Original Paper: Inflammatory Bowel Disease

Digestion

Digestion 2002;66:121–126
DOI: 10.1159/000065592

Received: January 30, 2002
Accepted: May 21, 2002

Candidate Genes Colocalized to Linkage Regions in Inflammatory Bowel Disease

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Key Words
Inflammatory bowel disease · Candidate gene · Vitamin D receptor · Tumor necrosis factor-α · Epidermal growth factor receptor · Crohn's disease · Ulcerative colitis

Abstract
Background and Aims: The genes encoding for tumor necrosis factor-α (TNF-α), epidermal growth factor receptor (EGFR) and the vitamin D receptor (VDR) are colocalized to inflammatory bowel disease-associated linkage regions on chromosomes 6, 7 and 12. An association study of these gene polymorphisms with ulcerative colitis or Crohn's disease and a stratification according to disease phenotypes was performed in order to identify gentically homogenous subgroups. ***Patients and Methods:*** 119 healthy, unrelated controls, 95 patients with Crohn's disease and 93 patients with ulcerative colitis were genotyped for the (G to A) –308 TNF-α promoter polymorphism on chromosome 6, the codon 497 EGFR polymorphism on chromosome 7 and the *Taq*I polymorphism of the VDR gene on chromosome 12. After genotyping, patients were stratified according to the respective disease phenotype. ***Results:*** A disequilibrium in the distribution of the VDR genotypes was found in patients with ulcerative colitis compared to controls (p = 0.024). In fistulizing and fibrostenotic Crohn's disease the 'TT' genotype was significantly reduced compared with other phenotypes (p = 0.006), whereas the 'tt' genotype was found more frequently (p = 0.04). The frequency of the WT allele of the EGFR gene was significantly higher in ulcerative colitis (p = 0.04) than in controls. Further significant differences, concerning the associations of the different polymorphisms and disease susceptibility or clinical phenotypes, were not observed. ***Conclusions:*** Regardless of the disease phenotype, the associations between the polymorphisms and inflammatory bowel disease investigated herein are modest, even after stratification for the disease phenotypes. Hence, these polymorphisms are unlikely to confer the reported linkage between inflammatory bowel disease and chromosomes 6, 7 and 12.

Introduction

One of the most attractive hypotheses concerning the etiology of inflammatory bowel disease comprises an aberrant regulation of the mucosal immune response which probably results in a loss of the immune tolerance against luminal antigens. Thus, genes involved in the regulation of the immune response and intestinal mucosal repair and defense are apparently candidates for genetic studies. Genome wide linkage analyses in large patient cohorts of different ethnic backgrounds have defined inflammatory bowel disease susceptibility loci located on chromosomes 3, 6, 7, 12 and 16 employing microsatellite markers in affected relative pairs [1–13], although linkage could not be confirmed by every group [7, 14, 15].

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The tumor necrosis factor-α (TNF-α) gene is located on chromosome 6 within the HLA class III region, telomeric to the class II and centromeric to the class I region. Linkage of inflammatory disease has been demonstrated recently to this region which is situated on the short arm of chromosome 6 [4]. The biallelic G to A transition polymorphism located at position –308 in the TNF promoter region defines the TNF1 (-308G) and the TNF2 (-308A) alleles [16]. Elevated circulating levels of TNF-α were described in individuals homozygous for TNF2 [17].

Epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein which mediates the effects of epidermal growth factor (EGF) and transforming growth factor -α (TGF-α). EGF enhances host natural defenses against mucosal injury by creating a dilutional barrier that restricts injury from caustic agents [18]. In animal models of colitis a marked increase in EGFR immunoreactivity has been noted [19], suggesting a role for EGF in intestinal mucosal repair and defense. Moriai et al. [20] identified a G to A transition at codon 497 of the EGFR resulting in an arginine to lysine substitution. The human EGFR maps to chromosome 7p12, a region which has been linked to inflammatory bowel disease [1].

The gene encoding for the vitamin D receptor (VDR) is situated within 3 cM to the microsatellite marker D12S85 on chromosome 12, which has been linked to ulcerative colitis and Crohn's disease by different groups [1, 3, 5, 21]. Vitamin D_3 affects calcium hemostasis and exhibits distinct immunomodulatory effects, by activating macrophages and monocytes and suppressing lymphocyte proliferation and immunoglobulin production [22, 23]. The proinflammatory transcription factor NFκB is inhibited by vitamin D_3 and subsequently the expression of proinflammatory cytokines is impaired [24].

The present study sought to address whether immunorelevant genes colocalized to previously described linkage regions are associated with inflammatory bowel disease. In addition, a stratification of the respective polymorphisms according to the disease phenotypes was performed in order to identify genetically homogenous subgroups.

Methods

Study Population

Blood samples were obtained from 95 patients with Crohn's disease (male:female ratio 33:62; age 40 ± 14 years), 93 patients with ulcerative colitis (male:female ratio 41:52; age 43 ± 14 years), and 119 healthy, unrelated controls. The diagnosis of Crohn's disease and ulcerative colitis and the respective disease phenotype, e.g. fistulizing, fibrostenotic, were assessed by conventional clinical, endoscopic, histopathological and radiologic criteria. Patients were recruited consecutively between 1986 and 2000 (urban, middle European population).

DNA Extraction

Genomic DNA was prepared from buffy coat layers employing a commercially available kit (QIAamp DNA Blood Mini Kit; Quiagen, Hilden, Germany).

Determination of the TNF-α Genotype

Two allelic forms were genotyped, referred to as TNF1 and TNF2, that are identical apart from a single base transition (G to A) at –308 of the TNF-α promoter. Typing was performed by PCR amplification using the primers as previously described [25]. The primer pair C1 5′-TCTCGGTTTCTTCTCCATCG-3′ was used in combination with either the primer C2 5′-TAGGTTTTGAGGGGCATGG-3′ for amplifying TNF1 or the 5′ primer C3 5′-ATAGGTTTTGAGGGGCATGA-3′ for amplifying the 184-bp TNF2 fragment. The primer pair GAPDHse/GAPDHas producing a 310-bp fragment out of the GAPDH gene was added to each reaction as an internal control. The 184-bp fragment of the TNF-α promoter region was amplified by a 32-cycle PCR with a hot start Taq polymerase (HotStar*Taq* DNA Polymerase, Qiagen GmbH) employing a Biometra-Thermocycler (Biometra, Göttingen, Germany). PCR was carried out under standard conditions. Annealing was performed for 60 s at 60°C. PCR products were analyzed using a 2% agarose gel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

EGFR (or HER) Polymorphism

Primer sequences 5′-GTTTGGGACCTCCGGTCAG-3′ and 5′-CTTGTCCACGCATTCCC TGC-3′ spanning the BstNI restriction site were identical to those described by Moriai et al. [20]. For PCR amplification (Cycler and *Taq* as above), standard conditions were used (annealing temperature 59°C for 1 min, 35 cycles). 20 µl of the 102-bp PCR product was digested with BstNI (New England Biolabs) at 65°C for 2 h. Alleles were separated on 4% NuSieve ethidium bromide-stained gels. In the case of a homozygous wild-type individual, one undigested 102-bp band was obtained. The G to A transition at codon 497 created a BstNI restriction site so that three bands (102, 54 and 48 bp) were present in heterozygous individuals. If a patient had a homozygous transition, only the 54- and the 48-bp band were seen.

Analysis of the Vitamin-D Receptor Genotype

Sequence-specific primers were used for typing the biallelic VDR polymorphism as described elsewhere [26]. The consensus primer 5′-CAGAGCATGGACAGGGAGC-3′ was either used with primer T 5′-CGGTCCTGGATGGCCTCA-3′ giving rise to the T allele or the primer t 5′-CGGTCCTGGATGgCCTCG-3′ for amplifying the 310-bp t fragment. As an internal control the primer pair F/R was added to each reaction producing a 700-bp fragment out of the GAPDH gene. At an annealing temperature of 66°C for 50 s, PCR was performed under standard conditions. PCR products were also visualized in 2% agarose gel.

Statistical Analysis

Statistical evaluation was performed by use of the Fisher's exact test.

Results

The genotype frequencies of the three polymorphisms investigated herein are depicted in table 1. The frequencies of the VDR genotypes in ulcerative colitis were found in significant disequilibrium (p = 0.024): The 'Tt' genotype was found less frequent (41 vs. 58%), whereas the 'tt' genotype was more prevalent when compared to controls (29 vs. 16%). The frequency of the WT allele in the EGFR gene was increased in ulcerative colitis (p = 0.04). Further significant differences concerning the frequency of the TNF-α and the EGFR polymorphism between patients and controls were not observed (see table 2).

The frequencies of the different polymorphisms which were observed after stratification according to the disease phenotype in Crohn's disease are listed in table 3. The 'TT' genotype shared a negative and the 'tt' a positive association with the fistulizing and fibrostenotic phenotype (p = 0.006 and 0.04, respectively). Further significant differences in the genotype distribution were not observed after stratification with regard to clinical phenotypes.

Table 1. Genotype frequencies in the different groups of the study population

	Controls	Crohn's disease 95 patients	Ulcerative colitis 93 patients
TNF-α			
TNF1/TNF1	87 (73%)	78 (82%) n.s.	74 (80%) n.s.
TNF1/TNF2	32 (27%)	17 (18%)	19 (20%)
EGFR			
WT/WT	62 (52%)	50 (53%) n.s.	60 (65%) n.s.
WT/EGFR2	47 (39%)	37 (39%) n.s.	30 (32%) n.s.
EGFR2/EGFR2	10 (9%)	8 (8%) n.s.	3 (3%) n.s.
VDR			
T/T	31 (26%)	37 (39%) n.s.	28 (30%) p = 0.024
T/t	69 (58%)	45 (47%)	38 (41%)
t/t	19 (16%)	13 (14%)	27 (29%)

TNF1 = Wild-type allele , TNF2 = G to A transition, WT = wild-type, T = wild-type allele, t = A to C substitution, n.s. = not significant.

Discussion

Inflammatory bowel diseases are heterogeneous with respect to clinical behavior, response to medical treatment and prognosis. The concept of genetic heterogeneity in inflammatory bowel disease is supplied by several observations [27, 28]. In accordance with this concept an appropriate understanding of the etiology of inflammatory bowel disease will only be possible after stratification of patients according to clinical or genetic markers in order to identify genetically homogenous subgroups.

TNF-α is a proinflammatory cytokine of paramount importance in inflammatory bowel disease. Increased concentrations of TNF-α were described in serum [29], stool [30] and the intestinal mucosa of patients with Crohn's disease [31, 32]. The administration of anti-TNF-α antibodies has profound effects in active Crohn's disease [33–35]. The individual variations in TNF-α pro-

Table 2. Allele frequency in the different groups of the study population

	Controls	Crohn's disease 95 patients/190 alleles	Ulcerative colitis 93 patients/186 alleles
TNF-α			
TNF1 allele	206/238 (87%)	173/190 (91%) n.s.	167/186 (90%) n.s.
TNF2 allele	32/238 (13%)	17/190 (9%)	19/ 186 (10%)
EGFR			
WT allele	171/238 (72%)	137/190 (72%) n.s.	150/186 (81%) p = 0.04
EGFR2 allele	67/238 (28%)	53/190 (28%)	36/186 (19%)
VDR			
T allele	131/238 (55%)	119/190 (63%) n.s.	94/186 (51%) n.s.
t allele	107/238 (45%)	71/190 (37%)	92/186 (49%)

TNF1 = Wild-type allele , TNF2 = G to A transition, WT = wild-type, EGFR2 = G to A transition, T = wild-type allele, t = A to C substitution, n.s. = not significant.

Table 3. Genotype frequencies according to the disease phenotype in Crohn's disease

	Fistulizing 15 patients	Stenotic 24 patients	Fistulizing + stenotic 40 patients	Nonfistulizing, nonstenotic 16 patients
TNF-α				
TNF1/TNF1	11 (73%) n.s.	20 (83%) n.s.	35 (88%) n.s.	12 (75%) n.s.
TNF1/TNF2	4 (27%)	4 (17%)	5 (13%)	4 (25%)
EGFR				
WT/WT	10 (67%) n.s.	14 (58%) n.s.	19 (48%) n.s.	7 (44%) n.s.
WT/EGFR2	4 (27%) n.s.	8 (33%) n.s.	16 (40%) n.s.	9 (56%) n.s.
EGFR2/EGFR2	1 (7%) n.s.	2 (8%) n.s.	5 (13%) n.s.	0 n.s.
VDR				
T/T	7 (47%) n.s.	13 (54%) n.s.	9 (23%) p = 0.006	8 (50%) n.s.
T/t	7 (47%) n.s.	9 (38%) n.s.	22 (55%) n.s.	7 (44%) n.s.
t/t	1 (7%) n.s.	2 (8%) n.s.	9 (23%) p = 0.040	1 (6%) n.s.

TNF1 = Wild-type allele, TNF2 = G to A transition,WT = wild-type, EGFR2 = G to A transition, T = wild-type allele, t = A to C substitution, n.s. = not significant. p values vs. controls.

duction are likely to be caused by regulatory polymorphisms in or near the TNF-α gene, such as the polymorphism at position –308 in the TNF promoter region. Several groups have shown that the TNF2 allele polymorphism is associated with increased transcription factor binding as well as increased transcriptional activity compared to the TNF1 allele [36–38]. This correlates with increased circulating concentrations of TNF-α in individuals homozygous for TNF2, as compared to individuals homozygous for TNF1, whereas heterozygous individuals display intermediate levels of TNF-α [17]. However, in contrast to previously reported data [39, 40], we were unable to detect an association between the –308 TNF-α polymorphism and ulcerative colitis or Crohn's disease. Such an association was also not observed after stratification according to the clinical phenotype in Crohn's disease.

Exogenous administration of EGF significantly reduces the mucosal damage and inflammation induced in animal models of colitis [41]. Although the mechanism by which EGF provides protection is currently unknown, it has been postulated that through its ability to stimulate mucin glycoprotein synthesis and secretion [42–44], EGF enhances host natural defenses against mucosal injury by creating a dilutional barrier, that restricts injury from caustic agents [18]. EGFR might therefore play an important role in intestinal mucosal repair and defense. In 1993, a variant EGFR was identified that had an arginine to lysine substitution at codon 497 [20]. Wild-type EGFR differs in contrast to mutant EGFR with respect to epithelial proliferation following the administration of EGF and TGF-α in rodents [45]. Thus, it is appears possible that mutations which influence the function or expression of EGFR might predispose to inflammatory bowel disease. A slight but significant association between the WT allele and ulcerative colitis was found. Further associations regarding the genotype or disease phenotype were not observed.

The immunoregulating effects of the cellular receptor for $1,25(OH)_2$ vitamin D_3 are complex and not yet finally understood. The *Taq*I polymorphism, located at the 3′ end of the VDR gene, has been associated with bone mineral density and osteoporosis in a number of different populations [46], as well as with the risk of prostate cancer [47]. The 'tt' genotype of the *Taq*I polymorphism seems to predispose to a variety of infectious diseases, such as tuberculosis, chronic hepatitis B and leprosy [48–50]. Since the *Taq*I polymorphism does not result in an amino acid coding change (it is an A to C base substitution at codon 352 of exon 8, both isoleucine), the mechanism of how the polymorphism might affect disease susceptibility remains unclear. In addition, the data reflecting the functional significance of the *Taq*I polymorphism are contradictory [51]. Under the assumption that the 'tt' genotype is associated with enhanced VDR transcription, a stronger cell-mediated immune response with increased mac-

rophage or monocyte activation could predispose to inflammatory bowel disease. Furthermore, an impaired mucosal antibody secretion resulting in increased susceptibility to bacterial infection which could trigger inflammatory bowel disease. In contrast to previously reported data [26], we were unable to detect a positive association between the 'tt' genotype and Crohn's disease, instead we found a disequilibrium in the distribution of the VDR genotypes in ulcerative colitis. Compared to the controls, the 'Tt' genotype was less frequent in patients with ulcerative colitis, whereas the 'tt' genotype was found more often. After stratification of patients with Crohn's disease regarding the clinical phenotype, we found a negative association of the 'TT' genotype, as well as a positive association of the 'tt' genotype with the fistulizing and fibrostenotic phenotype. However, concerning the conflicting nature of the data and the weakness of the associations, it appears unlikely that the *Taq*I polymorphism of the VDR gene is an important determinant of disease susceptibility.

In summary, with the exception of the associations between the VDR genotypes and inflammatory bowel disease and the weak association of the WT allele and the EGFR gene, further significant associations between the immunologically relevant gene polymorphisms which are colocalized to previously reported linkage regions in inflammatory bowel disease were not observed in the present study. Thus, it appears unlikely that linkage of chromosomes 6, 7 and 12 with inflammatory bowel disease is due to polymorphisms of the genes investigated herein.

Acknowledgments

This paper contains parts of the doctoral thesis of K. Martin and M. Radlmayr. The authors thank Dr. J. Klose for helpful advice in performing the statistical analysis. The project was supported by the grant of 'Förderung von Forschung und Lehre (FoFöLa Reg-Nr. 69)' and the Friedrich-Baur-Institut, Munich, Germany, to Ch. Folwaczny.

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