The Role of Cytotoxic T-Lymphocyte Associated Antigen 4 (CTLA4) +49A/G and Tumor Necrosis Factor Alpha (TNF-α) −308G/A Polymorphism in the Development of Celiac Disease in Jordanian Patients

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To test for the role of cytotoxic T-lymphocyte associated antigen 4 (CTLA4) +49A/G and tumor necrosis factor alpha (TNF-α) −308G/A polymorphisms in the development of celiac disease (CD) in Jordanian patients we investigated 85 patients, 30 CD families and 100 healthy matched controls. In this work we found that, the frequency of the CTLA4 +49GG genotype and G allele were significantly higher in patients when compared with controls. This significant increase in the frequency of both, GG genotype and G allele, is also demonstrated in CD families. No significant differences were found in TNF-α −308G/A polymorphisms in CD patients compared with control group. No significant differences in the frequency were found when the family group compared with the control group for both AA genotype and A allele. Our results demonstrated the importance of CTLA4 +49G allele in the development of CD among Jordanian CD patients and that no significant association of this disease with TNF-α −308A allele.

Key words —— celiac disease, genotype, polymorphism, cytotoxic T-lymphocyte associated antigen 4 (CTLA4) +49A/G, tumor necrosis factor alpha (TNF)-α −308G/A

INTRODUCTION

Celiac disease (CD) is a permanent intolerance to dietary gluten, mostly to the gliadin fraction, a protein contained in wheat, rye, barley and a multitude of prepared foods.[1] Genetic susceptibility to CD is conferred by genes in the Human Leukocyte Antigen (HLA) region on the short arm of chromosome 6. In most populations, more than 90% of CD patients carry the DQ2 heterodimer, while most of CD patients negative for DQ2 carry either DQ8 or they are positive for one of the alleles coding for DQ2.[2] Although DQ2 and DQ8 presence seems to be necessary for the development of CD, they are not enough to fully explain the genetic susceptibility to the disease. The frequent association of cytotoxic T lymphocyte antigen 4 (CTLA4) with CD made of it the best candidate gene to be studied.[3] CTLA4 is a protein expressed on the activated T cells which involved in the regulation of the immune response. CTLA4 confers an inhibitory effect on lymphocyte activation and proliferation. Therefore, it acts as a negative control of the immune response.[4] CTLA4 +49A/G polymorphism was found to be associated with many autoimmune disorders, such as Graves disease, type-1 diabetes, rheumatoid arthritis and multiple sclerosis.[5] This polymorphism was also tested for the association with CD in a number of populations such as, French, Scandinavian, Swedish and Italian where they found a strong association between CD and the presence of A allele of this gene.[6–8] Dutch and U.K. Caucasian showed a slight increase in the frequency of the G allele of the gene.[5] On the other hand, a number of populations showed no association with any of CTLA4 A/G alleles.[9–11] Tumor necrosis factor (TNF)-α is one of the cytokines family which is involved in the regulation of the immune response.[12] TNF-α gene located within class III region of Major Histocompatibility Complex (MHC) on chromosome 6p21.3.[13] The most extensively investigated polymorphism

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was that in the promoter region at −308 position. TNF-α −308G/A polymorphism has higher activity with A allele than G allele.[14] A number of studies demonstrated a significant association of this polymorphism with CD in different populations.[15–21] The aim of this study is to analyze and to test the role of CTLA4 +49A/G and TNF-α −308G/A polymorphisms in the development of CD in Jordanian patients.

MATERIALS AND METHODS

Samples —— A total of 85 (47 females and 38 males), biopsy confirmed, Jordanian CD patients mean age of 23 years, 30 families of CD patients with a total number of 56 individuals (32 females and 24 males) with no symptoms of CD and 100 (45 females and 55 males) healthy individuals, with mean age of 26 years, as a control group, were included in this study. All families were checked for celiac specific antiendomysial IgA/IgG antibodies and for IgA/IgG antigliadin antibodies and they found to be negative. Venous blood was obtained in EDTA tubes and used to extract the DNA.

Genotyping —— DNA was extracted using a special kit from Promega (Madison, WI, U.S.A.) according to the manufacturer's protocol. Polymorphisms CTLA4 +49A/G and TNF-α −308 were analyzed using Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism method (PCR-RFLP). The A/G polymorphism in exon one of the CTLA4 was amplified using a forward primer (5′-AAG GCT CAG CTG AAC CTG GT-3′) and a reverse primer (5′-CTG CTG AAA CAA ATG AAA CCC-3′). The forward primer was designed with a single base mismatch for the last nucleotide which corresponds to the +47 position to introduce a base change in the sequence of the PCR product. This substitution creates a BstEII restriction site in the A allele. PCR was carried out with the following cycles: hot start at 94°C for 5 min; 30 cycles consisting of 30 sec at 94°C for denaturaion, 30 sec at 58°C for annealing and 30 sec at 72°C for extension, and 10 min at 72°C for final extension.[22] 10 μl of the amplified product were incubated with 3U of the restriction enzyme BstEII (this enzyme recognizes and cuts double stranded DNA at the following sequence 5′-GGTNACC-3′ between two adjacent G residues) at 60°C for 4 hr then the digested products were run on a 3.5% agarose gel. The G/A polymorphism in TNF-α −308 were amplified using a forward primer (5′-AGG CAA TAG GTT TTG AGG GCC AT-3′) and a reverse primer (5′-ACA CTC CCC ATC CTC CCT GCT-3′). The TNF-α forward primer includes a single base mismatch which introduces an NcoI restriction site in the result of the amplification of the G allele at position −308. PCR was performed using the following amplification profile: hot start at 95°C for 2 min; 35 cycles of 30 sec at 95°C for denaturaion, 15 sec at 60°C for annealing and 30 sec at 72°C for extension, and 10 min at 72°C for the final extension. 10 μl of the PCR product was incubated with 3U of the restriction enzyme NcoI (this enzyme recognizes and cuts the double stranded DNA at the following sequence 5′-CCATGG-3′ between two adjacent C residues) at 37°C for 4 hr. 10 μl of the digested products were run on 4% agarose gel.[23] DNA leader of 50 bp was used to determine the size of the DNA bands. This work was approved by the university review committee for research on human.

Statistical Analysis —— analysis was performed using EPI info program. The p-value for the CTLA4 +49A/G polymorphism was calculated using Chi-square test and for the TNF-α −308G/A polymorphism using Fisher exact test.

RESULTS

As demonstrated in Figs. 1 and 2, the obtained PCR product for CTLA4 was 152 bp and that for TNF-α is 116 bp. In addition, it is demonstrated that the treatment of the PCR product for CTLA4 with BstEII restriction enzyme resulted in two fragments one of 152 bp and the other of 130 bp. The digested A allele yielded a fragment of 130 bp because of the presence of the restriction site for the restriction enzyme and the G allele yielded an intact 152 bp fragment because it contains no restriction site. It is also demonstrated that the treatment of the PCR product for TNF-α with NcoI restriction enzyme resulted in two fragments one of 96 bp and the other of 116 bp. The digested G allele yielded a fragment of 96 bp because of the presence of the restriction site for the restriction enzyme and the A allele yields an intact 116 bp fragment due to the absence of the restriction site in this amplified sequence.

Table 1 demonstrated the genotype and the allele frequencies of CTLA4 +49A/G polymorphism in 85 CD patients, 100 healthy controls and 30 CD families. The frequency of the GG genotype

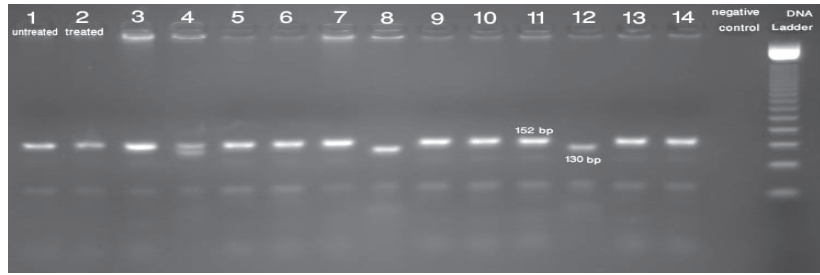

Fig. 1. Gel Electrophoresis of CTLA4 +49A/G Polymorphism

Lanes 2, 4, 6, 8, 10, 12, and 14 are PCR samples treated with BstEII restriction enzyme. Lane 1, 3, 5, 7, 9, 11, 13 are untreated samples. Lane 4 represents a sample for heterozygous AG genotype. Lane 6 represents a sample for mutant homozygous GG genotype. Lane 8 represents a sample for normal homozygous AA genotype.

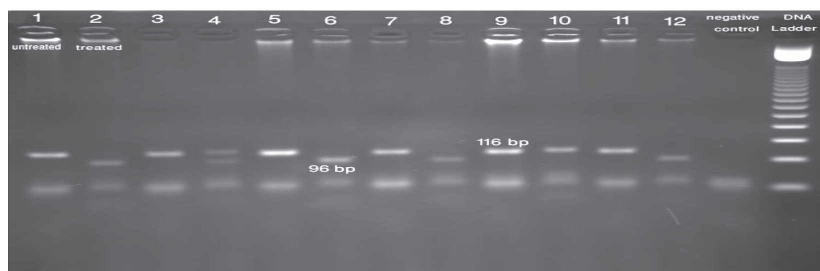

Fig. 2. Gel Electrophoresis of TNF-α −308G/A Polymorphism

Lanes 2, 4, 6, 8, 10 and 12 are PCR samples treated with NcoI restriction enzyme. Lane 1, 3, 5, 7, 9 and 11 are untreated samples. Lane 4 represents a sample for heterozygous AG genotype. Lane 10 represents a sample for mutant homozygous AA genotype.

Table 1. Genotypes and Allele Frequencies of the CTLA4 +49A/G Polymorphism

Genotype	Frequency		
	Patients (n = 85)	Control (n = 100)	Family (30 families, 56 members)
GG	25 (29.4%)[a)]	4 (4 %)	14 (25%)[b)]
AG	31 (36.5%)	39 (39 %)	28 (50%)
AA	29 (34.1%)	57 (57 %)	14 (25%)
Allele			
G	81 (47.6%)[c)]	51 (25.5%)	56 (50%)[d)]
A	89 (52.4%)	149 (74.5%)	56 (50%)

p-value using Chi-square test. *a)* p-value < 0.001. *b)* p-value < 0.001. *c)* p-value < 0.001. *d)* p-value < 0.001.

was significantly increased among patients as compared with the control group, 29% *vs*. 4%, p-value < 0.001. Also, G allele frequency was found to be significantly increased in the CD patients compared with controls, 48% *vs*. 25%, p-value < 0.001. In addition, both GG genotype and G allele frequencies in CD families were found to be significantly higher when compared with the control group. GG genotype in family group 25% *vs*. 4% in controls, p-value < 0.001 and G allele frequency was 50% *vs*. 25%, p-value < 0.001.

Table 2 shows the genotype and the allele frequencies of TNF-α −308G/A polymorphism in 85 CD patients, 100 healthy controls and 29 CD families. No significant differences were found in the distribution of the AA genotype between CD patients and control, 4.7% *vs*. 2%, p-value = 0.4158. Also the analysis of A allele frequency in patient compared to control group showed no significant association, 22.4% *vs*. 15.5%, p-value = 0.09168. In addition, no significant difference was found when the family group compared to control group both in AA genotype, 1.8% *vs*. 2%, p-value = 1.00 and in A allele frequency, 14.3% *vs*. 15.5%, p-value = 0.7736.

Table 2. Genotypes and Allele Frequencies of TNF-α −308G/A Polymorphism

Genotype	Frequency		
	Patients (n = 85)	Control (n = 100)	Family (30 families, 56 members)
AA	4 (4.7%)[a]	2 (2 %)	1 (1.8%)[b]
AG	30 (35.3%)	27 (27 %)	14 (25 %)
GG	51 (60 %)	71 (71 %)	41 (73.2%)
Allele			
A	38 (22.4%)[c]	31 (15.5%)	16 (14.3%)[d]
G	132 (77.6%)	169 (84.5%)	96 (85.7%)

p-value using Fisher exact test. *a*) *p*-value = 0.4158. *b*) *p*-value = 1.0000. *c*) *p*-value = 0.09168. *d*) *p*-value = 0.7736.

DISCUSSION

Although the HLA component of CD susceptibility is well characterized, little is known about the role of non-HLA genes. One or more non-HLA genes were found to be predisposed to CD, and they might be strong determinants of the disease susceptibility. A high concordance rate in monozygotic twins (71%) in non-HLA compared with only 30% in HLA identical siblings and the estimated proportion of sibling relative risk due to HLA, which is contributed at most to 40% of the sibling inherited risk, came to confirm the major role of non-HLA genes in CD susceptibility. CTLA4 is the most non-HLA gene studied in relation with CD, because the region contains this gene, 2q33, has repeatedly positive results in linkage with the disease.[3] CTLA4 confers an inhibitory effect on the lymphocyte activation and proliferation.[24] Disruption of this inhibitory role will lead to the disruption of the down regulation of the immune response.[25] This might contribute to the pathogenesis of autoimmune diseases including CD. The crucial role of CTLA4 in controlling autoreactivity has been demonstrated by CTLA4 knock out mice. CTLA4 −/− mice suffer from lymphoproliferative disorders characterized by polyclonal T cell proliferation and early lethality. This fact demonstrates a critical role of CTLA4 in down regulation of T cell response.[4] In our work we studied two non-HLA polymorphisms CTLA4 +49A/G and TNF-α −308G/A that might be involved in the etiology of CD. This study showed a strong association of the G allele of CTLA4 with CD. This association was found to be highly significant for both GG genotype and for G allele. This highly significant association was also demonstrated in CD families for both GG genotype and G allele. The obtained results are on the contrary with what have been reported by others in other parts of the world. Most of the previous studies supported the importance of the CTLA4 +49 polymorphism in CD, but most of these results showed a significant association of this disease with the A not with the G allele. In French, Scandinavian, Swedish, and Italian studies they found a strong association of CD with the A allele.[6–8] While only two studies (Dutch and U.K. Caucasian) showed a slight increase in the frequency of the G allele although this increase was not significant, and they referred that to different ethnic backgrounds.[5] However, a Basque, combined Italian, Tunisian and Irish studies had found no evidence of association with CTLA4 +49A/G polymorphism at all, neither A nor G allele.[9–11] Although several studies had linked the CTLA4 +49A allele with the susceptibility to CD, we did not find any association of this polymorphism with this disease in Jordanian population. In fact the A allele is over represented in non celiac subjects. This research is the only one that demonstrated a significant association of CD with the G allele. The variations between studies might be due to ethnic differences. Some authors refer this variation to the recombination event that might take place between the CTLA4 +49A/G variant and the disease causing variant in the 6.1 Kb 3′ region of CTLA4.[5] Despite the differences in the results concerning the causative allele, this work came to confirm the importance of the CTLA4 gene, especially the +49A/G polymorphism, in the increasing susceptibility to CD. The other non-HLA gene that we were studied is TNF-α gene. TNF-α has been linked to the susceptibility or severity of many inflammatory disease of intestine, such as Crohn's disease and ulcerative colitis. The implication of TNF-α in CD pathogenesis has been demonstrated, an increase in TNF-α expression either in mononuclear cells, in mesenchyme or in epithelial cells of the intestinal mucosa has been observed.[17] The contribution of TNF-α −308G/A promoter polymorphism, which is known for its effect on the increase in gene ex-

pression, has been studied in many European populations. McManus found a significant association of this polymorphism with CD patients in Ireland. The frequency of A allele was 50% in patients while it was 16.35% in controls.[14] A study on Spanish population showed a high frequency of the A allele in celiac families compared to healthy controls, 32.2% *vs*. 7.9%, respectively. Another study on unrelated Spanish patients with CD supported the significance of A allele in this disease. The reported frequency was 55.6% in patients and 21.5% in controls.[17] These results were supported by other studies performed on Italian, Swedish, and Sicilian populations where they demonstrated the increase in A allele frequency in celiac patients when compared to healthy controls. Less significance association of A allele with CD was found in Finnish Celiac families.[20] Our results on TNF-α −308G/A polymorphism did not show significant association of this polymorphism with CD in Jordanian patients when compared with healthy controls. The A allele frequency in patients was 22% *vs*. 15% in controls (p-value = 0.09168). Also the A allele frequency in families was 14% (p-value = 0.7736). As these results demonstrated, we do have a slight increase in the frequency of TNF-α −308G/A polymorphism in CD patients and families compared with the control group but this increase is not significant. The difference between the reported results and what we found in our work might be due to the differences in the ethnic background. This study is the only one in the Middle East that focused on the possible role of non-HLA genes in the etiology of celiac disease. In conclusion, we demonstrated the importance of CTLA4 +49G allele in the development of celiac disease among Jordanian CD patients and that no significant association of this disease with TNF-α −308A allele.

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