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ORIGINAL ARTICLE

Association of TNF-α-308 Polymorphism with Susceptibility to Autoimmune Hepatitis in Tunisians

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Abstract Autoimmune hepatitis (AIH) is a chronic hepatitis of unknown etiology and several proinflammatory cytokines are implicated in its pathogenesis. The association of TNF-α gene polymorphism with AIH onset is not fully elucidated especially in the Tunisian population. The aim of this study was to determine the association of TNF-α (-308 G > A) polymorphism with AIH susceptibility and with TNF-α expression or clinical manifestations of AIH. A total of 50 AIH patients and 150 controls were included. Evaluation of TNF-α polymorphism was performed by ARMS PCR method. A significantly higher frequence of the AA genotype was found in AIH patients compared to controls (34 vs. 8%, $p = 0.00002$, OR 5.88). The frequency of the A-allele was significantly higher in patients with AIH compared to controls (55 vs. 37.3%, $p = 0.002$, OR 2.05). The G-allele was significantly more frequent in healthy controls compared to AIH patients [43 vs. 61.3%, $p = 0.001$, OR 0.47 (0.3–0.75)]. There was a positive correlation between the A/A genotype and a higher serum expression of TNF-α. The TNF*A allele confer susceptibility to AIH in the Tunisian patients and is associated with increased production of TNF-α. Anti-TNF antibodies could be an alternative to the use of corticotherapy and may avoid the exacerbated immune response in AIH.

Keywords TNF-α gene polymorphisms · Tumor necrosis factor-α · Autoimmune hepatitis

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Abbreviations

AIH	Autoimmune hepatitis
TNF-α	Tumor necrosis factor-α
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
ARMS PCR	Amplification refractory mutation system polymerase chain reaction

Introduction

Autoimmune hepatitis (AIH) is a progressive inflammatory disease of the liver of unknown etiology, characterized by the increase of aminotransferase levels, hypergammaglobulinemia and the presence of autoantibodies in serum leading to acute liver failure. AIH results from a loss of tolerance against hepatocyte antigens which is considered as the main cause of the liver lysis and hepatocyte destruction (Liberal et al. 2015). The autoimmune response in AIH is favored via molecular mimicry, genetic predisposition and the impaired regulation of regulatory T-cells (Lohse and Mieli-Vergani 2011; Gatselis et al. 2015). AIH type 1 is characterized by the presence of antinuclear antibodies (ANA), smooth muscle antibodies (SMA), or both and is diagnosed more frequently in adults (Czaja and Manns 1995). Type 2 AIH is characterized by antibodies to liver-kidney microsome type 1 (LKM1) and occurs mainly in children (Manns and Vogel 2006). AIH occurs in all ethnic groups and in all geographical areas with a higher frequency in Northern Europe region (Lohse and Mieli-Vergani 2011), but it is considered relatively rare, as its annual incidence in Europe is 0.8–1.9 cases per 100,000 inhabitants with a maximum prevalence of 11.6–17 per 100,000 inhabitants (Invernizzi 2010). To date, no data were found concerning the incidence of AIH in the North African or Tunisian populations. AIH is diagnosed in 1/2 of the patients as a form of acute hepatitis, and in 5% of patients as a fulminant or subfulminant hepatitis (Wang et al. 2016). Susceptibility to AIH was linked to the main human leucocytes antigen genes HLA DRB1*0301 and HLA DRB1*0401 in North American and European patients (Czaja et al. 1997; Strettell et al. 1997). The role of HLA DRB1*03 was also demonstrated in conferring susceptibility to AIH in the Tunisian patients (Chaouali et al. 2017). These associations, however, could differ between the different ethnic groups, since HLA DRB1*04 was associated to AIH susceptibility in Japanese (Seki et al. 1992; Yoshizawa et al. 2005), Mexican (Vazquez-Garcia et al. 1998) and Korean (Lim et al. 2008) populations, and HLA DRB1*13 was linked to AIH susceptibility in Argentinians (Pando et al. 1999) and Indians (Kaur et al. 2013). AIH is a complex disease in which the

HLA genes alone may not explain the entirely mechanism of development and triggering of the autoimmune response. Multiple genes located outside the major histocompatibility complex could also play a significant role in susceptibility to type 1 AIH.

The main candidate genes at present are the polymorphic genes encoding the proinflammatory and immunoregulatory cytokines such as TNF-α, IL-10, TGFβ1 or INF-ɣ (Aizawa and Hokari 2017). Cookson et al. (1999) reported a significant association of TNF-α-308 polymorphism with autoimmune hepatitis susceptibility in white North American patients and a high frequency of the rare allele TNF*A was found in AIH patients compared to controls (56 vs. 26%, $p = 0.00008$). Polymorphisms in the tumor necrosis factor α (TNF-α) promoter gene sequence at position -308 have been also associated with a number of autoimmune liver diseases such as primary sclerosing cholangitis (PSC) (Bernal et al. 1999) and primary biliary cirrhosis (PBC) (Jones et al. 1999). The functional involvement of TNF-α-308 polymorphism in the expression and serum production of the TNF-α cytokine in AIH patients is still under investigation, although the previous studies have shown that the TNF*A promoter allele was associated with increased level of transcription of the TNFA gene and was also linked to a number of cohorts (Tang et al. 2012).

The TNF-α cytokine is produced by many cell types including lymphocytes and macrophages and has a wide range of proinflammatory and immunoregulatory effects. It belongs to a superfamily of molecules that modulate cellular proliferation, differentiation and cellular apoptosis (Kawasaki et al. 2002). The human TNF-α is a molecule consisting of 157 amino acids. The gene coding for TNF-α is present in a single copy of approximately 3 Kb, located on the short arm of chromosome 6p21 (Watts et al. 1997). The TNF-α overproduction in serum of patients with AIH favours the exacerbated activation of the Th1 immune response leading to increase the risk of developing autoimmunity (Bittencourt et al. 2001). More specifically, the TNF-α-308 A allele would be implicated in the TNF-A gene transcription leading to a higher constitutive and inductible production of TNF-α.

To our knowledge, this is the first report studying the association of TNF-α (-308 G > A) polymorphism with AIH in the Tunisian population and in North Africans. This study was therefore conducted in order to assess the role of the TNF-α (-308 G > A) polymorphism in conferring susceptibility to AIH in Tunisian patients and search for correlation between TNF-α-308 polymorphism and TNF-α expression and clinical and biological parameters in AIH.

Patients 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) (Hennes et al. 2008). 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 and fifty unrelated healthy donors were included in our study (47 women and 13 men, mean age 49.6 years ± 7.4) and matched for gender and age with AIH cases. Study participants have signed an informed consent before the study, and the Ethics Committee of the Pasteur Research Institute in Tunis 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.

Genotyping

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. TNF-α (-308) was amplified using amplification refractory mutation system polymerase chain reaction method (ARMS PCR). One hundred nanograms of genomic DNA that had been extracted from whole blood was amplified in reaction mixtures containing 200 µmol/L each of dATP, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate (Amersham Pharmacia-Biotech, St. Albans, UK), 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris–HCl pH 8.3, 50 mmol/L KCl, 0.01% gelatin, 0.5 µmol/L of each primer (Table 1) and 2–2.5 U Taq Polymerase (Quiagen). Amplified DNA fragments were separated on 1.5% ethidium bromide agarose gel, and visualized under ultraviolet light.

Cytokine Assessment

The serum TNF-α in AIH patients was detected by chemiluminescence method (Immulite 1000) according to manufacturer's instructions. The calibration slope is adjusted by the user using two sera (Adjustors; DPC) at least every 2 weeks. The adjustment was validated in each run using a high- and low-quality control (DPC). Quantitative analysis ranges were: for TNF, 4–1000 ng/L. The sample and the test units (one for each cytokine to be tested) were simply loaded on the platform. The test procedure was fully automated. The data acquisitions were completed within 40 min.

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. Variables with normal distribution were expressed as mean ± SD while those with non Gaussian

Table 1 Characteristics of Tunisian autoimmune hepatitis in cases and controls

Parameters	Cases ($n=50$)[a]	Controls ($n=150$)[a]	p value
Gender, *n* (%)			
Women	36 (84.0)	129 (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	–
Clinical presentations, *n* (%)		NA	–
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

[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))

distribution were expressed as median and interquartile range (IQR). The difference between means was calculated with *t* Student's. Correlations between genotypes and cytokine levels was calculated by the Kruskal–Walli non parametric test. The analysis TNF-α-308 genotype associations with clinical and biological

manifestations of AIH was realised by the Pearson correlation and linear regression tests.

Results

I-Clinical and Immunological Features in AIH Patients

Demographic and clinical characteristics of 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. (Table 1).

II-Association of TNF-α (-308 G > A) Polymorphisms with Susceptibility to AIH

Analysis of TNF-α polymorphism at position -308 revealed a significantly higher frequence of the AA genotype in autoimmune hepatitis patients compared to 150 healthy controls [34 vs. 8%, $p = 0.00002$, OR 5.92 for CI of 95% (2.5–13.5)].The presence of G/G or A/G genotypes was more frequent in the healthy controls 92% than in AIH patients 66% and this difference was statistically significant $p = 0.00002$, therefore the presence of the G/G or A/G genotypes could confer protection of AIH among the Tunisian population. Table 2 summarizes the frequencies of TNF-α genotypes found in AIH patients and controls.

The results showed also that the frequency of the A-allele was significantly higher in patients with AIH compared to controls [55 vs. 37.3%, $p = 0.002$, OR 2.05

Table 2 Distribution of TNF-α-308 genotypes in Tunisian AIH patients and controls

TNF-α-308 genotypes	AIH patients ($n = 50$)		Controls ($n = 150$)		p	OR (95% CI)
	n	GF	n	GF		
A/A	17	34	12	8	0.00002	5.92 [2.5–13.5]
A/G	22	44	90	60	0.05	0.52 [0.27–1.00]
G/G	11	22	48	32	0.21	–
A/A or A/G	39	78	102	68	0.21	–
G/G or A/G	33	66	138	92	0.00002	0.16 [0.07–0.38]

p calculated with Fisher's exact test

n number of subjects, *AIH* autoimmune hepatitis, *GF* genotype frequency, *OR* odds ratio, *CI* confidence interval

Table 3 Distribution of TNF-α-308 allelic frequencies in Tunisian AIH patients and controls

TNF-α-308 allelic frequencies	AIH patients ($2n=100$)		Controls ($2n=300$)		p	OR (95% CI)
	N	AF	N	AF		
A	55	55	112	37.3	0.002	2.05 [1.29–3.24]
G	43	43	184	61.3	0.001	0.18 [0.3–0.75]

p calculated with Fisher's exact test

n number of alleles, *AIH* autoimmune hepatitis, *AF* allele frequency

for CI of 95% (1.29–3.24)]. The G-allele was significantly more frequent in healthy controls compared to AIH patients [43 vs. 61.3%, $p=0.001$, OR 0.47 for CI of 95% (0.3–0.75)] Table 3.

III-Correlation Between TNF-α-308 Genotypes and Serum Levels of TNF-α in Tunisian Patients with AIH

We studied the correlation of serum levels of TNF-α in Tunisian AIH patients with the presence of different A/A, A/G and G/G genotypes in order to search for associations between the genotype and the clinical phenotype of AIH patients. The results showed a significant correlation of A/A genotype with the highest mean serum level of TNF-α (61.73 µL) $p=0.027$ compared to AIH patients who carried the A/G or G/G genotypes Table 4.

IV-Association of TNF-α-308 Genotypes and Clinical Manifestations of AIH

We studied the association of the A/A, A/G and G/G genotype presence at position -308 of the TNF-α gene promoter with the different clinical manifestations observed in AIH patients (jaundice, pruritus, hepatomegaly or splenomegaly.) but there was not any statistically significant results. The correlations and regression analysis were performed to detect the association between these genotypes and biological manifestations of AIH such as the levels of ASAT, ALAT, alkaline phosphatase and IgG

Table 4 Correlation between TNF-α-308 genotypes and serum levels of TNF-α in Tunisian patients with AIH

TNF-α-308 genotypes	AIH patients ($n=50$)	Mean serum level of TNF-α in µL	p
A/A	17	61.73 ± 12.6	
A/G	21	1.63 ± 2.6	0.027
G/G	11	14.21 ± 10	

p calculated by the Kruskal–Wallis test

n number of subjects, *AIH* autoimmune hepatitis, mean ± standard deviation

Table 5 Association of biological parameters with TNF-α-308 genotypes

	AST	Pearson correlation	*p*	ALT	Pearson correlation	*p*
AA	73.7 (16–199)	−0.04	0.75	76.06 (38–184)	−0.17	0.22
AG	68.2 (24–138)	0.09	0.49	67.3 (24–109)	0.04	0.77
GG	74.4 (45–101)	−0.04	0.78	58.8 (29–95)	0.15	0.28
	IG	Pearson correlation	*p*	PAL	Pearson correlation	*p*
AA	83.2 (15–202)	0.11	0.41	64.3 (34–98)	−0.02	0.04
AG	65.9 (23–161)	0.16	0.25	54 (23–98)	0.04	0.75
GG	77.3 (49–106)	−0.32	0.01	43.1 (12–88)	0.24	0.08

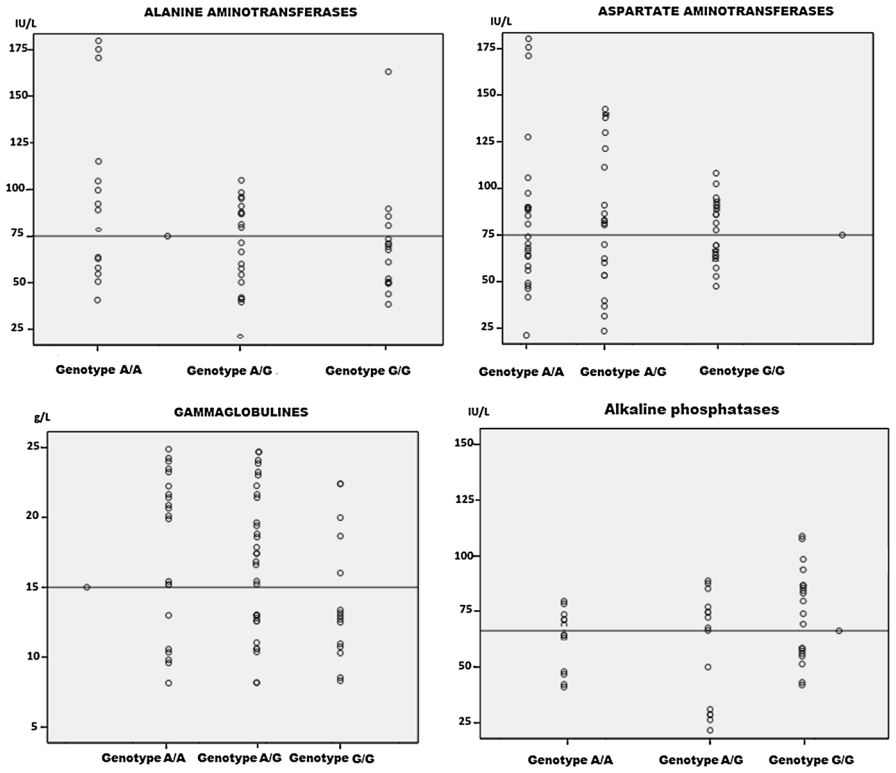

Fig. 1 Regression linear analysis plots for correlation of biological parameters with TNF-α-308 genotypes. *IU/L* international unity/litre

levels. The results showed no significant correlation for levels of ASAT and ALAT with TNF-α-308 genotypes. A significant negative correlation was found for AIH patients with higher alkaline phosphatase levels carrying the TNF-α-308 A/A genotype ($p = 0.04$). For the IgG levels, a significant negative correlation was also found

for AIH patients carrying the G/G genotype ($p=0.01$) Table 5, Fig. 1. There was no significant association between the AA genotype and the presence of the serum autoantibodies or the presence of autoimmune diseases associated with AIH.

Discussion

AIH is a rare chronic inflammatory hepatitis which could lead to cirrhosis and liver failure without the institution of corticotherapy (Francque et al. 2012). These severe complications impose the comprehension of AIH pathogenic mechanism and detection of genetic susceptibility biomarkers in order to allow early AIH diagnosis and management. Our study showed a significantly higher frequence of the AA genotype in AIH patients compared to healthy controls. The frequency of the TNF*A allele was significantly higher in patients with AIH compared to healthy controls. The G-allele was significantly more frequent in healthy controls compared to AIH patients. The A/A genotype in TNF-α-308 promoter could be considered as a genetic susceptibility biomarker triggering AIH onset. We can consider that there is a significant correlation between TNF-α-308 A/A genotype and an increased expression of the TNF-α in the serum of patients with AIH triggering a more intense inflammatory response. The GG/AG genotypes were significantly more frequent in controls than in AIH patients and could be considered as protective factors from AIH onset. Our findings were similar to those reported by Cookson et al. (1999) who showed significantly increased frequency of the TNF*A2 allele frequency in European and North American patients with AIH-1 when compared to controls (first set of patients: 34% of controls versus 49%, Pc = 0.014 and second set: 26 versus 56%, $p=0.00008$). The contribution of TNF-α-308 gene polymorphism to AIH pathogenesis could be proven by the association of this polymorphism to higher expression of the TNF-α cytokine that have been shown as a mediator of the inflammatory response in this disease (Wilson et al. 1994). A significant association of the TNF-α polymorphism located in position -308 with the development of AIH was also demonstrated by another study conducted in New Zealand and involved 77 patients with AIH. This study showed that the minor allele TNF -308A conferred susceptibility to AIH [OR 2.06 (1.41–3.01), $p=0.0001$] (Jing et al. 2013).

In contrast to these findings, other studies have failed to show any significant association of TNF-α (-308 G > A) polymorphism with AIH susceptibility and development. The study of Bittencourt el al (2001) showed that the distribution of the TNF*A alleles was similar in patients with AIH types 1 and 2, when compared with controls. This study did not show any strong association of this polymorphism with AIH clinical manifestations; however, higher gamma globulin levels were noticed in AIH patients carriers of the TNFA*2 allele. Another recent study showed that the 238 G > A polymorphism in promoter region of TNF-α gene was not associated with increased risk of non-alcoholic fatty liver disease in Iranian patients (Mohseni et al. 2016). Two other independent investigations in China have shown different results. The study of Ma and Qiu (2004) considered that the allele A in position -308 of the TNF-α gene might be involved in AIH pathogenesis, but the other study conducted by Fan et al. (2004) included 49

patients with AIH and 58 patients with PBC and did not find any significant association of the TNF-308 G/A polymorphism with AIH or primary biliary cirrhosis in the Chinese patients. The different results revealed by the different ethnic groups could be mainly due to different genetic backgrounds, different autoantigens presented to lymphocytes T cells, diversity of HLA susceptibility alleles and triggering factors affecting AIH susceptibility and development in these different populations (Hardtke-Wolenski et al. 2017). The association of TNF-α polymorphism with AIH susceptibility could therefore be elusive in these populations and it could therefore be possible that additional polymorphisms affecting other genes would be involved in AIH susceptibility and onset (Bittencourt et al. 2001).

Our study showed also a positive correlation between the presence of A/A genotype in AIH patients and higher serum levels of TNF-α compared to carriers of the G/G or A/G genotypes which is in accordance with previous studies that demonstrated an association of TNFA*2 with increased transcription levels of TNF-α (Cookson et al. 1999). In addition, higher level of TNF-α expression were revealed in AIH patients carriers of HLA DR3 and DR4 comparing to the patients who carried HLA DR2 (Jacob et al. 1990). This could be in part explained in the fact that HLA DR3 and DR4 are known susceptibility alleles for type 1 AIH while the HLA DR2 allele is associated with a reduced risk of the disease. TNFA*2 allele has been shown to be in strong linkage disequilibrium with the HLA haplotype of susceptibility to AIH A1-B8-DR3. Higher TNF-α levels found in patients with HLA DRB1*03-DQB1*02 could be related to the presence of TNF-α polymorphism, a combined effect of these two genes could also have a role in increasing the susceptibility of AIH development and the cytolytic mechanisms leading to hepatic failure and cirrhosis (Liberal et al. 2015). Czaja et al. (1999) have also demonstrated a significantly higher frequency of TNFA*2 allele in position TNF-α-308 in AIH patients compared with healthy subjects (56 vs. 26%; $p = 0.001$) and was also associated with increased production of TNF-α. The level of remission after the institution of corticosteroid therapy was less frequent in the patients carrying the high producer polymorphism with increased level of treatment failure and progression to cirrhosis.

Many deleterious effects could be originated from the exaggerated production of TNF-α by the A variant polymorphism. In fact, the TNF-α cytokine could increase the expression of class II HLA and adhesion molecules on the surface of cells in AIH leading to a more triggered disease and a perpetuation of the autoimmune hepatocyte destruction (Liberal et al. 2013). The TNF-α cytokine would generate a cascade of cytokine release that would increase level production of IL-1, IL-6, and TNF-α itself leading to the macrophage activation and cellular cytotoxicity enhancement. These actions could exacerbate the immune response in AIH and enhance its clinical expression and aggressiveness (Tang et al. 2012). Increased production of TNF-α cytokine in the serum of AIH patients as a consequence of the TNF-α (-308 G > A) polymorphism may intensify the immunoreactivity, affect AIH onset and alter corticosteroid responsiveness (Czaja et al. 1999).

Weiler-Normann et al. reported clinical and biological improvement in a first set of AIH patients that were treated with anti-TNF antibodies following the failure of standard treatments by corticotherapy (Weiler-Normann et al. 2013). This could lead

to the use of anti-TNF antibodies as an alternative to immunosuppressive or corticoid treatment which causes more aggravated side effects for AIH patients in the long term.

Conclusion

The TNF*A allele confers susceptibility to AIH in the Tunisian patients and is associated with higher production levels of the TNF-α cytokine in AIH. The TNF*G allele may confer protection from AIH in Tunisian patients. Anti-TNF antibodies could be an alternative to the use of corticotherapy and could therefore avoid the exacerbated immune response caused by excessive production of TNF-α in AIH. Further studies are needed to evaluate the efficacy and tolerance of anti-TNF-α in AIH.

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Compliance with Ethical Standards

Conflict of interest All authors have no conflict of interest.

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