NOD2 (CARD15) mutations in Crohn’s disease are associated with diminished mucosal α-defensin expression


Background: Mutations in NOD2, a putative intracellular receptor for bacterial peptidoglycans, are associated with a subset of Crohn’s disease but the molecular mechanism linking this protein with the disease pathogenesis remains unclear. Human α-defensins (HD-5 and HD-6) are antibiotic effector molecules predominantly expressed in Paneth cells of the ileum. Paneth cells also express NOD2. To address the hypothesis that the function of NOD2 may affect expression of Paneth cell defensins, we compared their expression levels with respect to NOD2 mutations in Crohn’s disease.

Methods: Forty-five Crohn’s disease patients (24 with NOD2 mutations, 21 with wild-type NOD2) and 12 controls were studied. Real time reverse transcription-polymerase chain reaction was performed with mucosal mRNA for HD-5, HD-6, lysozyme, secretory phospholipase A2 (sPLA2), tumour necrosis factor α, interleukin 8, and human hypoxanthine phosphoribosyltransferase (housekeeping gene). Immunohistochemistry with anti-HD-5 and histological Paneth cell staining were performed in 10 patients with NOD2 mutations or wild-type genotypes.

Results: Ileal expression of HD-5 and HD-6, but not sPLA2 or lysozyme, were diminished in affected ileum, and the decrease was significantly more pronounced in patients with NOD2 mutations. In the colon, HD-5, HD-6, and sPLA2 were increased during inflammation in wild-type but not in NOD2 mutated patients. In both the colon and ileum, proinflammatory cytokines and lysozyme were unaffected by NOD2 status. Immunohistochemistry identified Paneth cells as the sole source of HD-5.

Conclusion: As α-defensins are important in the mucosal antibacterial barrier, their diminished expression may explain, in part, the bacterial induced mucosal inflammation and ileal involvement of Crohn’s disease, especially in the case of NOD2 mutations.
frozen in liquid nitrogen. The study was approved by the local table 1. All patients gave written informed consent before wild-type genotypes. Patient characteristics are shown in systematic differences between patients with NOD2 mutated or according to their clinical requirements without any sys-mainly with aminosalicylates, corticosteroids, or azathioprine.

**METHODS**

**Patients**

Twelve patients with a routine colonoscopy lacking significant pathology were randomly selected as controls. A cohort of 68 Crohn’s disease patients from two inflammatory bowel disease centres (Stuttgart and Homburg) were screened for NOD2 mutations. Ileal biopsies were available from 26 of 68 patients with Crohn’s disease which were stratified into those with (n = 18) and without (n = 8) macroscopic ileal disease. Colonic biopsies were available from 39 of 68 patients. In the case of Crohn’s disease, samples were taken from macroscopically inflamed and not inflamed mucosa, if possible. The diagnosis was based on standard criteria using radiological and endoscopic findings in every case. Patients were treated mainly with aminosalicylates, corticosteroids, or azathioprine according to their clinical requirements without any systematic differences between patients with NOD2 mutated or wild-type genotypes. Patient characteristics are shown in table 1. All patients gave written informed consent before colonoscopy was performed. Samples were immediately snap frozen in liquid nitrogen. The study was approved by the local ethics committee.

**Ribonucleic acid preparation and reverse transcription**

Frozen biopsies were disrupted mechanically and total RNA was isolated using TRizol reagent (Invitrogen, San Diego, California, USA) according to the supplier’s protocol. RNA quality and quantity were determined by gel electrophoresis and photometry. Subsequently, 1 μg of total RNA was reverse transcribed with oligo dT primers and 200 U of Superscript (Invitrogen) according to routine procedures. These RNA preparations from each biopsy were used for assay of all defensins and cytokines tested.

**Mutation analysis**

Genotyping of genomic and/or cDNA for the functionally relevant NOD2 mutations (SNP8, SNP12, and SNP13) was performed using TaqMan technology (Applied Biosystems, Foster City, California, USA), as described previously. In brief, amplification reactions (25 μl) were carried out with 20 ng of template DNA, 1 × TaqMan Universal Master Mix buffer (Applied Biosystems), 900 nM of each primer, and 200 nM of each fluorogenic probe. Detection of fluorescence signals was performed using the ABI PRISM 7700 detection system and the results were analysed as described previously by use of the Sequence Detection System (SDS) Software Version 1.7 (Applied Biosystems).

**Real time polymerase chain reaction**

We studied 24 patients displaying a NOD2 mutation, a demographically comparable Crohn’s disease group of 21 patients randomly selected from those with a NOD2 wild-type genotype, and 12 non-IBD patients without significant pathology serving as controls. After isolation of mucosal RNA, real time reverse transcription-polymerase chain reaction (RT-PCR) was performed with specific primer pairs for HD-5, HD-6, lysozyme, secretory phospholipase A2 (sPLA2), proinflammatory cytokines tumour necrosis factor α (TNF-α) and interleukin 8 (IL-8), as well as human hypoxanthine phosphoribosyltransferase (HPRT) as a control, in a fluorescence temperature cycler (LightCycler, Roche Diagnostics GMBH, Mannheim, Germany) and normalised to the amount of total RNA, as recommended and described previously. This technique continuously monitors cycle by cycle accumulation of fluorescently labelled PCR product. Briefly, cDNA corresponding to 20 ng of RNA served as a template in a 20 μl reaction containing 4 mM MgCl2, 0.5 μM of each primer, and 1 × LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). Samples were loaded into capillary tubes and incubated in the fluorescence thermocycler (LightCycler) for initial denaturation at 95°C for 10 minutes followed by 45 cycles, each cycle consisting of 95°C for 15 seconds, “touchdown” of −1°C/cycle from the primer specific start to end annealing temperature (table 2) for five seconds, and 72°C for 10 seconds. At the end of each

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Primer sequences and temperatures for touchdown protocols</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sense</strong></td>
<td><strong>Antisense</strong></td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’TCT GGC CCA GGC AGT CAC ATG 3’</td>
</tr>
<tr>
<td>IL-8</td>
<td>5’TAC ACT TCC AAG GTG GCC GTG GC’</td>
</tr>
<tr>
<td>HD-5</td>
<td>5’GCC ATC CTG GCT GGC ATT C 3’</td>
</tr>
<tr>
<td>HD-6</td>
<td>5’CCT CAC CAT CAC TCG TGC TGC 3’</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5’AAA ACC CCA GGA GCA GGA AT 3’</td>
</tr>
<tr>
<td>sPLA2</td>
<td>5’TGA CCA GAG GAA AGG AAG CCC GAC 3’</td>
</tr>
<tr>
<td>HPRT</td>
<td>5’ATC AGA CTG AAG AGC TAT TGT AAT GAC CA 3’</td>
</tr>
</tbody>
</table>

T, temperature; TNF-α, tumour necrosis factor α; IL-8, interleukin 8; HD-5, human defensin 5; HD-6, human defensin 6; sPLA2, secretory phospholipase A2; HPRT, human hypoxanthine phosphoribosyltransferase.
run melting curve profiles were produced by cooling the sample to 65°C for 15 seconds and then heating slowly at 0.20°C/s up to 95°C with continuous measurement of fluorescence to confirm amplification of specific transcripts. Cycle to cycle fluorescence emission readings were monitored and analysed using LightCycler Software (Roche Diagnostics). Lysozyme and sPLA2 control plasmids were used for standard curves. Lysozyme primers were used as described previously. Specificity of the amplification products was further verified by subjecting the amplification products to electrophoresis on a 2% agarose gel. Fragments were visualised by ethidium bromide staining and the specificity of PCR products was verified by sequencing of representative samples. Standard curves were obtained for each primer set with serial dilutions of DNA. Primer sequences and specific temperatures were used as shown in Table 2.

**Immunohistochemistry**

Immunohistochemistry on colonic as well as ileal biopsies was performed with anti-HD-5 from 10 patients with Crohn’s disease (SNP8, 12, and 13 wild-type, n = 5; SNP13 heterozygotic, n = 4; SNP13 homozygotic, n = 1). The polyclonal anti-HD-5 was donated as a gift from T Ganz and E Porter (UCLA, USA) and immunohistochemistry was performed as described previously.18

**Paneth cell staining**

In order to identify Paneth cells, we used phloxine tartrazine histological staining, as described previously.16

![Figure 1: Ileal expression of Paneth cell defensins (A, B), lysozyme (C), secretory phospholipase A2 (SPLA2) (D), and the proinflammatory cytokines tumour necrosis factor α (TNF-α) (E) and interleukin 8 (IL-8) (F) with respect to involvement in Crohn’s disease and NOD2 genotype. Real time polymerase chain reaction was performed in controls and Crohn’s disease patients showing means of relative transcription levels. Values are mean (SEM). NS, not significant; C, controls; Wt, NOD2 wild-type; Mut, NOD2 mutation (number of patients).](http://www.gutjnl.com)
immunohistochemistry and histological staining were performed in parallel sections from all patients with available tissue.

Statistics
Mean expression values were obtained from duplicate or triplicate real time PCR measurements. Mean (SEM) values are presented. The U test of Wilcoxon, Mann-Whitney was used for statistical comparison of grouped data.

RESULTS
NOD2 mutation analyses
Fifteen of 68 patients were positive for SNP13 (12 heterozygotes and three homozygotes). Five were positive for SNP8 (four heterozygotes, one homozygote) and four patients displayed a mutation for SNP12 (heterozygotes only). Two of the 12 SNP13 heterozygote patients also displayed a SNP12 mutation. Three patients with a SNP13 mutation were also positive for SNP8 (compound heterozygote).

Gene expression of HD-5 and HD-6 was decreased in Crohn’s disease patients carrying a NOD2 mutation ileal expression
Of the Crohn’s disease patients with a NOD2 mutation, only one had a normal unaffected ileum. The group with ileal disease had diminished HD-5 expression compared with either controls or those with non-ileal Crohn’s disease (fig 1A). The level of expression was lowest in those with both the mutation and ileal disease. The expression pattern of HD6 was comparable (fig 1B) but did not attain statistical
significance. The group with ileal disease and NOD2 mutation had diminished HD-6 expression compared with either controls or those with non-ileal Crohn’s disease. One patient with a NOD2 mutation and a normal ileum displayed low HD-5 and HD-6 expression.

Compared with HD-5 and HD-6, expression of other Paneth cell products was not diminished by NOD2 mutations. Even though the difference failed to achieve statistical significance, lysozyme showed a tendency towards higher expression in NOD2 mutated patients (fig 1C). NOD2 mutated patients with ileal disease showed the highest levels of lysozyme (fig 1C). Ileal expression of sPLA2 was not influenced by NOD2 genotype. In common with HD-5 and HD-6, there was a tendency towards weaker expression in patients with ileal disease but this difference did not reach statistical significance (fig 1D).

**Colonic expression**

In non-inflamed colonic tissue, no difference was detected in expression of HD-5 or HD-6 in Crohn’s disease patients compared with controls. As expected, compared with ileal expression of Paneth cell defensins, expression levels in the colon were much lower. In samples with colonic inflammation, HD-5 was increased threefold in Crohn’s disease with the NOD2 wild-type genotype. However, Crohn’s disease patients with NOD2 mutations failed to show increased HD-5 or HD-6 expression in inflamed colonic tissue. Moreover, analysis of inflamed specimens showed that levels of HD-5 and HD-6 expression were lower in NOD2 mutated compared with wild-type Crohn’s disease patients (fig 2A, B). In both HD-5 and HD-6, the difference between wild-type NOD2 genotype and SNP13 mutated patients was even more pronounced (insert in fig 2A, B).

Similar to observations in the ileum, lysozyme showed higher expression in the colon during inflammation (fig 2C).

In comparison with control patients, expression of lysozyme was not altered in non-inflamed colonic tissue from Crohn’s disease patients. During inflammation, expression of lysozyme was higher but this increase was not statistically significant (fig 2C). The NOD2 genotype did not influence expression of lysozyme.

Similar to other products in non-inflamed colonic tissue, no difference was detected in expression of sPLA2 in Crohn’s disease patients compared with controls. In samples with colonic inflammation, sPLA2 was increased in Crohn’s disease with the wild-type NOD2 genotype (fig 2D). However, Crohn’s disease patients with NOD2 mutations failed to show increased sPLA2 expression in inflamed colonic tissue.

**Ileal and colonic gene expression of TNF-α and IL-8 were not affected by NOD2 mutations**

In contrast with HD-5 and HD-6, expression of the pro-inflammatory cytokines TNF-α and IL-8 were not significantly influenced by NOD2 genotype (fig 1E, 1F, 2E, 2F). A weak non-significant decrease in TNF-α and IL-8 gene expression in Crohn’s disease patients carrying a NOD2 mutation could be detected in ileal biopsies (fig 1E, 1F).

**Normal number of ileal Paneth cells in patients carrying NOD2 mutations**

Staining with phloxine tartrazine revealed a normal number of Paneth cells in ileal biopsies from four wild-type patients compared with three patients with a NOD2 mutation. Staining for a control and the SNP13 homozygote is shown in fig 3. A heterozygote patient exhibited metaplastic Paneth cells in the colon, similar in number to five NOD2 wild-type patients. However, the number of colonic biopsies with Paneth cell staining was too limited for a quantitative evaluation. Essentially, although with the same limitations,
the number of HD-5 positive cells was comparable between the groups. In parallel sections, Paneth cells also stained positive for HD-5, even in the homozygous patient, but the technique excludes quantitation of staining intensity (fig 3).

**DISCUSSION**

Crohn’s disease is a complex multifactorial disease whose pathogenesis is still not well understood. Recent research has focused on the role of luminal bacteria and their essential role in pathophysiology. A major advance for better understanding the disease is the discovery of mutations in the NOD2 gene in approximately one third of Crohn’s disease patients. NOD2 is a putative receptor of bacterial peptido-glycan which is also expressed by Paneth cells and crypt epithelial cells. Paneth cells are secretory cells expressing $\alpha$-defensins HD-5 and HD-6. These antibiotic peptides are predominantly expressed in the small intestine and by metaplastic Paneth cells during inflammation in the colon. The findings reported herein for the first time link a mutation in NOD2 to reduced $\alpha$-defensin expression in Crohn’s disease.

Mutations in the NOD2 gene are associated with ileal involvement in Crohn’s disease. As NOD2 and $\alpha$-defensins are co-expressed in ileal Paneth cells, their relative deficiency in Crohn’s disease may be related to ileal preference, especially in patients with NOD2 mutations. Although the defect in ileal $\alpha$-defensins is particularly pronounced in NOD2 mutated patients, HD-5 is also reduced in NOD2 wild-type patients with ileal disease. This is compatible with the concept that other signalling peptides may be affected in NOD2 wild-type patients. Diminished expression of $\alpha$-defensins, especially in patients with the NOD2 mutation, contrasts with the comparably enhanced levels of ileal mucosal TNF and IL-8 in both the NOD2 wild-type and mutated group with ileal involvement. Previous studies showed that Paneth cell populations as well as the secretory granule area are preserved or even increased in ileal Crohn’s disease, suggesting that Paneth cell function rather than number may be compromised in this disease. In the colonic mucosa, expression of HD-5 and HD-6 also seem to be regulated or influenced by NOD2 because lack of function mutations, especially SNP13, lead to diminished induction during inflammation. In contrast with diminished HD-5 and HD6 levels in the ileum of patients with a NOD2 genotype, enhanced levels of lysozyme and unchanged levels of sPLA2 in ileal Crohn’s disease patients. The observation that HD-5 and HD-6 are unchanged during pouchitis (Kiehne et al abstract DDW 2002, Fellermann et al submitted for publication) strongly argues against speculation that downregulation of HD-5 and HD-6 accompanies inflammation alone. Although the functional relevance of defensins has been shown in mice, it still has to be proven in Crohn’s disease. The fact that ileal and colonic expression of proinflammatory cytokines are not influenced by NOD2 mutations further supports the specificity of the partial lack of certain defensins. Therefore, these data support the hypothesis that Crohn’s disease may be a complex defensin deficiency syndrome.

**ACKNOWLEDGEMENTS**

This work was supported by the Robert Bosch Foundation, Stuttgart, Germany. We thank Dr N Homann for data collection as well as Drs T Ganz and E Porter for supplying human defensin-5 antibody. We also thank P Miller, K Siegel, D Weller, D Biegger, as well as R Feathers for excellent technical assistance.

**Authors’ affiliations**

J Wehkamp*, M Schlie, P Fritz, K R Herrlinger, K Fellermann, E F Stange, Departments of Internal Medicine I and Pathology, Robert Bosch Hospital, Stuttgart, Germany

M Schwab, E Schäffeler, Dr Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany

J Harder*, M Weichenthal, J M Schröder, Department of Dermatology, University of Schleswig Holstein, Campus Kiel, Germany

F Noack, Department of Pathology, University of Schleswig Holstein, Campus Luebeck, Germany

A Stallmach, Internal Medicine II, University of Saarland, Germany

C L Bevins, Department of Microbiology and Immunology, University of California, Davis, USA

*J Wehkamp and J Harder contributed equally to this study.

**REFERENCES**


cholestasis. [18F]fluoro-2-deoxy-D-glucose-positron emission tomography (FDG-PET) revealed avid uptake of FDG in the bile ducts. Retrograde cholangiography, no stents were detected and no remaining stricture of the bile ducts was noted. The patient gained a net weight gain of 9 kg. There were no abnormal laboratory findings. On endoscopic stent exchange, the stent was not well placed. The patient was admitted for stent exchange after seven months (he missed the three month stent exchange appointment). The patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first admission the patient is doing well without signs of a malignant disease. Despite all imaging and laboratory findings, the patient was considered eligible for cholecystectomy but refused surgery. Two years after the first adm
NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal α-defensin expression

J Wehkamp, J Harder, M Weichenthal, M Schwab, E Schäffeler, M Schlee, K R Herrlinger, A Stallmach, F Noack, P Fritz, J M Schröder, C L Bevins, K Fellermann and E F Stange

Gut 2004 53: 1658-1664
doi: 10.1136/gut.2003.032805

Updated information and services can be found at:
http://gut.bmj.com/content/53/11/1658

These include:

References
This article cites 38 articles, 13 of which you can access for free at:
http://gut.bmj.com/content/53/11/1658#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/