THE AMERICAN JOURNAL OF GASTROENTEROLOGY
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Published by Elsevier Science Inc.

Vol. 98, No. 3, 2003
ISSN 0002-9270/03/$30.00
doi:10.1016/S0002-9270(02)06009-4

CARD15 and Crohn's Disease: Healthy Homozygous Carriers of the 3020insC Frameshift Mutation

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OBJECTIVES: Single nucleotide variations in the *CARD15* gene have recently been shown to be associated with Crohn's disease (CD). Of special interest is a cytosine insertion at position 3020 of exon 11 (3020insC), which leads to a stop codon, truncation of the *CARD15* protein, and an altered function of *CARD15*. The aim of the study was to evaluate this frameshift mutation in Dutch, multiple-affected families with inflammatory bowel disease (IBD).

METHODS: Ninety-three Caucasian, multiple-affected families with IBD were recruited by interviewing patients attending our department. Sixty-one probands had CD, and 32 probands ulcerative colitis (UC). The diagnosis of probands and affected family members was verified according to standard criteria. In addition, 81 healthy, unrelated controls were included. Genomic DNA was isolated from venous blood of all participants to determine the *CARD15* 3020insC mutation by using an allele-specific polymerase chain reaction, followed by agarose gel electrophoresis and DNA sequencing.

RESULTS: Association with *CARD15* 3020insC was statistically significant for CD, but not for UC. In one of the multiple-affected families, middle-aged and elderly homozygous carriers were identified without CD.

CONCLUSIONS: Although *CARD15* 3020insC appears to be etiologically important in CD, homozygous carriage does not always lead to IBD. (Am J Gastroenterol 2003;98: 613–617. © 2003 by Am. Coll. of Gastroenterology)

INTRODUCTION

The etiology of inflammatory bowel disease (IBD), a chronic recurrent gastrointestinal disease, is complex and largely unknown. Genetic susceptibility, however, plays an important role, as demonstrated by several epidemiological studies (1). Additionally, during the 1990s several genome-wide linkage analyses with multiple, highly polymorphic microsatellite markers in multiple-affected families with IBD have revealed putative disease loci. The results of these studies are summarized in Table 1.

A pericentromeric locus on chromosome 16 was the first genomic susceptibility region of interest, described by Hugot *et al*. in 1996. This so called "*IBD1* locus" appeared to be linked with Crohn's disease (CD) (2). The findings were replicated by others (3–13). Absence of linkage of CD with the *IBD1* locus has also been reported (14, 15).

Linkage of CD with the *IBD1* locus appears to be based on genetic alterations in the *CARD15* gene. The *CARD15* gene is located on chromosome 16q12 and was described by Ogura *et al.* in 2001 (16). Three single nucleotide variants appeared to be highly associated with CD. One specific alteration involves a cytosine insertion at position 3020 of exon 11, leading to a stop codon and truncation of the *CARD15* protein. If affects the C-terminal leucine-rich repeats (LRRs), shortening this protein domain by 33 amino acids. This 3020insC frameshift mutation showed preferential transmission in families with CD but not in families with ulcerative colitis (UC) (17, 18). The spectacular findings have been subsequently confirmed by several other groups (19–26).

The goal of this study was to study the prevalence of *CARD15* 3020insC in Dutch, IBD-affected probands and their (non-)IBD-affected family members.

MATERIALS AND METHODS

Patients and Families

Multiple-affected families with IBD were identified by interviewing IBD patients attending the Departments of Gastroenterology, Internal Medicine, and Surgery of the Erasmus Medical Center Rotterdam. Probands, affected relatives, and available nonaffected relatives completed a questionnaire about IBD and other diseases. Based on information supplied by their physicians, the diagnosis was verified according to standard criteria (27).

The study was approved by the Ethics Committee of the Erasmus Medical Center Rotterdam. All participants gave written, informed consent.

Table 1. Summary of Genome Screens in IBD

Author	Significant Results of Linkage Analysis (33)	
	Chromosome	Disease
Hugot *et al.*, 1996 (2)	16 (IBD1 locus)	CD
Satsangi *et al.*, 1996 (34)	12q (IBD2 locus), 7q, 3p	CD and UC
Cho *et al.*, 1998 (8)	1p, 3q, 4q	CD and UC
	16	CD
Hampe *et al.*, 1999 (11)	1q, 6p (IBD3 locus), 22	CD and UC
	10, 12, 16	CD
	4, X	UC
Ma *et al.*, 1999 (35)	12, 14q	CD
Duerr *et al.*, 2000 (36)	14q (IBD4 locus)	CD
Rioux *et al.*, 2000 (37)	3p, 5q (IBD5), 6p, 19q	CD and UC

DNA Isolation

Genomic DNA was isolated from whole peripheral venous blood collected in ethylenediaminetetraacetic acid–anticoagulated tubes (Becton Dickinson, Leiden, The Netherlands) using standard techniques. In some instances, DNA was isolated from Epstein-Barr virus–transformed lymphoblastoid cell lines (28), derived from peripheral venous blood collected in acid citrate dextrose solution–containing tubes (Becton Dickinson, Leiden, The Netherlands).

Genotyping

The *CARD15* 3020insC mutation was determined by means of an allele-specific polymerase chain reaction (PCR), followed by separation on agarose gels, as described by Ogura *et al.* (18). Briefly, a multiplex PCR with four primers was performed. Two control intronic primers were used, including forward 5′-CTGAGCCTTTGTTGATGAGC-3′ and reverse 5′-TCTTCAACCACATCCCCATT-3′ resulting in a 533 base-pairs (bp) fragment including position 3020 of exon 11. Two additional exonic primers were used to discriminate between the wild-type and 3020insC allele, including reverse 5′-CGCGTGTCATTCCTTTCATGGGGC-3′ and forward 5′-CAGAAGCCCTCCTGCAGGCCCT-3′. These primers result in a 319 bp–containing wild-type fragment, or a 214 bp fragment in the case of a 3020insC allele. The PCR products were identified by electrophoresis on a 2% agarose gel and ethidium bromide staining. When participants appeared positive for *CARD15* 3020insC, the mutation was confirmed by DNA sequencing of a exon 11 PCR fragment amplified with the forward primers 5′-AGGATGTGTCTA-AGGGACAG-3′ and reverse primer 5′-CTGAATGTCA-GAATCAGAAGG-3′. Sequencing was performed on an ABI 310 genetic analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands).

In one family, non-IBD homozygous *CARD15* 3020insC mutation carriers were found. To exclude these results to be caused by an unknown sequence variation in the binding site of the primers, a second confirmation test was used. Exon 11 was amplified and sequenced with the two control intronic primers mentioned earlier.

Statistics

Association was determined by comparing the frequencies of the *CARD15* 3020insC frameshift mutation in cases with CD and controls, using the Fisher exact test. It was expressed as relative risk (RR) with a 95% CI. The genotype relative risk (GRR) was evaluated by comparing the genotype frequencies in cases and controls. For cases, the observed frequencies were used, whereas for controls the genotype frequencies were estimated assuming the genotypes to be in Hardy-Weinberg equilibrium proportions (*i.e.*, assuming no selection against the homozygotes) (18). The analyses were performed with SPSS 8.0 software (SPSS, Chicago, IL).

RESULTS

Ninety-three IBD-affected probands with either CD (n = 61) or UC (n = 32), and with a positive family history for IBD were screened for the *CARD15* 3020insC frameshift mutation. In addition, 81 unrelated healthy controls were examined.

Fourteen (23%) probands with CD, one (3%) proband with UC, and 3 (4%) healthy controls were carriers of the mutation. Three CD-affected probands were homozygous for the frameshift mutation. All other carriers, including UC-affected probands and controls, were heterozygous. The allele frequency of the *CARD15* 3020insC among patients with CD was 14% and approximately 2% in UC patients and in controls. The frequency of the frameshift mutation was significantly higher in CD patients than in controls ($p < 0.0001$, Fisher exact test). No association was found with UC.

In probands with CD and heterozygous for *CARD15* 3020insC, the GRR was 6.1 (95% CI = 1.6–23.7). As the *CARD15* 3020insC genotype frequencies in controls were assumed to be in Hardy-Weinberg equilibrium proportions ($p = 0.87$), we could estimate the expected frequency of homozygous frameshift carries in the control population. We used this to calculate the GRR for the homozygous carriers, which was 178 (95% CI = $0–\infty$).

Subsequently, available IBD-affected relatives of the 14 probands with CD who were positive for *CARD15* 3020insC were screened for the mutation. The data are summarized in Table 2. Most affected relatives were concordant for disease as well as for carriage of the mutation.

In addition, available non–IBD-affected relatives were screened for carriage of *CARD15* 3020insC when the proband had the frameshift mutation. In one family with a homozygous CD-affected proband, two sisters, the father, and an aunt (sister of the father) were also homozygous carriers. Except for one sister, all these relatives had no symptoms or signs of IBD or other major GI health problems. One relative (sister) was diagnosed with IBD, based on a normal colonoscopy and blood tests. Follow-up of these non-IBD family members was available until the beginning of 2002 (range of age 42–83 yr). The pedigree of

Table 2. *CARD15* genotype of IBD-Affected Relatives of Probands With CD Who Carried the *CARD15* 3020insC Frameshift Mutation

	CD-Affected Relatives		UC-Affected Relatives	
Familial Relation to Proband With CD	3020insC Positive	3020insC Negative	3020insC Positive	3020insC Negative
1st degree	4	1	1	0
2nd degree	1	1	0	1
3rd degree	4	2	0	0
>3rd degree	3	3	0	3
Not available	1*		1†	

* A 3rd degree family member was not available for testing.
† A 2nd degree family member died before starting this study.

the family is shown in Figure 1. Individual details are given in Table 3. In another family with a *CARD15* 3020insC homozygous CD-affected proband, the father was heterozygous for the mutation but appeared to be have UC.

DISCUSSION

The *CARD15* (formerly *NOD2*) gene is a recently described gene (16). Functionally, the *CARD15* gene product has been predicted to have similar activity to the apoptotic protease activating factor-1, which is a regulator of apoptosis (29). The structure of the *CARD15* protein is similar to that of *CARD4* (formerly *NOD1*) (16). *CARD15* is located on chromosome 16q12 within the *IBD1* locus. It seems to play an important role in the etiology of CD, as three single nucleotide variants are highly independently associated with the disease (17, 22).

CARD15 is a cytosolic protein expressed in monocytes. Its C-terminal region is presumed to be involved in recognition of bacterial lipopolysaccharide (LPS) through the LRRs. Binding or interaction of LPS with the LRRs domain triggers activation of the transcription factor nuclear factor kappa-B (NFκB) through two N-terminal caspase recruitment domains (CARD). NFκB is an important regulator of genes involved in a proinflammatory response (30).

One of the three CD-associated nucleotide variants is a 3020insC in exon 11, which results in a truncated *CARD15* protein missing the final 33 amino acids of the LRRs. It probably leaves the CARD motifs and the centrally located nucleotide-binding domain functionally intact (17, 18). Embryonic kidney cells (HEK293T) transfected with 3020insC-mutated *CARD15* plasmids showed a reduced activity of NFκB after exposure to LPS from various bacteria, compared with wild-type *CARD15*-transfected cells (18). Based on these experiments, the *CARD15* frameshift mutation is thought to result in an altered monocytic response to bacterial components in CD. Whether this reduced NF-κB response to LPS under artificial circumstances is relevant to the pathogenesis of Crohn's disease is not clear, as colonic tissue from CD patients has been found to have increased

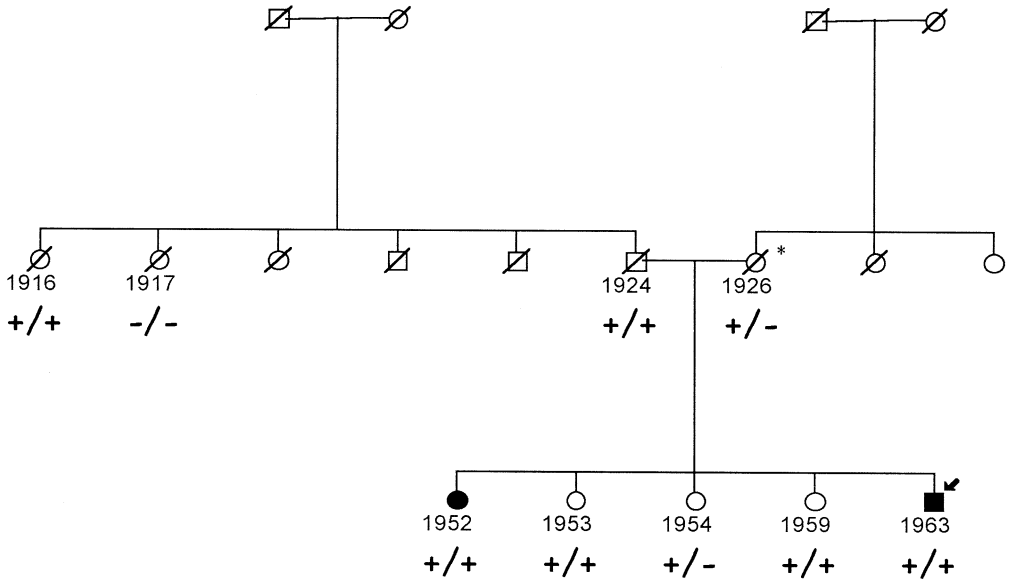

Figure 1. A multiple IBD-affected family with healthy homozygous *CARD15* 3020insC carriers. In one of the included multiple IBD-affected families, healthy homozygous *CARD15* 3020insC carriers were identified. ■ = CD-affected male; ● = CD-affected female; □ = non–IBD-affected male; ○ = non–BD-affected female; ↙ = proband; +/+ = homozygous for *CARD15* 3020insC; +/− = heterozygous for *CARD15* 3020insC; −/− = homozygous carrier of wild-type *CARD15*. Row below individuals = year of birth. *reconstructed genotype.

Table 3. Characteristics of an IBD Family With Affected and Nonaffected Homozygous *CARD15* 3020insC Frameshift Mutation Carriers

	Year of Birth (Year of Death)	*CARD15* 3020insC Genotype	Age of Diagnosis CD (yr)	Location of CD	Other Relevant Diseases
Proband	1963	+/+	16 (1979)	Small bowel, rectum	Uveitis, sacroiliitis
Sister of proband	1952	+/+	29 (1981)	Small bowel	
Sister of proband	1953	+/+			IBS, normal colonic barium study in 1995
Sister of proband	1954	+/−			IBS, endoscopy at early adolescence was normal
Sister of proband	1959	+/+			
Father of proband	1924 (1999)	+/+			No history of abdominal disorders Died of an acute myocardial infarction
Mother of proband	1926 (1988)	+/−*			No history of abdominal disorders Died due to metastatic cancer of unknown primary
Sister of father	1916 (2000)	+/+			No history of abdominal disorders Known with cardiovascular disease, diabetes mellitus, and chronic obstructive pulmonary disease
Sister of father	1917 (2000)	−/−			History of irritable bowel disease. Colonoscopy was normal in 1997. Also history of cerebral vascular accident

* Reconstructed.

rather than decreased NF-κB activity compared to that of controls (31).

Carriage of the *CARD15* 3020insC mutation results in an increased RR of developing CD and appears to be a risk factor for early onset of disease (20), location of disease in the small bowel (20, 21, 24–26), and development of stenosis (20, 22, 24, 25). Whether carriage is also associated with fistulas is not yet clear (20, 24). In the initial studies on the prevalence of *CARD15* 3020insC mutation in IBD, no homozygous healthy controls have been detected. In these studies, the RR of homozygous CD-affected individuals was estimated to be as much as 18–42-fold, assuming that the genotype frequencies in controls were in Hardy-Weinberg equilibrium proportions (18, 19). In this study also, no unrelated homozygous controls were found. However, we did find homozygous carriers of *CARD15* 3020insC within a family of two CD-affected siblings. These homozygous relatives had never had important GI complaints or features suggesting IBD. They remained asymptomatic 27–67 yrs beyond the age of diagnosis of the proband. Searching the literature for other reports of non-IBD homozygous carriers, we found only one other study on the prevalence of *CARD15* in psoriasis, which reported a 60-yr-old healthy homozygous carrier in the control group (32). These results demonstrate that homozygosity does not necessarily lead to manifest IBD, even at older age.

If the mutation by itself were a major determinant of CD, one would expect, by analogy with other genetic diseases, homozygosity to result in a more severe phenotype. Other factors, both genetic and environmental, must be involved in the pathogenesis of CD. As *CARD15* mutations have only been found in a minority of CD patients (17–19), it is likely that IBD is not a single disease but rather a group of etiologically and genetically distinct diseases with similar clinical presentations. The various results of genome-wide linkage studies in IBD (Table 1) also support this multigenetic pathogenesis.

In conclusion, this study confirms the association of *CARD15* 3020insC with CD. Homozygous carriage, however, does not always lead to disease. Further studies on the function of both wild-type and truncated *CARD15* protein will help unravel the role of this frameshift mutation in CD.

ACKNOWLEDGMENTS

This work was funded by a grant of the Revolving Fund of the Erasmus Medical Center Rotterdam, Rotterdam, The Netherlands.

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Received Apr. 8, 2002; accepted Sep. 27, 2002.

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