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CTLA-4, ICOS, PD1 and *PTPN22* Gene Polymorphisms and Susceptibility to Autoimmune Hepatitis Type 1

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Abstract: Autoimmune hepatitis type 1 (AIH-1) is a progressive inflammatory liver disorder in which HLA Class II gene polymorphism prevails as the most important genetic risk. However, other gene polymorphisms have been associated with this disease. The single nucleotide polymorphisms of four candidate genes (*CTLA-4* +49A/G, *ICOS* c.1564 T/C, *PD1.3* G/A, *PTPN22* 1858C/T) were selected in this study. One-hundred and ninety (190) mestizo Venezuelan unrelated individuals grouped in AIH-1 patients (n=70) and healthy subjects (n=120) were evaluated. Our results showed significantly increased frequency of the *PTPN22* 1858 C/T (p= 0.0014; pc= 0.0042; OR= 8.7; 99%CI: 1.82-41.54) and *ICOS* c.1564 T/C (p= 0.070; pc=0.21; OR= 2.08; 95%CI: 1.09-3.93) genotypes in the patient population compared to control group. There was no significant association between *CTLA-4* +49A/G and *PD1.3* G/A genotypes in both groups. In addition, the *PTPN22* 1858C/T polymorphism was associated to cirrhosis, treatment relapse, increased IgG levels and co-existence of other autoimmune diseases. Furthermore, the *ICOS* c.1564 T/C polymorphism was related to higher levels of globulins, IgG and presence of ANA. Conclusion: this data suggest for the first time, that *PTPN22* 1858 C/T and *ICOS* c.1564 T/C gene polymorphisms are associated with the development of AIH-1.

Keywords: Autoimmune Hepatitis Type 1, Mestizo Venezuelan, Genetics, Immunopathology, T Lymphocyte

1. Introduction

Autoimmune hepatitis type 1 (AIH-1) is a chronic inflammatory liver disease of unknown etiology, and is characterized by elevated serum transaminase levels, hypergammaglobulinemia predominantly of the immunoglobulin G (IgG) type, and serum autoantibodies such as antinuclear antibodies (ANA). The histological features of AIH-1 are defined by periportal or periseptal interface hepatitis due to immune cell infiltrate consisting of lymphocytes, macrophages and plasma cells [1, 2].

Although the etiology of AIH-1 is not fully understood, it has been recognized that interactions of susceptible genetic background and environmental risk factors contribute to the initiation and promotion of disease [3]. Furthermore, AIH-1 is a complex polygenic disease with a strong genetic association to the human leucocyte antigen (HLA) gene located within the major histocompatibility complex (MHC). Indeed, this disease has been associated with *DRB1*03*, *DRB1*04* and *DRB3* alleles in European and North American Caucasians; *DRB1*0405* in Japanese; *DRB1*0404* in Mexican; and *DRB1*1301* in Argentinean populations [3, 4]. Previous study also indicated that AIH-1 predisposition was associated with *DRB1*1301* and *DRB1*0301* alleles in a mestizo Venezuelan population [5]. Nonetheless, the genetic predisposition conferred by HLA is neither adequate nor necessary for disease development [1]. In this regard, several other loci outside the MHC region particularly those encoding proteins that regulate the function of T lymphocyte, could be involved in the pathogenesis of AIH-1 [2, 6].

The Cytotoxic T-Lymphocyte Antigen 4 (*CTLA-4*) gene is located in chromosome 2q33 and over 100 single nucleotide polymorphisms (SNPs) have been identified. One of the most extensively studied SNP is A/G substitution at position +49

in exon 1 [7]. Agarwal *et al.* firstly described this polymorphism (*CTLA-4* G allele) as a second susceptibility allele (besides *HLA-DRB1*) in northern European Caucasoid type AIH-1 patients [8]. In addition, they proposed that there might be synergy between the *HLA-DRB1* 0301* and the G/G genotypes in terms of disease risk [8]. Nevertheless, subsequent investigations focusing on this novel polymorphism showed different outcomes. For instance, several independent studies reported that this polymorphism did not confer susceptibility to AIH-1 in either Brazilian [9] or Japanese populations [10]. However, another study from Djilali-Saiah *et al.* showed differing results in Canadian and French populations [11]. This group presented a genetic analysis of the *CTLA-4/CD28* region in a panel of Caucasian AIH children and their families from Canada and France. Compared to Agarwal's data, Djilali-Saiah's results stressed that children carrying the A allele but not G allele at the +49 position of *CTLA-4* were predisposed to AIH-1 [11]. On the other hand, Fan *et al.* found that polymorphism of *CTLA-4* gene exon 1 (+ 49 A/G) was not associated with AIH in the Chinese population [12]. Because of these previous conflicting results, the first aim of the study was to assess the association between *CTLA-4* gene polymorphism and AIH-1 in mestizo Venezuelan patients.

Protein tyrosine phosphatases (PTPs) are critical regulators of T cell signal transduction [13]. In conjunction with protein tyrosine kinases, PTPs regulate the reversible phosphorylation of tyrosine residues and thereby play important roles in many different aspects of T cell physiology [14]. Abnormalities in tyrosine phosphorylation have been shown to be involved in the pathogenesis of numerous human diseases, from autoimmunity to cancer [15]. Due to their potential etiologic and pathogenic roles in human disease, PTPs have been considered good candidate genes in autoimmune diseases. The protein tyrosine phosphatase non-receptor 22 (*PTPN22)* gene is located on chromosome 1p13 [16]. A SNP has been identified within *PTPN22* at position 1858 in codon 620 that results in arginine (Arg) to tryptophan (Trp) (C to T) shift [16]. This allele shift has been associated with diabetes mellitus type 1 [17], rheumatoid arthritis (RA) [18], systemic lupus erythematosus (SLE) [19], autoimmune hypothyroidism and Graves' disease [20, 21]. In view of these findings, the second aim of this study was to assess the role of *PTPN22* 1858C/T gene polymorphism in the predisposition and clinical expression of AIH-1.

Programmed death-1 (PD-1) is a molecule expressed on activated T-, B- and myeloid cells [22]. PD-1 provides an inhibitory signal to T-cell activation like CTLA-4, resulting inhibition of T-cell proliferation and cytokine secretion [23]. The expression of PD-1, Programmed death-ligand 1 (PD-L1) and Programmed death-ligand 2 (PD-L2) have been demonstrated in autoimmune liver biopsies of AIH and primary biliary cirrhosis (PBC) [24, 25]. In the human *PD-1* gene, located on chromosome 2 (2q37 region), over thirty SNP shave been identified [26]. One of the SNPs, *PD-1.3G/A* (position 7146), has been associated with susceptibility to SLE in Swedish, European American and Mexican families [27, 28, 29]. Furthermore, this allele was associated with a protective effect in a Spanish patient population with SLE [30]. The presence of *PD-1.3 G/A* has also been associated with other autoimmune diseases as RA [31], diabetes mellitus type 1 [32], ankylosing spondylitis [33] and juvenile-onset systemic lupus erythematosus [34]. The polymorphism of this gene has not been investigated in AIH-1 patients. Therefore, the third aim of this study was to assess the role of this *PD-1.3G/A* SNP in the predisposition and clinical expression of AIH-1.

Inducible T-cell costimulator (ICOS) belongs to the family of surface molecules CD28 membrane type immunoglobulin associated with co-stimulatory functions. ICOS is expressed with CD28 on activated T cells; however unlike CD28, ICOS is not constitutively expressed but induced *de novo* [35, 36]. Experimental studies have concluded that ICOS plays a critical role for the efficient activation of T cells and T-helper type-2 (Th2) cytokines production [37]. There is also experimental evidence on the involvement of ICOS in the pathogenesis of AIH in animals. For instance, Aoki *et al.* showed that blocking ICOS in PD-1 mice (-/-) using monoclonal antibodies suppressed the development of AIH [38]. Therefore, given the crucial function of this molecule in the activation of T lymphocytes, it was also assessed the role of *ICOS* gene polymorphism c.1564 T/C in AIH-1 patients, and their possible association with clinical and immunodiagnostic expression.

2. Material and Methods

2.1. Study Population

This study was approved by the local Research Ethics Committee, and written informed consent was obtained from all subjects. The study included 190 unrelated mestizos Venezuelan subjects of the third generation, divided into two groups: AIH-1 patients and healthy subjects. Mestizo Venezuelans are the result of a mixture of European (60.6%), Amerindian (23%) and African (16.3%) ancestry [39]. Patients and controls of mestizo extraction came from different geographical regions of Venezuela.

Seventy patients (70) had a definitive diagnosis of AIH-1 according to the diagnostic criteria of the International Autoimmune Hepatitis Group [40]. There were 10 men (14,2%) and 60 women (85,7%) with an age range of 8-70 years. Since AIH-1 is a disease with low incidence in Venezuela, patients were recruited from nine different Liver Units throughout the country.

One-hundred and twenty (120) healthy subjects were enrolled from our healthy human donor database of the Genetic Immunology Section of the Immunology Institute (Universidad Central de Venezuela). This group included 58 males (48,4%) and 62 females (51,6%) with an age range of 1-76 years.

2.2. Genotype Analysis of CTLA-4, PTPN22, PD1.3 and ICOS

The polymorphic variant *CTLA-4*+49A/G, *PTPN22* 1858C/T, *PD1.3* and *ICOS* c.1564 T/C genes were studied by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) technique, using primers and restriction enzymes as previously reported [41, 18, 42, 43]. Genomic DNA was extracted from proteinase K treated peripheral blood leucocytes by a high-affinity column method (QIAmp DNA Mini Kit, Qiagen®, Germantown, USA).

2.3. Statistical Analysis

Allele and genotype frequencies were determined by direct counting. The Hardy-Weinberg equilibrium was calculated by Chi square test. The statistical significance of allele frequency differences between patients and controls was estimated by Chi square test, *p* values were corrected (*pc*) by multiplying the number of comparisons made (Bonferroni correction), and were considered significant when $p< 0,05$ [44, 45]. Relative risks with the corresponding 95% confidence intervals (95% CI) were calculated as odds ratios (OR) according to Woolf's formula [45]. Coefficient of Spearman (rS) correlation [45] was performed to determine whether *HLA* alleles susceptibility for AIH-1 and *CTLA-4* +49A/G, *PTPN22* 1858C/T, *PD1.3* G/A and *ICOS* c.1564 T/C genotypes were associated with disease. This analysis was done with the PAST program [46].

3. Results

3.1. Clinical Characteristics of Subjects

Table 1 shows the clinical and immunological characteristics of AIH-1patients.

Table 1. *Clinical and immunological characteristics of patients with autoimmune hepatitis type 1.*

	Patients n=70
Clinical features	
Age (years)*	32,7 (8-70)
Sex (female/male)	60 (85,7%) / 10 (14,2%)
Associated autoimmune diseases	18 (25,7%)
Biochemical features	
AST (normal, <40 IU/L)*	630,8 (34 - 2102)
ALT (normal, <36 IU/L)*	648,8 (28- 2332)
ALP (normal, <136 IU/L, children <450 IU/L)*	411,2 (56 - 1940)
TSB (normal, <1.3 mg/dL)*	7,3 (0,44 - 69)
Globulin (normal, <3.5 g/dL)*	4,5 (2,1 - 9)
Immunoserological features	
IgG (normal, < 1621 mg/dL)*	3379,3 (1330 - 7100)
IgA (normal, < 387 mg/dL)*	337,1 (17 - 650)
IgM (normal, < 200 mg/dL)*	257,5 (79 - 958)
ANA**	45,7
SMA**	54,3
ANA+ SMA**	45,7

*Expressed as an arithmetic value.
**Expressed as a percentage.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP alkaline phosphatase; TSB total serum bilirrubin; Ig, immunoglobulin; ANA, antinuclear antibody; SMA, anti-smooth muscle antibody.

3.2. Frequency of CTLA-4, PTPN22, PD1.3 and ICOS Polymorphisms in AIH-1 Patients and Healthy Controls

Our findings showed the existence of Hardy-Weinberg equilibrium for genotype distribution in healthy individuals. Table 2 shows the frequencies of *CTLA-4, PTPN22, PD1.3* and *ICOS* genotypes in AIH-1 patients and controls. Frequency of the heterozygous *PTPN22* 1858 C/T genotype was higher in AIH-1 patients than in the control group (12,86% vs. 1,67%, respectively; χ^2 (df:1)=11,51; $p= 0,0014$; $pc= 0,0042$; OR= 8,7; 99% CI: 1,82-41,54). In addition, there was a significantly increased frequency of homozygous *ICOS* c.1564 T/T genotype in healthy controls compared to AIH-1 patients (50% vs. 32,86%, respectively, χ^2 (df: 2)= 5,31 $p= 0,070$; $pc=0,21$; OR= 2,08; 95%CI: 1,09-3,93). However, we did not find any significant association between *CTLA-4* +49A/G and *PD1.3* G/A genotypes and AIH-1 patients (see Table 2).

Table 2. *Genotype and allele frequency distribution of CTLA4, PTPN22, PD1.3 and ICOS polymorphisms in AIH-1 patients and controls.*

CTLA4 +49A/G				
Genotypes	**AA**	**AG**	**GG**	**Total**
Controls (N° individuals)	43	62	15	120
% of total	22.63	32.63	7.9	63.16
% within condition	35.8	51.7	12.5	-
% within +49A/G	63.24	63.9	60	-

CTLA4 +49A/G				
Genotypes	**AA**	**AG**	**GG**	**Total**
AIH-1 Patients (N° individuals)	25	35	10	70
% of total	13.16	18.42	5.26	36.84
% within condition	35.71	50	14.29	-
% within +49A/G	36.76	36.1	40	-
Total	68	97	25	190
% of total	35.8	51	13.2	100
χ^2 (df:2)=1.31 p= 0.748; OR: 0.94; 95% CI 0.52-1.69				
PTPN22 1858C/T				
Genotypes	CC	CT	TT	Total
Controls (N° individuals)	118	2	0	120
% of total	62.11	1.05	0	63.16
% within condition	98.33	1.67	-	-
% within PTPN22 1858C/T	65.92	18.18	-	-
AIH-1 Patients (N° individuals)	61	9	0	70
% of total	32.10	4.74	0	36.84
% within condition	87.14	12.86	-	-
% within PTPN22 1858C/T	34.08	81.82	-	-
Total	179	11	0	190
% of total	94.21	5.79	0	100
χ^2 (df:1)=11.51p= 0.0014 pc= 0.0042; OR= 8.7; 99%CI: 1.82-41.54				
PD1.3 G/A				
Genotypes	GG	GA	AA	Total
Controls (N° individuals)	109	11	0	120
% of total	57.37	5.79	0	63.16
% within condition	90.8	9.2	0	-
% within PD1.3 G/A	64.12	57.89	0	-
AIH-1 Patients (N° individuals)	61	8	1	70
% of total	32.10	4.21	0.53	36.84
% within condition	87.14	11.43	1.43	-
% within PD1.3 G/A	35.88	42.11	100	-
Total	170	19	1	190
% of total	89.5	10	0.5	100
χ^2 (df:2)=2.01 p=0,366 OR= 1,29; 95% CI: 0,49-3,40				
ICOS c.1564 T/C				
Genotypes	TT	TC	CC	Total
Controls (N° individuals)	60	49	11	120
% of total	31.58	25.79	5.79	63.16
% within condition	50	40.83	9.17	-
% within c1564T/C	72.29	55.68	57.89	-
AIH-1 Patients (N° individuals)	23	39	8	70
% of total	12.1	20.53	4.21	36.84
% within condition	32.86	55.71	11.43	-
% within c1564T/C	27.71	44.32	42.11	-
Total	83	88	19	190
% of total	43.68	46.32	10	100
χ^2 (df: 2) = 5.31p= 0.070 pc=0.21OR= 2.08; 95% CI: 1.09-3.93				

3.3. Correlation HLA Alleles Conferring Susceptibility of Developing AIH-1 with CTLA-4, PTPN22, PD1.3 and ICOS Polymorphisms

A value of rS = 0,42 (p<0.0001) was observed for the association of homozygous *ICOS* c.1564 T/T genotype and *HLA DRB1*03* when analyzing the correlations between gene polymorphisms (*CTLA-4, PTPN22, PD1.3* and *ICOS*) and *HLA* alleles that confer susceptibility to develop AIH-1 (*DRB1*13, DRB1*03* and *DRB1*04*). This finding indicates that although the relationship is of medium intensity, it is significant and suggests a trend that when the *ICOS*c.1564 T/T polymorphism appears, the *HLA DRB1*03* allele also appears. The other polymorphisms did not maintain significant correlations with *HLA* alleles.

3.4. Clinical Characteristics of Patients and Their Possible Association with the Presence of Susceptible Polymorphisms

According to the results of correlating *PTPN22* 1858C/T with the patient clinical characteristics, it was observed that the presence of cirrhosis (p=0,039; pc=0,0858); treatment relapse (p=0,054; pc=0,109); increased IgG levels (p=0,072; pc=2,296) and autoimmune diseases (p=0,073; pc=1,132) were noted in patients with heterozygous genotype (C/T) with statistically significant differences at 10% but lost when corrected with Bonferroni.

The case of polymorphism *ICOS* c.1564 T/C showed that patients with heterozygous genotype had higher levels of globulin (p=0,064; pc=0,128); IgG (p=0,059; pc=0,654) and

presence of ANA (p=0,059; pc=0,654) than those with the T/T genotype. This was also statistically significant at 10% but lost when corrected with Bonferroni.

Unlike the polymorphisms observed with HLA alleles conferring susceptibility for AIH-1 in mestizo Venezuelan patients [5], the genes polymorphisms evaluated in this study had no association with different age groups.

4. Discussion

In this study, the association of *CTLA-4*+49A/G, *PTPN22* 1858C/T, *PD1.3* and *ICOS* c.1564 T/C gene polymorphisms with AIH-1 were assessed in a mestizo Venezuelan population. It was decided to study these candidate genes as they influence T lymphocyte regulation which seems to be involved in the immunopathogenesis of AIH-1 [2]. It was also explored the association with HLA alleles previously described by our group in mestizo Venezuelan AIH-1patients [5].

4.1. Frequency of CTLA-4, PTPN22, PD1.3 and ICOS Polymorphisms in Patients with AIH-1

These results showed significantly higher frequency of the *PTPN22* 1858 C/T and *ICOS* c.1564 T/C genotypes in the AIH-1 population than the control group. However, there was no significant association between *CTLA-4* +49A/G and *PD1.3* G/A genotypes with AIH-1. This is the first report showing that these genotypes *PTPN22* 1858 C/T and *ICOS* c.1564 T/C are associated with autoimmune hepatitis type 1 in a mestizo patient.

PTPN22 gene has been considered a good candidate genetic marker for susceptibility to autoimmunity [47]. This gene encodes for lymphoid tyrosine phosphatase (LYP), which is highly involved in preventing spontaneous T cell activation [48]. As discussed earlier, the variant of this gene at 1858 C/T has been associated with multiple autoimmune diseases including diabetes mellitus type 1 [17], RA [18], SLE [19] and autoimmune thyroiditis [20, 21]. These diseases have common autoimmune autoantibody phenotypes that appear frequently before the clinical onset of disease. Therefore, it has been suggested that *PTPN22* 1858 C/T could be associated with pathologies that have a significant humoral immune component [19]. The risk allele (T) of the *PTPN22* gene results in an amino acid change at residue 620 (Arg by Trp) which is located in the region P1of LYP rich in prolines that alter sites interaction with the SH3 domain of CSK kinase [49]. As the binding of CSK and LYP is crucial for down regulating T cell, it is expected that the altered molecule LYP provokes an increase in T lymphocytes activation. Furthermore, results of experimental [48] and clinical studies [50] have suggested that individuals with heterozygous or homozygous mutation for the gene *PTPN22* 1858 C/T (present on activated T cells over-expression of the mutant LYP molecule) can cause a reduced flow of intracellular calcium with inactivation of kinases and their own substrates signaling cascade. This, in turn, leads to increased inhibition of lymphocyte function.

Based on this evidence, polymorphism *PTPN22* 1858C/T could lead to the development of autoimmunity through multiple mechanisms: firstly, suppressing T cell receptor (TCR) signaling more efficiently during thymic development, resulting in the survival of autoreactive T cells that must be deleted by negative selection [47]; secondly, reducing activation of regulatory T cells which makes them less potent in suppressing the autoimmune response [47] and thirdly, there is evidence that this polymorphism can also contribute to autoimmunity by decreasing activation of B lymphocytes, via B-cell receptor (BCR) [50] causing the escape of autoreactive B cells. This could ultimately result in increased production of autoantibodies, supporting the observation that diseases associated with this polymorphism are those with high humoral response. The results found in the polymorphism LYP, which is associated with changes in receptor response of T and B cells, enable us to support the concept that there is a dysfunctional combination of both populations of lymphocytes within mechanisms of predisposition to AIH-1.

By exploring the distribution of genotype polymorphism *ICOS* c.1564 T/C, the genotypic frequencies of TT/CT was significantly different when comparing our patient population to the control group. Indeed, the heterozygous genotype (T/C) and mutated homozygote (C/C) were more prevalent in AIH-1 patients. In contrast, the T/T genotype (determining genotype protection) was significantly more prevalent in the control group. If we consider that the homozygous wild polymorphisms are associated with increased expression of ICOS molecule [51], it could be assumed that Th2 response might confer protection to the development of AIH in the mestizo Venezuelan population. This possible immunologic mechanism is supported by multiple reports describing improvement of AIH during pregnancy [52, 53], condition in which predominates Th2 response necessary to maintain immunological tolerance required in this condition [54]. Therefore, it seems that the presence of wild genotype (T/T) observed in the healthy population is associated with increased expression of ICOS. This, in turn, would favor the polarization of the response towards Th2 and preventing a Th1 prolonged response characteristically of AIH-1 patients, which may be given by the presence of heterozygote (T/C) genotype.

4.2. Clinical Characteristics of Patients and Their Possible Association with the Presence of Susceptible Polymorphisms

It was observed that the presence of cirrhosis, relapse during treatment, increased levels of IgG and concomitant autoimmune disease were more prevalent in patients with heterozygous genotype *PTPN22* C/T than in those wild homozygotes C/C with statistically significant differences at 10%. Although this association showed low statistical significance, this suggests that the presence of this polymorphism *PTPN22* 1858 C/T not only increases the susceptibility to AIH-1 but also seems to influence disease severity.

In addition, it was found an association of *ICOS* c.1564

gene polymorphism with the clinical characteristics of patients. Indeed, the heterozygous genotype T/C had higher levels of globulins, IgG and presence of ANA than those in patients with genotype T/T. Although this association also showed low statistical significance, the predominance of clinical alterations in the genotype T/C also suggests a correlation of this genotype with parameters of disease activity. Despite the fact that these statistical differences were lost when applying the Bonferroni correction, this was considered relevant to highlight these findings as both polymorphisms showed significantly increased frequency.

4.3. Correlation HLA alleles Conferring Susceptibility of Developing AIH-1 with CTLA-4, PTPN22, PD1.3 and ICOS Polymorphisms

It was found a trend of a positive correlation between presence of HLA *DRB1*03* allele and *ICOS* c.1564 T/T gene polymorphism, both considered to confer susceptibility to AIH-1. However, the other polymorphisms did not maintain significant correlations with *HLA* alleles. These findings suggest that the role of ICOS in the immunopathogenesis of this disease is very complex, and reflects the multiplicity of immunogenetic factors influencing the eventual appearance of this pathology specifically in this ethnic group.

5. Conclusions

In summary, this report to show that *PTPN22* 1858 C/T and *ICOS* c.1564 T/C gene polymorphisms are associated with predisposition to autoimmune hepatitis type 1 in a mestizo patient population. However, further research is required to establish the definitive role of ICOS/LYP and the humoral immune response in etiopathogenesis of this disease. The next step is to perform blood transcriptome analyses to assess the expression levels of ICOS and LYP in this patient population of autoimmune hepatitis type 1.

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