A Polymorphism of the Tnf- α Gene Promotes Steroid-Resistance In patients with Inflammatory Bowel Disease

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Abstract

Background and Aim: Crohn's disease (CD) and ulcerative colitis (UC) are promoted by a complex interaction of genetic, immunologic and environmental factors. Given the great efficacy of anti-TNF- α therapy, we aimed in this study to investigate the potential involvement of TNF- α polymorphisms for either pathogenesis or response to therapy.

Methods: 1221 patients, 586 CD (336 male, mean age 46 $\pm$ 14 yrs), 635 UC (394 male, mean age 49 $\pm$ 14 yrs), and 386 gender-matched healthy subjects were genotyped for G308A and C857T TNF- α polymorphisms. Allele-genotype frequencies and genotype/phenotype associations were evaluated by multivariate logistic regression.

Results: The frequency of -308A allele of the TNF- α gene was significantly increased in both CD (OR=1.6 CI=1.1-2.3; P=0.005), and UC patients (OR=1.6 CI=1.1-2.2; P=0.005), compared with controls (7%). Similarly, the homozygous and heterozygous carriers together, were significantly more frequent in both CD (20%; OR=1.7 CI=1.2-2.5; P=0.003) and UC patients (19%; OR=1.6 CI=1.1-2.3; P=0.01), compared to healthy controls (11%). In contrast, for the -G857T polymorphism no significant difference was found. CD carriers of -308A risk allele displayed a more aggressive clinical course, with an increased frequency of surgical resection (42%; P=0.03) and steroid resistance (19%; P=0.007). At the stepwise logistic regression, the correlation between the TNF- α risk genotype and reduced efficacy of steroid therapy in patients with CD was confirmed (OR=2.7; CI=1.2-6; P=0.015), also taking into account other potential confounders.

Conclusion: These observations, awaiting further confirms, leads us to hypothesize that carriers of the uncommon allele -308A, by producing more TNF- α , are more prone to become steroid-resistant.

Keywords: Inflammatory Bowel Disease (IBD), Ulcerative Colitis (UC), Crohn's disease (CD), genetic predisposition, Tumour necrosis factor-alpha (TNF- α)

Introduction

Ulcerative colitis (UC; MIM 191390) and Crohn's disease (CD; MIM 266600) are two similar yet distinct conditions, collectively referred as inflammatory bowel disease (IBD). Although the key mechanisms underlying the development of IBD are still unknown, available evidences suggest that it results from a complex interaction of genetic, environmental, and immunologic factors.

In the last years, with improved genotyping technologies and the completion of the human genome sequence, several genome-wide association studies (GWAS) have identified besides the known variants of CARD15 located at 16q12 [4,5] more than 150 loci that confer susceptibility or protection against IBD [6].

The IBD3 locus on chr 6p has been identified with consistence evidence in different genome wide scans [4-7] and meta-analyses [8,9]. This area encompasses the HLA region and the Tumour Necrosis Factor-alpha (TNF- α) gene. TNF- α encodes a pro-inflammatory cytokine, involved in the imbalance between regulatory and effector cell immune response that was found increased in the mucosa, serum and stool of patients. The role of TNF- α in IBD has been largely demonstrated in animal, clinical, and functional studies. In the TNF^{AARE} model, deletion of 30 regulatory elements leads to increased TNF synthesis and a Crohn's-like phenotype [10]. In contrast, TNF-/- mice show marked reduction to chemically induced intestinal inflammation compared with control mouse [11]. Convincing clinical evidence of the importance of TNF- α is seen in both CD and UC from the dramatic response following administration of anti-TNF- α monoclonal antibody [12,13]. Furthermore, TNF- α induces dysregulation of the intestinal barrier regulating the tight junctions leading to increased permeability [14].

A number of TNF promoter polymorphisms have been described raising the possibility that genetic factors might play a role in determining IBD susceptibility through altered TNF expression. Polymorphisms in the TNF- α gene and promoter region have been studied extensively on IBD susceptibility and/or clinical manifestations, although with conflicting findings [15, 36].

For instance, the -G308A polymorphism in the promoter region has been associated with inducible levels of TNF- α in vitro and functional effect on gene transcription activity; in addition, carriers of the TNF- α risk allele -308A presented higher constitutive and inducible transcriptional levels than -G308 allele carriers [16,17]

Moreover, the promoter TNF-857 C-->T single nucleotide polymorphism (SNP) is functional through the binding to the transcription factor octamer transcription factor-1 (OCT-1), and has been frequently associated with CD [16, 19, 25, 31, 33, 38].

Given the potential interest of TNF- α polymorphisms for both pathogenesis and response to therapy in IBD patients, and the contradictory results available, we planned to investigate the contribution -C857T and -G308A polymorphisms in disease susceptibility and more specifically the response to medical therapy in a large population of Italian IBD patients.

Materials and Methods

Study Population

IBD patients were consecutively recruited in three Italian referral centres: the IRCCS “Casa Sollievo della Sofferenza” Hospital of San Giovanni Rotondo, the University Hospital of Padua, and the Careggi University Hospital of Florence. Diagnosis of CD and UC was established according to accepted clinical, endoscopic, radiological, and histological criteria [18].

One thousand two hundred twenty-one IBD patients were included in the study; 586 were CD patients (336 male, mean age 46 ± 14 yrs), and 635 were UC patients (394 male, mean age 49 ± 14 yrs). Their clinical characteristics are summarized in Table 1. Three hundred eighty-six gender-matched healthy subjects were also evaluated. They were unrelated, asymptomatic subjects (blood donors, students, and staff members) recruited in the same centres, all of Caucasian and non-Jewish descent. Ethics approval was obtained for each of the participating centres. All the study participants were Caucasian, and gave their fully informed written consent.

Data Collection

The Montreal classification was used [19]; for the evaluation of localisation, the largest extent of the disease based on x-ray, endoscopy, or surgical report was considered. A detailed clinical questionnaire concerning different features of the disease was used. In all patients the following clinical features were recorded: family history, age at diagnosis, duration of follow-up, localization of CD (ileum, ileocolonic, colonic, upper GI tract), disease “type” (penetrating, stricturing, inflammatory

[non-penetrating and non-stricturing]), extent of UC (proctitis, left-sided colitis, pancolitis), presence of perianal fistulas, extra-intestinal manifestations (presence or absence of any extra intestinal manifestations and more specifically arthropathy or skin lesions), abdominal surgery (colectomy in UC or bowel resection in CD) and smoking habit (at least 1 cigarette/day). To account for the known modification of clinical characteristics during the disease course, only patients with at least 3 years of follow-up from symptom onset and at least 1 year of follow-up from firm diagnosis were included in the genotype/phenotype analysis. The standard investigations used in these patients were upper GI endoscopy, ileocolonoscopy, barium follow-through and entero-MR. For the purpose of the study, particular attention was paid to medical therapy and, more specifically, to the response to mesalamine (5-ASA), corticosteroids (CS), azathioprine (AZT), 6-mercaptopurine (6-MP), methotrexate, cyclosporine A and anti-TNF agents. Patients were classified as responder or non-responder on the basis of the review of medical records [20]. More specifically, for 5-ASA the clinical response was determined during the first course of treatment. First course was defined

Table 1: Clinical characteristics of CD and UC patients (EIM = extra-intestinal manifestations). (§ 4 patient had only upper G-I tract involvement).

	Crohn's disease (n=586)	Ulcerative Colitis (n =635)
Age (mean $\pm$ SD) (range)	46 $\pm$ 14 (22-85)	49 $\pm$ 14 (22-86)
Age at diagnosis	35 $\pm$ 13 (19-76)	37 $\pm$ 14 (19-76)
Duration of follow-up (yrs)	7 $\pm$ 6 (1-37)	8 $\pm$ 6 (1-36)
Sex (male/female)	336/250	394/241
Localization CD n, (%)		
ileum	237 (40)	
ileo-colon	174 (30)	
colon	171 (29)	
upper G-I tract	30 (5)	
Localization UC n, (%)		
rectum		102 (16)
left colon		292 (46)
pancolitis		241 (38)
Disease type CD n,(%)		
inflammatory	227 (39)	
stricturing	162 (28)	
fistulising	197 (33)	
Resective surgery y/n (%)	196/390 (33)	82/553 (13)
EIM y/n (%)	234/352 (40)	160/475 (25)
Perianal disease y/n (%)	99/487 (17)	21/614 (3)
Smoking		
yes	218 (37)	
no	261 (45)	
ex	107 (18)	
Family history y/n (%)	15/571 (3)	16/619 (3)
ANCA pos/neg n (%)	36/212 (15)	78/173 (31)
ASCA pos/neg n (%)	88/89 (50)	16/87 (16)
Steroids y/n (%)	410/176 (70)	419/216 (66)

as the period from the date that patients started therapy with 5-ASA to the date that the medication was discontinued or to the last follow-up if the medication was not discontinued. Response was defined as clinical remission or clear improvement (mild symptoms not requiring alternative therapies) by the initial 8 weeks of therapy [21]. Patients requiring oral/parenteral CS, immunosuppressive (IMS) drugs, or anti-TNF α during the disease course were considered non-responders to 5-ASA

Patients using CS were classified as CS responders (at least 1 course of systemic steroids with clinical remission reported in the medical history), CS dependent (according to accepted definition), or CS refractory (when an unsuccessful clinical response was achieved, leading to alternative therapies such as surgery, use of anti-TNF α or other IMS drugs) [22,23]. When a modification of response to therapy was recorded during the disease course (i.e., previous CS responder becoming CS dependent), only the most clinically relevant category was considered (i.e., refractory more relevant than dependent, more relevant than responder). Particular attention was paid to the dose and duration of the therapy used, according with current guidelines [24]. Patients with incomplete information or inadequate therapeutic schedule were excluded from analysis. Finally, patients were divided into anti-TNF α responder or non-responder according to short-term (12 weeks) response [25]. Many patients with IBD are currently treated with a combination of drugs during the disease course. A possible synergic effect of different drugs (i.e. 5-ASA and CS) was not assessed in this study; however, for the purpose of this analysis, the most clinically relevant category was chosen. For example, when a patient with a 5-year favourable response to 5-ASA eventually required steroid therapy, the patient was classified as 5-ASA non-responder, even if 5-ASA was continued. Accordingly, when the patient became steroid dependent and responsive to 6-MP after an effective course of steroid therapy, he or she was classified as a 5-ASA non-responder, steroid-dependent, 6-MP responder. Patients intolerant of any investigated drug were not included in this analysis.

Genotyping

Genomic DNA samples were extracted from peripheral blood leukocytes, according to standard protocols [26] and genotyped at the Molecular Laboratory of the Unit of Gastroenterology of the San Giovanni Rotondo Hospital, Italy.

The TNF- α gene promoter polymorphisms G308A and C857T were genotyped by a polymerase chain reaction (PCR) restriction fragment length polymorphism procedure. In brief, after PCR (annealing temperature, 55°C, 35 cycles), the 170 base pairs (bp) for 308 and 154 bp for 857 products were digested by the restriction enzyme NcoI and HpaCH4 IV (New England Biolabs, Ipswich, MA), respectively, and separated by agarose gel electrophoresis. The profile of the -G308A variant was characterised by 23 and 147 bp; for C857T, it was 21 and 128 bp. Genotyping for Arg702Trp and Leu1007 fsinsC common CARD15 variants was also performed by DHPLC, whereas for the Gly908Arg variant, a restriction fragment length polymorphism

assay was used. The 380-bp PCR product was digested with HhaI (New England Biolabs), yielding 2 fragments of 138 and 242 bp in the presence of the C allele, and then visualized on 2% (wt/vol) agarose gel. One hundred random samples were also confirmed by sequencing on an ABI 310 DNA sequence (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations.

Statistical Analysis

Comparison of allele-genotype frequencies and genotype/phenotype association was performed by the chi-square or Fisher exact tests, when appropriate; Student's T test was used to compare means of continuous variables by the SPSS software ver. 11.5. Test of Hardy-Weinberg equilibrium and marker linkage disequilibrium analysis was performed by the Arlequin software ver 2.0.

Genotype-phenotype associations were analysed by means of univariate and multivariate logistic regression with SPSS software; this approach allowed us to take into account a dose-response effect (heterozygote or homozygote), the possible interactions between genes and the effect of potential confounding variables (e.g. disease behaviour, disease localisation).

Results

All the investigated SNPs were found to be in Hardy-Weinberg equilibrium in the control group and in patients. Allele and genotype frequencies are presented in Table 2.

The frequency of -308A allele of the TNF- α gene was significantly increased in IBD (OR=1.6 CI=1.2-2.2; P=0.002), and in both CD (OR=1.6 CI=1.1-2.3; P=0.005), and UC patients (OR=1.6 CI=1.1-2.2; P=0.005), compared with controls (7%). Considering the homozygous and heterozygous states together, carriers were significantly more frequent in the whole IBD group (19%; OR=1.7 CI=1.2-2.3; P=0.003), in CD (20%; OR=1.7 CI=1.2-2.5; P=0.003) and UC patients (19%; OR=1.6 CI=1.1-2.3; P=0.01), compared to healthy controls (11%). In contrast, for either allele or genotype frequencies of the -G857T allele no significant difference in UC and CD patients compared with controls emerged.

When patients were stratified according to the presence or absence of at least 1 CARD15 variant to disclose possible gene-gene interaction, no significant difference in TNF- α was found (Table 3).

Genotype/Phenotype Analysis

For the analysis of possible correlation between genotype and phenotype, patients were classified on the basis of presence (TNF+= -308AA or AG genotypes) or absence (TNF-= GG308 genotype) of the risk genotype for TNF- α . At the univariate analysis of clinical features of patients with CD (Table 4), an increased frequency of surgical resection in TNF+(42%) compared with TNF- carriers was found (31%; OR= 1.58; CI=1.0-2.4; P=0.03). No correlation was found for age at diagnosis, family history, disease behaviour, perianal fistulas, and extra-intestinal manifestations. When the efficacy of medical

Table 2: Allele and genotype frequencies of TNF-α (-G308A and -C857T) variants in CD and UC patients compared with healthy controls. AA is the wild type, while aa is the variant.

Marker	Genotypes						Allele			
	AA	Aa	aa	Tot	p value	OR (95% CI)		Freq	p value	OR (95% CI)
TNF-α										
-G308A							A			
IBD	984 (81%)	220 (18%)	17 (1%)	1221	0.003	1.66 (1.19-2.31)		0.10	0.002	1.61 (1.18-2.19)
CD	469 (80%)	112 (19%)	5 (1%)	586	0.003	1.72 (1.20-2.46)		0.10	0.006	1.61 (1.15- 2.26)
UC	551 (81%)	108 (17%)	12 (2%)	635	0.01	1.60 (1.12-2.30)		0.10	0.005	1.61 (1.15-2.24)
Controls	337 (87%)	46 (12%)	3 (1%)	386	-			0.07		
-C857T							T			
IBD	792 (68%)	331 (28%)	43 (4%)	1166	ns	-		0.18	ns	-
CD	384 (70%)	151 (27%)	17 (3%)	552	ns	-		0.17	ns	-
UC	408 (67%)	180 (29%)	26 (4%)	614	ns	-		0.19	ns	-
Controls	259 (67%)	111 (29%)	16 (4%)	386	-			0.19		

Table 3: Genotype frequency of TNF-α -G308A variant in IBD cases stratified by CARD15 status.

CARD15	TNF	CD		UC		HC	
CARD15+	AA/AG (TNF ⁺)	32	6 %	21	3 %	8	2 %
	GG (TNF ⁻)	150	27 %	71	12 %	44	12 %
CARD15-	AA/AG (TNF ⁺)	76	14 %	94	15 %	41	11 %
	GG (TNF ⁻)	303	54 %	427	70 %	282	75 %
Total		561		613		375	

therapy was investigated, patients TNF+ were significantly more resistant to steroids (19%) compared with TNF- patients (9%; OR= 2.4; CI=1.26–4.8; P=0.007). No difference in the efficacy of mesalamine, IMS, and anti-TNFα was found.

In patients with UC (Table 4), no significant association of TNF-α polymorphisms with any clinical characteristics was demonstrated, with the exception of an increased frequency of surgical resection in TNF+(18%) compared with TNF- patients (12%; OR= 1.7; CI= 1–2.9; P=0.05).

At the stepwise logistic regression, the correlation between the TNF-α risk genotype and reduced efficacy of steroid therapy in patients with CD was confirmed (OR=2.7; CI=1.2–6; P=0.015), also taking into account other potential confounders such as disease localisation, and disease behaviour. When patients with surgical resection were studied, taking into account potential confounders such as disease localisation, disease behaviour and TNF genotypes, the disease type (i.e. stenosing/fistulising) but not TNF genotype increased the risk of surgical treatment (OR=18.7; CI=8.8–39.8; P<0.01) (Table 5).

Discussion

Chronic inflammation in IBD is primarily due to an immunological imbalance between pro- and anti-inflammatory cytokines, together with a defective apoptosis of lamina propria T cells [41]. Amongst the pro-inflammatory cytokines, clinical, functional, and animal studies candidate TNF-α gene as an attractive player in the inflammatory process of IBD. TNF-α is a pro-apoptotic cytokine largely secreted by monocytes/macrophages during innate immune responses and by effector Th1 cells.

Studies on TNF-α gene have pointed mainly on a limited number of promoter polymorphisms. Association of the C allele of the TNF -C857T polymorphism to IBD, with more significant association specifically to CD [17, 22, 28, 32] have been reported. In addition, carriers of the A risk allele of the TNF -G308A variant has been associated with stenosing and penetrating behaviour in CD [18,23,31,33] Moreover, by using a weighted linear combination of SNPs[27] the variation -308 was confirmed to be involved with CD to the same extent as markers of CARD15.

It has been also demonstrated that these variants are associated with increased constitutive and/or inducible levels of TNF protein production in vitro. However, whether this variant is itself functional or in LD with the actual causative allele is still unclear [41, 42]. In addition, with the exception of our pilot study in children with IBD [40] no evidence of correlation of TNF-α polymorphism with response to medical therapy has been demonstrated.

In our large cohort of 1221, IBD patients, the two more studied polymorphisms, -G308A and -C857T, in the TNF-α gene promoter have been analysed.

The -308A allele was significantly associated with both CD (10%; P=0.005) and UC patients (10%; P=0.005). Carriers of combined homo or heterozygote genotypes were more frequently

Table 4: Genotype frequencies of -G308A SNP in CD and UC patients stratified by phenotypic subgroups.

	CD			UC		
	AA_GA n=117	GG N=469	p value OR (95% CI)	AA_GA n=120	GG n=515	p value OR (95% CI)
Age at diagnosis (yrs)	36 ± 14	35 ± 12		38 ± 14	37 ± 12	
Smoking history Yes/No	64/53 (55)	261/208 (56)		47/73 (39)	243/272 (47)	
Disease location						
Ileum (L1)	47 (41)	190 (41)				
Colon (L2)	32 (32)	142 (30)				
Ileo-colon (L3)	37 (27)	134 (29)				
Upper GI (L4)	6 (5)	24 (5)				
Rectum				43 (36)	159 (31)	
Left colon				29 (24)	163 (32)	
Pancolitis				48 (40)	193 (38)	
Disease behavior						
Inflammatory (B1)	47 (40)	180 (38)				
Strictureing (B2)	37 (32)	125 (27)				
Penetrating (B3)	33 (28)	154 (35)				
Perianal disease Yes/no	19/98 (16)	80/389 (17)		5/115 (4)	16/499 (3)	
Family history Yes/no	0/117 (0)	15/454 (3)		3/117 (3)	13/502 (3)	
ANCA Pos/Neg	7/32 (18)	29/176 (17)		11/36 (23)	67/137 (33)	
ASCA Pos/Neg	10/18 (36)	78/71 (52)		1/16 (6)	15/71 (17)	
Surgery Yes/No	49/68 (42)	147/322 (32)	0.03 1.58 (1.04-2.39)	22/98 (18)	60/455 (12)	0.049 1.70 (1-2.91)
EIM Yes/No	44/73 (38)	190/279 (41)		34/86 (28)	126/389 (25)	
Therapy						
5-ASA resp/res	84/28 (75)	309/137 (69)		87/24 (78)	388/144 (77)	
Steroids resp/res	69/16 (81)	294/28 (91)	0.007 2.43 (1.25-4.75)	64/9 (88)	298/43 (87)	
AZT/6 MP resp/res	24/2 (92)	125/18 (87)		26/5 (84)	85/14 (86)	
Infliximab resp/res	12/5 (71)	56/16 (78)		0/0	7/3	
MTX resp/res	1/1	11/2		3/1	5/0	
Cyclosporine resp/res	0/0	1/4		1/1	5/3	

Table 5: Data obtained by multiple logistic regression analysis. Based on univariate analysis, selected outcomes were response to steroids and surgical resection. (Resp = Responder; Depend = Dependent; Resist = Resistent; TNF⁺ = genotype GG and TNF⁺ genotypes AA +GG).

Outcome	Covariates	p-values	OR (95%)
Steroid Response			
Resp+Depend vs Resist	Inflammatory vs Strictureing/ Fistulizing	0.047	2.75 (1.01-7.46)
	TNF ⁺ Vs TNF	0.015	2.69 (1.21-5.96)
Surgical Resection			
Yes vs No	Inflammatory vs Strictureing/ Fistulizing	< 0.01	18.76 (8.84-39.84)

CD (20%; P=0.003) and UC patients (19%; P=0.01) than healthy controls (11%). In contrast, for either allele or genotype frequencies of the -G857T allele no significant difference in patients with CD and patients with UC compared with controls

emerged.

Moreover, CD carriers of -308A risk allele displayed a more aggressive clinical course, with an increased frequency of surgical resection (42%; P=0.03) and steroid resistance (19%; P=0.007). No correlation was found for age at diagnosis, family history, disease behaviour, perianal fistulas, extra-intestinal manifestations and the efficacy of mesalamine, IMS, and anti-TNFα. To better understand the contribution of the TNF-α variant to the clinical heterogeneity of CD with respect to other factors (i.e., disease localisation, disease behaviour and TNF) a logistic regression analysis has been performed. The correlation between the TNF-α risk genotype and reduced efficacy of steroid therapy in patients with CD was confirmed (OR=2.7; CI=1.2–6; P=0.015), also taking into account other potential confounders such disease localisation and behaviour.

In contrast, when patients with surgical resection were studied, the disease type (i.e. stenosing/fistulising), but not the TNF polymorphism, significantly increased the risk of surgical

treatment (OR=18.7; CI=8.8–39.8; P<0.01).

When considering the use of corticosteroids, 11% of our 407 CD patients did not respond to therapy; specifically, carriers of -308AA and AG genotypes were significantly more frequent into the resistant group (19%) compared to patient responders (9%; OR= 2.4; CI=1.26–4.8; P=0.007). The value remains significant after correcting for disease localisation and disease behaviour (P=0.015).

Corticosteroids are still effective as induction therapy in moderate to severe active UC and CD patients. Epidemiological data have shown that up to a quarter of patients became steroid-dependent and 20% steroid-resistant within 1 year [28]. Although the mechanism of steroid resistance is unclear, it is known that TNF-α levels are increased in carriers of the uncommon -308A allele [26] and TNF-α was demonstrated to induce dysregulation of the intestinal barrier regulating the tight junctions (TJ) leading to increased permeability. A number of study have suggested that the elevated levels of TNF-α present in CD patients are responsible for the observed increases in intestinal permeability seen in these patients [27–29]. It was also proved that in vitro glucocorticoids inhibit the increase TJ permeability induced by TNF-α [28].

Taken together these observations leads us to speculate that carriers of the uncommon allele -308A, by producing more TNF-α, are more prone to become steroid-resistant probably because the drug does not exert the full activities to block the increased endogenous level of TNF-α of these patients. Therefore, pinpoint patients non-responsive to steroids based on TNF-α polymorphism might avoid prolonged and eventually ineffective therapy, reducing either the risks for the patient and a more prolonged exposure to drug.

Further studies are needed to confirm these data and discover the possible biological mechanism of steroid resistance and its correlation with TNF-α genotype and levels.

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