












### Association of HLA-DRB1, HLA-DQB1 Alleles, and TNF- $\alpha$ Promoter Polymorphisms with Multiple Sclerosis in the Cuban Population

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### ABSTRACT

Multiple sclerosis (MS) is an inflammatory, demyelinating, autoimmune disease of the central nervous system. It is known that the Major Histocompatibility Complex class II region produces the most potent effect on MS genetic susceptibility. In addition, the genetic polymorphism within the *TNF* locus has been involved in the pathogenesis of various autoimmune diseases. This study has the purpose of evaluating HLA-DRB1, HLA-DQB1 alleles and *TNF* promotor alpha gene polymorphism (SNP TNF-  $\alpha$  -238 G/A; -243G/A; -308 G/A; -375 G/A, -856 C/T; -862 C/A) in a sample of Cuban MS patients. Disease-associated HLA susceptibility alleles were genotyped by the SSP-PCR method. The TNF-  $\alpha$  genotypes were identified by sequencing. The association was found between HLA and MS, DRB1\*15:01, DRB1\*14:01, DQA\*01:02 and DQB1\*06:02 being susceptibility alleles. TNF- $\alpha$ -308 G (OR=1,6, P<0,01) and TNF-  $\alpha$  -238 G (OR=2,0, P<0,01) alleles had higher frequency among MS patients than control subjects. The odds ratio was increased among HLA-DRB1\*1501 positive individuals. Our results have shown that the combination of TNF- $\alpha$ -238 G, -308 G with HLA-DRB1\*15:01 and HLA-DQA1\*01:02 increased susceptibility to MS (p<0.05 OR=4.2) in the Cuban population.

**Keywords:** HLA, TNF-Alpha, polymorphism, SNP, Multiple Sclerosis, Cuban population

## INTRODUCTION

Multiple sclerosis (MS) is a disease of the human central nervous system, characterized by inflammation and accompanied by demyelination and axonal damage<sup>1</sup>. The overall prevalence of MS is about 70 per 100,000 individuals (range from 2 to 150)<sup>2</sup>. The first case of MS in Cuba was published in 1965<sup>3</sup>. The estimated prevalence of clinically defined MS in Cuba is 4.42 cases per 100,000 inhabitants, using as a source the case registries of all provinces and those from the Association of Multiple Sclerosis Patients in Cuba<sup>4</sup>.

Genetic and epidemiological studies have suggested that MS is a complex genetic disease where the interaction between genetic characteristics of individuals and environmental factors play essential roles in the risk of developing MS<sup>5</sup>. Several different genes contribute to disease susceptibility; among these, genes of the central histocompatibility complex region show the strongest association. The human leukocyte antigen (HLA) genes have been defined as the strongest in association with MS risk, with the most evidence coming from the HLA-DRB1 gene in the MHC Class II region as the central susceptibility locus for MS<sup>5</sup>. Genome-wide association studies have also provided information that over 200 alleles have also been associated with MS comprising immunoglobulin heavy chain (IgH), T-cell receptor (TCR), tumor necrosis factor (TNF) and myelin essential protein (MBP), among others<sup>6,7,8</sup>. Increased levels of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF- $\alpha$ ), have been found in the peripheral blood and brain lesions of patients with MS<sup>9,10,11</sup>. Numerous reports document the association between these cytokines and disease activity, thus implicating them as mediators of the immunopathogenesis of MS<sup>12,13,14</sup>.

Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) is a proinflammatory cytokine with a wide range of biological activities, including cellular immune response. The TNF- $\alpha$  gene is located on chromosome six. Some single nucleotide polymorphisms (SNP) at positions -243, -308, -375, -856, and -862 in the TNF- $\alpha$  gene promoter have been identified, and a large number of genetic studies have been conducted to investigate the association between those polymorphisms and MS. However, the results of these studies are inconclusive<sup>6</sup>. TNF- $\alpha$ 's roles seem related to the genetic polymorphisms in genes regulating its production and effect<sup>15</sup>.

Our study aimed to investigate the influence of TNF- $\alpha$  promoter polymorphisms and its interaction with HLA-DRB1 and DQ alleles on the susceptibility to MS in the Cuban population.

## MATERIAL AND METHODS

**Patients and controls.** Samples from 100 MS patients with Relapsing-Remitting (MS-RR) MS were diagnosed according to criteria defined by McDonald in 2001 and were analyzed in the present study<sup>17</sup>. Unrelated healthy controls (n=200), matched in age, sex and self-identified ethnicity with the group of patients were enrolled in the study. Written informed consent was obtained from each participant prior to sample collection. The Ethical Research Board from the International Center of Neurological Restoration (Havana, Cuba), according to the guidelines of the national legislation and the Code of Ethical Principles, approved the study protocol for Medical Research Involving Human Subjects of the World Medical Association<sup>16</sup>.

### DNA isolation and genotyping

According to the manufacturer's instructions, DNA was isolated from whole blood using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany).

**HLA Typing.** Selected HLA-DRB1, -DQA1 and -DQB1 alleles were genotyped by aPCR-SSP method as described previously<sup>17,18</sup>. The selection of these particular alleles was based on previous studies in which they

have been reported to be implicated as part of haplotypes conferring the highest risk or protection scores <sup>19</sup>. All primers were obtained from the Department of Oligonucleotide Synth at the Center for Genetic Engineering and Biotechnology (Havana, Cuba). The PCR mixture underwent 35 cycles (at 95, 65 and 72°C, for 30 s each) on a Mastercycler Personal (Eppendorf, Germany). Amplified products were separated by electrophoresis in 2% agarose gels containing ethidium bromide after adding a loading buffer and visualized using UV illumination.

Human TNF- $\alpha$  Promoter region polymorphisms (TNF- $\alpha$  SNP). The TNF- $\alpha$  -238 G/A, -243 G/A -308 G/A, -375 G/A, -857 C/T and -862 C/A polymorphisms were analyzed using DNA Sanger sequencing assay. The six polymorphisms were amplified with the following primers: 5-AGTGAGAACTTCCCAGTCTATCTAAG-3 and 5-CCGTGGGTCAGTATGTGAGA-3 at 0.25  $\mu$ M. The PCR mix underwent 35 cycles (at 95°C, 25°C, and 72°C, for 30 seconds each) on a Personal Mastercycler (Eppendorf, Germany). The amplified products were purified and sequenced with a BigDye Terminator Cycle Sequencing Kit and run on an ABI PRISM 3730XL DNA sequencing device. The data were analyzed with the STADEN package <sup>20</sup>, and the sequences were aligned with the consensus sequence of the human TNF- $\alpha$  promoter to identify SNPs in patients and controls.

Statistical analysis. The Chi-square test tested the fit of the genotype frequencies to the Hardy-Weinberg proportions. HLA alleles and pTNF- $\alpha$  SNP frequencies were estimated employing an expectation maximization algorithm <sup>22</sup>. All these analyses were carried out using Pypop software <sup>23</sup>. The frequencies of HLA class II alleles, TNF  $\alpha$  promoter alleles and haplotypes 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 was taken as p-value < 0.05. For allele comparisons, Bonferroni's method was used to correct multiple comparisons, multiplying the value of p obtained in the statistical test by the total number of alleles tested <sup>18</sup>.

## RESULTS

### Characteristics of the population

Most MS patients were mainly women (71,04%). The self-identified ethnicity corresponds to those of Spanish descent, 76%, of African descent, 16,9%, and of mixed descent, 13,3%. The mean age of MS patients was 40,01 $\pm$ 13,09 years, and all were diagnosed with relapsing-remitting. Participants' characteristics are shown in Table 1.

**Table 1. Demographic characteristics of study subjects**

## HLA DRB/DQ typing

In the Cuban sample, we assessed the association of selected HLA-DRB1/DQ alleles with MS. The association of selected HLA DR/DQ alleles with MS in the Cuban sample has shown a piece of evidence for susceptibility to MS for the presence of HLA-DRB1\*15:01, DRB1\*14:01, DQA1\*01:02 and DQB1\*06:02 alleles, with odds ratio (OR) more significant than 2,5. A modest protective effect of HLA-DRB1\*07:01 and HLA-DRB1\*11:01 alleles was found (OR 0.21 and 0.19, respectively). (Table 2).

OR, odds ratio; CI, confidence interval; p value < 0,05 corrected for Bonferroni was considered significant; ns= not significant

**Table 2. HLA Allelic association of MS patients vs controls**

## Human TNF alpha Promoter polymorphisms (pTNF- $\alpha$ SNPs)

The allele frequencies of Human TNF alpha promoter polymorphisms (TNF-  $\alpha$  SNPs) for patients and controls are shown in Table 3. There was no deviation from the HWE distribution for any TNF-  $\alpha$  SNPs evaluated ( $p > 0,05$ ). The frequencies of TNF- $\alpha$ -238 G/A, -308 G/A, and -376 G/A alleles increased in patients with MS compared with controls ( $p < 0,05$ ), showing a significant difference among the studied individuals.

TNF- $\alpha$  SNPs: Human TNF alpha Promoter polymorphisms, OR, odds ratio; CI, confidence interval; p-value < 0,05 corrected for Bonferroni was considered significant; ns= not significant

**Table 3. Frequencies of Human TNF alpha Promoter polymorphisms for MS compared with the control group**

Furthermore, the haplotype association of the HLA alleles and TNF- $\alpha$  SNPs for MS patients is compared to the control group (table 4). The highest association to MS was described to interactions between HLA-DRB1\*15:01, HLA-DQA1\*01:02 TNF- $\alpha$  -238 G/A and TNF- $\alpha$  -308 G/A ( $p < 0,0037$ , OR 4,22).

**Table 4. Haplotypes association analysis of HLA alleles / Human TNF alpha Promoter polymorphisms with MS**

## DISCUSSION

The human leukocyte antigen (HLA) is the exclusive consistent genetic association identified with MS <sup>24</sup>. Other immune-mediated genes such as immunoglobulin heavy chain (IgH), T-cell receptor (TCR), tumor necrosis factor (TNF) and myelin essential protein (MBP) also have been described in association with MS <sup>6, 7</sup>. Classic linkage and affected sib-pair linkage studies have examined these genes as potential risk factors, but the results have conflicted <sup>25,26</sup>.

This study examined the association of six SNPs in the TNF alpha promoter, HLA-DRB1 and HLA DQ alleles in Cuban patients with MS. In addition, we investigate interactions between TNF-alpha promoter polymorphism and HLA with MS. Our results have indicated the evidence for susceptibility to MS with the presence of HLA-DRB1\*15:01, DRB1\*14:01, DQA1\*01:01 and DQB1\*06:02 alleles with higher odds ratio

(OR 3,4; 4,96; 2,53; 2,77 respectively) compared to healthy controls. Numerous genetic studies identify HLA-DRB1\*1501 as the highest susceptibility allele in MS. However, the close genetic proximity with HLADQ and strong linkage disequilibrium between them make it almost impossible to distinguish the contribution of these genes except through functional studies<sup>27</sup>. Up today, the HLA class II DRB1\*1501/DQA1\*0102/-DQB1\*0602 (DR2) haplotype is the only region repeatedly confirmed as being associated with MS in most European populations<sup>28,29</sup>

The current experiment enhances evidence for the association of TNF- $\alpha$  promoter gene polymorphism and MS in Cuban individuals. The frequencies association have resulted from the evaluation of seven SNP polymorphisms located in the TNF alpha promoter (pTNF-  $\alpha$ ) and have shown a possible role against MS for pTNF-a SNP -238 (G/A), -308 (G/A), -376 (G/A) polymorphisms ( $p < 0.01$ ). Goertsches et al. developed a similar research on the Spanish population<sup>30</sup>. They have referred to their results as corresponding with the Genetic Analysis of MS in Europeans (GAMES) project reports in which these three alleles have been described as the ones for the genetic MS risk factor<sup>30,31</sup>.

Among them, The -308 G/A TNF-alpha promoter polymorphism has been described as having a condition highly associated with the development of MS<sup>32,33,34</sup>. However, some discrepant results have been recorded without any MS significant association<sup>35,36</sup>.

The results of meta-analysis have shown that the bearing of the A allele of TNF-a -308 is associated with a reduced risk of MS, probably because a high level of TNF alpha and increasing activity of the TNF-a pathway interfere with the onset of CNS inflammation, and then reduce the risk of MS. This group has suggested that larger-scale case-control studies stratified by ethnicity are required to confirm their results<sup>37</sup>.

Rahmanian and Kargar have described the association of TNF- $\alpha$  gene polymorphism and MS in south Iranian native people with included 81 patients with MS according to the problem criteria and 82 healthy controls (HC) with the same age, sex, social, ethnical and geographical features. They detected some noteworthy alleles of TNF- $\alpha$  microsatellite significantly more than usual in MS groups, as compared with the HC groups, and then they concluded a possible genetic predisposition to MS in Iranian patients<sup>38</sup>.

While Elahi et al. have alleged that it is difficult to make general announcements about the associations of TNF polymorphisms and TNF $\alpha$  production or pathology. They have emphasized that the TNF polymorphisms are found in a region of significant polymorphic variation and are in linkage disequilibria with the HLA genes and each other. Because of differences in the distribution of HLA alleles, one might expect variations in associations between TNF polymorphisms and conditions in different geographical areas. Indeed, there is a geographical variation in the frequency of the -308A allele. It is present in approximately 5% of the population of South Africa compared with 30% in white Caucasians in the United Kingdom<sup>39</sup>.

The TNF-alpha promoter -308 (G/A) is correlated with transcriptional activation and the amount of protein production<sup>40</sup>. Moreover, some studies have found the high expression levels of TNF-alpha linked to the rare

allele (A) and the extended haplotype TNF- $\alpha$ -308(AA) -HLA-A1-B8-DR3-DQ2 to be associated with autoimmunity and high TNF- $\alpha$  production <sup>41</sup>.

A study in men of Russian and Tatar ethnic origin found that susceptibility to MS was influenced by the HLA DRB1\*1501 SNP rs909253 and TNF  $\alpha$  SNP rs1800629 <sup>42</sup>. Another case-control study conducted with an Iranian population reported an interaction between HLA-DRB1\*1501 and TNF  $\alpha$  SNP rs1800629, with a 7-fold increased odds of MS among individuals with at least one copy of the risk allele <sup>33</sup>.

Our results have shown the haplotype HLA DRB1\*1501, HLA DQA1\* 0102, TNF-  $\alpha$  -238 G/A, -308 G/A, which have exhibited the heightened association ( $p < 0.05$ , OR = 4.1) with MS in our sample.

Farorova et al. have developed a study of Russian descent and have identified a combination of more than two alleles that confers a genetic predisposition to MS as a complex polygenic disease <sup>43</sup>. They have described two new allelic variant's patterns as "trios" of allelic variants: the first one corresponding to the SNP -509 of the TGFB1\*C gene, DRB1\*18 44, and the G allele of cytotoxic T lymphocyte antigen 4 (CTLA4) gene (trio 1) and the second one grouping the alleles -238 TNF\*B1, -308TNF\*A2 and CTLA4\*G (trio 2) ( $p < 0.01$ , OR 18 trio1, 17.4 tri 2) <sup>44</sup>. Besides, the authors have emphasized the contribution of several others influencing alleles involved in the autoimmune inflammatory response as well <sup>43</sup>.

The impact of the HLA-DRB1\*1501 allele and TNF- $\alpha$  -308 G/A single nucleotide polymorphism and their interaction in the susceptibility to MS in the Iranian population was evaluated by Shahbazia et al. from 366 MS Patients and 414 control subjects <sup>45</sup>. Their results have shown that the HLA-DRB1\*1501 allele and TNF- $\alpha$  -308 G/A polymorphism are associated with the risk of multiple sclerosis in those populations. They observed an interaction between these two loci that supports the role of HLA alleles and cytokine genes and gene-gene interaction in the development and pathogenesis of MS <sup>45</sup>.

DNA sequence variations in the regulatory region might interfere with TNF gene transcription, influencing TNF's circulating level and susceptibility to autoimmune and other neurodegenerative diseases <sup>46</sup>. In addition, clinical observations have implicated dysregulation of the TNF- $\alpha$  pathway in susceptibility to MS. In a clinical trial, patients who received lenercept, a p55 tumor necrosis factor receptor fusion protein, had increased relapse activity compared to patients who received a placebo <sup>47</sup>. A clinical trial of an anti-TNF antibody was associated with increased disease activity, and demyelinating disease has emerged in persons treated with monoclonal antibodies to TNF- $\alpha$  for indications such as inflammatory bowel disease and rheumatoid arthritis <sup>41, 48</sup>.

It is unclear whether the interaction between HLA and TNF  $\alpha$  is biological or simply that the weaker effect of TNF  $\alpha$  is more easily detected when the HLA risk allele is absent. However, HLA haplotypes are known to affect the synthesis of TNF- $\alpha$ , with some haplotypes increasing TNF  $\alpha$  synthesis and others decreasing TNF  $\alpha$  synthesis <sup>49, 50</sup>.

MS is a multifactorial disease; thus, determining the genetic polymorphisms of genes might be an essential strategy as a marker of genetic predisposition to MS. Then, our results suggest that the evaluation of those

genetic polymorphisms could be considered a prognostic mechanism to identify individuals at higher risk of developing MS. However, future structural and functional genetic studies on larger populations can help to understand the importance of polymorphisms at the TNF alpha promoter gene in the onset of the disease. Additionally, evaluating the potential effects of an interaction between TNF-  $\alpha$  and HLA may offer insights into disease mechanisms.

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## CONCLUSIONS

In conclusion, our study provides substantial evidence for the association of HLA alleles and TNF- $\alpha$  promoter polymorphisms with MS susceptibility in the Cuban population. Specifically, the HLA-DRB115:01, DRB114:01, DQA101:02, and DQB106:02 alleles were identified as susceptibility alleles, while the TNF- $\alpha$  -238 G and -308 G alleles were found to be more frequent in MS patients compared to controls. These findings align with previous studies highlighting the crucial role of HLA genes in MS susceptibility and reinforce the complex interplay between genetic and environmental factors in disease development.

Furthermore, our study underscores the importance of examining gene-gene interactions in understanding MS susceptibility. The combined effect of the HLA-DRB11501, HLA-DQA101:02, TNF- $\alpha$  -238 G/A, and TNF- $\alpha$  -308 G/A haplotype was found to significantly increase the risk of MS, suggesting that the interaction between HLA alleles and TNF- $\alpha$  polymorphisms may play a crucial role in disease pathogenesis. This finding adds a new dimension to our understanding of the genetic basis of MS. It emphasizes the need for further research to unravel the complex mechanisms underlying gene-gene interactions in MS.

Our study contributes to the growing evidence implicating both HLA alleles and TNF- $\alpha$  promoter polymorphisms in MS susceptibility. These findings have important implications for developing novel diagnostic and therapeutic strategies for MS, as they highlight potential targets for intervention. However, further research is needed to validate our findings in more extensive and diverse populations and to elucidate the precise mechanisms by which these genetic factors contribute to MS pathogenesis.

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