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Association of *STAT4*, *TGFβ1*, *SH2B3* and *PTPN22* polymorphisms with autoimmune hepatitis

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ABSTRACT

The physiopathology of autoimmune hepatitis (AIH) is complex and still not fully elucidated. The genes localized outside the histocompatibility complex involved in regulation and signal transduction of the immune system *SH2B3*, *TGFβ1*, *STAT4* and *PTPN22* could be associated to the susceptibility and hepatocyte lysis mechanism of this lethal autoimmune disorder.

Patients and methods: We investigated four polymorphic sites in *SH2B3* (rs3184504), *TGFβ1* (rs1800471), *STAT4* (rs7574865) and *PTPN22* (rs2476601) in 45 AIH patients and 150 healthy controls from Tunisia using real-time PCR.

Results: Significant associations were found for *SH2B3* T allele (OR = 1.861; p = 0.015, pc = 0.366) and *PTPN22* A allele (OR = 7.070; p = 0.026; pc = 1.00) and AIH with opposite homozygous being protective against the disease (CC genotype with OR = 0.420, p = 0.025; GG genotype with OR = 0.136, p = 0.025, respectively). No statistically significant associations were found for the *TGFβ1* and *STAT4* polymorphisms with AIH susceptibility.

Conclusion: Our work enlarges information on non-HLA genes that are associated with AIH by focusing in a region of the world that was poorly molecularly characterized for this disease.

1. Introduction

Autoimmune hepatitis (AIH) is an immuno-mediated inflammatory liver disorder of unknown etiology characterized by hypergammaglobulinaemia, circulating autoantibodies and interface hepatitis. AIH is expected to evolve progressively towards liver cirrhosis and end-stage liver failure. (Muratori and Longhi, 2013) The diagnosis is based on histological abnormalities, specific clinical and biochemical findings, abnormal levels of serum globulins and the presence of specific autoantibodies. (Wang et al., 2016) The serological markers of the disease consist mainly of anti-nuclear antibodies (ANA), anti-smooth muscle antibodies (SMA), liver/kidney microsomal antibody 1 (LKM1), anti-liver cytosol type 1 (LC1) and anti-soluble liver antigen/liver pancreas (SLA/LP). (Liberal et al., 2011) AIH is considered relatively rare, as its mean annual incidence in northern Europeans is 0.85 to 1.9 per 100,000 inhabitants. The incidence of AIH ranges from 0.67 to 2.0 per 100,000 people per year with a value of 0.83 per 100,000 in Spain (Wang et al., 2016). Its prevalence ranges from 4.0 to 42.9 per 100,000 people with a higher prevalence in Alaska and northen Europe (Liberal et al., 2015). To date, no information was found concerning the incidence of AIH in the North-African populations. The predisposition to AIH has been linked to a strong female preponderance and a significant contribution of genetic factors in the occurrence, pathogenesis and progression of the autoimmune response. (Liberal et al., 2015) The strongest associations are located within the human leukocyte antigen HLA-DRB1 locus, with HLA-DRB1*03:01 and HLA-DRB1*04:01 conferring susceptibility in European and North American populations (Czaja et al., 1997; Strettell et al., 1997). A significant association of HLA DRB1*03:01 was also found with AIH susceptibility and clinical manifestations in Tunisian patients. (Chaouali et al., 2017a, 2017b) Although the etiology of autoimmune hepatitis has not been completely elucidated, there are several genes described that code not only for HLA

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molecules, but also for cytokines, transcription factors, or signal transduction molecules that could interact together to promote the development of autoimmunity. Many of these genes are so far unknown, which demonstrates that AIH is a multifactorial polygenic disease with a complex genetic susceptibility background. (Aizawa and Hokari, 2017) These genetic promoters are located outside the MHC complex and could trigger AIH onset by interacting synergistically with the major HLA susceptibility alleles (epistasis) or by acting alone (Barrett et al., 2009; Tomer et al., 2015).

Previous genome-wide studies indicated a strong association between the genes encoding the immunoregulatory molecules such as the transcription factor *STAT4*, the tyrosine phosphatase *PTPN22*, the adaptation protein *SH2B3* or the transforming growth factor *TGFβ1* with susceptibility to autoimmune hepatitis. (De boer et al., 2014; Tang et al., 2012) Different combinations of these genes could be involved in triggering the pathogenic mechanism of autoimmune hepatitis. The *SH2B3* adaptation protein is a negative regulator of T cell activation, tumor necrosis factor, and protein kinase 2 and 3 signaling. A recent study showed that the polymorphism affecting the gene encoding the protein SH2B3 was associated with increased susceptibility to AIH. (Li et al., 2017) *STAT4* plays an important role in the activation of dendritic cells and macrophages, and in the signal transductions within activated peripheral blood monocytes while *PTPN22*, located in the cytosol of immune cells, is involved in many signaling pathways associated with the immune response such as lymphocyte activation and proliferation. (Tang et al., 2012) The association of *STAT4* and *PTPN22* genes with AIH onset as well as some autoimmune diseases such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) has been previously described (Beltran Ramirez et al., 2016; Li et al., 2017). TGFβ is a cytokine that plays an integral role in the regulation of immune responses. Two functional polymorphisms affecting the coding region of the *TGFβ1* gene (codon 10 and codon 25) have been associated with increased *TGFβ1* expression levels and severe progression of AIH.

The polymorphisms in these genes could contribute to structural modifications of these proteins, or could modulate the intracellular signaling pathways that regulates the immune responses, which would lead to the amplification of autoreactive T lymphocyte response against hepatocytes and the lysis mechanism of hepatic cells observed during AIH. However, other studies did not find a significant association between the polymorphism of these candidate genes and the occurrence of AIH. This work is the first study conducted in Tunisia to investigate possible associations between *SH2B3*, *TGFβ1*, *STAT4* and *PTPN22* genes and susceptibility to autoimmune hepatitis.

2. Material and methods

2.1. Patients and controls

A total of 45 unrelated patients with definite AIH were recruited from the Gastroenterology department of Military hospital of Tunis, Charles Nicolle, La Rabta and Habib Thameur Hospitals, between September 2014 and April 2016. AIH was diagnosed based on International AIH Group criteria using a scoring calculator (10–15). A score of > 15 was taken as definite AIH, and ≥ 10 as probable AIH. Patients with a score of < 10 were excluded from the analysis. Clinical and biological features were obtained from the medical records of patients. One hundred fifty three unrelated healthy donors were included in our study (113 women and 40 men, mean age 49.6 yrs. ± 7.4) and matched for gender and age with AIH cases. Study participants have signed an informed consent before the study, and ethics committees approved the study protocol. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in *a priori* approval by the institution's human research committee.

Genotype frequencies in several worldwide populations for the four SNPs were extracted from 1000 Genomes database (Abecasis et al., 2012).

2.2. Genotyping by real time PCR

Genomic DNA was extracted from lymphocytes separated from whole blood using a Ficoll–Paque solution (density 1.077 ± 0.001 g/ml). DNA extraction was performed using QIAamp® DNA Blood Mini Kit (Qiagen®), following the manufacturer's instructions. Single Nucleotide Polymorphism (SNP) genotyping was realized in four different gene positions; *SH2B3* C > T (rs3184504), *TGFβ1* C > G (rs1800471), *STAT4* G > T (rs7574865) and *PTPN22* A > G genes (rs2476601) and performed by TAQMAN PCR Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, USA). The TaqMan PCR is based on the real time amplification of target DNA sequences by hybridization of the specific Taqman probe and elongation by the Taq polymerase. PCR amplification using 10 ng of genomic DNA and TaqPath™ ProAmp™ Master Mix (Applied Biosystems, Carlsbad, USA) was performed by a thermocycler BioRad ™ with an initial step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for min. The fluorescence profile of each well was directly proportional to the fluorophore release and therefore to the amount of PCR products and the analysis was performed by the TaqMan® Genotyper ™ software.

2.3. Statistical analysis

Genotype frequencies of all SNPs was assessed by using the software Arlequin version 3.5.2.2 [2]. Compliance of alleles at individual loci with the Hardy-Weinberg equilibrium was measured at the level of the control population using a $\chi 2$ test (level of significance set to p-value $< .05$) implemented in the software Arlequin version 3.5.2.2 (Excoffier, 2010). The comparison of allele frequencies between cases and controls was expressed as P values (two tailed Fisher exact test), odds ratios (ORs), and 95% confidence intervals (CIs) and was performed at vassarStats (http://vassarstats.net/); p-value $< .05$ was considered as statistically significant.

3. Results

3.1. Clinical and immunological parameters in AIH Tunisian patients

Our AIH cohort has an excess of women (84.4%), as expected for this disease. The mean age at disease onset was 50.48 ± 16.05, while the mean age of the control group was 46.74 ± 14.17. The disease duration was presented as median (IQR), 4.0 (3.0–7.7). The most frequently observed clinical manifestations were jaundice (88.8%), asthenia (66.6%), splenomegaly (57.7%) and pruritus (51.1%). The immunological parameters were mainly specific serum antibodies such as ANA (77.7%) and SMA (68.8%). Three patients had an infection with cytomegalovirus (CMV) and only one patient presented Epstein-Barr virus (EBV) infection. The main clinical and immunological characteristics of AIH patients are shown in Table 1.

3.2. Overall worldwide frequencies of the four SNPs

The *STAT4* variant was the most widely polymorphic across the globe (Fig. 1C), followed by *SH2B3 variant (Fig. 1A) which is more polymorphic in European, Tunisian and European-descendant American populations.* Both variants in *TGFβ1* (Fig. 1B) and *PTPN22* (Fig. 1D) *genes are less polymorphic in all populations, with minimum allele frequency variant in the range of 0.027–0.048 average worldwide frequency*.

3.3. Association of SH2B3 T/C polymorphism with susceptibility to AIH

The study of the T/C polymorphism (rs3184504) in SH2B3 gene revealed a higher frequency of TT genotype in patients with AIH

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