard Genomic DNA Purification Kit according to the manufacturer's instructions (Promega, Madison, WI, USA). All primers were obtained from the Department of oligonucleotide synthesis, at Center for Genetic Engineering and Biotechnology (Havana, Cuba).

Single nucleotide polymorphism genotyping

Seventeen ancestry informative marker (AIMs) single nucleotide polymorphisms (SNPs) and 20 R-NMO SNPs were genotyped by PCR amplification, using sequence-specific primers (SSP-PCR), as previously described [21]. Amplification reactions were performed in a final volume of 25 µL containing 100 ng of genomic DNA, 1.5 - 2.0 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris HCl, 0.2 mmol/L of each dNTP, 1 mmol/L of both primers, 10% DMSO, and 1UTaqDNApolymerase (Promega). All loci were scored after electrophoresis through agarose gels.

Ancestral informative markers (AIMs) were identified from previous studies as having large differences in allele frequency (d. 30%) between the Native American, West African, and European ancestral populations [22].

HLA. Selected HLA DRB1 and DQ alleles were determined by SSP-PCR as previously described [23,24]. The selection of these particular alleles was based on studies reporting their involvement as part of haplotypes conferring higher risk or protection scores [25,26]. Briefly, they are described as: DRB1* 04:01 - 04:11 - 03:01 - 15:01 - 15:02 - 01:01 - 01:03 - 01:02 - 07:01 - 07:02 - 10:01 - 11:01 - 11:04 - 14:01 - 14:04 - 14:03, DQB1*06:02, DQA1*03:01, *01:02. A primer pair to amplify the third intron of DRB1 genes was included in each PCR reaction as the internal positive control. These two primers matched non-allelic sequence [24,26]. The primers were used at 0.25 µM. Amplified products were separated by electrophoresis in 2% agarose gels containing ethidium bromide after the addition of the loading buffer and visualized using UV illumination.

Human TNF alpha promoter alleles

PCR was used to amplify the alleles with the following primers (5-AGTGAGAACTTCCCAGTCTATCTAAG-3) and (5-CCGTGGGTCAGTATGTGAGA-3) at 0.25 µM. PCR

Table 1: The association between the HLA DR/DQ alleles and NMO.

HLA	Alleles	*P**	*OR*	95% CI
DRB1	*01:01 - 01:03	NS	0.2	(0.01 - 0.92)
	*01:02	NS	0.36	(0.04 - 2.8)
	*03:01	NS	2.1	(0.7 - 5.8)
	*07:01 - 07:02	NS	0.79	(0.27 - 2.35)
	***10:01**	**< 0.01**	**0.17**	**(0.03 - 0.79)**
	***11:01 - 11:04**	**0.01**	**0.27**	**(0.10 - 0.7)**
	*14:01 - 14:04	NS	0.69	(0.08 - 1.7)
	*14:03	NS	3.2	(0.7 - 13.0)
	*15:01 - 15:02	NS	0.8	(0.3 - 3.0)
DQA1	*01:02	NS	0.8	(0.30 - 2.17)
	***03:01**	**0.01**	**0.13**	**(0.01 - 1.5)**
DQB1	*06:02	NS	2.77	(1.57 - 4.86)

OR: Odds Ratios; CI: Confidence Interval; *P*: P value* corrected for population stratification; NS: Non-Significant.

mixture underwent 35 cycles (at 95 °C, 25 °C, and 72 °C, during 30 second each) on a Personal Mastercycler (Eppendorf, Germany). The amplified products were purified and sequenced by BigDye Terminator Cycle Sequencing Kit and run on an ABI PRISM 3730XL DNA sequencing device. The data were transformed for the STADEN package [27,28], and sequences were aligned together with the consensus human promoter TNF-alpha sequence for all NMO patients and controls.

Detection of anti AQP4 antibodies

Serum samples were collected and kept at -20 °C until the runs of the assay were made. A commercial kit from RSR limited (England vendor) was used. The assay was carried out following the instructions from the supplier. Briefly, undiluted serum samples, calibrators and controls were left to interact with AQP4 bound to the well surfaces. The AQP-Biotin conjugate, followed by the streptavidin-peroxidase conjugate, the TMB substrate, and the stopping solution were added. Absorbance was read at 450 nm and 405 nm. Data analysis was achieved following the instructions and calculations of results based on the read-off from the calibration curve provided. Samples were considered positive when the concentration of anti-AQP4 antibodies was equal or above 3 u/mL.

TNF-alpha serum concentrations were quantified using a Quantikine HS ELISA from R&D Systems, USA. In brief, standards or samples were first added to the pre-coated solid phase with *E. coli*-derived recombinant human TNF-alpha. After washing to remove unbound molecules, a specific polyclonal antibody for human TNF-alpha, conjugated to alkaline phosphatase, was added. After washing, a chromogenic substrate was added to the wells followed by the amplification solution after incubation time. A stopping solution was added and the intensity of the color, which is proportional to the amount of TNF of the samples, was measured. Sample protein levels were calculated using the standard curve provided. All samples were analyzed undiluted and duplicated.

Statistical analysis

The goodness of fit of genotype frequencies to the Hardy-Weinberg distribution was given by the Chi-square test. Haplotype frequencies were estimated by an expectation maximization algorithm [29]. The parental population frequencies used were those reported [22] for samples of Spanish, average Amerindian (Mayan, southwestern US Native Americans), and average West African (Central African Republic, Nigeria, and Sierra Leone) populations (Table 1). The frequencies of the HLA class II alleles and TNF alpha promoter alleles were compared between patients and controls using the chi-square test or the two-tailed Fisher's exact probability test. *Odds Ratio* (OR) (95% CI) was also estimated (SPSS 16.0 software). The level of significance taken was the *p value* < 0.05. For allele comparisons, Bonferroni's method was used for the correction of multiple comparisons, multiplying the value of *p* obtained in the statistical test by the total number of alleles tested [23]. In order to control potential confounding because of population stratification as a result of admixture, the ADMIXMAP program was used [30]. This program performs an association analysis by means of a logistic regression for the relation of NMO, as the dependent variable, to HLA alleles, TNF alpha promoter alleles, and the individual ancestry estimates as covariates. The admixture proportions of groups were estimated by the gene identity method [31] using the Admix95 software provided by Bertoni B (http://www.genetica.fmed.edu.uy/software.htm).

Results

The characteristics of the population

R-NMO patients studied (n = 20) were mainly women (78%) from Havana, Cuba. The self-identified ethnicity corresponds to those of Spanish descent, 78%, of African descent, 5%, and of mixed descent, 17%. Age ranged between 15 and 54-years-old (mean = 33.10 ± 13.34; median = 37). The onset of symptoms of the disease was at between 12 and 47 years of age (mean = 29.89 ± 11.86; median = 29). NMO was found to be associated with an autoimmune disease, myasthenia gravis (5%), in only

Table 2: Ancestral informative markers (AIMs) allele frequency in the parental populations [22]. AIMs allele frequencies for NMO cases and controls are also shown. Asterisks precede the name of the largest allele.

AIM	*European*	*West Africans*	*Amerindian*	*Cases*	*Controls*
GC-*1F	0.356	0.853	0.339	**0.550**	**0.586**
AT3*ins	0.273	0.858	0.061	**0.500**	**0.430**
LPL*ins	0.494	0.971	0.442	**0.625**	**0.630**
APOA1*ins	0.917	0.420	0.977	**0.900**	**0.768**
MID154*ins	0.333	0.806	0.420	**0.325**	**0.415**
MID187*ins	0.342	0.759	0.301	**0.500**	**0.442**
D11S429*T	0.440	0.087	0.119	**0.555**	**0.375**
TSC-11020*T	0.921	0.487	0.137	**0. 777**	**0.824**
FY-null*T	0.999	0.001	1.000	**0.750**	**0.715**
OCA2*A	0.636	0.115	0.488	**0.600**	**0.563**
WI-7423*T	0.517	0.000	0.058	**0.630**	**0.340**
GS*1S	0.607	0.931	0.931	**0.675**	**0.758**
WI-14867*C	0.558	0.976	0.418	**0.470**	**0.571**
WI-16857*G	0.180	0.751	0.181	**0.310**	**0.390**
PV92*ins	0.171	0.225	0.792	**0.289**	**0.196**
CYP19-E2*T	0.287	0.332	0.741	**0.394**	**0.419**
TYR 192*A	0.485	0.005	0.034	**0.475**	**0.359**

one person, and one person of the patient population died in the course of the study. Transverse myelitis (TM) and optic neuritis (ON) were the most frequent acute events at the onset of the disease (n = 11/55%). The disease, in all patients, showed a recurrent clinical course (n = 20/100%). The progression of the disease took between one and 20 years (mean = 7.10 ± 4.54; median = 5). Magnetic Resonance Imaging (Magneton AERA 1.5 T, CIEMENS) of the brain and spinal cord was used to study all patients. T2, FLAIR T2, T13D at 1 mm, DWI and magnetic susceptibility weighted sequences for the cranial studies were carried out in axial, coronal and sagittal sights, and T2, T1 STIR TSE and myelography sequence was performed for the cervical and dorsal column.

All patients had different degrees of spinal cord injury, while the MRI of the brain was also positive in 95% of the patients. The most frequent lesions were those of the posterior fossa (4/20), periventricular (2/10), and cerebral atrophy (2/10). The NMO-IgG antibody was analyzed in all patients by the ELISA method, where there were eight positive (40%) and 12 negative cases out of 20 (60%).

Allele frequencies for the ancestral informative markers (AIMs) in ancestral populations

We evaluated the allele frequencies for the 17 AIMs in ancestral populations, NMO, and healthy controls and the results are shown in Table 2. There was no deviation from the Hardy-Weinberg distribution ($p > 0.05$).

Genetic ancestry estimates showed that the European contribution was prevalent in the cases and control groups and were of 97% and 76% respectively. West African origin was of 2% and Native American was 1% for cases, while West African origin was of 21.7% and Native American was 2.3% for the controls.

HLA DRB/DQ typing

The association between the HLA DRB/DQ alleles and NMO was assessed in the sample. The HLA-DRB1*, HLA-DQA1* and DQB1* allelic associations for NMO patients and controls are shown (Table 1). The association of HLA alleles to a susceptibility to NMO was not observed although there was a slight protective effect for HLA DQA1*03:01, DRB1*10:01 followed by DRB1*11:01 - 11:04 alleles, among NMO patients (*OR* 0.13, 0.17, 0.27 respectively) when compared to healthy controls (Table 1).

Relationship between HLA alleles and the NMO IgG status

The association between HLA alleles and the anti-AQP4 status was evaluated (Table 3). No significant differences were observed in HLA allele frequency patterns between the two NMO subgroups (NMO IgG-positive patients and NMO-IgG-negative patients).

Human TNF alpha promoter polymorphisms (pTNF-a SNPs)

The allele frequencies of Human TNF alpha promoter polymorphisms (pTNF-a SNPs) for patients and controls are shown in Table 4. There was no deviation from the Hardy Weinberg distribution for any of the pTNF-a SNPs ($p > 0.05$). No significant differences between NMO and control groups were found in relation to pTNF-a - 243 G/A, - 574 A/C, - 856 C/T and - 862 C/A. However, an association was found between the pTNF-a - 308 G/A as well as a possible NMO protective role (*OR = 0.29, p values p < 0.001, Bonferroni corrected chi-square test*). These results suggest that the presence of the G allele in the genotype could protect against NMO. Finally, we found one genotypic combination between pTNF-a **308A/A** and HLADR1*03:01 that showed higher frequencies in the NMO patient group than in the control group (*p < 0.05 OR = 16.6*).

TNF alpha protein expression levels in NMO patients

TNF alpha protein expression level was measured to study an association with pTNF-a SNPs. The secretion of TNF protein was similar between cases and controls groups, no statistical differences were found (Figure 1). The association with SNP could not be evaluated.

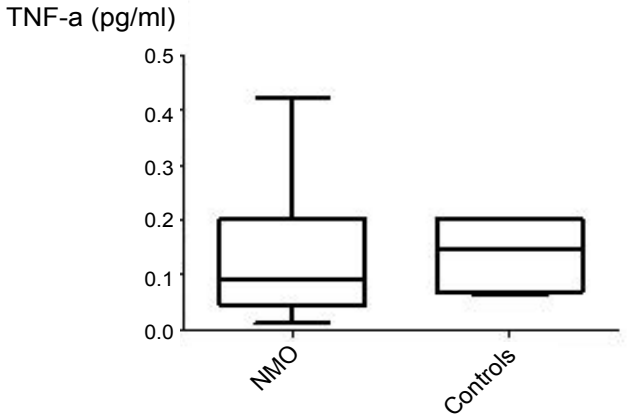

Figure 1: Serum TNF expression measured on NMO patients and controls. No statistical differences were found among groups (p = 0.904).

Table 3: Relationship between HLA alleles and AQP4 autoantibodies in NMO patients.

		Allele frequencies (%)		
HLA	**Alleles**	***N = 8**	****N = 12**	***P value***
DRB1*	03:01	25	10	0.21
	15:01 - 15:02	5	5	0.7
	01:01 - 01:03	0	0	0.7
	01:02	0	0	0.7
	07:01 - 07:02	15	5	0.3
	10:01	5	0	0.5
	11:01 - 11:04	15	25	0.2
	14:01 - 14:04	0	0	0.7
	14:03	5	10	0.4
DQA1*	03:01	0	10	0.24
	01:02	15	10	0.57
DQB1*	06:02	5	5	0.7

*N: Number of patients with autoantibodies against AQP4, **N: Number of patients without autoantibodies against AQP4.*

Linkage of significant HLA alleles and SNPs in the clinical variable of NMO patients

The main clinical characteristics of NMO patients such as: age at onset, duration of disease, number of relapses, number of patients with Transverse myelitis or with Optic neuritis was evaluated with in relation to the significant HLA alleles and SNPs determination (Table 5). The results showed no statistically significant differences between any clinical parameters and the significant HLA alleles and SNPs evaluated.

Discussion

In 2009 Cabrera-Gomez, et al., [32] published a study where 58 NMO patients (7 males and 51 females) showed a prevalence rate of 0.52 per 100,000 (95% CI 0.39 - 0.67) in Cuba for an estimated average annual incidence rate of 0.053 per 100,000 individuals. This study describes that the disease was equally prevalent in whites, blacks, and mixed (black and white) subjects.

Table 4: Allelic association of the Human promoter TNF *alpha* in NMO patients and controls.

Human promoter TNF *alpha* Alleles	**Patient**	**Controls**	***P****	***OR***	**95% CI**
238G/A	0.88	0.92	NS	2.1	(0.7 - 5.8)
243G/A	0.94	0.95	NS	0.8	(0.3 - 3.0)
308G/A	**0.69**	**0.84**	**< 0.01**	**0.29**	**(0.13 - 0.68)**
376A	0.91	0.96	NS	0.36	(0.04 - 2.8)
574A/C	0.99	0.99	NS	0.79	(0.27 - 2.35)
856C/T	0.83	0.91	NS	0.17	(0.03 - 0.79)
862C/A	0.99	0.99	NS	0.27	(0.10 - 0.7)

OR: Odds Ratios; CI: Confidence Interval; P: P value corrected for population stratification; NS: Non-Significant.*

Table 5: Clinical characteristics of NMO patients with in relation to the significant HLA alleles and Human promoter TNF alpha polymorphisms.

Variables	**HLA DRB1 *10:01**		**HLA DRB1 *11:01 - 11:04**		**HLA DQA1 *03:01**		**pTNF-a SNPs 308G/A**	
	(+) N = 2	(-) N = 18	(+) N = 9	(-) N = 11	(+) N = 3	(-) N = 17	A vs. G F = 0.38	F = 0.77
AO (years)*	29.8 ± 11.3	37 ± 2	27.7 ± 10.3	13.3 ± 4.2	19.0 ± 5.6	11.8 ± 2.8	25.5 ± 13.9	29.7 ± 11.4
P value	*P = 0.446*		*P = 0.47*		*P = 0.17*		*P = 0.430*	
DOD (years)*	6.7 ± 4.4	7.4 ± 2.2	7.2 ± 1.94	7.0 ± 1.05	4.6 ± 0.66	7.47 ± 1.13	8.42 ± 2.43	7.33 ± 1.18
P value	*P = 0.316*		*P = 0.66*		*P = 0.14*		*P = 0.783*	
NR*	3.31 ± 2.10	2.5 ± 0.7	3.44 ± 5.8	3.20 ± 7.85	3.5 ± 1.5	3.29 ± 5.26	2.21 ± 0.78	3.4 ± 0.6
P value	*P = 0.84*		*P = 0.31*		*P = 0.84*		*P = 0.891*	
TM (%)	1 (5)	9 (45)	3 (33.3)	6 (60)	2 (10)	7 (35)	3 (15)	12 (60)
P value	*P = 0.526*		*P = 0.242*		*P = 0.211*		*P = 0.15*	
ON (%)	1 (5)	6 (30)	3 (33.3)	3 (30)	1 (5)	6 (30)	4 (20)	8 (40)
P value	*P = 0.684*		*P = 0.630*		*P = 0.456*		*P = 0.88*	

*: Means ± SD, pTNF-a: TNF alpha polymorphisms; F: Frequencies of alleles A and G; AO: Age at Onset; DOD: Duration of Disease; NR: Number of Relapses; TM: Transverse Myelitis; ON: Optic Neuritis.

The official racial/ethnic classification of the Cuban population which provides three groups officially cited by "skin color" [33] was used by the authors.

The population structure of Havana in the multiethnic Cuban population was reported by Cintado, et al., [34] where ancestral informative markers (AIMs) were evaluated showing that 85% is of Spanish descent and the African contribution is of 15%, thereby suggesting that admixture must be considered when evaluating traits in this population. Ancestry Informative Markers (AIMs) have been applied as a robust tool to adjust for population mixture avoiding spurious associations in case control studies [35].

Aditionally Marcheco-Teruel B, et al., [36] reported an analysis of the proportions of admixture in a large Cuban sample using a combination of AIMs, where the average European, African and Native American contributions were of 72%, 20% and 8%, respectively, which confirmed previous results.

The typing of 17 AIMs having large differences in frequencies between European, West African and Native American populations was applied to this study. The association of ancestry proportions with NMO disease in accordance with suggestions of Cabrera-Gomez on disease incidence in our population [32] was measured. We observed that European contributions were predominates in patient and control groups.

NMO is considered a rare disorder in Caucasians, but this view is based on a few studies with small patient populations from tertiary hospitals. No population-based studies have been carried out so far in Caucasians [9,37,38]. However, in 2011, Asgari, et al., estimated the incidence and prevalence of NMO in a Caucasian population [13], and found that the yearly incidence of NMO in southern Denmark was of 0.4 per 10^5 person-years (95% CI 0.30 - 0.54) and the prevalence was 4.4 per 10^5 (95% CI 3.1 - 5.7).

In addition, Brum, et al., [39] demonstrated that the genetic contribution of Europeans predominates in NMO patients in Brazil. This is a population with a genetically diverse background after five centuries of the interethnic admixture of individuals of European, African and Amerindian ancestry. Several associations between HLA alleles and autoimmune disorders, as well as other diseases have been established over the past decades [40]. HLA association studies in NMO patients are limited and based on small cohorts. A few papers have indicated that HLA alleles determine either the susceptibility or resistance to NMO [14,16,41].

According to Asgari, et al., they could play a prime role in the genetic risk of NMO and may provide an important insight in the profound understanding of NMO pathogenesis; also, they can be used for the differential diagnosis mainly from MS and other demyelinating diseases [42].

Positive associations observed between HLA-DRB1*03 alleles and NMO were reported in French Caucasian, Brazilian mixed, and French Afro-Caribbean patient populations [14,16,41,43]. In the Asian population the HLA DPB1*05:01 is the most common allele in NMO patients, but this is rarely found in Caucasians [44].

HLA association could not be significantly demonstrated in our study due perhaps to the small sample size, while a modest protective effect was found for HLA DQA*03:01, DRB1*10:01 followed by DRB1*11:01 - 11:04 alleles. Moreover, after correcting for population stratification in our sample, allele disease associations continued coherent. Furthermore, in our study, a link was not found between the HLA alleles mentioned above and clinical variables such as age of the patients at onset, duration of disease, number of relapses, and the predominant clinical events (transverse myelitis and optic neuritis).

According to Wingerchuk, et al., [5] for the diagnosis of NMO an important criterion is the presence of the pathogenic antibody directed against the aquaporin-4 (AQP4) water channel [45]. Since antibodies against AQP4 were established as serum biomarkers for NMO [46], the understanding of the immunopathogenesis of the disease has improved [7].

Yoshimira, et al., [47] reported a positive association with alleles DPB1*16:02 and anti-AQP4 antibody positivity in an Asian population study. Furthermore, in French Caucasian NMO patients, DRB1*03 alleles were also found to be associated with AQP4 seropositivity [48].

Moreover, when we analyzed our cohort according to anti-AQP4 seropositivity in association with HLA alleles, no significant differences were observed in HLA allele frequency patterns between seropositive and seronegative individuals. This agrees with other studies [16,43]. The difference in HLA patterns between the two NMO subgroups (AQP4-Ab-positive patients and AQP4-Ab negative patients) was not statistically significant, probably due to the small sample size.

The association of seven SNPs polymorphisms located in the TNF alpha promoter, and NMO in Cuban patients, was evaluated. TNF-alpha gene is located within the Class III region of the human Major Histocompatibility Complex (MHC) on chromosome 6 (6p21.31) and it encodes a pro-inflammatory cytokine that plays a critical role in certain inflammatory diseases. Three single-nucleoide polymorphisms, -238 (G/A), -308 (G/A), and -376 (G/A), were involved in several association studies [49-51] suggesting that these polymorphisms could affect the expression, and hence, the level of TNF alpha [52,53]. In the present paper, we only found an association between TNF-alpha -308 (G/A) and the NMO disease ($p < 0.05$), and a possible protective role against NMO was observed (OR = 0.37). We noticed

that the G allele was significantly more common in the control (0.84) than the patient group (0.69). The TNF-alpha -308 (G/A) have been found to be correlated with transcripcional activation and the amount of protein production [54]. Some studies have found the high expression levels of TNF-alpha linked to the rare allele (A) and the extended haplotype TNF-a-308(AA) -HLA-A1-B8-DR3-DQ2 to be associated with autoimmunity and high TNF-alpha production [55]. Also, in our study, a link between pTNF-a 308 A/A and HLADR1*03:01 was found.

In addition, Penton-Rol G, et al., [56] demonstrated that the TNF-alpha levels of NMO patients were undetectable, suggesting the possible neuroprotective role of this molecule. In the current study, regarding protein TNF-alpha levels, no significant differences were found between NMO patients and controls. We should point out that the patients in a chronic, disabling and long-term evolution disease were receiving medication such as immunosuppressive drugs at the time of the serum samples were taken. Moreover, no association was found between pTNF-a SNPs and the clinical variables assessing.

Conclusion

The results of this study show that there was a prevalence of European descent in NMO Cuban patients and a slight protective effect of alleles HLA DQA*03, DRB1*10 followed by DRB1*11 alleles. An association between TNF-alpha -308 (G/A) and the NMO disease was observed, suggesting a possible protective role against NMO. HLA alleles as well as pTNF-a SNPs in this sample of NMO Cuban patients differ from other populations since there was no association between HLA alleles, pTNF-a SNPs and clinical variables.

To our knowledge, this is the first study where the association between the polymorphism of promoter TNF-alpha, HLA alleles and clinical variables in NMO patients was developed taking to account the ancestrally informative markers.

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