Tumor necrosis factor-α and interleukin-6 gene polymorphism association with susceptibility to celiac disease in Italian patients

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ABSTRACT. The aim of this research was to study polymorphisms in the genes encoding cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in patients with celiac disease (CD) antigens DQ2 (DQ2-positive) or DQ8 (DQ8-positive). We compared the results with healthy controls to determine whether any of the polymorphisms have a role in susceptibility to CD. A case-control of 192 patients with CD (96 DQ2-positive and 96 DQ8-positive) and 96 healthy controls from northeast Italy were

included in the study. Analysis of single nucleotide polymorphisms (SNPs) was carried out using the polymerase chain reaction-restriction fragment length polymorphism method. Significant differences for the *TNF-α* (-308 G>A) polymorphism were observed when we compared the flowing groups: DQ2-positive with controls [odds ratio (OR) = 0.45, P = 0.0002]; DQ8-positive with controls (OR = 3.55, P < 0.0001); and DQ2-positive with DQ8-positive (OR = 0.12, P < 0.0001). We did not observe a statistically significant association between *IL-6* (-174 G>C) polymorphism and CD (P > 0.05). Our results suggest that *TNF-α* (-308 G>A) polymorphism may play a role in susceptibility to CD in Italian patients.

Key words: Celiac disease; IL-6; TNF-α; HLA-DQ

INTRODUCTION

Celiac disease (CD) is an autoimmune condition with strong heritability and is characterized by inflammation of the small intestine (Trynka et al., 2010). The disorder is triggered by the ingestion of a peptide component of dietary gluten, gliadin, and is characterized by villous atrophy of the jejunal mucus membrane, crypt cell hyperplasia, and an increase in intraepithelial lymphocytes (Clot and Babron, 2000). In genetically predisposed individuals, gliadin triggers a cascade of innate and adaptive immune responses and leads to the destruction of the intestinal epithelium and mucosa. This peptide is recognized by cells that present human leukocyte antigen (HLA) class II molecules. Upon activation, macrophages release pro-inflammatory cytokines, which activate intraepithelial lymphocytes and result in the characteristic histologic alterations of CD (Clot and Brabon, 2000; Manavalan et al., 2010). Studies have shown that inflammatory gluten-reactive T cells recognizing gluten selectively in the context of the HLA region (alleles coding the heterodimers HLA-DQ2 or HLA-DQ8) are presented only in the small intestinal mucosa of individuals with CD (Abadie et al., 2011). Pro-inflammatory cytokines are also known to play an important role in disease progression and tissue damage in autoimmune diseases such as multiple sclerosis (Wen et al., 2012), rheumatoid arthritis (Kishimoto, 2006), and systemic lupus erythematosus (Dienz and Rincon, 2009).

Interleukin-6 (IL-6) is a multifunctional pro-inflammatory cytokine that regulates the immune response, hematopoiesis, the acute phase response, and inflammation. IL-6 has been extensively studied in the context of several inflammatory or autoimmune conditions, and different results have been obtained (Dema et al., 2009). Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine involved in cellular and inflammatory immune reactions. This cytokine has been implicated in the severity of different immune-regulated diseases including autoimmune and inflammatory diseases, lymphoma, and transplantation (Hajeer and Hutchinson, 2000; Bel Hadj Jrad et al., 2007). The production and/or function of cytokines are at least partially regulated by polymorphisms in their gene sequences (Mosaad et al., 2012). Polymorphisms within the promoter regions of cytokine genes may play a role in the genetically determined production of both T helper cell types 1 and 2 (Th1 and Th2 cells), influencing the susceptibility of a broad spectrum of immune-mediated diseases, including CD (de la Concha et al., 2000).

In CD patients, exposure of the mucosa to gluten stimulates the expression of IL-6 and TNF-α *in vitro* (Nilsen et al., 1998). In addition, serum levels of these interleukins significantly increase in patients with active CD compared with healthy individuals, and decrease only after

a year on a gluten-free diet (Clot and Brabon, 2000; Romaldini et al., 2002). In this way, genetic polymorphisms that modify the levels of these cytokines can make an important contribution to susceptibility to CD (de la Concha et al., 2000; Mosaad et al., 2012).

Among the various polymorphisms described, the genetic variant most extensively investigated is located at position -308 (G>A) of the *TNF*-α gene promoter (Hajeer and Hutchinson, 2000). Independent studies have shown that the -308A *TNF* allele has higher transcriptional activity compared with the -308G *TNF* allele (Wilson et al., 1997; Batikhan et al., 2010). When considering the inflammatory conditions of the gastrointestinal tract, a positive association of the -308A allele has been demonstrated; conversely, the same polymorphism has not been associated with ulcerative colitis (Elahi et al., 2009). The polymorphism at position -174 (G>C) of the *IL-6* gene promoter seems to affect *IL-6* transcription (Ishihara and Hirano, 2002) and IL-6 plasma levels (Dema et al., 2009). For this polymorphism, G/G and G/C genotypes are associated with the production of high levels of IL-6, while the C/C genotype is associated with low levels (Santhosh et al., 2010). However, contradictory results for these interleukin polymorphisms influencing autoimmune inflammatory diseases have been found in different populations (Schotte et al., 2001; Mirowska-Guzel et al., 2011; Angelo et al., 2012; Mosaad et al., 2012).

The purpose of the present study was to evaluate whether both *TNF*-α (-308 G>A) and *IL-6* (-174 G>C) promoter polymorphisms are associated with CD susceptibility in an Italian population. Moreover, a HLA-stratified association analysis among HLA-DQ genes and *IL-6* and *TNF-a* was performed.

MATERIAL AND METHODS

Subjects

The study group comprised 192 (120 females and 72 males; mean age: 17.08 ± 15.41 years, range 2-70) patients with CD (96 DQ2-positive and 96 DQ8-positive), and 96 healthy individuals (62 females, 34 males; mean age 27.5.08 ± 12.45 years, range 18-60). CD patients were enrolled at the Gastroenterology Service of IRCCS Burlo Garofolo (Trieste, Italy). After the evaluation of clinical symptoms, patients were diagnosed on the basis of positivity for both antibody tests: anti-tissue transglutaminase antibodies (anti-tTG) [using the enzyme-linked immunosorbent assay (ELISA) Eu-tTG kit; Eurospital, Trieste, Italy]; and anti-endomysial antibodies (EMA) (using the anti-endomysium kit; Eurospital). Molecular typing for HLA was performed using the I-DQ kit (Eurospital) to confirm the serological diagnosis and exclude celiac patients in doubtful cases. An intestinal biopsy was also performed for all CD patients and the presence of histological infiltrative lesion was considered predictive of CD. Both the collection of biological material and the diagnostic tests for the disease were performed at IRCCS Burlo infantile maternal Garofolo, Trieste, Italy. The healthy controls were blood donors form the Transplant Service of the “Ospedale Maggiore” of Trieste (Italy), all were negative for DQ2 and DQ8 variants, and had negative serology for anti-transglutaminase, anti-gliadin, and anti-endomysial antibodies.

The study was approved by the Research Ethics Committee - CEP/UPE in Brazil, with the registry CAEE 0213.0.097.000-11.

DNA extraction

The DNA was extracted from peripheral blood using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to a standard procedure.

Genotyping

IL-6 polymorphism (*rs 1800795*)

IL-6 polymorphism was detected by polymerase chain reaction (PCR) amplification followed by restriction fragment length polymorphism (RFLP), using PCR primers and amplification conditions, as previously described by Depboylu et al. (2004). For RFLP analysis, 5 µL of the PCR product was digested overnight with 2U of *Hsp92*II restriction enzyme (Promega) in a total volume of 20 µL. The RFLP-generated profile comprised two fragments (244 base pairs (bp), 56 bp) for allele G, and three (133, 111, and 56 bp) for allele C. Approximately 10% of the PCR products were randomly selected for DNA sequencing by a MegaBACE 1000 capillary sequencer (Amersham Pharmacia Biotech).

TNF-α *polymorphism* (*rs 1800629*)

Genotyping of *TNF*-α -308 G>A promoter polymorphism was performed by PCR-RFLP, as previously described by Bel Hadj Jrad et al. (2007). For RFLP analysis, 5 µL of the PCR product was digested overnight with 2U *Nco*I restriction enzyme (Promega) in a total volume of 20 µL. The RLFP profile comprised one fragment of 107 bp for allele A, and two fragments of 87 and 20 bp for allele G. About 10% of the PCR products were randomly selected for DNA sequencing by a MegaBACE 1000 capillary sequencer (Amersham Pharmacia Biotech) to double-check the PCR-RLFP genotyping results.

Statistical analysis

All genotype groups were tested for Hardy-Weinberg equilibrium for each variant. The chi-square (χ^2) test was used for the calculation of genotypic and allelic frequency. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated between all groups. The differences were considered significant if $P \leq 0.05$. Statistical analyses were performed using BioEstat software 5.0.

RESULTS

Genotype and allele frequency distributions of the TNF-α G>A polymorphism in CD patients (DQ2- and DQ8-positive) and healthy controls are presented in Table 1. No significant difference was observed between CD patients and healthy controls for allelic frequency and genotype.

When we parsed the variants of the HLA gene, we observed that the frequency of the G allele was higher in the DQ8 group than in the DQ2 group or in the healthy controls, and the same was true of the A allele in the DQ2 group *vs* the DQ8 group or the controls. For both allele A (high producer) and AA genotype, there was a highly significant difference between the DQ2 group and the control group. The DQ8 group showed a high significant difference with the low producer, G allele. Thus, the analysis of the two HLA variants (DQ2 *vs* DQ8) showed a high significant difference between them, both allelic and genotypic.

Genotypes and allele frequency distributions of the IL-6-174 C>G polymorphism in CD patients (DQ2- and DQ8-positive) and healthy controls are presented in Table 2.

Table 1. Genotypic and allelic frequency of the TNF-α promoter polymorphism (-308) in patients with celiac disease (CD) (DQ2 and DQ8 groups) and healthy controls, and chi-square test (χ^2) statistics for genotypic and allelic frequency.

	CD patients % (N = 192)	DQ2+ patients % (N = 96)	DQ8+ patients % (N = 96)	Healthy controls % (N = 96)	χ^2	P	OR (95%CI)	P (OR)
Genotypes								
GG (low)	52 (100)	24 (23)	80 (77)	44 (42)	Reference			
GA	33 (63)	49 (47)	17 (16)	48 (46)	χ^2 1 = 4.32	P1 = 0.0377	1.74 (1.029-2.935)	P1 = 0.0519
					χ^2 2 = 3.56	P2 = 0.0001	0.54 (0.279-1.028)	P2 = 0.0847
					χ^2 3 = 24.69	P3 < 0.0001	5.27 (2.665-10.42)	P3 < 0.0001
					χ^2 4 = 42.00	P4 < 0.0001	0.10 (0.0488-0.2119)	P4 < 0.0001
AA (high)	15 (29)	27 (26)	3 (3)	8 (8)	χ^2 1 = 0.92	P1 = 0.3367	0.66 (0.278-1.555)	P1 = 0.4503
					χ^2 2 = 15.07	P2 = 0.0001	0.17 (0.066-0.432)	P2 = 0.0002
					χ^2 3 = 5.96	P3 = 0.0146	4.89 (1.231-19.42)	P3 = 0.0342
					χ^2 4 = 42.40	P4 < 0.0001	0.03 (0.0096-0.1243)	P4 < 0.0001
G	68 (263)	48 (93)	89 (170)	68 (130)	Reference			
A	32 (121)	52 (99)	11 (22)	32 (62)	χ^2 1 = 0.77	P1 = 0.6812	1.04 (0.715-1.503)	P1 = 0.9244
					χ^2 2 = 19.43	P2 < 0.0001	0.45 (0.296-0.678)	P2 = 0.0002
					χ^2 3 = 32.39	P3 < 0.0001	3.55 (2.153-6.307)	P3 < 0.0001
					χ^2 4 = 95.97	P4 < 0.0001	0.12 (0.072-0.206)	P4 < 0.0001

1 = CD patients *vs* healthy controls; 2 = DQ2-positive patients *vs* healthy controls; 3 = DQ8-positive patients *vs* healthy controls; 4= DQ2-positive patients *vs* DQ8-positive patients.

Table 2. Genotypic and allelic frequency of the IL-6 promoter polymorphism (-174) in patients with celiac disease (CD) (DQ2 and DQ8 groups) and healthy controls, and chi-square test (χ^2) statistics for genotypic and allelic frequency.

	CD patients % (N = 192)	DQ2+ patients % (N = 96)	DQ8 + patients % (N = 96)	Healthy controls % (N = 96)	χ^2	P	OR (95%CI)	P (OR)
Genotypes								
GG (high)	52 (99)	49 (47)	54 (52)	43 (41)	Reference			
GC	40 (76)	40 (38)	40 (38)	53 (51)	χ^2 1 = 3.48	P1 = 0.0619	1.62 (0.975-2.694)	P1 = 0.0822
					χ^2 2 = 2.03	P2 = 0.1538	1.54 (0.850-2.784)	P2 = 0.2020
					χ^2 3 = 3.18	P3 = 0.0746	1.70 (0.947-3.060)	P3 = 0.1022
					χ^2 4 = 0.11	P4 = 0.7404	0.90 (0.0497-1.644)	P4 = 0.8582
CC (low)	9 (17)	11 (11)	6 (6)	4 (4)	χ^2 1 = 0.95	P1 = 0.3296	0.57 (0.180-1.792)	P1 = 0.4751
					χ^2 2 = 2.07	P2 = 0.1504	0.42 (0.123-1.410)	P2 = 0.2475
					χ^2 3 = 0.06	P3 = 0.8045	0.85 (0.224-3.196)	P3 = 0.9299
					χ^2 4 = 1.72	P4 = 0.1893	0.49 (0.1691-1.4374)	P4 = 0.2937
G	71 (274)	69 (132)	74 (142)	69 (133)	Reference			
C	29 (110)	31 (60)	26 (50)	31 (59)	χ^2 1 = 0.27	P1 = 0.6047	1.11 (0.757-1.613)	P1 = 0.6740
					χ^2 2 = 0.01	P2 = 0.9121	0.98 (0.633-1.504)	P2 = 1.0000
					χ^2 3 = 1.04	P3 = 0.3084	1.26 (0.806-1.966)	P3 = 0.3652
					χ^2 4 = 1.27	P4 = 0.2590	0.77 (0.4970-1.2075)	P4 = 0.3097

1 = CD patients *vs* healthy controls; 2 = DQ2-positive patients *vs* healthy controls; 3 = DQ8-positive patients *vs* healthy controls; 4 = DQ2-positive patients *vs* DQ8-positive patients.

In the CD group, for both DQ2 and DQ8 variants the frequency of the G allele was much greater than that of the C allele. The same was observed in the control group. When we examined the genotypic frequencies, the GC and GG genotypes were at higher frequency than the CC genotype.

The results of statistical analysis did not show significant differences between the CD patients and the healthy controls for both genotypic and allelic frequencies. When we compared the groups DQ2 *vs* healthy controls, DQ8 *vs* healthy controls, or DQ2 *vs* DQ8, for allelic and genotypic frequencies, no significant differences were found.

DISCUSSION

Celiac disease is a complex disorder and genetic factors play an important role in the development of the condition; the most important genetic risk factors that have been studied are the HLA class II genes (Trynka et al., 2010). It is believed that HLA is necessary for the development of CD, but further investigation is required. Expression of HLA-DQ accounts for up to 40% of the genetic component of this disorder, of which 40-90% of patients with CD express the HLA-DQ2 heterodimer; the small proportion that do not express it are HLA-DQ8-positive. Therefore, other factors must be involved in the development of the disease, such as genes coding for several molecules related to the immune system response, which have been implicated in CD susceptibility (Garrote et al., 2005; Trynka et al., 2010).

Several studies have indicated that the HLA region might contain an additional genetic factor that affects disease susceptibility independently of the DQ genes (Woolley et al., 2005). Since the *TNF*-α gene has been located on chromosome 6, within the major histocompatibility complex (MHC) and between HLA class I and II regions, there has been much speculation concerning a genetic association between *TNF*-α polymorphisms, TNF production, immunologic response, and susceptibility to disease (Cataldo et al., 2003). Some polymorphisms in the promoter region of the gene for TNF-α have been associated with the development of the disease (Angelo et al., 2012), but the allele -308A is the most reported, inducing, especially in homozygosis, higher levels of *TNF-α* transcription and facilitating the inflammatory response to gluten (Kroeger et al., 2000). In the present study, allelic distribution of the *TNF*-α promoter region (-308) polymorphism did not show a significant difference between celiac patients and the control group ($P = 0.9244$). Similarly, Kekik et al. (2011) did not find an association in a Turkish population ($P > 0.05$).

When we analyzed separately the distribution of allelic frequencies, as DQ2-positive patients *vs* the control group, DQ8-positive patients *vs* the control group, or DQ2-positive *vs* DQ8-positive patients, significant differences were observed. These results suggest that DQ2-positive and DQ8-positive patients together show a difference in the modulation of CD susceptibility. This can be seen in the high frequency of allele A in DQ2-positive patients and the high frequency of allele G in DQ8-positive patients (Table 1).

Moreover, several studies have found a relationship between the *TNF-α* rs1800629 allele A and susceptibility to CD (de la Concha et al., 2000; Lio et al., 2005), as well as other inflammatory and autoimmune diseases, such as systemic lupus erythematosus (Angelo et al., 2012) and type 1 diabetes (Garrote et al., 2002). In our study, a higher difference in the frequency of the allele A (high producer) was observed when we compared DQ2-positive CD patients with the healthy controls. However, different results were observed when we compared DQ2-positive patients with DQ8-positive patients for both allelic and genotypic frequencies. Supporting our results, Garrote et

al. also found in a Spanish population an increased frequency of the *TNF-α* -308A allele within the group of DQ2-positive patients with respect to DQ2-negative cases, suggesting the allele A as an additional risk marker for susceptibility to CD in DQ2-positive individuals (Cherñavsky et al., 2008).

To date, most studies associating the TNF-α -308A polymorphism have been conducted on DQ2-positive individuals (McManus et al., 1996; Capilla et al., 2007; Henderson et al., 2007), whereas studies associating the TNF-α -308 polymorphism only with DQ8-positive patients have not yet been performed. Nevertheless, Garrote et al. (2005) reported that 10% of CD patients express the DQ8 heterodimer with a similar ability to link gluten peptides or other heterodimers that share one of the chains with DQ2, but they usually do not carry the *TNF -308A* allele.

This feature was also found in our study, where 89% of DQ8-positive individuals had the G allele, whereas only 11% had the A allele. The frequency of individuals carrying the GG genotype for *TNF*-α (-308) polymorphism was different among DQ2-positive *vs* healthy controls, and DQ8-positive *vs* healthy controls. As a result, the frequency of individuals carrying the GA genotype was different among DQ8 positive *vs* healthy controls. Thus, the GG genotype could confer protection from CD in DQ2-positive patients. In contrast, GG and GA genotypes could cause susceptibility to CD in DQ8-positive patients.

It is important to note that studies involving the relationships of HLA-DQ refer mainly to HLA-DQ2. Consequently, more is known about HLA-DQ2-associated CD than HLA-DQ8-associated CD (Henderson et al., 2007). Therefore, studies stratifying CD patients into DQ2 and DQ8 variants are less frequent.

To our knowledge, this is the first study that has looked specifically at a group of DQ8-positive patients in relation to polymorphism in the TNF-α gene. Unfortunately, it was not possible to obtain information about the same distribution of polymorphism in other populations. Here, we observed a higher frequency of the G allele (low producer) in individuals carrying HLA-DQ8 than in those carrying HLA-DQ2, suggesting this allele may be an additional risk marker for CD in DQ8-positive patients.

Another gene encoding molecules involved in the immune system and possibly involved in susceptibility to CD is the gene encoding for interleukin-6, a cytokine essential for regulation of the immune process. Moreover, overproduction of this cytokine leads to inflammation and has been correlated with inflammatory autoimmune diseases (Kishimoto, 2006). Some polymorphisms in the promoter region of the gene for *IL-6* have been studied, but the polymorphism at position -174 is one of the most important, according to the literature, since it affects *IL-6* expression (Barisani et al., 2006). This polymorphism has been investigated in a variety of diseases, such as pemphigus (Mosaad et al., 2012), multiple sclerosis (Mirowska-Guzel et al., 2011), and CD (McManus et al., 1996; Depboylu et al., 2004; Dema et al., 2009).

In our study, genotypic and allelic distributions for polymorphism at position -174 of the *IL-6* gene promoter showed no significant difference between celiac patients *vs* the healthy control group. The same results were observed in previous studies in Italian (McManus et al., 1996) and Spanish populations (Dema et al., 2009). Furthermore, studies relating this polymorphism to other inflammatory and autoimmune diseases, such as systemic lupus erythematosus (Schotte et al., 2001) and rheumatoid arthritis (Arman et al., 2011), also did not show a relationship to the development of the disease.

In contrast, Garrote et al. showed that the ancestral allele G (higher producer) in DQ2-negative patients was associated with the development of CD compared with both the control group and the DQ2-positive patients (Garrote et al., 2005). The relationship between the G allele

and susceptibility to diseases was also observed for irritable bowel syndrome (Barkhordari et al., 2010) and lupus erythematosus (Hamdy et al., 2012). We also evidenced higher frequencies of the GG and GC genotypes, as well as the G allele in the region -174 of the *IL-6* gene in both CD patients and healthy controls, but these differences were not significant. The same results were found by Abbas et al. (2011) in a study on systemic lupus erythematosus in an Egyptian population. However, Mirowska-Guzel et al. (2011) found an association of the allele C (low producer) with the development of sclerosis in a Polish population ($P < 0.00001$), showing that the polymorphism in the region -174 of the *IL-6* gene varies depending on the population studied.

When we analyzed the -174 G>C *IL-6* polymorphism according to HLA gene variants (DQ2 and DQ8) for DQ2-positive *vs* the control group, DQ8-positive *vs* the control group, and DQ2-positive *vs* DQ8-positive, no significant differences were found (Table 2). As with *TNF*-α, the influence of *IL-6* polymorphisms in celiac HLA-DQ8 individuals has not yet been disclosed.

In conclusion, our findings suggest that *TNF*-α rs1800629 allele A possibly influences CD susceptibility in DQ2-positive patients, and *TNF*-α rs1800629 allele G may be considered an additional risk marker for CD susceptibility in DQ8-positive patients.

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