Association between insertion mutation in *NOD2* gene and Crohn's disease in German and British populations

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Summary

Background Genetic predisposition to inflammatory bowel disease (IBD) has been shown by epidemiological and linkage studies. Genetic linkage of IBD to chromosome 16 has been previously observed and replicated in independent populations. The recently identified *NOD2* gene is a good positional and functional candidate gene since it is located in the region of linkage on chromosome 16q12, and activates nuclear factor (NF) κB in response to bacterial lipopolysaccharides.

Methods We sequenced the coding region of the *NOD2* gene and genotyped an insertion polymorphism affecting the leucine-rich region of the protein product in 512 individuals with IBD from 309 German or British families, 369 German trios (ie, German patients with sporadic IBD and their unaffected parents), and 272 normal controls. We then tested for association with Crohn's disease and ulcerative colitis.

Findings Family-based association analyses were consistently positive in 95 British and 99 German affected sibling pairs with Crohn's disease (combined p<0·0001); the association was confirmed in the 304 German trios with Crohn's disease. No association was seen in the 115 sibling pairs and 65 trios with ulcerative colitis. The genotype-specific disease risks conferred by heterozygous and homozygous mutant genotypes were 2·6 (95% CI 1·5–4·5) and 42·1 (4·3–∞), respectively.

Interpretation The insertion mutation in the *NOD2* gene confers a substantially increased susceptibility to Crohn's disease but not to ulcerative colitis.

Lancet 2001; **357:** 1925–28
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Introduction

Epidemiological and genetic linkage studies have shown the cause of inflammatory bowel disease (IBD) and its constituent clinical phenotypes Crohn's disease and ulcerative colitis to have a genetic component. Epidemiological investigations have consistently shown familial clustering[1] and an increased concordance of the IBD phenotype in monozygotic twins.[2,3]

An IBD1 susceptibility region on chromosome 16 was detected by genome-wide linkage analysis,[4] and this finding has been replicated in several independent populations,[5–7] and by the International IBD Consortium.[8] This region of linkage is therefore the most widely and consistently replicated in IBD.

Previous pathophysiological research has shown that signalling by tumour necrosis factor and activation of nuclear factor (NF) κB in mononuclear cells have a key role in IBD.[9,10] Therefore, genetic variants that would lead to increased or persistent NFκB activation are of particular interest. *NOD2*—a member of the *NOD1*/*APAF1* gene family—has been identified and mapped to chromosome 16q12.[11] This gene family has a role in inflammatory responses to bacterial triggers, especially lipopolysaccharides, through activation of NFκB,[12,13] and a submission to an electronic database suggested the presence of *NOD2* polymorphisms in Crohn's disease (NCBI locus ID 64127).

NOD2 is expressed exclusively in monocytes. Creation of truncation variants of the protein product in the original identification study[11] showed that only the deletion of the leucine-rich region leads to increased activation of NFκB. We therefore selected this leucine-rich region as a functional and positional candidate region for further exploration.

Participants and methods

Participants

The family cohorts investigated in this study were recruited by an international group of IBD investigators at the Charité University Hospital (Berlin, Germany), the Department of General Internal Medicine at the Christian-Albrechts-Universität (Kiel, Germany), St Mark's, Guy's and King's College Hospitals (London,

Cohort	Crohn's disease	Ulcerative colitis	Total
Cases			
British families*	95 (159)	53 (79)	148 (238)
German families*	99 (176)	62 (98)	161 (274)
German trios	304	65	369
Total	639	242	881
Controls	..	..	272

*Number of families (number of affected offspring).

Table 1: **Cohorts investigated**

Cohort investigated	Crohn's disease			Ulcerative colitis		
	Number of affected individuals	Observed/expected transmissions	p	Number of affected individuals	Observed/expected transmissions	p
Families						
British	159	33/23·9	0·0043	79	2/4·9	0·03
German	176	48/37·4	0·0048	98	8/9·5	0·38
Total	335	81/61·4	<0·0001	177	10/14.4	0·04
German trios	304	97/61	<0·0001	65	4/4·1	0·95

Table 2: **Family-based linkage and association tests in UK and German families and German trios**

UK), and other European centres. These cohorts have been used in previous studies within the collaborative group.[5,14–16] We also recruited German patients with sporadic IBD and their two unaffected parents (referred to as German trios). Normal controls were recruited through the Department of Transfusion Medicine at the Kiel University Hospital or the European Collection of Animal Cell Cultures (ECACC, Wiltshire, UK).

The diagnosis of either ulcerative colitis or Crohn's disease was confirmed by clinical, radiological, and endoscopic (type of lesions, distribution) analysis,[17,18] and histological findings also had to be confirmative or compatible with this diagnosis. In cases of uncertainty, indeterminate colitis was assigned and the patient was excluded from the study. A venous blood sample was obtained from the affected siblings and their parents, if possible. Informed, written consent was obtained from all study participants. Recruitment protocols were approved by ethics committees at participating centres before commencement of the study.

Methods

The *NOD2* gene was screened for mutations by genomic resequencing or denaturing high-performance liquid chromatography in unrelated individuals with IBD and controls. Primers for exon amplification were designed on the basis of the Genbank sequence NT_019610. 12 mutations were identified (accession numbers to dbSNP: ss2992219–24 and ss2992237–42). A C-insertion mutation identified in exon 11 in the region coding for the leucine-rich region leads to a premature stop codon at aminoacid 1007 of the encoded protein. On the basis of biochemical evidence presented by Ogura and colleagues,[11] this mutation is thought to lead to a dysfunctional leucine-rich repeat domain and thus to altered activation of NFκB. We therefore selected this mutation for genotyping under the primary hypothesis.

The C-insertion polymorphism was genotyped by use of the Taqman system (Applied Biosystems, Foster City, CA, USA) with the following primers: 5′-GTCC-AATAACTGCATCACCTACCTAG-3′ (forward), 5′-CTTACCAGACTTCCAGGATGGTGT-3′ (reverse), and 5′-CCCTCCTGCAGGCCCTTGAAA-3′ (wild-type probe), and 5′-CCTCCTGCAGGCCC-CTTGAAA-3′ (mutant probe). Genomic DNA was prepared from whole blood with the Puregene system (Gentra Systems, Minneapolis, MN, USA). Taqman-PCR products were read directly in an ABI 7700 analyser (Applied Biosystems). The data were managed and checked for Mendelian inheritance errors by means of an integrated database system.[19]

Statistical analysis

Genetic analyses were done with the two standard diagnostic categories Crohn's disease and ulcerative colitis. Family-based linkage and association statistics were calculated by use of the TRANSMIT program[20,21] with the robust variance estimator option. Significances were verified within TRANSMIT by 10 000 bootstrap samples. Case–control analyses were done with χ^2 statistics or Fisher's exact test. CIs for the odds ratios were calculated by Gaussian approximation or reference to the exact conditional distribution underlying cell counts.[22] The population attributable risk was calculated according to Hennekens and colleagues.[23]

Results

512 affected individuals from 309 multiplex families of German or British descent, 369 German patients with sporadic IBD, and 272 healthy controls were genotyped for the C-insertion mutation in exon 11 of *NOD2* (table 1). For the Crohn's disease phenotype, family-based tests showed a significant association for the British and German cohorts alone, and for both cohorts combined (table 2). A confirmatory analysis in the German trios (sporadic cases and their parents) yielded a similar result. Analyses for the ulcerative colitis phenotype were negative in both familial and sporadic cases (table 2).

To estimate the genotype relative risk, we did a case–control analysis of the patients with sporadic IBD and the 272 normal controls. The *NOD2* mutation was highly associated with the Crohn's disease phenotype (table 3).The population-attributable risk of the homozygous mutated genotype was 6·6%, whereas the population attributable risk of carriership of the mutation was 18·1%.[23] The ulcerative colitis phenotype was not associated with the *NOD2* mutation, confirming the results of the family-based association study.

A point estimate of the genotype relative risk of the homozygous mutant genotype was obtained as follows: assuming a population prevalence for Crohn's disease of 4/10 000,[24] the data in table 3 yield estimates of 0·033% for the penetrance of genotype −/− (wildtype), and 0·085% for the penetrance of genotype −/+ (heterozygous). Since

Genotype	Controls (n=272)	Crohn's disease (n=304)	Odds ratio (95% CI)	Ulcerative colitis (n=65)	Odds ratio (95% CI)
−/−	248 (91·2%)	227 (74·7%)	1·00	61 (93·8%)	1·00
−/+	24 (8·8%)	57 (18·8%)	2·6 (1·5–4·5)	4 (6·2%)	0·7 (0·2–2·2)
+/+	0	20 (6·5%)	42·1* (4·3–∞)	0	··

+ denotes mutation, −denotes wildtype. *Formally, this variable would be estimated as ∞. The quoted odds ratio has been derived under the assumption that genotype frequencies in the control population are in Hardy-Weinberg equilibrium and that the population prevalence of Crohn's disease is 4/10 000.

Table 3: **Mutation frequency in cases and controls**

Crohn's disease is rare, the frequency (f) of the mutant allele in the general population is about equal to the frequency seen among controls—ie, f=24/544=0·044. Then, under Hardy-Weinberg equilibrium, $p_{-/-}(1-f)2+p_{-/+}2f(1-f)+p_{+/+}f^2=4/10\ 000$ and thus $p_{+/+}=1{\cdot}389\%$. This low penetrance of genotype +/+ results from the fact that the population frequency of homozygotes ($0{\cdot}044^2=0{\cdot}002$), as expected at Hardy-Weinberg equilibrium, is five times higher than the prevalence of Crohn's disease. The lack of homozygote controls (table 3) would imply that the relative risk of genotype +/+ cannot be reliably approximated by its odds ratio. Under Hardy-Weinberg equilibrium, however, $p_{-/-}/p_{+/+}=42{\cdot}1$ provides a reliable alternative estimate for the relative risk of homozygotes.

Discussion

The results of our study provide evidence for association of a *NOD2* frameshift mutation with Crohn's disease. Consistent results were obtained in two different populations by means of family-based association analyses (estimation of transmission distortion) and case–control analysis. The mutation is quite rare—only about 6·5% of Crohn's disease patients are homozygous for it. We estimate that about 18% of the genetic risk in the population can be attributed to this mutation. The *NOD2* mutation confers a high degree of risk in homozygous individuals. We did not find any homozygotes in 272 control individuals.

NOD2 is a good candidate gene for inflammatory bowel disease in terms of position and function. It is located on chromosome 16q12 under the peak of the 16q linkage, and is a member of the *NOD1/APAF1* gene family.[11] It is expressed primarily in monocytes, and it activates NFκB after stimulation with lipopolysaccharides.[9,10] The frameshift mutation leads to truncation of the transcript in the leucine-rich repeat region. Truncation variants lacking this region have been shown to exhibit a five-fold greater activation of NFκB than the wildtype.[11] This truncation variant could thus lead to inappropriate activation of NFκB in response to bacterial products such as lipopolysaccharide. This hypothetical mechanism supports the current pathophysiological understanding of Crohn's disease. Increased activation of mononuclear cells[25] and activation of the NFκB system[10,26] have been consistently shown in IBD. The most effective treatment of steroid-refractory Crohn's disease with infliximab[27] also critically depends on suppression of the NFκB system.[28] However, further experiments need to be done to investigate the pathophysiological mechanisms induced by variations in the *NOD2* gene in the intestinal mucosa. Continuing studies are investigating the role of variants in the *NOD2* gene on the natural course of disease and response to therapies. The lack of effect of the described mutation on the ulcerative colitis phenotype might be caused by use of different pathways in this disorder. NFκB activation is also stronger in Crohn's disease than ulcerative colitis.[10] Two other groups have now reported associations between mutations in the *NOD2* gene and susceptibility to Crohn's disease.[29,30]

Contributors

A Cuthbert, P J P Croucher, M M Mirza, S Mascheretti, H Frenzel, K King, A Hasselmeyer, and J Hampe did the mutation detection and genotyping. S Fisher, M Krawczak, C Lewis, and J Hampe analysed the data. A J S MacPherson, S Bridger, S van Deventer, A Forbes, S Nikolaus, J E Lennard-Jones, U R Foelsch, and S Schreiber recruited the patients and contributed to the study design. J Hampe drafted the paper. S Schreiber and Christopher G Mathew coordinated the experiments and patient recruitment and edited the paper.

Acknowledgments

We thank the physicians, IBD patients, and their families for participating in this study. We also thank the German Crohn's and Colitis Foundation (DCCV), Prof Raedler (Hamburg), Prof Kruis (Köln), Dr Theuer (Heilbronn), Dr Meckler (Gedern), Prof Lochs, Dr Wedel, T Herrmann (Berlin), Dr Herchenbach (Recklinghausen), Prof Scheurlen (Würzburg), Dr Demharter (Augsburg), Dr Simon (Munich), Dr Purrmann (Moers), Dr Jessen (Kiel), Dr Zehnter (Dortmund), Dr Lübke, Dr Weismüller (Koblenz), Dr Eiche (Denkendorf), Dr Schönfelder (Aachen), Prof Fleig (Halle), Dr Wewalka (Linz, Austria), Prof Knofloch (Wels, Austria), Dr Hodgson, Dr Sanderson, Dr Pounder, Dr Forgacs (London, UK), Dr Bird (Maidstone, UK), Dr Hines (Haywards-Heath, UK), Dr Cairns, Dr Ireland (Brighton, UK), Dr Barrison (St Albans, UK), and Dr Smith-Lang (Sidcup, UK). Special acknowledgments go to John C W Lee for his role in patient recruitment.

In the UK, this work was supported by the Wellcome Trust, the Generation Trust, the National Association for Colitis & Crohn's disease, Crohn's in Childhood Research Association, and the Sir Halley Stewart Trust. In Germany, support was from the Deutsche Forschungsgemeinschaft (For 423), a Training and Mobility of Research (TMR) Network grant from the European Union (ERB-4061-PL-97-0389), a competence network "Chronisch-entzündliche Darmerkrankungen", the German Human Genome Project (DHGP), and the National Genome Research Network (all funded by the German Federal Department for Research and Education).

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