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CARD15 Genotype and Phenotype Analysis in 55 Pediatric Patients With Crohn Disease From Saxony, Germany

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ABSTRACT

Objectives: Crohn disease is a chronic inflammatory bowel disorder that is caused by environmental and genetic factors. Mutations in the *CARD15* gene have been recently identified to be associated with the disease. Until now no genetic study has focused directly on a pediatric population.

Methods: The authors sequenced all 12 exons of the *CARD15* gene in 55 pediatric patients with Crohn disease from Saxony. Their average age at onset was 11.2 years (1–17.5 years). The authors also evaluated the genotype-phenotype relationship in the patients.

Results: Fourteen different polymorphic and/or disease-related nucleotide alterations have been identified in the patients. Sixty-five percent of their genomic DNA samples harbored at least one of six mutations within the *CARD15* gene, which previously has been identified as being associated with Crohn disease. The authors found that the cytosine insertion mutation 3020insC was significantly more common in their pediatric population than in patients with Crohn disease (26% versus 11% of the alleles) whose results were reported in the literature. The genotype–phenotype analysis showed that the authors' patients with at least one of the six *CARD15* disease-associated mutations had a high risk of inflammation located in the terminal ileum and ascending colon. In 10 of 19 patients with two mutations, intestinal resection surgery was necessary because of stricturing.

Conclusions: In the authors' pediatric patients, the genetic influence on Crohn disease was more pronounced than that reported in any other study, and it strongly affected the clinical phenotype. ***JPGN 37:492–497, 2003.*** **Key Words:** Crohn disease—*CARD15*—*NOD2*— Mutation—Stricturing—Surgery. © 2003 Lippincott Williams & Wilkins, Inc.

INTRODUCTION

Crohn disease (CD [OMIM 266600]) is a chronic relapsing intestinal inflammatory disorder. It may involve any part of the gastrointestinal tract but is most frequently located in the terminal ileum and colon. CD is primarily a disease of adolescence and young adulthood, with peak incidence occurring between 15 and 30 years of age (1). The incidence of CD has been steadily increasing in the pediatric age group (2).

With the use of genome-wide linkage analyses and positional cloning methods, one gene on chromosome 16 (16q12) was identified to be associated with CD (3,4); the gene was named *NOD2*, but later the name was changed to *CARD15* (5). It is a member of the Apaf-1/Ced4 superfamily, expressed in monocytes and important for inflammatory signal transduction. The *CARD15* gene product can activate the transcription factor NF-κB upon stimulation with the bacterial cell membrane component lipopolysaccharide (6). A disregulation of this process caused by alterations in the *CARD15* gene has been proposed as an important factor that contributes to CD. The normal gene encodes a protein (Fig. 1) that contains two N-terminal CARDs (caspase recruitment domain), a centrally located NBD (nucleotide-binding oligomerization domain), and 10 C-terminal LRRs (leucine-rich repeats) (7).

LRRs are regarded as primary sensors of the innate immune system (8). In *CARD15*, it is required to sense lipopolysaccharide and to activate an NF-κB signaling pathway (9). NF-κB regulates apoptosis and promotes proinflammatory gene expression. Many patients with CD have the insertion of a cytosine (3020insC), which leads to an early stop codon and the deletion of 33 amino acids at the C terminal end of the leucine-rich region. The mutation might increase NF-κB translocation and thereby elicit spontaneous inflammation. However, the

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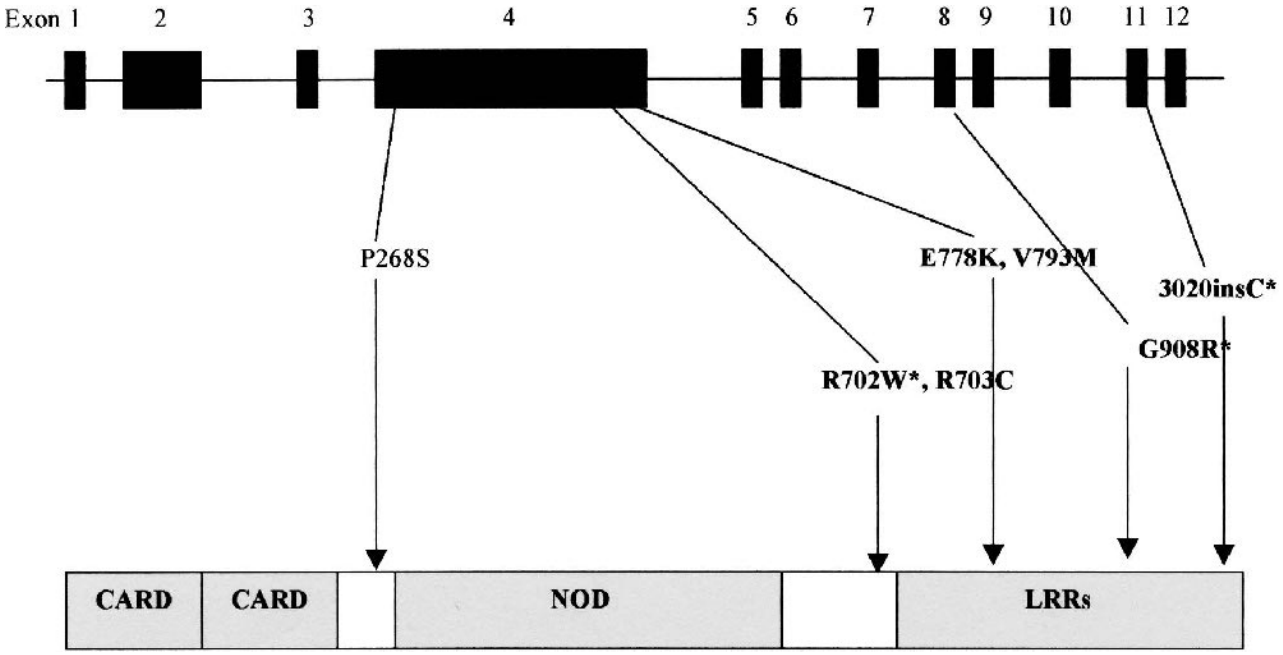

FIG. 1. Distribution of the nonconservative mutations along *CARD15.* Note: the three main disease-associated mutations are marked with an asterisk, the remaining mutations in boldface are likely to be disease associated, and P268S is not disease associated (15).

mechanism by which the mutations affect the pathway of apoptosis or NF-κB activity remains unknown (8).

It has been suggested that genetic factors are particularly important in early onset inflammatory bowel disease (IBD) (5), but until now, no large groups comprising only children have been studied. The purpose of our study was to examine the *CARD15* genotype in pediatric patients and to identify associations with their phenotypes.

MATERIALS AND METHODS

From January 1, 1982, to March 1, 2002, in the Pediatric Clinic of the Technical University of Dresden, 108 patients younger than 18 who had diagnoses of IBD agreed to participate in the genetic study. Fifty-five of them had CD. Venous blood samples were obtained from these patients (all parents gave written informed consent) and from 101 healthy control subjects with the same ethnic background; the control subject blood samples came from a German blood bank in Saxony. All patients were seen by two gastroenterologists (J. Henker, U. Winkler), and all medical records were reviewed by two investigators (A. Thürigen, L. Sun). The diagnosis of CD was confirmed by clinical, radiologic, endoscopic, and histologic analysis according to the criteria of Lennard-Jones (10). We excluded patients in cases of uncertainty or indeterminate colitis.

Genotype analysis

Purification of genomic DNA

For isolation of DNA the Invisorb® Spin Blood Kit (Berlin-Buch, Germany) was used according to the protocol provided.

Polymerase chain reaction conditions

A pair of primers was designed for each exon of *CARD15* (GenBank accession number AF178930) except for exons 2, 4, 5, and 6. For exon 2, two overlapping fragments were amplified; and for exon 4, four overlapping fragments were amplified; exon 5 and 6 used the same amplified fragment (Table 1). Thermostable DNA polymerase, Ampli Taq Gold, and 10 × buffer containing 15 mM MgCl2 were purchased from Perkin Elmer (Weiterstadt, Germany). Five microliters of buffer were mixed with 2.5 μL dioxyribonucleotides (dNTPs) (10 mM of each), 2.5 μL of each primer (250 ng), 5 μL genomic DNA solution, 0.5 μL polymerase (2.5 U), and 37 μL double-distilled H_2O (ddH_2O). The cycle program included 10 minutes at 94°C to denature the DNA, 35 cycles of 45 seconds at 94°C, 45 seconds at annealing temperature adapted to the primer pairs (54°C–62°C), 90 seconds at 72°C, and 5 minutes final extension at 72°C. Synthesis of appropriately sized polymerase chain reaction (PCR) products was confirmed by agarose gel electrophoresis. The PCR product was cleaned from primers and other low–molecular-weight compounds by Microcon-50 filter devices (Amicon, Witten, Germany).

Cycle sequencing

The BigDye Terminator Cycle Sequencing Reaction was purchased from Perkin Elmer to determine mutations in the *CARD15* gene according to the protocol provided. Briefly, after mixing 0.5 μL of the described PCR products (approximately 50 ng DNA) with 1.0 μL primer (50 ng), 4 μL terminator ready reaction mix (containing Mg^{2+}, pyrophosphatase, and polymerase), and ddH_2O to a final reaction volume of 20 μL, the following cycle conditions were applied: ×25, 96°C for 30 seconds, at annealing temperature adapted to the primer

TABLE 1. *Primers used for PCR-based direct genomic sequencing*

Exon	Primer		Size of PCR product (bp)
	Forward	Reverse	
1	TTG TGC CAG AAT TGC TTG GAA TT	GCC CTG GAG TGG TTC TCA GCT	513
2a	GGG GAG CTT GGA TTG GGT AAT	GCC GGT GAC TCT GCA GGT CT	484
2b	GCC AGC CTC TCT CCC ACT T	TAG CCA GGG TAA CAG AGT GAG AA	531
3	TTG GAT GCA TGC GTT CAA TTC	CGG GGT GGT GCT AGC CTT AA	451
4a	GGC TCT CCT ATC CCT TCA GTT AT	AAG AGC AGG GTC TGG ACA GAG	689
4b	GCA GGG CAA GAC TTC CAG GAA TT	CAG GCC CCA CAG AGC CAG TCT	723
4c	GTG TCC AAA TGC CAC CAG GAA CTG T	CCA GAC ACC AGC GGG CAC AG	661
4d	TTC CTG GCA GGG CTG TTG T	GCT GGG CCC AGA CTT CTA GAG	530
5,6	GCT GGC ACT TCA GGG ATG AAT GAA	CAG TGC CAG GCC AAG ACC AGA TC	574
7	CCT CCC GGG CAG GTC TTC AAT	GGG GCC TCC TGG CTG AAG AGT	292
8	CTG CCC CTC TGG CTG GGA CTG	TGG CCC CAG CTC CTC CCT CTT	258
9	CCA CGA ATT TTG CCC TCC ATA G	GGC CAG GGG ATC AAC AGA GAT	230
10	AAT CCC CAC AAC GTA CTT TAT CT	AGA CCA AGG CAA GCT GTT CTA	365
11	CAT TGG GAA TCT CAG ACA TGA G	GAA GGG ATC CTC AAA ATT CTG	336
12	CTG CAG CTG GGC CAG AGA GTC	AAG AGG CTG CCA ACA CAT GTC AC	286

pairs (54°C–62°C) for 15 seconds, and 60°C for 4 minutes, 4°C forever (Trio Thermoblock, Biometra, Göttingen, Germany).

The sequence was determined using the ABI 310 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The whole sequence of the coding regions, including adjacent intronic regions, was determined in each patient and control subject. The DNA sequence of each patient was compared with the published *CARD15* gene sequence (GenBank accession number AF178930) and the sequence of a healthy control subject using the ABI 310 sequence navigator software, version 1.0.1 (Applied Biosystems).

Phenotype analysis

The phenotypic classification was based on disease localization and behavior. Family history was defined as having at least one first- or second-degree relative with CD. Localization was determined by radiologic, histologic, or endoscopic examination. It was divided into three categories: upper gastrointestinal tract (including jejunum or upper ileum), ileocolonic (terminal ileum or terminal ileum and ascending colon), and colon (transverse colon, descending colon, sigmoid, or rectum). Complications included stricturing and fistulizing disease. Extraintestinal manifestations included eye, skin, joint, lung, hair, bone, and liver involvement (e.g., arthritis, arthralgia, uveitis, erythema nodosum). Surgical intervention was defined as any operative procedure for fistulizing or perforating disease, intractable disease, and the need for gut resection.

Statistics

Data were evaluated with the SPSS 10.0 and Epi Info 5.0 (SPSS Inc., Chicago, IL, USA, and WHO, Gent, Switzerland) software. The following tests were performed to analyze possible associations with mutations: univariate logistic regression for age at onset of CD, χ^2 method (cross-table analyses) and Fisher exact test for family history, disease localization, complications, extraintestinal manifestations, and intestinal resection. χ^2 testing was also used to compare the published mutation frequency with the respective frequency in our patients and to compare the 3020insC frequency in our patients with that of the control subjects.

RESULTS

Genotype

We have sequenced all 12 exons of the *CARD15* gene (including 80–200 bp of the adjacent introns) in 55 patients with CD and exon 11 in 101 control samples from healthy donors. Fourteen different nucleotide alterations, as compared with the published standard sequence, were found in the patients (Table 2). Most of them were located in exon 4 (length of 1816 bp); no variations were seen in exon 3 (106 bp), exon 5 (84 bp), exon 6 (86 bp), exon 7 (84 bp), or exon 12 (70 bp). Of the 14 sequence alterations, 3 occurred in the noncoding regions (UTR) and were even more common than the standard sequence. Except for the cytosine insertion mutation (3020insC), the variants resulted in single base-pair substitutions. Of these, eight were caused by transition-type changes (5 G→A, 5 C→T). Of the 11 mutations in the coding regions, three (S178S, R459R, R587R) were silent, one was conservative (V955I), and the other seven were all nonconservative missense mutations. However, one of these, P268S, was not considered to cause CD because of its tight linkage disequilibrium with other mutations (3,5).

The allele frequencies of the three main disease-associated mutations, R702W, G908R and 3020insC, were calculated to be 14%, 5%, and 26%, respectively. The frequencies for the other three missense mutations, R703C, E778K, V793M, which are likely to be disease associated (5), were all 2% (two patients each carried one of the mutations). Nine patients were homozygous for 3020insC, 2 for R702W, and 1 for G908R, but no patient was homozygous for one of the three rare mutations. In addition, 7 patients had two different mutations (Table 3); 17 had one mutation; and 19 of the pediatric patients with CD had none of the six mutations.

We confirmed that 3020insC in exon 11 was significantly more common among patients with CD (allele

TABLE 2. *CARD15 genetic variants observed in 55 patients with CD*

Location and nucleotide change	Peptide change	Polymorphic marker	Protein domain	No (%) of variant alleles (n = 110)	No (%) of patients (n = 55)
IVS 1					
5′ UTR -59 G → A	Unknown	SNP-EX1.59		62 (56%)	41 (74%)
IVS 2					
5′ UTR -25 G → T	Unknown	SNP-I1.26		60 (55%)	41 (75%)
Exon 2					
534 C → G	S178S		CARD2	39 (35%)	29 (53%)
Exon 4					
802 C → T	P268S	SNP5		63 (57%)	41 (75%)
1377 C → T	R459R	SNP6	NBD	60 (55%)	40 (73%)
1761 T → G	R587R	SNP7		36 (33%)	29 (53%)
2104 C → T	R702W*	SNP8		15 (14%)	13 (24%)
2107 C → T	R307C+			2 (2%)	2 (4%)
2332 G → A	E778K+		LRR2	2 (2%)	2 (4%)
2377 G → A	V793M+		LRR2	2 (2%)	2 (4%)
Exon 8					
2722 G → C	G908R*	SNP12	LRR6	5 (5%)	4 (7%)
Exon 9					
2863 G → A	V955I		LRR8	12 (11%)	11 (20%)
IVS 10					
5′ UTR +64 A → T				85 (77%)	46 (84%)
Exon 11					
3020insC	1007fs*	SNP13	LRR10	29 (26%)	20 (36%)

Note: The seven nonconservative mutations are indicated in boldface, the three main disease-associated mutations are marked by an asterisk, and the three mutations likely to be disease associated are marked by +.

frequency 26%) than among healthy control subjects (2%), ($P < 0.00000001$; OR = 17.72). Twenty (36%) patients, but only 4 (4%) healthy donors, had this mutation. All of these four control subjects were heterozygotes, and no homozygote has ever been discovered (Table 4).

Two of the disease-associated mutations (R702W, R703C) detected in our patient population were located between the nucleotide-binding oligomerization domain and the LRR domains; the other four mutations (E778K, V793M, G908R, 3020insC) were found within the LRR domain (Fig. 1).

Genotype–phenotype relationship

The medical records of the 55 patients with CD were reviewed in detail. Patient age at onset, family history, disease localization, complications, and intestinal resection surgery were analyzed (Table 5). The average age at onset was 11.2 years (1–17.5 years).

Patients were divided into three groups (A0, no mutation; A1, one mutation; A2, two mutations). Table 5 shows the mutation distributions in the different subgroups of CD and the genotype–phenotype relationships. A significant association was found between *CARD15* mutations and a localization in the ileocolonic region ($P = 0.005$). In addition, there was a nonsignificant trend for a higher risk to acquire CD in younger patients with disease-associated mutations and for stricturing in patients with such mutations. The involvement of the transverse colon, left colon, or rectum and extraintestinal manifestations were relatively less common in this patient subgroup, but these trends did not reach significance. In contrast, a positive family history, the involvement of the upper gastrointestinal tract, and fistulizing were in the same range in all groups (A0, A1, or A2). We also evaluated a possible association between intestinal resection surgery and *CARD15* mutations. As shown in

TABLE 3. *Pediatric patients with more than one of the six disease-associated mutations*

Genotype	Patients
3020insC homozygous	9
R702W homozygous	2
G908R homozygous	1
R702W/3020insC	3
G908R/3020insC	1
G908R/V793M	1
V793M/3020insC	1
G908R/E778K	1
Total	19 (35%)

TABLE 4. *C-insertion mutation (3020insC) frequency in patients and control subjects*

Genotype	Patients (n = 55)	Control subjects (n = 101)
–/–	35 (64%)	97 (96%)
–/+	11 (20%)	4 (4%)
+/+	9 (16%)	0 (0%)

Note: +: mutation, –: wildtype.

TABLE 5. *Genotype–phenotype relationship*

Phenotype	Genotype no. (%) A0	A1	A2	P one sided	OR	95% CI	P¶ two sided
Age of onset	13 y (4.1–17.5 y)*	12 y (1–16 y)	11.8 y (3.6–15.6 y)	0.152†	0.991†	0.978–1.003†	
Family history							
+	5 (38.5%)‡	4 (30.8%)	4 (30.8%)	0.490§	0.800	0.221–2.902	0.93
–	14 (33.3%)	13 (31.0%)	15 (35.7%)				
Localization							
UGT							
+	11 (36.7%)	9 (30.0%)	10 (33.3%)				
–	8 (32.0%)	8 (32.0%)	9 (36.0%)	0.470§	0.813	0.265–2.495	0.93
Ileocolon							
+	8 (21.6%)	13 (35.1%)	16 (43.2%)				
–	11 (61.1%)	4 (22.2%)	3 (16.7%)	0.005§	5.696	1.667–19.471	0.014
Colon							
+	16 (40.0%)	12 (30.0%)	12 (30.0%)				
–	3 (20.0%)	5 (33.3%)	7 (46.7%)	0.142§	0.375	0.091–1.543	0.33
Complication							
Stricturing							
+	6 (25.0%)	8 (33.3%)	10 (41.7%)				
–	13 (34.5%)	9 (29.0%)	9 (29.0%)	0.153§	2.167	0.674–6.962	0.40
Fistulizing							
+	5 (41.7%)	3 (25.0%)	4 (33.3%)				
–	14 (32.6%)	14 (32.6%)	15 (34.9%)	0.397§	0.676	0.182–2.512	0.82
Extraintestinal manifestations							
+	12 (42.9%)	8 (28.6%)	8 (28.6%)				
–	7 (25.9%)	9 (33.3%)	11 (40.7%)	0.150§	0.467	0.149–1.460	0.40
Surgery							
+	4 (23.5%)	3 (17.6%)	10 (58.8%)				
–	15 (39.5%)	14 (36.8%)	9 (23.7%)	0.032‖	5.185	1.114–24.142	

* Median age of onset (youngest, oldest); †Logistic regression (age as quantitative variable); ‡Number (%) of patients; §χ^2 testing, A1 + A2 vs. A0; ‖Unadjusted, A2 vs. A1; OR: odds ratio; CI: confidence interval; ¶χ^2 testing, A0 vs. A1 vs. A2; A0: no mutation; A1: one mutation; A2: two mutations; +: present, –: not present. Family history: having a first- or second-degree relative with CD; UGT: upper gastrointestinal tract; ileocolon: terminal ileum or terminal ileum and ascending colon; colon: transverse colon, descending colon, sigmoid, and rectum; surgery: intestinal resection.

Table 5, more than half of the patients (10 of 19) with two mutations had intestinal resections ($P = 0.032$, A2 versus A1), indicating that this genotype (A2) could cause a more severe clinical course of CD.

DISCUSSION

Mutations in the *CARD15* gene have been found to predispose patients to CD (3,4). Our study confirmed the strong association of the *CARD15* frameshift mutation (3020insC) with this disorder (Table 4); 36% of our patients, but only 4% of the control blood donors, had the mutation. The 55 pediatric patients and 101 healthy blood donors to a Saxonian blood bank were ethnically homogeneous because of the political situation of the former German Democratic Republic, which highly restricted the mobility of Saxons. The allele frequency (2%) of this mutation in our control samples was the same as in the healthy population studied by the French group (5). They examined the genotypes of 453 patients with CD who were recruited from a large European consortium involving clinicians from Belgium, Denmark, France, Germany, Ireland, Italy, Spain, and Sweden (5). The average age at disease onset was 19.4 years (range, 2–56 years). We found that the 3020insC mutation was significantly more common in our pediatric patients than in their patient group, which had a majority of adults, although the background populations were comparable (26% versus 11% of the alleles, $P = 0.000002$). Two other mutations, R702W and G908R, had similar allele frequencies in our pediatric patient and the patients reported in the literature (Table 6). In contrast, for three rare mutations (R703C, E778K, V793M), which were also found in our pediatric patients, there is no firm evidence they cause CD. Nevertheless, according to Lesage et al. (5), they are likely to induce the disease.

We used direct sequencing of the *CARD15* gene, whereas other groups partially relied on screening methods (5,11,12). Although we have sequenced all coding and adjacent intronic regions of the *CARD15* genes of our patients, we did not find a new mutation associated with the disease. All genetic alterations found, other than the six already known to be disease related, occurred frequently and were located in intronic regions without known motives (e.g., for splicing) or were silent, conservative, or have already been described as a mere polymorphism (P268S) (3). Therefore, the number of different disease-associated mutations in the *CARD15* gene is probably limited. Interestingly, the six disease-related mutations (3020insC, R702W, G908R, R703C, E778K,

TABLE 6. *Allele frequencies comparison between pediatric and published patients*

Mutations	Allele frequency	
	Current study pediatric patients	Published patients
3020insC	26%	11%
R702W	14%	11%
G908R	5%	6%
R703C	2%	1%
E778K	2%	0.3%
V793M	2%	0.4%

Note. Lesage et al. (5) studied 453 patients with CD from Europe, both adults and children were included, but the majority were adults.

and V793M) found in our patients and those reported in the literature were all located in or adjacent to the LRRs region of *CARD15* (Fig. 1), indicating a common functional defect (see Introduction).

Another striking difference between our pediatric patients and those reported in the literature was the much higher frequency of two mutations in the pediatric group (35% versus 17%, Table 3). This confirmed that the patients with early age at disease onset had a greater degree of genetic influence. However, it was not always possible to clarify if the two mutations found were located on one or on two alleles. Homozygous patients clearly had two affected alleles. We had no cDNA samples available from patients with two different mutations to separate the alleles by cloning. Therefore, it can only be assumed that they were compound heterozygous. At least, it has already been shown by haplotyping that the variants R702W, G908R, and 3020insC were never found on the same haplotype (3).

Consistent with the findings of previous studies (5,11–14), our results confirmed that there was a strong association between these disease-related mutations and CD localization in the terminal ileum and ascending colon (ileocolon, Table 5). It is tempting to hypothesize a connection between this localization at a junction of a sterile gut region with a region populated with bacteria, components of these bacteria such as lipopolysaccharide, and the mutational alterations in the *CARD15* protein responsible for regulation of inflammation. Stricturing, which necessitates intestinal resection, is an indication of a severe clinical course of CD. Such severe disease occurred more frequently in patients with two mutations (Table 5) than in those with no or one mutation and thus was more common in pediatric than in adult patients.

The trends we found in clinical associations, which did not reach significance, were also in accordance with the findings of others (Table 5) (5,11,12). Younger age at disease onset and stricturing were associated with CARD15 mutations, whereas CD localization proximal to the terminal ileum or distal to the ascending colon and extraintestinal manifestations may be linked to other IBD susceptibility genes, e.g. tumor necrosis factor (TNF)-α(-857C) (15).

Future studies are needed to clarify the connection between functional alterations of proteins involved in inflammation and different manifestations of CD.

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