Mechanisms of disease

Association of *NOD2* (*CARD 15*) genotype with clinical course of Crohn's disease: a cohort study

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Summary

Background Crohn's disease is a heterogeneous disorder for which *NOD2* (*CARD 15*) has been identified as a susceptibility gene. We investigate the relation between *NOD2* genotype and phenotypic characteristics of patients with Crohn's disease.

Methods Hypotheses about the relation between *NOD2* genotype and Crohn's disease phenotype were generated retrospectively from a group of 446 German patients with this disorder. Positive findings ($p<0{\cdot}10$) were verified in prospectively established cohorts of 106 German and 55 Norwegian patients with Crohn's disease. All patients were genotyped for the main coding mutations in *NOD2*, denoted SNP8, SNP12, and SNP13, with Taqman technology.

Findings In the retrospective cohort, six clinical characteristics showed noteworthy haplotype association: fistulising, ileal, left colonic and right colonic disease, stenosis, and resection. In the German prospective cohort, these haplotype associations could be replicated for ileal ($p=0{\cdot}006$) and right colonic disease ($p\leqslant 0{\cdot}001$). A similar trend was noted in the Norwegian patients.

Interpretation We recorded a distinct relation between *NOD2* genotype and phenotype of Crohn's disease. Test strategies with *NOD2* variations to predict the clinical course of Crohn's disease could lead to the development of new therapeutic paradigms.

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Introduction

Crohn's disease is a chronic intestinal inflammatory disorder that is characterised by a dysregulated mucosal immune response. Results of epidemiological and genetic linkage studies[1–3] suggest that there is a genetic component that causes this disorder, and the pericentromeric region of chromosome 16 has proved the most probable location of this component. Results of positional cloning and candidate-gene analysis of chromosome 16 have identified NUCLEOTIDE OLIGOMERISATION DOMAIN (NOD) 2 as a gene linked to Crohn's disease.[4–6] Recently, the nomenclature of *NOD2* has been changed to CASPASE ACTIVATING RECRUITMENT DOMAIN (CARD) 15. *NOD2* has been shown to have a role in the cause of Crohn's disease in four independent and ethnically different groups of patients.[4–6]

The association of high risk of Crohn's disease with *NOD2* mutations suggests a potential diagnostic applicability. Furthermore, no patient with ulcerative colitis who is homozygous for SNP13—the most important truncating *NOD2* mutation—has so far been identified, which suggests that the range of mutations of *NOD2* might facilitate differential diagnosis of Crohn's disease and ulcerative colitis.[4]

Crohn's disease has a range of clinical manifestations, and its heterogeneity has been highlighted by results of several epidemiological studies aimed at identification of subgroups or clusters of phenotypes of this disorder.[7–10] Age at diagnosis, location (terminal ileum, colon, ileocolon, upper gastrointestinal tract), and development of complications (stricturing, penetrating) are useful variables for clinical classification.[7] Such classification schemes would, however, benefit further from associated genetic factors, since clinical classifications can change over time.[11]

Phenotype clustering in families and in homozygotic twins[12,13] suggests that genetic factors affect clinical outcome in Crohn's disease. Since mutations in *NOD2* are an important factor in cause of Crohn's disease, investigation of the range of mutations of *NOD2* for possible genotype-phenotype relations seems worthwhile.

NOD2 is a member of the NOD gene family, and has a role in mediation of inflammatory response to bacterial antigens. Therefore, *NOD2*-related clinical manifestations can be assumed to show the pathophysiological effect of intestinal flora—eg, ileocolonic or fistulising disease.

Investigation of the genotype-phenotype relation in patients with Crohn's disease is methodologically challenging, since many clinical characteristics have to be assessed. We have therefore used a two-stage study design, in which a set of hypotheses was first generated by exploratory analysis of a retrospectively characterised group of patients. Hypotheses emerging as potentially important were then validated in a second, prospectively established, verification cohort.

GLOSSARY

CASPASE ACTIVATING RECRUITMENT DOMAIN (CARD)
Caspases are important enzymes in the apoptosis process.

HAPLOTYPE
A specfic set of alleles present at two or more linked loci on a chromosome.

NFkB
Nuclear factor kappa B, a transcription factor that regulates expression of most inflammation genes.

NUCLEOTIDE OLIGOMERISATION DOMAIN (NOD)
A structural characteristic of the *NOD* gene family. Members of the family include *APAF1*, *CED4*, *NOD1*, *NOD2*, and some cytosolic products of plant disease-resistance genes. *NOD2* is the first gene linked to Crohn's disease. *NOD2* contains a CARD, the *NOD* motif, and a leucine-rich region. In accordance with new nomenclature, *NOD2* is now called *CARD 15*.

SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS)
Caused by a base exchange or insertion of an additional base into the DNA sequence.

We investigated the importance of the three major functional variants of *NOD2*—SNP8, SNP12, and SNP13—on the clinical course of Crohn's disease. Presence of stenoses, fistulae, arthritis, other extraintestinal complications, and resections, relapse frequency, and location of disease (ileal, right colon, left colon) were examined.

Patients and methods

Study populations

We analysed two groups of patients. Patients in all cohorts were recruited consecutively. For the retrospective group, we invited—through the German Crohn's and Colitis Foundation—about 9000 people to take part in the study, and we received over 6000 responses. Of these, we included the first 446 patients with Crohn's disease who fulfilled the criteria listed below. Patients were eligible for the study if inflammatory bowel disease had been diagnosed with standard clinical criteria and by endoscopy, radiology, or both.[14]

All responders completed a multi-item questionnaire with their doctor. Between July, 1996, and January, 1998, we made available a telephone helpline for patients and doctors at the Charité University Hospital in Berlin, Germany, to provide assistance with completion of the questionnaire.

Clinical information for every patient was obtained with the following questions: type of disease (Crohn's disease, ulcerative colitis, indeterminate colitis); method used to confirm diagnosis (endoscopy, histology, radiology); and presence of stenoses, overt fistulae, or extraintestinal manifestations (coded as presence or absence of any extraintestinal manifestations, including arthritis or severe chronic arthralgia), arthritis, pattern of disease (ileum, right or left colon), previous resections (coded as yes or no), and course of disease (more than one relapse every 2 years). If continuous follow-up could not be documented, this variable was coded as unknown. We coded patients whose diagnosis had changed during the course of illness as indeterminate colitis, and excluded them from the study.

Venepuncture, which was done at the same time as the questionnaire was completed, ensured interaction with the family doctor or gastroenterologist (the blood samples were used for DNA preparation).

The prospective cohort included 106 patients with Crohn's disease from Germany and 55 from Norway. Individuals had been followed up prospectively for 2–10 years before recruitment. The diagnosis of Crohn's disease was made with standard clinical, radiological, and endoscopic criteria.[14] Histological findings had to be compatible with diagnosis; in cases of uncertainty, we excluded patients from the study.

The German patients were recruited consecutively through the competence network inflammatory bowel disease, which is coordinated at Christian-Albrechts-University, Kiel, Germany. We selected the first 106 patients who fulfilled the criteria. Clinical variables, which we recorded at every outpatient visit, included course of disease (stenosis, overt fistula, resection), location of disease (ileal, right and left colon), arthritis, extraintestinal involvement, age at diagnosis, and relapses. To establish disease location or stenosis, an endoscopic or radiological study within the past 24 months was needed. We ascertained disease location by macroscopic appearance of mucosa on endoscopy. If the respective procedure was done more than 24 months ago, we coded the outcome as unknown and excluded the patient from the study. Continuous follow-up over the past 24 months was needed to establish whether relapse had happened during that period.

Norwegian patients with Crohn's disease (from four counties in southeastern Norway) were registered prospectively between Jan 1, 1990, and Dec 31, 1993.[15,16] Two gastroenterologists at the Rikshospitalet, Oslo, Norway independently reviewed diagnostic and clinical data. At 2 and 5 years after diagnosis, all patients underwent clinical reassessment.[17] The first 55 patients who entered 10-year follow-up—a timepoint at which clinical data and blood samples were obtained—were included in the study.

Doctors and patients were aware of the aim of the study—identification of genetic risk factors for inflammatory bowel disease, or its clinical characteristics—but not of the specific set of hypotheses to be investigated. All patients gave informed consent to participation in a genetic study investigating the causes and clinical course of inflammatory bowel disease. The test procedures and data handling were approved by the ethics committee and the regional government data-protection agencies (Landesdatenschutzbeauftragter).

Demographic and clinical data from both cohorts are summarised in table 1. We aimed to summarise the complete clinical course in the phenotype. If, for example,

	Retrospective (n=446)	Prospective	
		German (n=106)	Norwegian (n=55)
Demographics			
Men	145 (33%)	36 (34%)	26 (47%)
Age at diagnosis	23·5 (8·0)	28·3 (11·9)	32·6 (16·0)
Clinical			
Stenosis*	288 (65%)	45 (42%)	7 (13%)
Fistulae†	246 (55%)	57 (54%)	9 (16%)
Extraintestinal‡	340 (76%)	66 (62%)	24 (44%)
Arthritis	275 (62%)	61 (58%)	22 (40%)
Previous resection	256 (57%)	63 (59%)	15 (27%)
Frequent relapse	213 (66%)§	70 (66%)	29 (53%)
Location			
Ileal*	368 (83%)	80 (75%)	30 (55%)
Right colon*	308 (69%)	72 (68%)	16 (29%)
Left colon*	319 (72%)	73 (69%)	25 (45%)

Data are number (%) or mean (SD). *Established by endoscopy or radiology within 24 months. †Any past fistulising disease, including perianal fistulae. ‡Including arthritis and severe chronic arthralgia. §Measured in 324 patients followed up continuously over the past 24 months.

Table 1: **Demographic and clinical characteristics of patients**

Primers and probes used in genotyping of *NOD2* polymorphisms

Polymorphism	Primers	Probes
NOD2-SNP8	TTCCTGGCAGGGCTGTTGTC	Fam-CCTGCTCCGGCGCCAGGC-Tamra
	AGTGGAAGTGCTTGCGGAGG	Tet-CCTGCTCTGGCGCCAGGCC-Tamra
NOD2-SNP12	ACTCACTGACACTGTCTGTTGACTCT	Fam-TTTTCAGATTCTGGGGCAACAGAGTGGGT-Tamra
	AGCCACCTCAAGCTCTGGTG	Tet-TTCAGATTCTGGCGCAACAGAGTGGGT-Tamra
NOD2-SNP13	GTCCAATAACTGCATCACCTACCTAG	Tet-CCTCCTGCAGGCCCCTTGAAA-Tamra
	CTTACCAGACTTCCAGGATGGTGT	Fam- CCCTCCTGCAGGCCCTTGAAAT-Tamra

Probes were labelled with a fluorescent dye (Fam or Tet) at the 5′ end and a quencher at the 3′ end (Tamra).

a patient had fistulae and resection without further relapse 10 years ago, he or she was coded as positive for both fistulae and resection. The phenotype was established before commencement of genotyping. The phenotypic variables listed in table 1 were used as target variables for the phenotype-genotype analysis. Identical classification criteria were used in the prospective and retrospective cohorts.

We recruited controls from the blood-donor system at the university hospitals of Kiel and Berlin (for German controls, n=373) and the Rikshospitalet, Oslo (for Norwegian controls, 202). We obtained a medical history and did a laboratory investigation and physical examination for every individual to exclude pre-existing disorders.

Raw data for patients can be obtained on request from the corresponding author.

Genotyping

We prepared genomic DNA from 10 mL fresh or frozen blood samples with the blood Gigakit (Invitec, Berlin, Germany). We assayed mutations with Taqman (Applied Biosystems, Foster City, CA, USA) as described.[18] In brief, genomic DNA was arrayed and dried on 96-well and 384-well plates. Taqman PCR was set up with Genesis pipetting robots (Tecan, Männedorf, Switzerland). We amplified samples with ABI9700 PCR machines (Applied Biosystems), and measured fluorescence with ABI7700 and ABI7900 fluorometers (Applied Biosystems). The primers and probes we used are listed in the panel and SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) elsewhere.[19] We managed and analysed all data with an integrated laboratory information system.[20]

Statistical analysis

We tested qualitative clinical characteristics for significant SNP HAPLOTYPE association with a log-likelihood ratio test. We estimated haplotype frequencies of the three *NOD2* SNPs in patients with or without the specific characteristic, with an expectation maximisation algorithm as implemented in the HAPMAX computer program.[21] Twice the log-likelihood ratio between the two data models that do or do not distinguish between phenotypic categories roughly follows a χ^2 distribution, with k–1 degrees of freedom. Here, k denotes the number of haplotypes considered. We also validated p values by randomisation (p_r) with 1000 replications per test. The p values that are a result of these randomisations have therefore a precision of 0·001. Thresholds for significant p values were 10% in the first exploratory stage and 5% in the second confirmatory stage, corrected for the number of hypotheses tested (ie, six) with a Bonferroni approach. Haplotype frequencies in patients and controls were tested for significant differences between the German and Norwegian population with a similar approach.

Haplotype			Controls		Patients	
SNP8	SNP12	SNP13	German (n=373)	Norwegian (n=202)	German* (n=552)	Norwegian (n=55)
2	2	2	0·9043	0·9504	0·7108	0·9304
1	2	2	0·0478	0·0249	0·0992	0·0352
2	1	2	0·0072	0·0123	0·0403	0·0086
2	2	1	0·0406	0·0095	0·1372	0·0179
1	1	2	..	..	0·0052	..
1	2	1	..	0·0028	0·0007	0·0079
2	1	1	..	..	0·0066	..

1=mutant; 2=wild-type. *Retrospective and prospective groups pooled.

Table 2: **Single nucleotide polymorphism (SNP) haplotype frequency estimates in German and Norwegian patients with Crohn's disease and controls**

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

446 patients were recruited for the retrospective sample, and 161 for the prospective group. Both groups had a closely similar distribution of sex and age at diagnosis (table 1).

Haplotype frequencies for SNP8, SNP12, and SNP13 were estimated for controls and patients (table 2). These frequencies differed significantly between the two geographic populations, both in controls (p_r=0·006) and in patients ($p_r \leq 0{\cdot}001$). Therefore, possible associations between genotypes and clinical variables were analysed separately for German and Norwegian patients.

Clinical manifestations in the retrospective group of patients with Crohn's disease were tested for SNP haplotype association with the phenotype categories listed in table 1. Six potentially relevant hypotheses emerged at the 10% significance level (table 3) including presence of stenoses, fistulae, previous resections, ileal disease, and left or right colonic disease. Since these results could potentially have been confounded by multiple testing, we verified the emerging hypotheses in the cohort of 106 prospectively established German patients, with a Bonferroni corrected threshold of 5% for the significance level. Two haplotype associations were confirmed, namely ileal and right colonic disease.

	Retrospective		Prospective German	
	p*	p_r	p†	p_r
Clinical variable				
Stenosis	0·019	0·022	0·538	0·568
Fistulae	0·069	0·071	0·455	0·475
Extraintestinal	0·839	0·822	..	..
Arthritis	0·322	0·305	..	..
Frequent relapse	0·296	0·304	..	..
Previous resection	0·048	0·046	0·222	0·261
Location				
Ileal	0·023	0·030	0·007	0·005
Right colon	0·062	0·061	0·004	0·003
Left colon	⩽0·001	⩽0·001	0·068	0·100

*Only seven haplotypes considered in likelihood maximisation. †Only six haplotypes considered in likelihood maximisation.

Table 3: **Analysis of the genotype-phenotype relation of three *NOD2* polymorphisms**

Haplotype			Retrospective		Prospective German		Prospective Norwegian	
SNP8	SNP12	SNP13	Without (n=78)	With (n=368)	Without (n=26)	With (n=80)	Without (n=25)	With (n=30)
2	2	2	0·8119	0·6694	0·9423	0·7461	1·0000	0·8637
1	2	2	0·0663	0·1187	..	0·0565	..	0·0696
2	1	2	0·0279	0·0413	..	0·0563	..	0·0167
2	2	1	0·0768	0·1549	0·0577	0·1245	..	0·0363
1	1	2	0·0105	0·0063	..	..	..	..
1	2	1	0·0065	0·0001	..	0·0141	..	0·0137
2	1	1	..	0·0095	..	0·0025	..	..

1=mutant; 2=wild-type.

Table 4: **Single nucleotide polymorphism (SNP) haplotype frequency estimates in patients with Crohn's disease with and without ileal disease**

For ileal disease (table 4), all three haplotypes with one mutation were characterised by a relative risk of about two. A similar effect was noted in the two prospective cohorts, although mutant haplotypes were rare in both the German and Norwegian groups. The SNP13 mutation was more strongly associated with right-colonic disease in the prospective than in the retrospective populations (table 5). Furthermore, although the haplotypes containing the SNP8 and SNP12 mutations had an increased relative risk in both German samples, this increase was not seen in the small Norwegian prospective cohort.

Discussion

We have shown that haplotypes with SNP13 are associated with ileal disease (table 4) whereas, at least in German patients, all three SNPs seem to be associated with increased risk for right-colonic disease (table 5). These results suggest that *NOD2* genotype has an effect on the clinical presentation of Crohn's disease. No association with general markers of disease severity (as assessed by relapse frequency, age of onset, and need for resections) was noted. This absence of association highlights the specificity of the observed genotype-phenotype relation. We postulate that identification of additional disease-genes in inflammatory bowel disease will further promote development of paradigms for early and preventive treatment.

Identification of genetic risk factors that cause polygenic disorders promises new approaches to dissect these complex diseases. Crohn's disease is an especially heterogenous disorder, with a wide range of sub-phenotypes and clinical presentations being subsumed under the same diagnosis. Heterogeneity affects both clinical diagnosis and management of the disease. Thus, efficacy of anti-inflammatory therapies is often limited to subgroups without clinical predictors.[22] To develop preventive clinical paradigms, however, identification of patients at risk of development of certain complications is essential.

Correlation between genetic risk—ie, whether the disease was linked to chromosome 6, 12, or 16—and the presence of certain subphenotypes of Crohn's disease was noted[23] before *NOD2* was established as an actual disease gene. The subsequent availability of susceptibility mutations rather than linkage data rendered analysis of genotype-phenotype relations even more powerful. Furthermore, since clustering of subphenotypes has been noted in families and in homozygotic twins,[12,13] a substantial causal relation between genetic factors and disease characteristics was to be expected.

Investigation of genotype-phenotype relations is a major methodological challenge, and we used a two-tier design to control for inflation of type 1 error because of multiple testing. Also, by the nature of the recruitment process, clinical information obtained prospectively is usually of higher quality than retrospective data. In our study, the retrospective sample was therefore only used to generate hypotheses that were then validated in a prospective cohort. Phenotypes in different cohorts were established at different centres, and clinical assessment of patients with Crohn's disease is known to vary between centres and doctors. Such differences also could have affected phenotyping in our study, and could thereby have reduced the power of our analysis to detect potentially relevant clinical associations. The specificity of the observed associations, however, will probably not be affected, since a conservative two-tier approach with correction for multiple testing was used.

SNP13 causes truncation of the NOD2 protein before the leucine-rich region, which is important for activation of the NFκB system in response to bacterial lipopolysaccharide. The NOD family of proteins is mainly expressed in monocytes, macrophages, and B cells.[24,25] The exact mechanism of NFκB activation is unclear, but mutations in the leucine-rich region of the gene could disturb activation.[5,25] NOD2 could therefore be an especially important part of innate immunity for maintenance of the intestinal barrier. The reported association with ileal and right-colonic disease accords with the hypothetical involvement of the intestinal flora in Crohn's disease.

We have shown the possible use of genetic factors as diagnostic markers for complex diseases. Genetic susceptibility could establish the clinical phenotype of

Haplotype			Retrospective		Prospective German		Prospective Norwegian	
SNP8	SNP12	SNP13	Without (n=148)	With (n=308)	Without (n=34)	With (n=72)	Without (n=39)	With (n=16)
2	2	2	0·7633	0·6648	0·9306	0·7240	0·9359	0·9062
1	2	2	0·0548	0·1304	0·0139	0·0575	0·0513	..
2	1	2	0·0238	0·0447	..	0·0652	0·0128	..
2	2	1	0·1186	0·1496	0·0556	0·1360	..	0·0625
1	1	2	0·0159	0·0027	..	..	..	..
1	2	1	0·0090	..	..	0·0159	..	0·0312
2	1	1	0·0065	0·0078	..	0·0014	..	..

1=mutant; 2=wild-type.

Table 5: **Single nucleotide polymorphism (SNP) haplotype frequency estimates in patients with Crohn's disease with and without right-colonic disease**

Crohn's disease,[9] and we have shown how the definition of distinct clinical manifestations in a complex disease could relate to different variants of a susceptibility gene. Our analysis could thus represent a step towards primary characterisation of clinical subgroups on the basis of genetic data. In this respect, genetic variables will be especially useful, since genetics-based classification is more stable than exclusive use of clinical features (which depend themselves on disease duration and activity).

Contributors

All authors contributed to study design. J Hampe and J Grebe contributed equally to recruitment of patients, phenotype characterisation, data analysis, and drafting of the report. S Nikolaus was responsible for cohort assembly, phenotype ascertainment, and analysis. C Solberg, J Jahnsen, and B Moum did phenotype analysis and patient follow-up. P J P Croucher, S Mascheretti, and M M Mirza did genotyping. B Klump did patient follow-up. M Krawczak designed analysis methods, did data analysis, and drafted the report. U R Foelsch was responsible for planning experimental design and discussion of the report. M Vatn was responsible for planning of the Norwegian phenotype ascertainment schema and discussion of the report. S Schreiber was responsible for overall experimental plan, establishment of German cohorts, and drafting of the report.

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Conflict of interest statement

None declared.

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