CLINICAL SCIENCE

The TNF-α -308 polymorphism may affect the severity of Crohn's disease

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OBJECTIVE: The goal of this project was to analyze the association between Crohn's disease, its clinical features, and the tumor necrosis factor alpha (TNF-α) -308 polymorphism.

METHODS: This is a case-control and cross-sectional study that enrolled 91 patients with Crohn's disease and 91 controls. Patients with Crohn's disease were characterized according to the Montreal Classification, along with their clinical and surgical treatment history. Analysis of the TNF-α -308 polymorphism was performed using a commercial kit. A stratified analysis was applied using an OR (odds ratio) with a 95% confidence interval. The chi-square and Fisher's exact tests were utilized for analysis of the association between the polymorphism and the clinical features of Crohn's disease.

RESULTS: The low producer predicted phenotype was present in 76.9% of Crohn's disease cases and 75.8% of controls (OR 0.94 [0.45-1.97]). The *TNF2* allele and the high producer predicted phenotype were more frequent among patients with Crohn's disease penetrating behavior ($p = 0.004$). The *TNF2* allele and the high producer predicted phenotype were also associated with a history of colectomy ($p = 0.02$), and the *TNF2* allele was associated with small bowel resection ($p = 0.03$).

CONCLUSIONS: The TNF-α -308 polymorphism appears to affect the severity of the disease. However, TNF-α -308 polymorphism does not appear to be important for the susceptibility in the development of Crohn's disease.

KEYWORDS: Crohn's disease; Inflammatory bowel disease; Tumor necrosis factor alpha; Genetic polymorphism; Mixed-race.

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INTRODUCTION

Crohn's disease (CD) is a chronic illness of unknown etiology. Current studies suggest that a combination of environmental and genetic factors contribute to its pathogenesis. The inflammatory process in CD is partially due to the dysregulation of an immune reaction to gut bacteria. Monocytes and macrophages in the gastrointestinal tract of CD patients often secrete high amounts of tumor necrosis factor alpha (TNF-α), which has an essential role in the disease activity.[1] Crohn's disease appears to be more common among whites than individuals of African descent.[2] Some studies suggest that different racial groups present different characteristics of CD, which could be due to unique pathogenetic mechanisms in patients of different ethnic backgrounds. Overall, there is a slight predominance of CD in females.[3] Additionally, smoking is a potential risk factor for development of CD.[4]

TNF-α is mainly secreted by cells of the immune system, including monocytes, macrophages, neutrophils, NK cells, and T lymphocytes. The TNF-α gene is located on the short arm of chromosome 6 in the HLA region. Several polymorphisms in its promoter region have been described.[5] The most important of these polymorphisms is a G→A substitution at position -308, where the *TNF1* (G) and *TNF2* (A) alleles originate.[6] The GG genotype is associated with a low TNF-α producing predicted phenotype, and the other two genotypes (GA+AA) are associated with a high TNF-α producing predicted phenotype.[7,8] The association between the TNF-α -308 polymorphism and the susceptibility to IBD has been previously evaluated, with conflicting results.[9,10] A European study found that the frequency of the *TNF2* allele was lower in patients with CD than in healthy controls.[11] Another study found a lower frequency of the *TNF2* allele in ulcerative colitis patients compared with controls.[12] However, a Japanese study

observed a fourfold increase in the frequency of the *TNF2* allele among patients with ulcerative colitis compared with controls.[13] Another study detected a similar frequency of the *TNF2* allele between IBD and control patients.[14] Polymorphisms at the -308 site of TNF-α are also associated with the clinical presentation of CD.[15] A study of European CD patients detected a connection between the frequency of the *TNF2* allele and fistulizing, steroid-dependent and colonic disease.[11]

The aim of this study was to analyze the association between the incidence of CD and the TNF-α -308 polymorphism and to examine the association between this polymorphism, severity criteria and the clinical features of Crohn's disease, such as age at diagnosis, behavior, and location of the disease.

MATERIALS AND METHODS

This was a cross-sectional and case-control study. We evaluated patients from outpatient gastroenterology clinics at the Professor Edgard Santos University Hospital and at the Roberto Santos General Hospital in Salvador. Patients and controls were enrolled between March 2006 and May 2007.

Patients 18 years or older were enrolled if they had an established diagnosis of CD, according to the criteria of Lennard-Jones.[16] All included patients were subjected to at least one small bowel follow-through and one colonoscopy with biopsy. When a colonoscopy could not be performed because of a suspected stricture, patients underwent a radiological evaluation of the colon. In cases with symptoms of the upper GI tract, the patients were subjected to an upper endoscopy. The classification of CD was based on the Montreal criteria,[18] which include age at diagnosis, behavior, and location. Race was evaluated according to the criteria of Krieger.[19] Patients were considered smokers if they had smoked seven cigarettes or more per week for at least one year. Only one patient from each family was enrolled. The complete clinical history of CD, including clinical and surgical treatment, was obtained from each patient.

Controls were individuals 18 or older who were evaluated at the listed clinics during the same period of time. These patients may have been diagnosed with dyspepsia or esophageal reflux disease. However, these subjects were required to not have had peptic ulcers, diarrhea, hematochezia, abdominal pain, fistulae, or a family history of inflammatory bowel disease. A patient's family history was considered positive for IBD if it occurred in a first cousin or closer relative.

To obtain genomic DNA, 10 milliliters of whole blood was collected from each patient and stored at -4℃ in EDTA tubes until DNA extraction was performed. Genomic DNA was purified with the commercial EZ-DNA Kit (Biological Industries, Kibbutz Beit Haemek, Israel), according to the manufacturer's instructions. The TNF-α -308 polymorphism was genotyped with the Cytokine Genotyping Tray Kit (One Lambda, Canoga Park, CA) according to the manufacturer's instructions. The results were interpreted using maps of the genotyping plates supplied by the manufacturer.

Sample size was calculated to detect an odds ratio of 2, considering a frequency of 18% for the *TNF2* allele among controls and a frequency of 36% among cases. The study was designed to have a power of 80%, with a 5% probability of an alpha standard error. Using these parameters, it was determined that each group should contain 91 individuals.

To analyze the results, databases were generated and analyzed with the SPSS software, version 11.0, and Epi Info, version 6.04. The study groups were evaluated to determine whether the *TNF* alleles were in Hardy-Weinberg Equilibrium. The main independent variable was carrying the *TNF2* allele, which corresponds with a high TNF-α producing predicted phenotype. The dependent variable was the diagnosis of CD. A 2×2 table was constructed with the main independent and the dependent variable to obtain odds ratios (OR) and 95% confidence intervals. The co-variables of interest were gender, racial group (evaluated as whites or African descent), and smoking (evaluated as the presence or absence of a smoking history). To analyze effects of confounding factors and modifiers on the main association, we performed a stratified analysis using estimated ORs with 95% confidence intervals, Mantel-Haenszel adjusted ORs and the Mantel-Haenszel chi-square test. The co-variables were also evaluated for the possibility of confounding effects by verifying the difference between crude OR and Mantel-Haenszel adjusted OR. The chi-square test or Fisher's test was used to examine the associations between the presence of the *TNF2* allele and the CD characteristics. The differences were considered statistically significant when the probability of the standard error was <0.05.

Informed written consent was obtained from all subjects. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hospital Ethics Committee.

RESULTS

In total, 91 patients with CD and 91 control patients were enrolled in the study. Of these 182 patients, 64 cases and 62 controls were from the Professor Edgard Santos University Hospital, while 27 cases and 29 controls were from the Roberto Santos General Hospital. The control group comprised 81 patients with gastroesophageal reflux disease and 10 patients with functional dyspepsia.

The genotype frequencies of both cases and controls were found to be in Hardy-Weinberg equilibrium. The mean age of patients with CD was 38.0 ± 12.8 years, and that of controls was 50.3 ± 13.6 years. Levene's test was used to evaluate the homogeneity of variances, and they were determined to be equal. The difference between the mean age of cases and controls was statistically significant ($p<0.001$).

Three individuals with CD were of Jewish ancestry. Otherwise, the distribution of racial groups was similar between cases and controls. Only two patients with CD had a family history of IBD.

The distribution of the allelic and genotypic frequencies, along with the predicted phenotypes, for the -308 G/A polymorphism in the TNF-α promoter region for both patients with CD and controls is shown in Table 1. The crude OR and its respective 95% confidence interval for the presence of the A allele and CD were 1.09 [0.58–2.05], while the OR for displaying the high producing predicted phenotype and CD was 0.94 [0.45–1.97]. A positive association was observed between whites (1.25 [0.45–3.50]), male gender (1.91 [0.98–3.76]) and Crohn's disease, and a

Table 1 - Distribution of alleles, genotypes and predicted phenotype frequencies of the TNF-α -308 polymorphism in controls and CD patients.

	Cases	Controls
Allele	**n = 182**	**n = 182**
G, n (%)	155 (85.2)	157 (82.3)
A, n (%)	27 (14.8)	25 (13.7)
Genotype	**n = 91**	**n = 91**
GG, n (%)	70 (76.9)	69 (75.8)
GA, n (%)	15 (16.5)	19 (20.9)
AA, n (%)	6 (6.6)	3 (3.3)
Predicted Phenotype	**n = 91**	**n = 91**
Low Producer, n (%)	70 (76.9)	69 (75.8)
High Producer, n (%)	21 (23.1)	22 (24.2)

negative association was identified between a smoking history (0.67 [0.32–1.37]) and Crohn's disease. However, these results were not statistically significant.

Table 2 summarizes the results of the crude ORs with their respective 95% confidence intervals for displaying the high producing predicted phenotype and CD and the stratified analysis for gender, racial group, and smoking. The results of the Mantel-Haenszel adjusted OR are also listed in Table 2. Notably, the adjusted OR did not differ from the crude OR for any of the variables.

The mean time from the onset of initial symptoms to the establishment of a CD diagnosis was 40.2 ± 59.2 months (range 1–324 months), and the median time was 34 months. The mean age at diagnosis was 32.3 ± 12.5 years. The median age at diagnosis was 33 years old, with a range from 5 to 63 years old. The mean time since CD diagnosis was 68.8 ± 66.2 months.

The CD description, according to the Montreal classification, is outlined in Table 3. The disease behavior was defined in 90 patients, and location was identified in 82 patients. In 81 patients, it was possible to classify both location and behavior. Most patients were diagnosed between 17 and 40 years old and displayed non-stricturing, non-penetrating behavior, and an ileocolic location.

A history of perianal fistula was detected in 45.1% (41/91) of the patients, and a rectovaginal fistula occurred in 5.6% (3/54) of women. Two patients (3.3%) had an enterocutaneous fistula.

Table 3 - Montreal classification for Crohn's disease.

Montreal Classification	n		n	Total n (%)
Age at diagnosis, n = 91				
A1 (≤16)				12 (13.2)
A2 (17 - 40)				55 (60.4)
A3 (>40)				24 (26.4)
Behavior, n = 90				
B1 – Non-stricturing, non-penetrating	31	B1+p	31	62 (68.9)
B2 – Stricturing	12	B2+p	1	13 (14.4)
B3 – Penetrating	7	B2+p	8	15 (16.7)
Location n = 82				
L1 – Terminal ileum	17	L1+L4	_	17 (20.7)
L2 – Colon	21	L2+L4	_	21 (25.6)
L3 – Ileocolic	36	L3+L4	7	43 (52.4)
L4 – Upper gastrointestinal	1			1 (1.3)

The alleles, genotypes, and predicted phenotype frequencies were not associated with age at diagnosis or location (data not shown).

Patients with penetrating behavior showed a greater frequency of the high producing phenotype and of the A allele. Specifically, they were mainly of the AA genotype. This difference was statistically significant. A complete description of the frequencies of the alleles, genotypes and predicted phenotypes according to CD behavior is shown in Table 4. There was no association between perianal disease and the TNF-α -308 polymorphism.

The analysis of the severity criteria showed associations between the *TNF2* allele, colectomy ($p = 0.04$) and small bowel resection ($p = 0.03$). Furthermore, the high producing predicted phenotype was associated with colectomy ($p = 0.02$) (Table 5).

DISCUSSION

Recent advances in studies of genetic polymorphisms and CD are evident, in that the trend for the molecular classification of this disease is based on studies of genes associated with particular clinical phenotypes. For example, the influence of HLA regions appears to be more important in determining colonic and/or perianal disease, while mutations in *NOD2/CARD15* seem to establish ileal disease in people diagnosed at a younger age.[20] The first study from Brazil evaluating genetic polymorphisms in IBD patients

Table 2 - Stratified analysis of association between TNF-α high producing predicted phenotypes and Crohn's disease with regard to gender, racial group and smoking.

Variable	Case n (%)	Control n (%)	OR_S † [95% CI]	OR_A ‡ [95% CI]	*p-value* *
Gender	54 (59.3)	67 (73.6)	1.60 [0.63–4.06]	0.93 [0.44–1.96]	NS
Female	37 (40.7)	24 (26.4)	0.32 [0.08–1.24]		
Male					
Racial Group	80 (87.9)	82 (90.1)	0.84 [0.38–1.86]	0.93 [0.44–1.96]	NS
African descent	11 (12.1)	9 (9.9)	2.0 [0.19–23.4]		
Whites					
Smoking	20 (22)	27 (29.7)	2.33 [0.55–10.14]	0.98 [0.46–2.05]	NS
Yes	71 (78)	64 (70.3)	0.67 [0.27–1.66]		
No					

Note: Crude odds ratio (OR) for having high producing predicted phenotype and CD = 0.94 [0.45–1.97].
†stratified OR.
‡adjusted OR.
*Mantel-Haenszel chi-square to test equality of the OR in strata. NS, not statistically significant.

Table 4 - Frequencies of the alleles, genotypes and predicted phenotypes for the TNF-α -308 polymorphism according to CD behavior.

Variable	B1+p (Non-stricturing non-penetrating +perianal)	*p*-value*	B2+p (Stricturing +perianal)	*p*-value*	B3+p (Penetrating +perianal)	*p*-value*
Alleles	**n = 124**	0.003	**n = 26**	NS	**n = 30**	0.004**
G, n (%)	112(90.3)		21 (80.8)		20 (66.7)	
A, n (%)	12(9.7)		5(19.2)		10(33.3)	
Genotypes	**n = 62**		**n = 13**		**n = 15**	
GG, n (%)	52 (83.9)		9 (69.2)		8 (53.3)	
GA, n (%)	8(12.9)		3 (23.1)		4 (26.7)	
AA, n (%)	2(3.2)		1 (7.7)		3 (20.0)	
Predicted Phenotypes	**n = 62**	0.02	**n = 13**	NS	**n = 15**	0.02
Low Producer, n (%)	52 (83.8)		9 (69.2)		8 (53.3)	
High Producer, n (%)	10 (16.2)		4 (30.8)		7 (46.7)	

Number of alleles = 180; number of patients = 90.
*Comparison with the other behaviors. ** Fisher's exact test.

found that *NOD2/CARD15* confers susceptibility to CD but does not influence the disease phenotype.[21]

TNF-α is a key cytokine in the inflammatory response of CD and appears to be important in the digestive and systemic manifestations of the disease. Research on the polymorphism at position -308 in the promoter region of the TNF-α gene has demonstrated its importance with regard to the clinical presentation of CD. Vatay et al.[22] found that carriers of the *TNF2* allele had higher C-reactive protein levels in the active phase of the disease compared with the inactive phase. In a study by González et al.,[15] 50 patients with the penetrating form of CD were evaluated, and the authors detected an association between the presence of the A allele and increased serum levels of TNF-α, interleukin 1ß and proteins of the acute phase. A more intense inflammatory response occurred in GA individuals compared with GG individuals, along with a higher frequency of arthritis. Their study included no AA patients.

In an analysis stratified by gender, racial group, and smoker/non-smoker variables in the current study, none of these factors were found to modify the effect or confound the association between the studied polymorphism and CD. This result reinforces the possibility that this polymorphism does not have relevance as a risk factor for CD in the studied population, and it is also in agreement with previous studies performed in other populations.[15,23] In agreement with our findings, another study of Brazilian IBD patients showed no association between this polymorphism and susceptibility to CD or UC.[24] Although our study was conducted in another state, those results are still comparable with ours because a multicentric study evaluating genetic ancestry in the Brazilian population showed a perfect genetic admixture from Portuguese, Amerindian and African genetic ancestries in all states studied, regardless of the different skin colors of the individuals.[25]

Some considerations are relevant in studies that evaluate the association of genotype and phenotype using a case-control design. Saito et al. demonstrated the necessity for a plausible biological rationale when studying cytokine polymorphisms. It is not justifiable to examine polymorphisms only because of their availability, without any suggestion for a possible association between the studied cytokine and the pathogenesis of the disease.[26] This study aimed to evaluate the TNF-α polymorphism, as this cytokine is involved in the pathogenesis of CD. In addition, it is important to include a detailed characterization of the control group, which may sometimes not be comparable with the disease group.[27] To better match our groups, we only included control subjects who were treated at the same centers where the study patients were evaluated and managed.

Individuals under 40 years old at diagnosis presented with a lower frequency of the high producing predicted phenotype. It is possible that the TNF-α gene contributes more to the inflammatory response in older individuals.

Table 5 - Frequencies of the alleles and predicted phenotypes for the TNF-α -308 polymorphism according to severity criteria.

Severity Criteria	Allele		*p*-value	TNF-α predicted phenotype		*p*-value
	A n (%)	G n (%)		High producer n (%)	Low producer n (%)	
Immunosuppressive treatment	10 (37.0)	60 (38.7)	NS	8 (30.1)	27 (38.6)	NS
Steroid treatment	19 (70.4)	101 (65.2)	NS	15 (71.4)	45 (64.3)	NS
Fistula surgery	8 (29.6)	24 (15.5)	NS	5 (23.8)	11 (15.7)	NS
Colectomy	10 (37.0)	30 (19.4)	0.04	9 (42.9)	11 (15.7)	0.02
Small bowel resection	5 (18.5)	13 (8.4)	0.03*	5 (31.3)	4 (5.7)	NS
Hospitalization in the last year †	8 (29.6)	48 (31.4)	NS	6 (28.6)	22 (31.9)	N

†90 patients, with 27 A alleles, 153 G alleles, 21 high producers and 69 low producers.
*Fisher's exact test. NS, not statistically significant.

Other genes, such as *NOD2/CARD15*, might contribute to the occurrence of the disease in the youngest individuals.[20]

In another study, an association was detected between the penetrating form of CD and a high TNF-α producing predicted phenotype.[11] We obtained similar results, showing that the frequency of a high producing predicted phenotype was greater among individuals with the penetrating behavior and that this difference was statistically significant. Reinforcing this finding, the non-stricturing, non-penetrating behavior was associated with the low producing predicted phenotype. Furthermore, the *TNF2* allele was associated with the presence of colectomy and small bowel resection, both of which are associated with more severe disease. Another study of Brazilian IBD patients (43 CD patients and 42 UC patients) found no association between disease phenotype and this polymorphism, but the authors did not report the classification applied, which prevents a valid comparison with our results.[24]

The uniform description of this complex disease may contribute to the recognition of the real mechanisms involved in its pathogenesis. We postulate that the lack of a single classification could be the reason for the absence of an association between the studied polymorphism and the severity of CD in other studies. It is also possible that this polymorphism is in linkage disequilibrium with other genetic markers. A detailed description of the evolution and treatment of patients with CD in genetic studies would be useful in understanding many of the currently unclear pathogenetic mechanisms of CD.

CONCLUSIONS

In conclusion, the TNF-α -308 polymorphism does not appear to predispose individuals to the development of CD. This observation is in agreement with research from other centers. However, this polymorphism might affect the behavior of CD. Additionally, the *TNF2* allele was associated with prior colectomy and small bowel resection, reinforcing the notion that the TNF-α -308 polymorphism might affect the severity of CD.

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