Association of Fas/Apo1 gene promoter (-670 A/G) polymorphism in Tunisian patients with IBD

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AIM: To detect a possible association between the polymorphism of the (-670 A/G) Fas/Apo1 gene promoter and susceptibility to Crohn's disease (CD) and ulcerative colitis (UC) in the Tunisian population.

METHODS: The (-670 A/G) Fas polymorphism was analyzed in 105 patients with CD, 59 patients with UC, and 100 controls using the polymerase chain reaction restriction fragment length polymorphism method.

RESULTS: Significantly lower frequencies of the Fas -670 A allele and A/A homozygous individuals were observed in CD and UC patients when compared with controls. Analysis of (-670 A/G) Fas polymorphism with respect to sex in CD and UC showed a significant difference in A/A genotypes between female patients and controls ($P$ corrected = 0.004 in CD patients and $P$ corrected = 0.02 in UC patients, respectively). Analysis also showed a statistically significant association between genotype AA of the (-670 A/G) polymorphism and the ileum localization of the lesions ($P$ corrected = 0.048) and between genotype GG and the colon localization ($P$ corrected = 0.009). The analysis of inflammatory bowel disease patients according to clinical behavior revealed no difference.

CONCLUSION: Fas-670 polymorphism was associated with the development of CD and UC in the Tunisian population.

Key words: Fas/Apo1; Gene polymorphisms; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract characterized by immune dysregulation and leukocyte recruitment[1]. IBD may manifest as either Crohn's disease (CD) or ulcerative colitis (UC), which are two distinct forms of IBD with some common clinical, epidemiological, and immunological features, but they can be distinguished by anatomical and histological features as well as by serologic markers[2,3].

The etiology of IBD is unknown, but the condition seems to be the result of a combination of environmental, genetic, and immunologic factors in which an uncontrolled immune response within the intestinal lumen leads to inflammation in genetically predisposed individuals[4].

Genetic factors are known to play an important role in determining an individual's susceptibility to IBD. Significant linkages in chromosomes 1, 3, 5, 6, 7, 12, 14, 16 and 19 have been reported[5-8]. However, IBD-1, a susceptibility locus in chromosome 16, was the first gene to be clearly associated with CD[9].

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Although the underlying genetic and environmental causes remain to be elucidated, CD4+ T helper 1 (Th1) lymphocytes play a pivotal role in the pathogenesis of these diseases. A Th1-like phenotype, with its signature cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α, is shared among many colitis models and is found in patients with active CD[11,12,13]. Moreover, IFN-γ not only cooperates with TNF-α to cause colonic cell death, it also modulates epithelial Fas expression and sensitizes colonic epithelial cells to Fas-induced apoptosis[14].

Programmed cell death, or apoptosis, is regulated by tightly controlled intracellular signaling events in response to pathological cytotoxic stimuli including TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), and Fas[15-17]. Fas, a member of the tumor necrosis factor superfamily, is constitutively expressed by the basolateral membrane of normal colon and small intestinal epithelium[11,12,16-19]. Fas ligation induces apoptosis in colonic epithelial cells and is implicated in the epithelial damage seen in ulcerative colitis[11,12,16-19]. The ligand for Fas (FasL) is expressed by intraepithelial and lamina propria lymphocytes, and its expression is increased in the lamina propria of UC patients, suggesting that Fas-FasL-induced apoptosis participates in the mucosal damage of UC[11,12]. Inhibition of cytokine-regulated apoptosis may therefore be useful in preventing or treating intestinal lesions in patients with IBD.

The Fas/Apo1 gene has been mapped to the chromosome 10q24.1 region[20]. The gene consists of nine exons and eight introns. Two polymorphisms located in the promoter region of the Fas gene have been reported[21]. One of these polymorphisms is a single nucleotide substitution (A/G) at the -670 position. Several studies have shown an association between this A/G single nucleotide polymorphism (SNP) and autoimmune diseases such as type 1 diabetes[22], multiple sclerosis[23], Sjögren’s syndrome[24], rheumatoid arthritis[25], and systemic lupus erythematosus[26].

In this study, we have analyzed the Fas/Apo1 gene promoter (-670 A/G) polymorphism in unrelated Tunisian patients with CD and UC to evaluate the contribution of the Fas/Apo1 (CD95) gene to genetic susceptibility to IBD.

**MATERIALS AND METHODS**

**Patients**

Blood samples were obtained from 164 subjects with IBD, composed of 13 cases of familial forms and 151 cases of sporadic forms. These patients were classified into 105 patients with CD (50 men, 55 women) with a mean age of 36.07 years (range, 23-60 years), and 59 patients with UC (17 men, 42 women) with a mean age of 37.89 years (range, 25-74 years). A total of 100 healthy individuals (52 men and 48 women) matched for age, sex and ethnicity were included as controls. None of the healthy controls 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[28]. Data obtained from each patient included age at diagnosis, disease location, disease characteristics, and extraintestinal location, which were used to group the patients according to the Vienna classification[29]. All patients and controls gave informed consent to participate in this study which was approved by the Ethics Committee of Charles Nicolle Hospital in Tunis.

**Methods**

Transition A/G, in position -670 of the promoter of the Apo1/Fas gene, was analyzed by the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. The Apo1/Fas marker frames an area of 232 Pb in the area of the promoter and was able to contain a site of cut for the enzyme Bst I (Promega). The digestion of the amplified fragment gave rise to two types of allele: the allele G marked by the presence of two fragments 188 Pb and 232 Pb and the allele A characterized by the absence of cut (A), therefore a fragment of 232 Pb. The PCR was carried out in a final volume of 20 μL containing 5 μL of genomic DNA at 40 ng/μL, 1 U of Taq polymerase, 1.5 mmol/L of MgCl2, 0.2 mmol/L of dNTP and 10 pmol of each primer.

The primers used were: Fas (1); 5’-CTACCTAAGGCTATCTACCGTTC-3’ and Fas (2); 5’-GGCGTGTCCATGGTTGGGCTGC-3’. The amplification of product was carried out on 2% agarose gel and visualized under UV. Ten microliter of the amplified fragments were then digested by 5 U of Bst I in a final volume of 20 μL and the mixture was left 2.5 h at 60°C. The separation of the products of digestion was performed on 3% agarose gel and visualized under UV.

**Statistical analysis**

Frequencies of the genotypes, alleles, and phenotypes were analyzed by using the χ² 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 χ² for reliability 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 by Bonferroni correction for multiple comparisons, taking into account the number of alleles studied.

**RESULTS**

As shown in Table 1, Crohn’s disease (64%) was more frequent than ulcerative colitis (36%) in the study. The peak of the disease is between 20 and 40 years. The familial forms represent 8.57% of the CD cases and 6.77% of the UC cases. The principal clinical manifestations observed during evolutionary flares of
Table 1 Clinical characteristics of study subjects (n (%))

|                        | CD (n = 105) | UC (n = 59) | In remission (46) |
|------------------------|--------------|------------|-------------------|
| Sex (Male/female)      | 50/55        | 17/42      |                   |
| Age average            | 36.07        | 37.89      |                   |
| Age (Min/max)          | 23.60/60     | 25/74      |                   |
| Familial forms         | 8 (5.75)     | 4 (6.77)   |                   |
| Surgical history       | 11 (10.47)   | 4 (6.77)   |                   |
| Activity of the disease | In remission | In remission |                   |
| Cortico dependency     | 1 (1.69)     |           |                   |
| Cortico-resistance     | 0            |           |                   |
| Location of lesion     | Severe (0)   |            |                   |
| Stenosing              | 43 (40.95)   |            |                   |
| Distal                 | 21 (19.67)   |            |                   |
| Left-sided colitis     | 6 (5.77)     |            |                   |
| Forms, n (%)           | Stenosing, 43 (40.95) | Favorable, 58 (98.30) | |
| Extra-intestinal       | Fistula, 37 (35.23) | Favorable, 58 (98.30) | |
| Evolution, n (%)       | 5 (8.47)     |            |                   |
| In remission           | 75 (71.43)   |            |                   |
| Treatments             | 5-ASA, 32 (30.47) | 5-ASA, 58 (98.30) | |

Table 2 Allelic and genotypic frequency of (-670 A/G) Apo1/Fas gene polymorphisms in patients with inflammatory bowel disease and controls (n (%))

| Apo1/Fas (-670) | Controls (n = 100) | IBD (n = 164) | UC (n = 59) | CD (n = 105) |
|-----------------|--------------------|---------------|-------------|-------------|
|                 |                    |               |             |             |
| Genotypic       |                    |               |             |             |
| frequencies     |                    |               |             |             |
| A/A             | 49 (49)            | 46 (28.04)    | 15 (25.42)  | 31 (29.52)  |
| A/G             | 32 (32)            | 70 (42.68)    | 30 (50.85)  | 40 (38.10)  |
| G/G             | 19 (19)            | 48 (29.26)    | 14 (23.73)  | 34 (32.38)  |
| Allelic frequencies |                |               |             |             |
| A               | 130 (70)           | 162 (49.39)   | 60 (50.84)  | 102 (48.57) |
| G               | 70 (35)            | 166 (50.61)   | 58 (49.16)  | 108 (51.43) |

When comparing CD patients with the control group, the frequency of the -670 A allele was found to be significantly lower in CD than in controls. Also, the distribution of the -670 A/A genotype was significantly lower in CD patients than in controls. When comparing UC patients with the control group, the frequencies of the -670 A allele and the homozygous -670 A/A genotype were lower in UC patients than in controls and these differences were statistically significant. When we analyzed the Fas/Apo1 gene promoter (-670 A/G) polymorphism with respect to gender (Table 3), we found that Fas/Apo1 genotype frequencies were similar in controls of both sexes (48 females and 52 males). However, the frequency of CD female patients carrying the -670 A/A genotype was significantly less when compared with the control group. The same result was obtained in women with UC when compared with those in the control group. Thus, the homozygosity for the A allele could be a protective factor for development of IBD in female patients. An analogous analysis has been done on male patients with CD and UC. The results did not show a statistically significant association for either CD or UC patients compared with 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 found a statistically significant association between genotype AA of the -670 A/G polymorphism and the ileum localization of the lesions. In addition, we found a statistically significant association between genotype GG of the polymorphism and the colon localization of the lesions. Analysis of IBD patients according to clinical behavior revealed no difference between those carrying...
Fas genotypes were similar in controls of both sexes: 52 males and 48 females: $\chi^2 = 0.06$, $P = 0.8$, two degrees of freedom; $\chi^2$ test of heterogeneity between CD females and controls ($\chi^2 = 8.15$, two degrees of freedom, $P$ corrected = 0.004) odds ratio for AA genotype is 0.36 (0.16 < OR < 0.77, CI = 95%); $\chi^2$ test of heterogeneity between UC females and controls ($\chi^2 = 5.04$, two degrees of freedom, $P$ corrected = 0.02) odds ratio for AA genotype is 0.42 (0.18 < OR < 0.96, CI = 95%); $\chi^2$ test of heterogeneity between UC females and controls ($\chi^2 = 6.46$, two degrees of freedom, $P$ corrected = 0.013) odds ratio for AG genotype is 2.57 (1.15 < OR < 5.76, CI = 95%).

or not carrying the (-670 A/G) allele (data not shown). We did not find an association between (-670 A/G) polymorphism and the severity of disease in any of the IBD patients, as defined by the need for surgery (data not shown).

**DISCUSSION**

There are several candidate genes potentially involved in the pathogenesis of IBD because of chromosomal location or function within inflammatory processes or both[27]. Much investigation has surrounded the FasL system, a major pathway responsible for inducing apoptosis of T cells and enterocytes in the colonic mucosa[28]. Fas is a type I transmembrane protein and a member of the TNF receptor superfamily that may be expressed constitutively by gut lamina propria T (LPT) cells[29]. FasL is a type II transmembrane protein that is expressed on cytotoxic T cells and that induces apoptosis of cells expressing Fas[30]. Defective apoptosis of LPT cells may be a factor in mucosal immune disregulation and tissue inflammation. Rioux et al[31] found that 15% of LPT cells underwent apoptosis in normal individuals. There was a marked reduction in apoptosis of LPT cells in patients with UC and CD and those with specific colitis. In the present study, we genotyped Fas-670 polymorphisms in Tunisian patients with IBD and healthy controls, and found that the frequencies of the A allele and the AA genotype were significantly lower in patients with IBD compared with those in the control group, and in UC patients compared to CD patients, but here the difference was not significant. Thus, we have demonstrated an association between a promoter polymorphism of the (-670 A/G) Fas/Apo1 gene and IBD in this study. The significance of this association may differ according to the population studied and the type of inflammatory bowel disease. In fact, no association of (-670 A/G) Fas/Apo1 SNP with IBD in Chinese, Dutch, Australian and Korean patients exists[12-15]. Confusingly, in some studies the A variant was found as a susceptibility factor for rheumatoid and multiple sclerosis disease. Stratification analyses revealed that the association was stronger in males than in females. Sex effects in IBD have already been reported by Huang et al[30] 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. Moreover, oral contraceptives have been shown to be associated with increased risk for CD[37]. It has been proposed that epigenetic factors play an important role in the pathogenesis of IBD[38], 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 Fas/Apo1 gene polymorphisms decrease susceptibility to CD and UC in females in the Tunisian population. However, because there was no evidence of linkage of the Fas/Apo1 gene with the X chromosome, it is possible that this difference could be a random variation. On the other hand, it appears that CTLA-4 +49 gene polymorphisms increase susceptibility to CD in females in the Tunisian population and in the same patients as have Fas/Apo1 polymorphisms. No explanations have been presented for the discrepancy between positive findings, like ours, and the negative findings of others. The most plausible is the known genetic diversity of the different populations at the haplotype level. Our present results and the lack of previous studies on the sex distribution of Fas/Apo1 polymorphisms in patients with IBD warrant further investigation. The reason for this divergence is not clear but might reflect an ethnic difference in the contribution of genetic factor(s).

Fas-670 polymorphism within the promoter region is situated at a transcriptional binding site (the $\gamma$ interferon activation site) and may potentially have a functional effect on gene regulation of factors such as STATs as well as on expression of proapoptotic proteins (Fas, FasL). In fact, in patients with UC, concentration of a systemic soluble form of Fas was found to be significantly lower in active UC than in controls[39]. Garcia Rodriguez et al[40] have studied Fas/FasL expression on activated colonic T
cells of UC patients, as well as the susceptibility of such T cells to activation-induced cell death, and have reported that CD4+ and CD8+ T cells in UC mucosa expressing FasL were significantly enhanced, suggesting that T cells in UC are less sensitive to apoptotic signals mediated by Fas. A few studies reported the association between the Fas/Apo1 gene promoter (−670 A/G) polymorphism and the localization of the lesions in IBD. Xia et al observed that CD8+ T cells were related to the expression of the Fas/Apo1 gene promoter (−670 A/G) polymorphism.

Regarding the Fas-670 polymorphism, it has been hypothesized that either increased apoptosis of intestinal epithelium or decreased apoptosis of lamina propria lymphocytes may induce inflammation of the gut and, although expression and functional effects of the Fas antigen have been found to be associated with IBD, the relationship between Fas-670 polymorphism and IBD has not been reported yet. However, in this study, a Fas-670 polymorphism was associated with the development of CD and UC, providing strong support for an IBD susceptibility gene in the region surrounding Apo1/Fas. It remains to be determined precisely how the Fas alleles influence the pathogenesis of IBD. Finally, it is possible that haplotypes exist in our population which we may have missed. We conclude that our results must await confirmation by other investigators.

**COMMENTS**

**Background**

In recent years, a few studies have been published addressing the question of where and under what conditions ligand for Fas (FasL) is produced in the gut in the normal and neoplastic situation. Some of these studies have considerably influenced our view of the role of the CD95/CD95L system. That is why it is necessary to analyze the Fas/Apo1 polymorphism in unrelated Tunisian patients with Crohn's disease (CD) and ulcerative colitis (UC) to evaluate the contribution of the CD95 gene to genetic susceptibility to inflammatory bowel disease (IBD).

**Research frontiers**

Inhibition of cytokine-regulated apoptosis may be useful in preventing or treating intestinal lesions in patients with IBD.

**Innovations and breakthroughs**

The relationship between Fas-670 polymorphism and IBD has not been reported yet. This is probably the first report on the association of Fas-670 polymorphisms in Tunisian CD patients. However, this polymorphism was associated with the development of CD and UC, providing strong support for an IBD susceptibility gene in the region surrounding Apo1/Fas.

**Applications**

By understanding how the Fas-670 polymorphism is associated with the development of CD and UC, this study may indicate a future strategy for therapeutic intervention in patients with IBD.

**Peer review**

The manuscript by Ben Aleya et al reports on the association of a Fas/Apo1 promoter polymorphism with IBD in Tunisian patients. The experiments are performed well and the results are interesting.

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