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FULL LENGTH ARTICLE

Cytotoxic T lymphocyte antigen-4 gene polymorphisms and susceptibility to type 1 autoimmune hepatitis in the Tunisian population

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Abstract Genetic factors and gene polymorphisms leading to the onset of autoimmune response in autoimmune hepatitis (AIH) are still not full elucidated. Since the CTLA-4 molecule is a key modulator of the lymphocytes responses we hypothezied that deficiencies or mutations in the gene encoding CTLA4 protein may be involved in AIH susceptibility and trigger the autoimmune response. We investigated 3 distinct polymorphic sites (+49A > G, CT60 G > A and −318C > T) of the CTLA4 gene in 50 AIH patients and 100 healthy controls using the KASP genotyping technology. A significant positive association with AIH susceptibility was found for the GG genotype in +49 position of the CTLA4 gene which was significantly higher in AIH patients compared to controls (28% vs 9%, $p = 0.003$, OR = 3.93 [1.56–9.88]). The CTLA4 A/A genotype in position CT60 was more significantly frequent in controls comparing to AIH patients and could be considered as a protective genotype for the tunisian patients. CTLA4 genotyping in position −318 did not show any statistically significant difference in genotype or allele distribution. The CTLA4 gene polymorphism in position +49 is associated to AIH susceptibility in the

Abbreviations: AIH, Autoimmune hepatitis; CTLA4, Cytotoxic T-lymphocyte antigen 4; *AMA-M2*, Anti-mitochondrial *antibody-M2*; *EBV*, *Epstein–Barr virus*; ANA, Anti-nuclear antibodies; *CMV*, *Cytomegalovirus*; HLA, Human leucocyte antigen; LKM1, Anti-Liver/Kidney Microsomal Antibodies Type 1; PBC, *Primary biliary cirrhosis*; PSC, Primary sclerosing cholangitis; SLA, *Antibodies* against *soluble liver antigen*; SMA, Smooth-muscle antibodies; KASP PCR, Competitive allele-specific real-time PCR.

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Tunisian population. Mutation in the CTLA4 gene may lead to a modification of the CTLA4 protein structure that could have functional relevance in AIH pathogenesis and onset.

Introduction

Autoimmune hepatitis (AIH) is a chronic and progressive liver inflammatory disorder characterized by the presence of elevated levels of serum transaminases, hypergammaglobulinemia, serum autoantibodies and interface hepatitis.[1] The diagnosis is based on a combination of clinical, biological and histological characteristics and the exclusion of viral or metabolic causes.[2] The etiology of AIH involves the interaction of immunogenetic background and environmental factors in triggering the autoimmune response and mediating the hepatocyte lysis. Liver destruction leads to severe fibrosis and cirrhosis.[3] The pathological mechanism of hepatocyte lysis is based on the lymphocyte mediated cytotoxicity, apoptotic pathways activation, infiltration of autoreactives cells within the liver parenchyma and numerical and functional deficiency of lymphocytes T regulators.[4] The mechanism of molecular mimicry implicates a prior infection with viruses such as Hepatitis B, C viruses, cytomegalovirus or herpes simplex type 1 virus and contributes to AIH autoimmune response in genetically predisposed individuals.[5] Autoimmune hepatitis is divided into two types according to the type of autoantibodies present in the serum; the type 1 AIH characterized by the presence of antinuclear antibodies (ANA), smooth muscle antibodies (SMA), or both and is the major form of autoimmune hepatitis among white adults. Type 2 AIH is characterized by antibodies to liver-kidney microsome type 1 (LKM1) and its more frequently found in children.[6] AIH is a relatively rare disease, although its incidence is higher in northern Europe.[7] Boberg et al found a prevalence of 16.9 cases per 100,000 people in Norwegian population.[8] 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 3. Hurlburt et al[9] reported an AIH prevalence ranging from 4.0 to 42.9 per 100,000 people in Alaska and Europe. To date, there is no data concerning the incidence of AIH in the Tunisian or the North-African populations. A significant implication of the genes located within the human leukocyte antigen (HLA) region and single nucleotide polymorphisms (SNP) within regulatory genes in increasing the risk and development of autoimmune hepatitis were demonstrated by multiple studies.

The implication of major histocompatibility complex (MHC) genes in the susceptibility and onset of HAI has been well established in distinct populations and multiple studies have confirmed the association of HLA alleles DRB1*0301 and/or HLA DRB1*0401with susceptibility to autoimmune hepatitis.[10,11] These genes encode for HLA antigens involved in the presentation of antigenic peptides to T lymphocytes and in triggering the autoimmune response. The role of HLA DRB1*03 was also demonstrated in conferring susceptibility to AIH in the Tunisian patients.[12] However, HLA alone does not explain the whole genetic predisposition to AIH, because at least 30—50% of patients with the disease do not carry the most common susceptibility alleles.[13] In this regard, genetic promoters located outside the MHC complex, particularly those encoding for immunoregulatory proteins, have been implicated in disease susceptibility and could also influence the occurrence of autoimmune hepatitis, either by acting alone or in synergy with the main HLA susceptibility alleles (epistasis). Potential candidates include most genes encoding immunoregulatory proteins, especially cytokine genes, genes encoding adhesion molecules, and genes encoding proteins involved in antigen presentation, processing and signaling.[14] The cytotoxic T lymphocyte antigen-4 (CTLA-4) is a T-cell surface molecule that interacts, in competition with the costimulatory molecule CD 28, with the ligands B7-1 and B7-2 on antigen presenting cells. The CTLA4 gene is located on chromosome 2q33. Recently, an A—G base exchange polymorphism in exon 1 of the CTLA-4 gene at position 49 has been associated with susceptibility to AIH-1. The CTLA-4 +49 A/G single nucleotide polymorphism (SNP) results in an amino acid substitution of Threonine with Alanine at position 17 in the CTLA-4 protein and has been shown to affect the expression of the CTLA-4 molecule.[15] A deficient function and lower production level of CTLA 4 protein would be associated with the CTLA-4 gene polymorphism.[16,17] A decreased number of regulatory T cells was also observed in individuals carriers the +49A > G variation.[18] A second polymorphism in the promoter region, −318C > T may favor upregulation of CTLA4 upon T-cell activation but its effects are less completely studied.

The aims of this study were to determine the association of CTLA4 gene polymorphisms and susceptibility to autoimmune hepatitis in the Tunisian population by genotyping three distinct polymorphic sites; A/G polymorphism in position +49 of the CTLA4 promoter (rs 231775), C/T polymorphism in position 60 of CTLA4 gene (rs 3087243) and T/C polymorphism in position −318 of CTLA4 gene (rs 5742909) in 50 adult Tunisian patients suffering from autoimmune hepatitis in comparison to 100 healthy control subjects.

Material and methods

Patients and controls

A total of 50 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 less than 10 were excluded from the analysis. Clinical and biological features were obtained from the medical records of patients. One hundred unrelated healthy donors were included in our study (86 women and 14 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.

CTLA-4 genotyping by KASP PCR

Genomic DNA was extracted from lymphocytes separated from whole blood using a Ficoll–Paque solution (density 1.077 ± 0.001 g/ml) (24). DNA extraction was performed using QIAamp® DNA Blood Mini Kit (Qiagen®), following the manufacturer's instructions.

The study of the CTLA4 gene polymorphism was carried out at 3 different positions; CTLA-4 exon-1 + 49A > G (rs 231775), the CTLA4 CT60 C > T polymorphism (rs 3087243) and the CTLA4 -318 T > C polymorphism (rs 5742909) by the competitive allele-specific real-time PCR technique or KASP PCR. A volume of 5 μL of patient and control DNA samples was deposed in a 96 well plate, 10 ng of DNA were required by PCR reaction, 5 μL of negative control was included on each plate, 14 μL of KASP Assay mix containing 3 specific primers; Two alleles specific forward primers; one labeled with the fluorochrome FAM and the other labeled with the fluorochrome HEX and one reverse primer with a common sequence tail were added in 500 μL of KASP Master mix containing the two universal FRET (resonance energy transfer), the KASP Taq polymerase, nucleotides, Mgcl2, a ROX™ passive generic dye in an optimized solution. The 5 μL of the mixture obtained was added in each well of the KASP PCR. The PCR KASP was run in a Fast Real-time PCR thermocycler 7500 (Applied Biosystem). Amplified DNA fragments were visualized as fluorescent spots in a KlusterCallerTM (LGC) Software designed to read the FRET plates (v.2.3 Applied Technologies).

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 software (IBM, Armonk, NY). The comparison of alleles and haplotype frequencies between cases and controls was expressed as *p* values, odds ratios (ORs), and 95% confidence intervals (CIs) and was performed by vassarStats (http://vassarstats.net/). A *p*-value < 0.05 was considered as statistically significant. P values were calculated by the Fisher exact probability test.

Results

Clinical and immunological features in AIH patients

Demographic parametres of AIH patients are shown in Table 1. AIH is more frequent in women (84%). The mean age is 52.9 ± 14.1; the mean age at disease onset is 46.2 ± 15.2 and the disease duration is presented as median (IQR), 4.0 (3.0–7.7). Patients with type 1 AIH are present with a frequency of 96% and those with type 2 AIH with 4%. The most frequent Clinical presentations are jaundice (80%), asthenia (66%), splenomegaly (52%) and Pruritus (46%). Specific antibodies were found in patients' sera such as ANA (70%) and SMA (58%). Three patients had an infection with cytomegalovirus (CMV) and only one patient presented Epstein–Barr virus (EBV) infection. Clinical characteristics of AIH patients are shown in Table 2.

Table 1 Characteristics of Tunisian autoimmune hepatitis cases and controls.

Parameters	Cases[a] (n = 50) n (%)	Controls[a] (n = 100) n (%)	*p*-value
Gender, n (%) Women	36 (84.0)	86 (86.0)	0.728
Age[b]	50.48 ± 16.05	46.74 ± 14.17	0.112
Smoking, n (%) Yes	6 (12.0)	32 (21.30)	0.145
Alcohol use, n (%) Yes	2 (4.0)	19 (12.30)	0.083
Age at onset[b]	44.22 ± 14.33	NA	–
Disease duration[c]	5.0 (2.00–8.00)	NA	–

[a] A total of 50 AIH cases and 150 healthy controls were included.
[b] t-Student's test for the variable "Age" with normal distribution (mean ± standard deviation).
[c] Variable without normal distribution (median (interquartile range)).

Table 2 Clinical characteristics of AIH patients.

Clinical presentations, n (%)	n (%)
Asthenia	33 (66)
Arthralgia	10 (20)
Nausea	8 (16)
Anorexia	10 (20)
Weight loss	13 (26)
Abdominal pain	13 (26)
Jaundice	40 (80.0)
Pruritus	23 (46)
Splenomegaly	26 (52)
Hepatomegaly	17 (34)
Antibodies presence, n (%)	
ANA	35 (70)
SMA	29 (58)
LKM1	2 (4)
SLA	2 (4)
AMA-M2	11 (22)
Infections, n (%)	
EBV infection	1 (3.3)
CMV infection	3 (6)

ANA: anti-nuclear antibodies, SMA: Smooth-muscle antibodies, LKM1: Anti-Liver/Kidney Microsomal Antibodies Type 1, SLA: *Antibodies* against *soluble liver antigen*, *AMA-M2*: Anti-mitochondrial *antibody-M2*, *EBV*: *Epstein–Barr virus*, *CMV*: *Cytomegalovirus*, *NA*: *not applicable*.

Association of CTLA4 (A/G +49) polymorphism with susceptibility to AIH

The genotyping analysis of the CTLA4 (A/G +49) gene polymorphism revealed a significantly higher frequency of the G/G genotype in AIH patients compared to controls (28% vs 9%, $p = 0.003$, OR = 3.93 [1.56–9.88]). The frequency of A/G genotype was higher in AIH patients compared to controls, although this result was not statistically significant, (57% vs 48%, $p = 0.30$). The A/A genotype frequency was higher in controls compared to HAI patients without statistical significance (34% vs 28%, $p = 0.26$) Table 3 demonstrates the differences in distribution of G/G, A/G and A/A genotypes of CTLA4 +49 gene polymorphism in AIH patients and controls. We then performed an analysis of the allelic distribution frequency of CTLA4 (A/G +49) polymorphism in the Tunisian patients. The results showed a significant higher frequency of the A allele in controls compared with HAI patients (62.5% vs 48%, $p = 0.018$, OR = 0.55 [0.34–0.90]). The G allele was also more frequently represented in patients with HAI compared to controls and this difference in distribution was statistically significant, (52 % vs 37.5%, $p = 0.018$; OR = 1.80 [1.11–2.93]).

Association of CTLA4 (A/G -CT60) polymorphism with susceptibility to AIH

The genotyping of the CTLA4 gene in position CT-60 revealed the presence of 3 differents genotypes A/A, A/G and G/G. The genotype G/A was the most frequent among AIH patients compared to A/A or G/G genotypes (58% vs 34% or 8% respectively). The frequency of A/A genotype in position CT-60 was significantly higher in healthy controls compared to AIH patients (22% vs 8%, $p = 0.039$, OR = 0.30 [0.1–0.95]). Patients suffering from AIH and carriers of the G/G or A/G genotypes did not show any statistically significant results when comparing patients to controls (Table 4). Analysis of the allelic frequency distribution of CTLA4 (G/A - in patients compared to controls and this difference between patients and controls.

Association of CTLA4 (C/T-318) polymorphism with susceptibility to AIH

Analysis of CTLA-4 polymorphism at position −318 revealed a higher frequency of the CC genotype in AIH patients and controls comparing to the C/T or T/T genotypes. The frequency of the CC genotype was higher in AIH patients compared to healthy controls but this difference was not statistically significant (94% vs 91%, $p = 0.75$). The C/T genotype was more frequently detected in healthy controls that in AIH patients but was not statistically significant (9% vs 6%, $p = 0.75$). The allelic frequencies analysis of the CTLA4 C/T-318 polymorphism revealed a significantly higher frequency of the C allele in the CTLA4 -318 position in patients with HAI 97% compared to controls 95.5%, without any statistical significance. The distribution of the T allele between patients and controls showed no significant difference between patients and controls ($p = 0.75$) (Table 5).

Discussion

Genetic susceptibility to type 1 autoimmune hepatitis was strongly associated not only with human leukocyte antigen (HLA) alleles DRB1*0301 and DRB1*0401 but also with genes located outside the histocompatibility complex. The main candidate currently is the exon 1 of the CTLA-4 coding gene that could increase the susceptibility to AIH and be involved in the pathologic autoimmune response of this immune disorder.[15] Our results revealed a significant positive association of the GG genotype in +49 position of the CTLA4 gene with AIH which was significantly higher in AIH patients compared to controls (24% vs 9%, $p = 0.003$, OR = 3.93 [1.56–9.88]). As previous studies have shown a significant association of HLA DRB1*03 with AIH [12], the GG genotype in CTLA4 +49 position could be considered as an additional susceptibility factor to AIH in the Tunisian population. Polymorphisms in CTLA4 gene were also detected for two other positions (G/A CT60) and (C/T −318) in 50 AIH patients and 100 controls. The CTLA4 A/A genotype in position CT60 was more significantly frequent in controls comparing to AIH patients and could be considered as a protective genotype for the tunisian patients. CTLA4 genotyping in position −318 did not show any statistically significant difference in genotype or allele distribution. A significant difference in the GG genotype distribution between 155 AIH patients and controls was also found by the study of Agarwal et al[15] in the United States (GG = 21/155 patients vs. 13/102 controls, $p = 0.011$). The G allele was over-represented in AIH patients compared to controls (105/155 patients vs. 51/102 controls, $p = 0.004$, odds

Table 3 Distribution of CTLA4 (A/G +49) genotypes and alleles in Tunisian AIH patients and controls.

CTLA 4 (A/G + 49) Genotypes	AIH Patients (n = 50)		Controls (n = 100)		p	OR (95% CI)
	n	GF (%)	n	GF (%)		
G/G	14	28	9	9	0.003	3.93 [1.56–9.88]
A/G	24	48	57	57	0.30	–
A/A	12	24	34	34	0.26	–
CTLA 4 (A/G + 49) Alleles	AIH Patients		Controls		p	OR (95% CI)
	2n	AF (%)	2n	AF (%)		
G	52	52	75	37.5	0.018	1.80 **[1.11–2.93]**
A	48	48	125	62.5	0.018	0.55 [0.34–0.90]

n: number of subjects, AIH: Autoimmune Hepatitis, GF: Genotype frequency, AF: Allelic frequency, OR: odds ratio, CI: Confidence interval. P value calculated by the Fisher exact probability test.

Table 4 Distribution of CTLA4 (-CT60) genotypes in Tunisian AIH patients and controls.

CTLA4 (-CT60) Genotypes	AIH Patients (n = 50)		Controls (n = 100)		p	OR (95% CI)
	n	GF (%)	n	GF (%)		
A/A	4	8	22	22	0.039	0.30 [0.1—0.95]
A/G	29	58	50	50	0.38	—
G/G	17	34	28	28	0.57	—
CTLA 4 (-CT60) Alleles	AIH Patients		Controls		p	OR (95% CI)
	2n	AF (%)	2n	(95% CI)		
G	63	63	106	53	0.10	-
A	37	37	94	0.47	0.10	

n: number of subjects, AIH: Autoimmune Hepatitis, GF: Genotype frequency, OR: odds ratio, CI: Confidence interval. P value calculated by the Fisher exact probability test.

Table 5 Distribution of CTLA4 -318 genotypes in Tunisian AIH patients and controls.

CTLA4 -318 Genotypes	AIH Patients (n = 50)		Controls (n = 100)		p	OR (95% CI)
	n	GF (%)	n	GF (%)		
C/C	47	94	91	91	0.75	—
C/T	3	6	9	9	0.75	—
T/T	0	0	0	0	0	—
CTLA 4 (C/T −318) Alleles	AIH Patients		Controls		p	OR (95% CI)
	2n	AF (%)	2n	AF (%)		
C	97	97	191	95.5	0.75	
T	3	3	9	4.5	0.75	—

n: number of subjects, AIH: Autoimmune Hepatitis, GF: Genotype frequency, OR: odds ratio, CI: Confidence interval. P value calculated by the Fisher exact probability test.

ratio = 2.12). The presence of the GG genotype in AIH patients was also associated with a significantly higher mean serum aspartate transaminase level ($p = 0.03$), greater frequency of antibodies to thyroid microsomal antigens ($p = 0.004$) and more commonly detected in patients carriers of HLA DRB1*0301 ($p = 0.02$). Treatment outcomes, however, were not affected by the nature of the genotypes revealed. Our study also showed that the frequency of the +49 A allele was significantly higher in controls than in AIH patients (62.5% vs 48%, $p = 0.018$, OR = 0. 55 [0.34—0.90]), which means that the +49 A allele would be considered as a protective factor for AIH but this hypothesis is no longer valid by the observation of other results showing a higher frequency of allele A in patients with HAI compared to controls and this different distribution was statistically significant, $p = 0.03$; OR = 1.6 [1.05—2.63]. CTLA-4 exon 1 gene polymorphism could have a functional involvement in susceptibility to type 1 AIH. Indeed, CTLA-4 is a cell surface molecule which acts as a negative regulator of T-cell activation and proliferation and plays an important role in maintaining the immune homeostasis by the modulation of T cells response.[19] Waterhouse and al[20] showed that the functional role of CTLA4 is emphasized by the development of lethal autoreactive lymphoproliferative disease in CTLA-4 deficient mice. Previous studies demonstrated the development of antigen-specific autoreactive T-cell clones in the physiopathologic mechanism leading to the onset of type 1 AIH.[21]The commitment of the CTLA-4 molecule and its binding to B7 is necessary to initiate the phenomenon of anergy and inhibit the development of autoreactive T lymphocytes. Although, functional abnormalities affecting the CTLA-4 T-cell interaction and costimulatory signal generated by this molecule would contribute to autoreactive T-cell responses genesis and could initiate the immunopathogenesis of AIH. The Thr/Ala substitution in the leader peptide may have an effect on post-translational processing or localization of the CTLA-4 protein.[15] The study of Kouki et al reported that the G/G genotype in position +49 of the CTLA gene was the cause of a modification occurring in CTLA4 gene sequence (threonine to alanine) leading to diminished inhibitory effects on T cell proliferation and stimulated hyperactivity of the lymphocytes T.[22] A recent meta-analysis study including reported that the A/A genotype located in position +49 of the CTLA4 gene conferred protection against type 1 autoimmune hepatitis (OR = 0.66, 95% CI 0.50—0.86) which seems in accordance with our results, although the G/G homozygosity was not significantly associated with AIH susceptibility.[23] In contrast to the previous findings, other studies found a significant association of the A allele rather than G with AIH, in fact, Djilali-Saiah et al[24] showed a preferential transmission of the CTLA-4 A allele from parents to offspring with AIH type 1 (62.5%) and a higher transmission of (AT)8 while no increase in the transmission of these alleles was observed in offspring affected with AIH type 2. Other studies have reported deficient inhibitory

effects of CTLA-4 on T cells proliferation for patients who present the G/G genotype rather than A/A genotype in CTLA-4 +49 gene promoter.[22] Multiple studies failed to show any significant association of the +49 A/G polymorphism with autoimmune hepatitis susceptibility and onset. In fact, a study realized[25] in New Zealand included 77 autoimmune hepatitis patients and showed no statistically significant association between the CTLA4 +49 gene polymorphism and the occurrence of autoimmune hepatitis. Other studies that have examined the implication of +49 A > G polymorphism CTLA4 (rs 231775) with AIH onset have failed to show a definitive association between CTLA4 A/G polymorphism and disease susceptibility in Brazilian, Chinese, Japanese or Netherlandic populations (Bittencourt et al. 2003[26]; Fan et al. 2004[27]; Schott et al. 2007[28]; Umemura et al. 2008,[29] Van Gerven et al. 2013[2]). Therefore, different genes could be involved in susceptibility to AIH in these distinct populations. Polymorphisms in exon 1 CTLA-4 gene are not major determinants of susceptibility to AIH in these populations. These differences possibly reflects regional differences between the genetic background and potential underlying etiologic factors to AIH (diet, infection, toxic chemicals, or drug history).[30] These difference also suggest a varying level of CTLA4 polymorphism risk among different ethnic populations.[2] Another polymorphism represented by a cytosine to thymine single base exchange at position −318 of the CTLA4 gene promoter have been described in autoimmune hepatitis. Ligers et al described increased mRNA expression and protein level production of CTLA-4 in patients who carried the CTLA4 T−318 genotype with the co-existence of the A/A genotype in position CTLA-4 + 49 in the same patients ($p = 0.0026$).[16] This study suggests that the −318C > T polymorphism may be the cause of a higher expression of CTLA-4 molecule during the activation of the immune responses.[16] However, the study performed by Scott et al[28] showed different results since the −318 T allele was underrepresented in patients with chronic liver diseases compared with healthy controls (19.6 vs. 25.7%, $p = 0.043$). Few studies have been conducted on the impact of this polymorphism on the cellular mechanisms underlying AIH pathogenesis and whether the functional effect of CTLA4 is caused by this polymorphism or not is still under investigation. Other unknown loci could be also involved in the expression and regulation of the CTLA-4 molecule. Another chinese study carried out by Fan et al[31] showed no significant difference in the genotype distribution of CTLA-4 promoter −318 T/C polymorphisms between AIH patients and controls, but the C allele frequency was significantly increased in patients with AIH, compared to controls ($p = 0.02$, OR = 2.43). The distribution of CTLA-4 gene exon 1 + 49 A/G genotypes exhibited significant difference between PBC patients and controls ($p = 0.006$), and the frequency of G allele showed a significant increase in PBC patients compared with healthy controls ($p = 0.0046$, OR = 1.8). The occurrence of the GG-CC genotype was also increased in the two groups of patients (AIH: 32.3%, PBC: 37.7%; control: 22.5%). The CTLA4 -318 T/C polymorphism would act in synergy with other mutations affecting the CTLA4 gene or be in strong linkage disequilibrium with the genes causing strong susceptibility to AIH.[30] Few studies have been reported for the association between CTLA4 CT60 G/A single nucleotide polymorphism with AIH susceptibility and onset. Our study showed that the A/A genotype in CT-60 position of the CTLA4 gene could be considered as a protective factor from AIH for tunisian patients ($p = 0.039$, OR = 0.30 [0.1−0.95]). Nevertheless, an italian study did not show any significant difference in the frequency distribution of CT60 gene polymorphism between patients and controls.[32] Another study conducted in Japan failed also to find any significant association of the CTLA4 CT60 G/A gene polymorphism with PBC susceptibility and onset.[33]

Autoimmune hepatitis is considered as an heterogenous and complex disease with varying degrees of genetic and environmental influence. Alteration in CTLA-4 signaling could contribute to type 1 AIH susceptibility. Recent evidence suggests that signaling through CTLA-4 may play a role in regulating the balance between Th1 and Th2 cell differentiation. Therefore, this mechanism may have different effects on the pathogenesis of type 1 and type 2 AIH by activating a cascade of events that trigger specific autoimmunity. Alternatively, CTLA-4 polymorphisms might be a marker of disease susceptibility or could be in linkage disequilibrium with other genes influencing AIH onset in close proximity to CTLA-4 on chromosome 2q33.[34]

Conclusion

To our knowledge, this is the first report studying the association between CTLA 4 + 49 A/G, CT60 G/A and −318 C/T gene polymorphisms with AIH susceptibility in North-Africans; Our results showed a significant association of the GG genotype in position +49 of the CTLA4 gene with AIH susceptibility, the presence of A/A or A/G could therefore confer protection from AIH in the Tunisian population. No significant differences in genotype frequencies between patients and controls were found for CT60 G/A and −318 C/T gene polymorphisms. Large-scale studies and stratified clinical groups could be performed to further verify this association.

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Conflict of interest

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References

1. Liberal R, Vergani D, Mieli-Vergani G. Update on autoimmune hepatitis. *J Clin Transl Hepatol*. 2015;3:42—52.
2. Van Gerven NM, De Boer YS, Zwiers A, et al. Cytotoxic T lymphocyte antigen-4 +49A/G polymorphism does not affect susceptibility to autoimmune hepatitis. *Liver Int*. 2013;33:1039—1043.
3. Wang Q, Yang F, Miao Q, Krawitt EL, Gershwin ME, Ma X. The clinical phenotypes of autoimmune hepatitis: a comprehensive review. *J Autoimmun*. 2016;66:98—107.
4. Chaouali M, Kochkar R, Tezeghdenti A, et al. Hépatite autoimmune chronique de l'adulte: étude clinique de 30 patients Tunisians. *Rev Franc Lab*. 2017;491:60—66.
5. Duclos-Vallée JC, Ballot É, Johanet C. Pathogenesis of autoimmune hepatitis: new concepts. *La lettre de l'hépato gastroentérologue*. 2003;5:179—182.
6. Muratori L, Muratori P, Granito A, Pappas G, Cassani F, Lenzi M. Current topics in autoimmune hepatitis. *Dig Liver Dis*. 2010;42:757—764.
7. Gronbaek L, Vilstrup H, Jepsen P. Autoimmune hepatitis in Denmark: incidence, prevalence, prognosis, and causes of death. A nationwide registry-based cohort study. *J Hepatol*. 2014;60:612—617.
8. Boberg KM, Aadland E, Jahnsen J, Raknerud N, Stiris M, Bell H. Incidence and prevalence of primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis in a Norwegian population. *Scand J Gastroenterol*. 1998;33:99—103.
9. Hurlburt KJ, McMahon BJ, Deubner H, Hsu-Trawinski B, Williams JL, Kowdley KV. Prevalence of autoimmune liver disease in Alaska Natives. *Am J Gastroenterol*. 2002;97:2402—2407.
10. Aizawa Y, Hokari A. Autoimmune hepatitis: current challenges and future prospects. *Clin Exp Gastroenterol*. 2017;10:9—18. https://www.ncbi.nlm.nih.gov/pubmed/28176894.
11. Strettell MDJ, Donaldson PT, Thompson LJ, et al. Allelic basis for HLA-encoded susceptibility to type 1 autoimmune hepatitis. *Gastroenterology*. 1997;112:2028—2035.
12. Chaouali M, Kochkar R, Messadi A, Tezeghdenti A, Ben Azaiez M, Ben Abdallah H. Distribution of HLA-DRB1/DQB1 alleles and DRB1-DQB1 haplotypes among Tunisian patients with autoimmune hepatitis. *Egypt J Med Hum Genet*. 2017;18:335—339, 2017.
13. Donaldson PT, Czaja AJ. Genetic susceptibilities for immune expression and liver cell injury in autoimmune hepatitis. *Immunol Rev*. 2000;174:250—259.
14. Czaja AJ, Cookson S, Constantini PK, Clare M, Underhill JA, Donaldson PT. Cytokine polymorphisms associated with clinical features and treatment outcome in type 1 autoimmune hepatitis. *Gastroenterology*. 1999;117:645—652.
15. Agarwal K, Czaja AJ, Jones DE, Donaldson PT. Cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphisms and susceptibility to type 1 autoimmune hepatitis. *Hepatology*. 2000;31:49—53.
16. Ligers A, Teleshova N, Masterman T, Huang WX, Hillert J. CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. *Gene Immun*. 2001;2:145—152.
17. Van Belzen MJ, Mulder CJ, Zhernakova A, Pearson PL, Houwen RH, Wijmenga C. CTLA4 +49 A/G and CT60 polymorphisms in Dutch coeliac disease patients. *Eur J Hum Genet*. 2004;12:782—785.
18. Atabani SF, Thio CL, Divanovic S, et al. Association of CTLA4 polymorphism with regulatory T cell frequency. *Eur J Immunol*. 2005;35:2157—2162.
19. Bluestone JA. Is CTLA-4 a master switch for peripheral T cell tolerance. *J Immunol*. 1997;158:1989—1993.
20. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science*. 1995;270:985—988.
21. Lohr HF, Schaak JF, Gergen G, Fleischer B, Dienes H-P. Meyer zum Buschenfelde K-H. Phenotypical analysis and cytokine release of liver infiltrating and peripheral blood T lymphocytes from patients with chronic active hepatitis of different etiology. *Liver*. 1994;14:161—166.
22. Kouki T, Sawai Y, Gardine CA, Fisfalen ME, Alegre ML, DeGroot LJ. CTLA-4 gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves' disease. *J Immunol*. 2000;165:6606—6611.
23. Miyake Y, Ikeda F, Takaki A, Nouso K, Yamamoto K. +49A/G polymorphism of cytotoxic T-lymphocyte antigen 4 gene in type 1 autoimmune hepatitis and primary biliary cirrhosis: a meta-analysis. *Hepatol Res*. 2011;41:151—159.
24. Djilali-Saiah I, Schmitz J, Harfouch-Hammoud E, Mougenot JF, Bach JF, Caillat-Zucman S. CTLA-4 gene polymorphism is associated with predisposition to coeliac disease. *Gut*. 1998;43:187—189.
25. Ngu JH, Wallace MC, Merriman TR, Gearry RB, Stedman CAM, Roberts RL. Association of the HLA locus and TNF with type I autoimmune hepatitis susceptibility in New Zealand Caucasians. *SpringerPlus*. 2013;2:355.
26. Bittencourt PL, Palacios SA, Cancado EL, et al. Cytotoxic T lymphocyte antigen-4 gene polymorphisms do not confer susceptibility to autoimmune hepatitis types 1 and 2 in Brazil. *Am J Gastroenterol*. 2003;98:1616—1620.
27. Fan LY, Tu XQ, Cheng QB, et al. Cytotoxic T lymphocyte associated antigen-4 gene polymorphisms confer susceptibility to primary biliary cirrhosis and autoimmune hepatitis in Chinese population. *World J Gastroenterol*. 2004;10:3056—3059.
28. Schott E, Witt H, Pascu M, et al. Association of CTLA4 single nucleotide polymorphisms with viral but not autoimmune liver disease. *Eur J Gastroenterol Hepatol*. 2007;19:947—951.
29. Umemura T, Ota M, Hamano H, et al. Association of autoimmune pancreatitis with cytotoxic T-lymphocyte antigen 4 gene polymorphisms in Japanese patients. *Am J Gastroenterol*. 2008;103:588—594.
30. Djilali-Saiah I, Ouellette P, Caillat-Zucman S, Debray D, Kohn JI, Alvarez F. CTLA-4/CD28 region polymorphisms in children from families with autoimmune hepatitis. *Hum Immunol*. 2001;62:1356—1362.
31. Fan LY, Zhu Y, Zhong RQ, et al. Study on the relationship of CTLA-4 -318, +49 polymorphisms with autoimmune hepatitis and primary biliary cirrhosis in a Chinese population. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 2004;21:440—443.
32. Donaldson P, Veeramani S, Baragiotta A, et al. Cytotoxic T-lymphocyte-associated antigen-4 single nucleotide polymorphisms and haplotypes in primary biliary cirrhosis. *Clin Gastroenterol Hepatol*. 2007;5:755—760.
33. Yang XC, Fujino M, Cai SJ, Li SW, Liu C, Li XK. Genetic polymorphisms of cytotoxic t-lymphocyte antigen 4 in primary biliary cholangitis: a meta-analysis. *J Immunol Res*. 2017;2017,5295164.
34. Umemura T, Katsuyama Y, Yoshizawa K, et al. Human leukocyte antigen class II haplotypes affect clinical characteristics and progression of type 1 autoimmune hepatitis in Japan. *PLoS One*. 2014;23(9), e100565.