Association between CTLA-4 Gene Promoter (49 A/G) in Exon 1 Polymorphisms and Inflammatory Bowel Disease in the Tunisian Population

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ABSTRACT

Background/Aim: To investigate the possible association between the polymorphism of the CTLA-4 exon 1 +49 A/G and susceptibility to Crohn’s disease (CD) and ulcerative colitis (UC) in the Tunisian population. Methods: The +49 A/G dimorphism was analyzed in 119 patients with CD, 65 patients with UC, and 100 controls by the polymerase chain reaction–restriction fragment length polymorphism method. Results: Significantly higher frequencies of the CTLA-4 +49A allele and A/A homozygous individuals were observed in patients with CD when compared with controls (pc = 0.0023 and pc = 0.0003, respectively). Analysis of CTLA-4 A/G polymorphism with respect to sex in CD showed a significant difference in A/A genotypes between female patients and controls (pc = 0.0001 and pc = 0.038, respectively). There were no differences in the subgroups of patients with CD. Conclusions: Forty-nine A alleles and AA genotype are associated with CD susceptibility in Tunisians. Other genes involved in the T-cell regulation remain strong candidates for IBD susceptibility and require further investigation.

Key Words: Crohn’s disease, CTLA-4, gene polymorphism, inflammatory bowel disease, ulcerative colitis

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Several studies have shown an association between an A/G single nucleotide polymorphism (SNP) in exon 1, position +49 of the CTLA-4 gene, and autoimmune diseases such as type 1 diabetes, multiple sclerosis, Grave’s disease, HLA-DR4-rheumatoid arthritis, and celiac disease. However, studies on the polymorphisms of the CTLA-4 gene in IBD have shown conflicting results in different populations.

In this study, we have analyzed the CTLA-4 exon 1 polymorphism (position 49 A/G) in unrelated Tunisian patients with CD and UC to evaluate the contribution of the CTLA-4 gene to genetic susceptibility to IBD.

**PATIENTS AND METHODS**

**Patients**

Blood samples were obtained from 119 patients with CD (62 male and 57 female) with a mean age of 34.65 years (range = 23–60 years), and 65 patients with UC (18 male and 47 female) with a mean age of 39.94 years (range = 25–74 years). A total of 100 healthy individuals (52 male and 48 female) matched for age (mean age = 32.4 years, range = 24–55 years), sex (40 male and 60 female), and ethnicity were included as controls. None of the healthy samples had any evidence of autoimmune diseases such as inflammatory bowel disease, diabetes, or other autoimmune diseases. All subjects were unrelated Tunisians treated at the Department of Gastroenterology of Charles Nicolle and La Rabta Hospitals in Tunis. The diagnoses of CD and UC were determined according to conventional endoscopic, radiological, and histological criteria. Data obtained from each patient included age at diagnosis, disease location, behavior, and extraintestinal location, which were used to group the patients according to the Vienna classification. None of them had any evidence of autoimmune diseases (type 1 diabetes, celiac disease, multiple sclerosis, Graves disease). All patients and controls gave informed consent to participate in this study that was approved by the Ethics Committee of Charles Nicolle Hospital in Tunis.

**Methods**

Genomic DNA was extracted from peripheral blood leukocytes from all patients and controls using proteinase K and the phenol/chloroform method. Typing of the CTLA-4 exon 1 A/G transition at position +49 was achieved by the polymerase chain reaction–restriction fragment length polymorphism method. The amplification reaction was carried out using primers: 5’CAAGGCTCAGCTGAACCTGGGT 3’ and 5’TACCTTTAACTTCTGCTTTG 3’ and previously described cycling conditions. The amplification products were digested with the restriction enzyme KpnI (Promega), and the fragments were separated on a 4% agarose gel. The A allele corresponds to the uncleaved fragment of 195pb.

**Statistical analysis**

Frequencies of the genotypes, alleles, and phenotypes were analyzed by using the $\chi^2$ test. The odds ratio (OR) and 95% confidence interval were calculated to measure the strength of the association observed. Hardy–Weinberg equilibrium was tested by calculating the $\chi^2$ for goodness of fit. Calculations were made by using Internet programs from www.myatt.demon.co.uk/epicalc.htm. Statistical significance was defined as $P < 0.05$. $P$ values were corrected (pc) by Bonferroni correction for multiple comparisons, taking into account the number of alleles studied.

| Table 1: Demographic characteristics and clinical features of Tunisian patients with Crohn’s disease and ulcerative colitis |
|----------------------------------|
| Crohn’s disease | n (%) | |
| Number (N) | 119 | |
| Men | 59 (49.5) | |
| Women | 60 (50.5) | |
| Sex ratio | 1.08 | |
| Age at onset | | |
| Age < 40 years | 91 (76.4) | |
| Age > 40 years | 28 (23.6) | |
| Location | | |
| Ileum | 30 (25.2) | |
| Colon | 26 (21.8) | |
| Ileocolon | 63 (53) | |
| Disease behavior | | |
| Nonstricturing | | |
| Nonpenetrating | 66 (55.5) | |
| Stricturing | 38 (32) | |
| Penetrating | 15 (12.5) | |
| Extraintestinal manifestations | | |
| Ulcerative colitis | n (%) | |
| Number (N) | 65 | |
| Men | 19 (29.2) | |
| Women | 46 (70.8) | |
| Sex ratio | 2.4 | |
| Age at onset | | |
| Age < 40 years | 28 (43) | |
| Age > 40 years | 37 (57) | |
| Location | | |
| Ulcerative proctitis | 17 (26.1) | |
| Left-sided | 30 (46.1) | |
| Pancolitis | 18 (27.7) | |
| Extraintestinal manifestations | | |
| Arthritis | 7 (10.7) | |
| Dermatological | 1 (1.06) | |
| Arthritis | 22 (18.5) | |
| Dermatological | 6 (5) | |
RESULTS

Table 1 shows several clinical characteristics of 119 CD patients and 65 UC patients. We have genotyped 184 Tunisian IBD patients (119 CD, 65 UC) and 100 matched healthy controls for CTLA-4 +49A/G polymorphism [Table 2]. All populations were in Hardy–Weinberg equilibrium. As the +49A/A genotype of CTLA-4 was associated with IBD in the entire patient population, and as there was no significant difference in the +49A/G CTLA-4 genotype frequencies in gender-grouped controls, we have compared the A/A genotype frequencies in various subgroups of IBD patients with those in the entire control population. When comparing CD patients with the control group, the frequency of the +49 A allele was found to be significantly higher in CD than in controls. Also, the distribution of the +49 A/A genotype was significantly higher in CD patients than in controls. On the other hand, when comparing UC patients with the control group, the frequencies of the +49A allele and the homozygous +49 A/A genotype were higher in UC patients than in controls, but those differences were not statistically significant when Bonferroni correction was applied. When we have analyzed the CTLA-4 +49 A/G polymorphism with respect to gender [Table 3], we found that CTLA-4 genotypes were similar in controls of both sexes (48 females and 52 males). However, the frequency of CD female patients carrying the +49 A/A genotype was significantly higher compared with that in the control group. The same result was obtained in women with UC when compared with that in the control group. Thus, the homozygosity for the A allele could be a risk factor for development of IBD in female patients. A similar analysis has been done on male patients with CD and UC. The results showed a weak significant association in only CD patients compared with that in the control group. Subsequently, we sought to investigate whether this polymorphism could be linked to a particular clinical phenotype. When stratifying CD patients according to the Vienna classification, we were unable to find significant differences in allelic and genotypic +49 A/G CTLA-4 frequencies compared with those in the control group. Likewise, subdivision of the UC patients according to the localization of the disease led to very small subgroups, too small to provide valid evidence for relevant observations. We did not find an association of +49 A/G polymorphism with the severity of disease in any of the IBD patients, as defined by the need for surgery (data not shown).

DISCUSSION

The frequencies of the A allele and the AA genotype were significantly higher in patients with CD compared with those in the control group. Thus, we have demonstrated an association between a promoter polymorphism of the CTLA-4 exon 1 A+49G and CD in this study. The significance of this association may differ according to the population studied and the type of inflammatory bowel disease, as also demonstrated for the NOD2/CARD15 gene polymorphisms. Indeed, several studies have reported an association between CD and NOD2/CARD15 mutation in Caucasians but not in UC patients.[17,30] In addition, evidence...
indicates that the type of inflammatory response occurring in the intestine of patients with CD differs from that in the UC patients. This probably reflects distinct pathways of immune activation and different types of cell recruitment in CD mucosa, where a Th1 response prevails with high IL-12 levels, whereas humoral immunity appears to be predominant in UC.\textsuperscript{[1,10,13]} Stratification analyses revealed that the association was stronger in females than in males. Sex effects in IBD have been already reported by Fisher et al., who have identified several putative regions of sex-specific linkage. Regions on chromosomes 6, 11, 14, and 18 showed strong evidence of linkage in male-affected families but not in female-affected families.\textsuperscript{[31]} Moreover, oral contraceptives have been shown to be associated with increased risk for CD.\textsuperscript{[32]} Similar sex-specific linkage occurs in other immune-mediated diseases including type 1 diabetes, multiple sclerosis, and rheumatoid arthritis.\textsuperscript{[33,34]} although the molecular basis for such effects is unknown. It has been proposed that epigenetic factors play an important role in the pathogenesis of IBD,\textsuperscript{[35]} and that sex effects are mediated by sexual hormones, which have an effect on gene expression and consequently could lead to differential expression of disease susceptibility genes in males and females. According to our findings, it appears that CTLA-4 gene polymorphisms increase susceptibility to CD in females in the Tunisian population. However, because there was no evidence of linkage of the CTLA-4 gene with the X chromosome, it is possible that this difference could be a random variation. On the other hand, because there was no statistical difference in the A allele frequencies between male and female subjects, it is possible that the AA genotype could be a predisposing factor to CD in females. It is worthy of note that Yang et al. reported that females with allergic rhinitis had a significantly higher frequency of the A/A genotype in the CTLA-4 +49 polymorphism than those without atopic diseases. On the other hand, males with and without allergic disorders exhibited no significant difference in CTLA-4 +49 polymorphisms.\textsuperscript{[36]}

Our present results and the lack of previous studies on the sex distribution of CTLA-4 polymorphisms in patients with IBD warrant further investigation. Previous studies assessed the prevalence of CTLA-4 A+49 G polymorphism in IBD patients with conflicting results.\textsuperscript{[25,27-30]} Only two reports found an association of CTLA-4 gene polymorphism with CD and/or UC. One study carried out on Japanese subjects with IBD found a high frequency of GG genotype at the A+49 G polymorphism in CD patients with fistula compared with those without it \( (P = 0.0388; \text{OR} = 2.67) \).\textsuperscript{[26]} Another study carried out on Chinese patients with UC reported an association between the longer allele (122 bp) of the CTLA-4 gene microsatellite polymorphism (AT)\textsubscript{n} and UC patients.\textsuperscript{[25]} All other studies carried out on Caucasian British, Spanish, Dutch, Iranian,\textsuperscript{[27,37,39]} or Chinese populations\textsuperscript{[27,38]} did not find any association between CTLA-4 gene polymorphisms and IBD. Not enough data are available to permit the conclusion that CTLA-4 polymorphism is or is not associated with IBD. In this study, a strong association was found between the CTLA-4 +49A allele and CD but not with UC, suggesting that the genetic factors for CD are distinct from those of autoimmune diseases. CD is not a true autoimmune disease, and has different associations at the CTLA-4 exon 1 +49 G>A from all other autoimmune disorders. The reason for this discrepancy is not clear but might reflect an ethnic difference in the contribution of genetic factor(s). The association of several autoimmune diseases with the CTLA-4 +49G allele has been explained as the result of a reduced inhibitory function associated with the G allele. Recently, Kouki et al. demonstrated that individuals homozygous for the G allele of +49 AG have reduced control of T-cell proliferation compared with A/A homozygotes.\textsuperscript{[16]} On the other hand, it has been shown that both the CTLA-4 protein and mRNA levels are significantly higher in individuals homozygous for the G allele.\textsuperscript{[30]} Similarly, subjects that carry the G/G genotype have a greater T-cell proliferation response when suboptimally stimulated, compared with A/A homozygotes.\textsuperscript{[41]} These findings might correlate with decreased negative regulation of T-cell proliferation, and therefore, predispose the individual to a greater risk of development of autoimmune diseases. The CTLA-4 A/G polymorphism is unlikely to affect the function of the gene because 49 A/G is located on the peptide leader.\textsuperscript{[35]} However, it is possible that the CTLA-4 gene polymorphism affects CTLA-4 mRNA stability and subsequent CTLA-4 expression.\textsuperscript{[42]} CTLA-4-deficient mice develop a lethal lymphoproliferative disease by massive, uncontrolled T-cell proliferation.\textsuperscript{[43]} Although a study reported by Kouki et al. shows important evidence for functional differences among CTLA-4 variants, it is premature to conclude anything in the absence of data on all linked polymorphisms. CTLA-4 has a significant but relatively small effect and does not seem to determine the phenotypic expression of CD. The chromosome 2q33 region contains also CD28 and ICOS, which are two other important immune regulatory genes that participate in regulating the T-lymphocyte-mediated immune response. Detailed analyses within the chromosomal region showed that linkage disequilibrium (LD) exists between CD28, CTLA-4, and ICOS, and a common haplotype is found very frequently among patients with autoimmune diseases and celiac disease.\textsuperscript{[44-46]} As the existence of LD between CTLA-4 and these two closely linked genes may mask the true causative risk allele for autoimmune disease, we cannot exclude that a single true disease-causing allele in CTLA-4 or another neighboring gene might be found in this chromosome region. The role of ICOS in modulating T-cell function by coactivation, combined with its close physical proximity to CTLA-4 and CD28, makes it an excellent candidate as a CD predisposition locus. Some polymorphisms have been detected in noncoding regions of the ICOS gene.\textsuperscript{[38]} It is clear that further studies of this
region are required to support or refute the possible location of a putative linked gene. Other possible candidates may be found among members of the CFLAR-CASP10-CASP8 gene cluster that is positioned about 2.7 Mb distal to the CD28-CTLA-4-ICOS gene cluster. This cluster encodes caspase 8, FADD-like apoptosis regulator (CFLAR), and caspase 10. Both caspases have been shown to be involved in CD95-mediated cell apoptosis. In addition, mutations in the caspase 10 gene lead to the breakdown of lymphocyte homeostasis and the development of autoimmune lymphoproliferative syndrome.[48] As the apoptosis of activated T cells is an important mechanism of peripheral immune tolerance and a defect in apoptosis of mucosal T cells is an important contributing factor in the pathogenesis of IBD,[49] caspase-encoding genes are attractive candidates, but further studies are needed to pinpoint the causal polymorphism.

Our study had certain limitations: (i) we did not genotype all known CTLA-4 SNPs but limited our study to the single polymorphism (CTLA-4 exon 1 +49 A/G); (ii) it is possible that haplotypes exist in our population and we may have missed such haplotypes; therefore, more detailed linkage analysis of this chromosome region is required to identify the IDB susceptibility genes. In this study, a CTLA-4 exon 1 +49A polymorphism was associated with the development of CD but not UC, providing a strong support for an IDB susceptibility gene in the region surrounding CTLA-4. It remains to be determined precisely how the CTLA-4 alleles influence the pathogenesis of IBD. Certainly, our results must await confirmation by other investigators. Because of the important role of CTLA-4 in the control of the inflammatory process and immune responses in IBD, our finding might, however, be compatible with the role of CTLA-4 gene as a potential candidate gene.

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