

A Chromosome 8 Gene-Cluster Polymorphism with Low Human Beta-Defensin 2 Gene Copy Number Predisposes to Crohn Disease of the Colon

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Defensins are endogenous antimicrobial peptides that protect the intestinal mucosa against bacterial invasion. It has been suggested that deficient defensin expression may underlie the chronic inflammation of Crohn disease (CD). The DNA copy number of the beta-defensin gene cluster on chromosome 8p23.1 is highly polymorphic within the healthy population, which suggests that the defective beta-defensin induction in colonic CD could be due to low beta-defensin-gene copy number. Here, we tested this hypothesis, using genomewide DNA copy number profiling by array-based comparative genomic hybridization and quantitative polymerase-chain-reaction analysis of the human beta-defensin 2 (*HBD-2*) gene. We showed that healthy individuals, as well as patients with ulcerative colitis, have a median of 4 (range 2–10) *HBD-2* gene copies per genome. In a surgical cohort with ileal or colonic CD and in a second large cohort with inflammatory bowel diseases, those with ileal resections/disease exhibited a normal median *HBD-2* copy number of 4, whereas those with colonic CD had a median of only 3 copies per genome ($P = .008$ for the surgical cohort; $P = .032$ for the second cohort). Overall, the copy number distribution in colonic CD was shifted to lower numbers compared with controls ($P = .002$ for both the surgical cohort and the cohort with inflammatory bowel diseases). Individuals with ≤ 3 copies have a significantly higher risk of developing colonic CD than did individuals with ≥ 4 copies (odds ratio 3.06; 95% confidence interval 1.46–6.45). An *HBD-2* gene copy number of < 4 was associated with diminished mucosal *HBD-2* mRNA expression ($P = .033$). In conclusion, a lower *HBD-2* gene copy number in the beta-defensin locus predisposes to colonic CD, most likely through diminished beta-defensin expression.

Crohn disease (CD [MIM 266600]) is a severe chronic inflammatory bowel disease characterized by intestinal ulceration that affects predominantly the ileum and colon.^{1,2} The cause of the disease is unknown. Recent findings have suggested that the mucosal immunological reaction is directed against the resident bacterial flora rather than against tissue antigens.³ This loss of tolerance to the normal flora may be due to a dysregulation of the gut mucosal immune response⁴ or, alternatively, a break in the antibacterial barrier where microbiota can trigger a deleterious immune response.⁵

CD appears to be a consequence of both genetic and environmental influences. The 50% concordance rate in MZ twins, who often exhibit the same phenotype,⁶ suggests a rather balanced impact of genetic and such environmental factors as smoking^{7,8} or childhood hygiene.⁹ During a genomewide search, several susceptibility loci and genes—including *NOD2* (*CARD15*) (MIM 605956),^{10,11} *DLG5* (MIM 604090),¹² and *OCTN* (MIM 604190 and 603377)¹³—have been found to be associated with CD. The best replicated is *NOD2*, which is involved in intracellular sensing of bacterial muramyl dipeptide, promi-

nently expressed in macrophages and in particular small intestinal Paneth cells.^{14,15} Despite these significant advances, the multiple susceptibility loci and other genetic factors hitherto identified¹⁶ do not satisfactorily explain the inheritance rates.

The clinical syndrome of CD is variable with respect to age at diagnosis, location (small and/or large intestine), and disease behavior (inflammatory, stricturing, or penetrating disease). Therefore, these behavior parameters were used in the Vienna classification,¹⁷ recently modified in Montreal.¹⁸ Location of disease involvement proved to be stable over time in individual patients, although the biological basis of small- versus large-intestinal involvement is unclear. *NOD2* mutations have been shown to be predominantly associated with ileal disease and have been reported to be related to a relative lack of ileal Paneth-cell alpha-defensins HD-5 (MIM 600472) and HD-6 (MIM 600471).¹⁹ Defensins are endogenous antibiotic peptides that form a chemical barrier at the epithelial surface, and their relative deficiency may lead to bacterial adherence to the mucosa, slow invasion, and secondary mucosal inflammation.^{5,20}

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In contrast to ileal disease, colonic CD is characterized by an impaired induction of the epithelial beta-defensins *HBD-2* (MIM 602215), *HBD-3* (MIM 606611), and *HBD-4*.^{21–23} This relative deficiency of several beta-defensins is unlikely to be explained by multiple coincident mutations or other genetic alterations of all these genes. Detailed studies of the defensin locus on chromosome 8 have uncovered an extensive DNA copy number polymorphism (CNP) of a gene cluster, including, among others, the human beta-defensin genes *HBD-2* (*DEFB4*), *HBD-3* (*DEFB103*), and *HBD-4* (*DEFB104*).²⁴ It has been shown that the number of gene copies is positively correlated with the expression of *HBD-2* in leukocytes.²⁴ Since all these defensins were found to be coordinately under-expressed in colonic CD,²¹ we hypothesized that this particular phenotype may be associated with a low beta-defensin gene cluster copy number. We therefore measured DNA copy number in the beta-defensin cluster in two independent cohorts with inflammatory bowel diseases and related it to mucosal *HBD-2* gene expression.

Material and Methods

Patients

The patients from three separate cohorts received diagnoses, with use of the same standard criteria, in Cleveland, Stuttgart, and Vienna and were treated in specialized tertiary-care out- and in-patient centers. Patients gave their informed consent, and local ethics committees approved the study protocols. A small cohort—of patients with colonic CD ($n = 10$) and healthy control individuals ($n = 10$)—was recruited in Stuttgart. Their blood-derived genomic DNA was subjected to the microarray analysis described below. The exploratory cohort was a surgical group of patients who underwent surgical resection at the Cleveland Clinic for treatment of ileal or colonic Crohn disease (table 1). The confirmatory cohort were all whites with CD or ulcerative colitis (UC [MIM 191930]) who were treated at the Robert-Bosch-Hospital (Stuttgart) or the University Hospital (Vienna) (table 1). Diagnostic and (Vienna) classification criteria were the same at both European centers.¹⁷ In this classification, L1 is defined as ileal disease only, L2 as colonic disease only, and L3 as ileal as well as colonic disease. Finally, the Stuttgart control group was combined with blood donors ($n = 103$), individuals unaffected by inflammatory bowel disease who underwent surveillance colonoscopy ($n = 46$), and 20 control individuals unaffected by intestinal disease from Cleveland. All colonoscopies that included biopsies were performed on patients from the Stuttgart cohort.

Array CGH (Array-Based Comparative Genomic Hybridization) Analysis

DNA preparation, labeling, hybridization, and analysis procedures were performed as published elsewhere.^{25,26} In brief, genomic DNA from fresh-frozen blood obtained from 10 patients with colonic CD and from 10 healthy control individuals was isolated using the Blood and Cell Culture Kit (Qiagen) following the instructions of the supplier. Sample DNA and reference DNA (pooled DNA from six healthy individuals) were labeled differentially with use of the Bioprime Labelling Kit (Invitrogen) and were hybridized on a DNA microarray consisting of ~8,000 genomic fragments

Table 1. Number of Patients in the Cohorts Examined for *HBD-2* Gene Copy Number

Cohort	No. of Subjects		
	Cleveland	Stuttgart	Vienna
Control	20	149	
CD:	85	54	111
Ileal (L1)	60	22	38
Colonic (L2)	25	10	36
Ileal and colonic (L3)		22	37
UC		38	37

covering the human genome at a resolution of ~0.5 Mb.²⁷ For the beta-defensin locus at 8p23.1, all additional genomic fragments that were available at the time were added to the microarray to enhance the resolution in the region of interest. The chromosomal mapping information was based on the Ensembl Genome Browser release 36.35i (December 2005). Arrays were scanned, and fluorescence intensities of all spots were filtered (intensity/local background >3; mean/median intensity <1.3; SD of genomic fragment log ratios <0.25) and were normalized blockwise. Chromosomal breakpoints delimiting regions of different copy number status were detected by GLAD (gain and loss analysis of DNA).²⁸

Determination of *HBD-2* Gene Copy Number

A TaqMan real-time PCR assay, specifically for amplification of genomic *HBD-2*, was established by using a specific set of amplification primers (forward 5'-CACCTGTGGTCTCCCTGGAA-3'; reverse 5'-AGCTTCTTGGCCTCCTCATG-3') and a probe (6-FAM-ATGCTGCAAAAAG-MGB). Quantitative *HBD-2* amplification data were normalized to *ALB* (*albumin* [MIM 103600]) as an internal reference gene, which was coamplified simultaneously in a single-tube biplex assay. The primers and probe for *HBD-2* were designed using Primer Express software, version 1.5 (Applied Biosystems). For *albumin*, we used the primers and probe that were published elsewhere.²⁹ Primers were purchased from MWG-Biotech, and probes were obtained from Applied Biosystems. Real-time PCR was performed using the ABI Prism 7700 sequence-detection system. Amplification reactions (25 μ l each) were performed in triplicate with 20 ng of template DNA, 1 \times TaqMan Universal Master Mix buffer (Applied Biosystems), 300 nM of each primer, and 200 nM of each fluorogenic probe. Thermal cycling was initiated with a 2-min incubation at 50°C, followed by a first denaturation step of 10 min at 95°C and then by 40 cycles for 15 s at 95°C and for 1 min at 60°C. In each assay, a standard curve was recorded and a no-template control was included. To amplify *HBD-2* and *albumin* in a one-tube biplex assay, limiting primer conditions were identified, to avoid competition of the two reactions. Quantification was performed by both the standard-curve method and the comparative C_T (threshold cycle) method, as described elsewhere.²⁹ The assay was validated with a selection of DNA samples, genotyped elsewhere (kindly provided by E. J. Hollox, Nottingham, United Kingdom), that contained 3, 4, 5, and 7 *HBD-2* gene copies.²⁴

Real-Time Quantitative PCR

Frozen biopsies were disrupted in 1 ml of Trizol (Gibco BRL) until complete fragmentation occurred. Total RNA was extracted according to the supplier's protocol. RNA quality was determined

by electrophoresis and was quantified by photometry. Subsequently, 2 μ g of RNA was reverse transcribed with oligo-dT primers and 200 U Superscript (Gibco BRL), according to the routine procedure.

cDNA samples were subjected to real-time PCR as outlined elsewhere.²¹ In brief, an aliquot corresponding to 50 ng of RNA was set up in a 20- μ l reaction mixture containing 4 mM MgCl₂, 0.5 μ M of each primer (for *HBD-2*, forward 5'-ATCAGCCATGAGG-GTCTTGT-3' and reverse 5'-GAGACCACAGGTGCCAATT-3' [annealing temperature 60°C; product 172 bp]; for *HBD-3*, forward 5'-TGAAGCCTAGCAGCTATGAGGATC-3' and reverse 5'-CCGCC-TCTGACTCTGCAATAA-3' [annealing temperature 62°C; product 128 bp]; for *interleukin 8* [*IL-8* (MIM 146930)], forward 5'-ATGACT-TCCAAGCTGGCCGTGGC-3' and reverse 5'-TCTCAGCCCTTCAAAAACCTC-3' [annealing temperature touch down protocol 66°C–60°C; product 292 bp] [Sigma]) and 1 \times LightCycler-Fast-Start DNA Master SYBR Green I Mix (Roche Molecular Biochemicals) and was loaded in capillary columns. PCR was performed for 45 cycles in a LightCycler (Roche Molecular Biochemicals). After each cycle, fluorescence emission readings reflecting the increase in PCR products were monitored and analyzed using LightCycler software (Roche Molecular Biochemicals).

DNA Sequencing

Exons 1 and 2 of the *HBD2* gene and ~50 bp of adjacent non-coding regions were PCR amplified from genomic DNA and were sequenced on an Applied Biosystems 3100 capillary sequencer with use of Big-Dye chemistry. The sequences of the amplification and nested sequencing primers are available on request.

Statistics

Statistical comparisons of copy numbers of (1) ileal and colonic subsets in the Cleveland cohort as well as (2) patients with L1, L2, and L3 UC and (3) control individuals from the European collective were performed with the (two-sided) Mann-Whitney test. Additionally, Kruskal-Wallis analysis of variance of ranks, including post hoc assessment by Dunn's test, was performed to correct for multiple testing. Differences in copy number distribution among the clinical cohorts were assessed by the Pearson χ^2 test, with continuity corrections. Again, the Mann-Whitney test was used to assess differences in *HBD-2* mRNA expression with increasing *HBD-2* gene copy numbers.

Results

Microarray Analysis

Using an array CGH with ~8,000 genomic fragments covering the human genome, with an average resolution of ~0.5 Mb, we screened 10 patients with colonic CD and 10 healthy control individuals for DNA copy number variations. No gross chromosomal aberrations were present in either group (data not shown), but we detected several known regions of CNP, such as the *IGHG1* gene cluster at 14q32.33 (MIM 147100) (data not shown) or the *amylase* gene cluster at 1p21.1 (MIM 104700) (fig. 1A). Except for the beta-defensin gene cluster at 8p23.1 (fig. 1B), however, no region showed a bias toward copy number loss or gain in patients versus controls. In the Ensembl Genome Browser (release 36.35i), the beta-defensin cluster is shown to be

organized as a pair of inverted repeats separated by a sequence gap (fig. 1C). This probably represents the minimal size of the locus in humans. In the patient and control samples that showed copy number variation compared with the control DNA, the size of the variable region was always the same, covering ~900 kb (megabase 7.1–8.0), including BAC clones RP11-278P18, RP11-1005B13, and RP11-52B19 (Clone Registry). This region contains both copies of the beta-defensin cluster but not the alpha-defensin cluster, as is shown for patient X1604 in figure 1C. When comparing the copy number profiles of the patient and control groups, it became evident that patients with CD, on average, seemed to have fewer copies than did healthy individuals. Relative to a DNA pool from 6 healthy control individuals, 8 of 10 patients with CD displayed small copy number losses, and none displayed copy number gains (fig. 1D). In control individuals, no such bias was observed (fig. 1E). The neighboring alpha-defensin gene cluster (fig. 1C), which mapped to megabase 6.76–6.90, and other beta-defensin-like loci—identified on chromosomal bands 6p12, 20q11.1, and 20p13 by sequence similarity search³⁰—did not show any copy number variation in patients or control individuals (data not shown).

HBD-2 Gene Copy Numbers in Inflammatory Bowel Diseases

Our array CGH analyses clearly indicated that low DNA copy number at the main beta-defensin locus might be connected to CD; however, the size of this initial sample was much too small for reliable correlation analysis. Furthermore, whereas array CGH is extremely useful for whole-genome scans, its quantitative performance is suboptimal at loci that are rich in low copy number DNA repeats (LCRs), such as the beta-defensin cluster. At such loci, the reported copy number ratios can be severely affected by cross-hybridization of the LCRs.²⁵

Therefore, we decided, as an alternative, to use quantitative PCR analysis of the *HBD-2* gene to estimate the DNA copy number of the beta-defensin cluster. This *HBD2* gene-specific approach was applied to a control population and to two patient cohorts, one from the United States and one from Europe (table 1). In the control population ($n = 169$) the copy numbers had a range of 2–10 per genome, with a median number of 4 copies (fig. 2A). The numbers of control individuals who carry the median (4), below-median (<4), or above-median (>4) number of copies were about equal. Details of the copy number frequencies of all cohorts and subgroups are given in table 2.

The U.S. patient cohort from the Cleveland Clinic consisted of 85 surgical patients with CD who had indications for ileal versus colonic resection. In patients with ileal resections (fig. 2B), the median number of copies was identical to that of the control group (4 copies); also, the frequency distribution of the three subgroups (with <4, with 4, and with >4 gene copy numbers) was not significantly

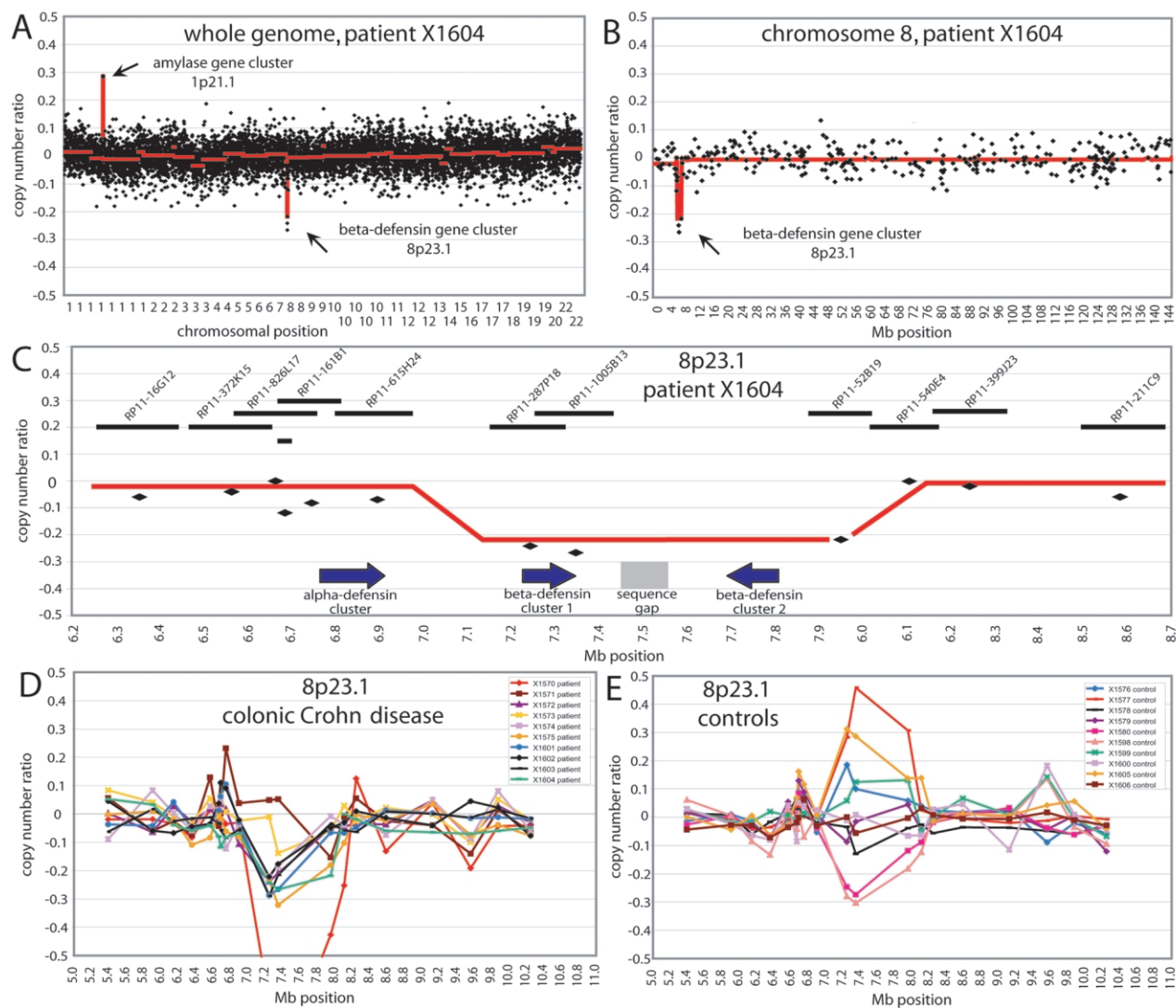


Figure 1. A, Copy number ratios of ~8,000 clones covering the whole genome, with a resolution of ~0.5 Mb, are shown for patient X1604. The loci of the amylase and beta-defensin gene clusters are indicated. The red lines in panels A and B show the smoothed copy number ratio, as calculated by the GLAD algorithm. B, Copy number ratios of all clones on chromosome 8 of patient X1604. The beta-defensin locus on 8p23.1 is indicated. C, Detail of copy number ratios on 8p23.1 for patient X1604. The alpha-defensin and the two beta-defensin loci are shown by arrows, indicating the genomic orientation of the loci; “sequence gap” indicates a region where no human reference sequence is available. The genomic position, length, and name of genomic fragments covering the region of interest are displayed at the top. The red line indicates the average genomic position of the region that shows a CNP in our 20 hybridizations. D, Copy number ratios in the 8p23.1 region of 10 patients with colonic CD. E, Copy number ratios in the 8p23.1 region of 10 healthy control individuals.

different from that of the control group. In contrast, the majority (72%) of patients with colonic resections had a copy number <4, with a median of 3 copies (fig. 2C). The difference in copy numbers was highly significant ($P = .008$ [Mann-Whitney]) between the ileal and colonic subgroups with CD. This was maintained when tested by Kruskal-Wallis analysis of variance ($P < .01$ for both post hoc Dunn test ileal vs. colonic and colonic vs. control). Similarly, the proportion of the three copy number groups in the two subgroups with CD was significantly different ($P = .018$ [Pearson χ^2]).

An independent second cohort of European patients with CD ($n = 165$) from Stuttgart and Vienna was classified according to the Vienna classification of location into those with ileal disease only (L1), with colonic disease only (L2), or with ileal plus colonic disease (L3). Again, ileal CD (L1) exhibited a copy number distribution similar to controls ($P > .05$), with a majority in the group with 4 gene copies (fig. 3). The median was 4 copies in L1 and controls compared with 3 in L2 ($P = .032$ and $P = .001$, respectively [Mann-Whitney]). Analysis of variance and post hoc test identified a significant difference between L2 and controls

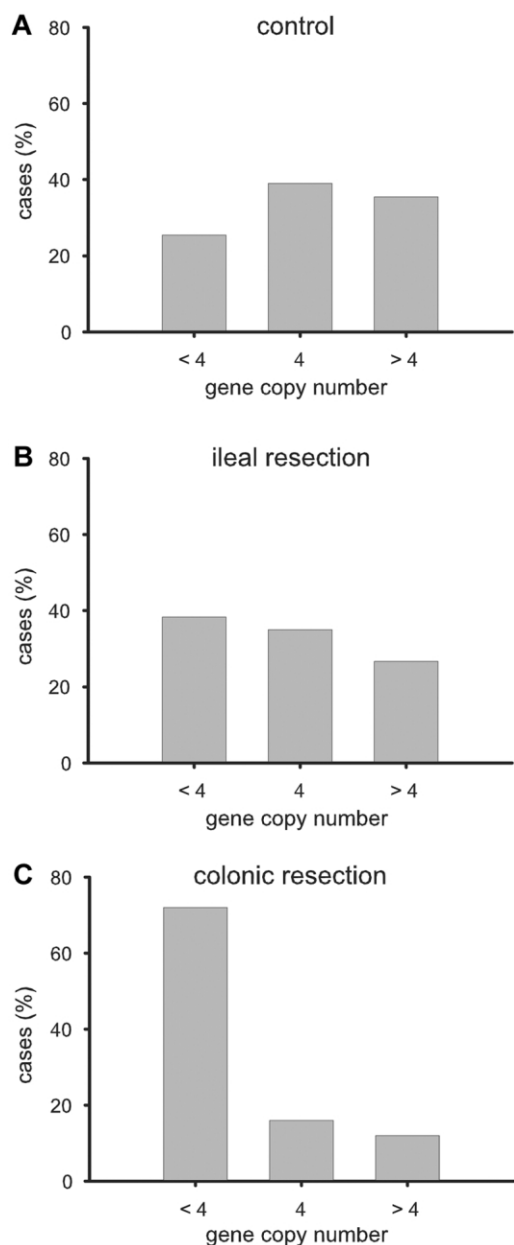


Figure 2. Distribution of *HBD-2* gene copy numbers in the 169 controls from Stuttgart and Cleveland (A) and both surgical CD cohorts with ileal (B) and colonic resection (C) allocated to <4, 4, or >4 copies per genome. The difference in distribution between ileal and colonic resection was significant ($P = .018$ [χ^2 test]).

only ($P < .05$). Colonic CD only (L2) was characterized by a shift to lower copy numbers, with the majority (52%) carrying <4 copies of *HBD-2* ($P = .002$ vs. controls; $P = .037$ vs. L1 [Pearson χ^2]). Individuals with ≤ 3 copies have a significantly higher risk of developing colonic CD than do individuals with ≥ 4 copies (odds ratio 3.06; 95% CI 1.46–6.45). Patients with L3 showed an intermediate distribution pattern between controls and L2, and, although the median was the normal 4 copies, the shift in distri-

bution was significant ($P = .034$ [Mann-Whitney]). In UC, differences with controls were not significant. Detailed noncategorized copy number data are given in table 2.

HBD-2 Expression Related to *HBD-2* Gene Copy Numbers in Inflamed Mucosa

Mucosal biopsies were analyzed for *HBD-2*, *HBD-3*, and *IL-8* expression in patients from the Stuttgart cohort. Since *HBD-2* expression is negligible in normal mucosa but is enhanced during inflammation, the biopsy samples were taken from a subgroup of 44 patients with inflammation due to CD ($n = 17$) or UC ($n = 27$). *HBD-2* and *HBD-3* expression was highly correlated ($r = 0.84$ in CD; $r = 0.86$ in UC). When the mucosal mRNA expression of *HBD-2* was related to the *HBD-2* gene copy number in the same patients (fig. 4), *HBD-2* mRNA expression was significantly diminished in the group with <4 compared with 4 copies ($P = .023$ [Mann-Whitney]) or ≥ 4 ($P = .033$), whereas *IL-8* expression in these copy number groups was not significantly different (data not shown).

HBD-2 Gene Sequencing

Finally, to investigate the possibility that *HBD-2* gene expression might be affected by the presence of gene copies that are inactivated by point mutations, we sequenced exon 1 and exon 2 of the *HBD-2* gene in eight patients with colonic CD and eight control individuals. We did not find any nonsense or missense mutations in the coding region. The only sequence variations present were one synonymous SNP in exon 2 (*rs2740090*) and four SNPs in noncoding sequences (*rs2740086*, *rs2740091*, *rs2737912*, and *rs2737913*). These SNPs occurred without predominance in either patients or control individuals (data not shown; SNPs were retrieved from dbSNP).

Discussion

CNPs and their effect on phenotypic variation and disease recently have become a major focus of attention.^{31–33} At present, >200 loci of large-scale CNPs have been detected

Table 2. *HBD-2* Gene Copy Number Frequencies

Cohort	Percentage of Cohort with <i>HBD-2</i> Gene Copy Number of									
	2	3	4	5	6	7	8	9	10	
Control:										
Cleveland	5.0	15.0	45.0	25.0	5.0	5.0				
Europe	2.7	23.5	38.3	23.5	10.1	.7	.7		.7	
Cleveland:										
Ileal	1.7	36.7	35.0	20.0	6.7					
Colonic	12.0	60.0	16.0	8.0	4.0					
European:										
L1	5.0	23.3	43.3	21.7	1.7	5.0				
L2	2.2	50.0	32.6	10.9	2.2	2.2				
L3	8.5	32.2	35.6	15.3	5.1	3.4				
UC	5.3	33.3	33.3	14.7	9.3	2.7			1.3	

NOTE.—For the number of patients in these subgroups, see table 1.

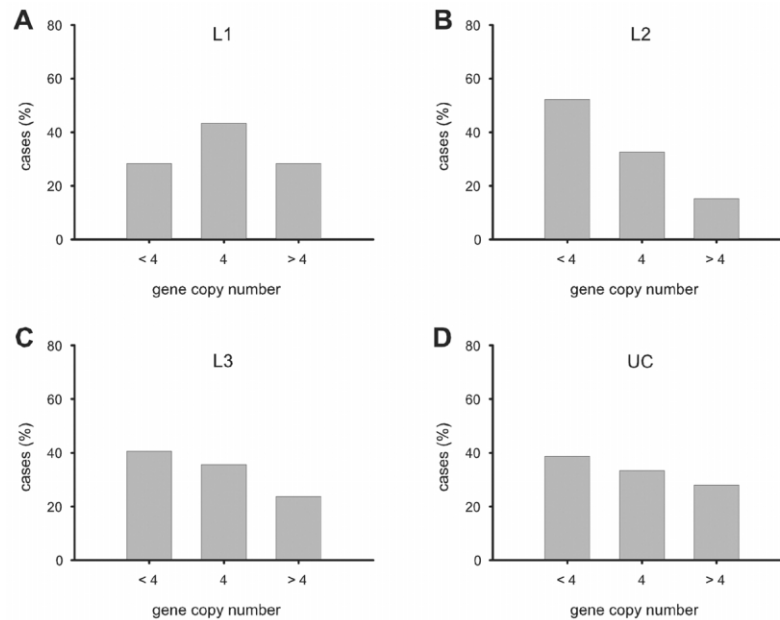


Figure 3. Distribution of *HBD-2* gene copy numbers (<4, 4, or >4 copies per genome) in the European cohort as categorized into ileal disease (L1) (A), colonic disease (L2) (B), ileal and colonic disease (L3) (C), and UC (D). L2 (colonic CD) differed significantly from L1 (ileal CD) and controls ($P = .037$ and $P = .002$, respectively [χ^2 test]).

using BAC arrays (Clone Registry)^{32,33} or oligonucleotide arrays.³¹ With use of SNP genotyping data, recent studies have found ~1,000 fine-scale deletion variants in the human genome.^{34–36} The role of these genetic alterations and their impact on disease or disease susceptibility is not clear. Several examples show that copy number alterations can lead to altered gene expression and disease; for example, copy number increase at the *alpha-synuclein* locus (4q21 [MIM 163890]) causes Parkinson disease (MIM 168600),³⁷ and duplication of *PMP22* (17q11.2 [MIM 601097]) causes Charcot-Marie-Tooth disease (MIM 118220).³⁸ Recently, a study showed that some cases of early-onset Alzheimer disease (MIM 104300) resulted from a duplication of the *APP* locus (MIM 104760).³⁹ CNPs can also lead to disease susceptibility, which was shown, for example, for the possession of a low copy number of the *CCL3L1* gene (MIM 601395) that is associated with markedly enhanced HIV/AIDS (MIM 609423) susceptibility.⁴⁰ Gene CNPs, which are associated with variable drug response, have also been described for drug-metabolizing enzymes. For example, inherited amplification of an active gene in the cytochrome P450 *CYP2D* locus is a cause of ultrarapid metabolism of debrisoquine.⁴¹ We have recently reported the development of an RT-PCR-based assay to genotype *CYP2D6* (MIM 124030) with respect to its gene copy number,²⁹ similar to the technique used in the present study. A previous study of beta-defensin gene copy number in cystic fibrosis (MIM 219700) had negative results.⁴²

Recently, the finished sequence and a gene catalogue of chromosome 8 were reported.⁴³ A unique feature of the chromosome is a vast region of ~15 Mb on distal 8p that

appears to have a strikingly high mutation rate. Within this region, the CNPs in the defensin cluster on chromosome 8p23.1 are of special interest, since they may be linked to immune function and disease. The copy number variation in this region is complex, with two different clusters of defensins—alpha- and beta-defensins—apparently showing an independent variation of their copy numbers.⁴⁴ In the alpha-defensin cluster, the genes *DEFA1* (MIM 125220) and *DEFA3* (MIM 604522) code for the neutrophil defensins HNP-1 and HNP-3, respectively. Their copy numbers, with a range of 5–14, were related to neutrophil HNP 1–3 levels.⁴⁴ In contrast, copy numbers of *DEFA5* (MIM 600472) and *DEFA6* (MIM 600471)—coding for the alpha-defensins HD-5 and HD-6, respectively—appear to be stable, with two per diploid genome.⁴⁴ This was also confirmed for the patients with CD, which suggests that the low HD-5 and HD-6 levels in ileal CD are not related to low copy numbers.⁴⁵

Prompted by the diminished induction of beta-defensins in the colonic mucosa of patients with CD compared with patients with UC,^{21–23} we asked whether the copy numbers in the variable beta-defensin locus were low with respect to those in both healthy control individuals and subjects with inflammatory bowel disease (UC and ileal CD). The initial experiments, with use of high-resolution array CGH for genomic copy number profiling, suggested that, compared with controls, there indeed was a lower copy number of the beta-defensin locus in patients with colonic CD. All other regions of CNPs covered by the microarray showed equal copy number distributions between patients with colonic CD and control individuals. A se-

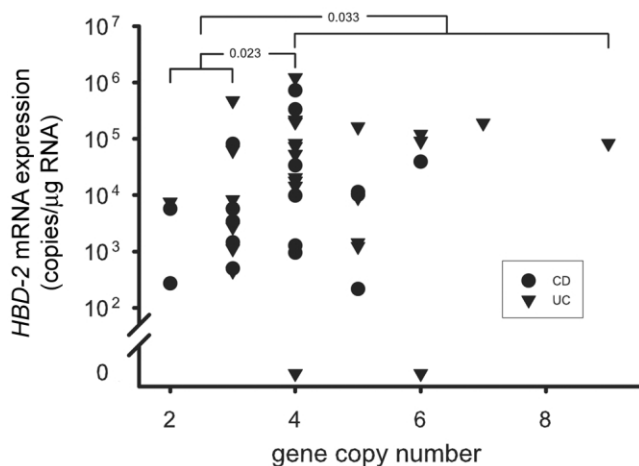


Figure 4. *HBD-2* mRNA expression, with respect to *HBD-2* gene copy number, in mucosal specimens from patients with CD and UC with rectal inflammation.

quencing approach of the *HBD-2* gene in a limited number of patients with colonic CD and control individuals revealed several known SNPs but no nonsense or missense mutations. None of the SNPs was preferentially found in either patients or control individuals. Further haplotype analysis of the *HBD-2* gene was not performed because of the immense difficulties of such analyses.⁴⁶

It might seem surprising that the beta-defensin locus on chromosome 8 has not been identified in any of the previous linkage studies,^{10,11} in particular since deletions recently were shown to be in linkage disequilibrium with SNPs.³⁵ The relevance of this finding for the present study is questionable, however, since the beta-defensin cluster consists of multiple gene copies and is expected to be much less stable than a simple deletion. Therefore, it is not clear whether gene CNPs are at all detectable by linkage analyses. Furthermore, previous studies might have overlooked a possible linkage, since they did not distinguish colonic and ileal CD.

Since DNA microarrays allow full coverage of the human genome but are less suitable for high-throughput studies in large patient cohorts, we developed a real-time PCR-based technique to quantitate *HBD-2* copy number variations. Since *HBD-2* and *HBD-3* copy numbers covary in tandem, it may be assumed that the measured *HBD-2* copy number reflects the whole beta-defensin gene cluster.^{24,44} This is also consistent with the close correlation of *HBD-2* and *HBD-3* expression demonstrated in the present and a previous study.²¹ A similar PCR-based technique was recently published by Chen et al.⁴⁷ The normal median number of 4 copies found in the present study confirms the finding of Chen et al.⁴⁷ and that of Hollox et al.⁴² but is slightly lower than the median of 5 observed by Linzmeier et al.⁴⁴

Since the surgical patients with CD usually have a par-

ticularly severe disease course, we first studied patients with ileal versus colonic resections as typical phenotypes. A clear-cut difference by 1 gene copy was observed and was then confirmed in a second, larger cohort of patients classified clinically according to the Vienna classification.¹⁷ We focused on localization as a stable factor during the disease course and noted that the patient cohort with colonic disease experienced a shift to lower copy numbers. Although this shift was significant, there was clearly an overlap between the groups, which suggests that additional, still unknown factors of genetic or environmental origin impact CD. Interestingly, patients with both ileal and colonic CD (L3) had an intermediate copy number distribution.

Notably, this relatively small difference in *HBD-2* copy numbers appeared to have an influence on mucosal *HBD-2* expression by gene dosage effect,⁴⁸ which was significantly higher in patients with 4 compared with those with fewer gene copies. The biological consequence will likely be an impaired antimicrobial defense in individuals with <4 copies. This is consistent with the previous finding of Hollox et al.²⁴ that *HBD-2* expression in lymphoblastoid cell lines was correlated linearly with the *HBD-2* gene copy number in the range of 2–7 copies. In some contrast, we did not observe a linear increase in the higher range of copy numbers >4, which may be related to the present complex patient situation in which many other factors, including bacterial flora and inflammation, may affect defensin expression. Alternatively, the plateau in defensin gene expression we observe at copy numbers of ≥ 4 might be due to some unknown mechanism limiting gene expression in the native colon tissue, which might not be active in lymphoblastoid cells. At any rate, despite these confounding factors, the gene dosage effect on *HBD-2* expression prevailed for low-to-normal copy numbers.

In conclusion, the low beta-defensin gene copy number in CD was clearly associated with colonic disease localization—that is, a specific phenotype of CD. A normal beta-defensin copy number distribution was seen in CD of the ileum and also in UC. This study may provide the genetic basis for the diminished induction of beta-defensins in colonic CD compared with UC in which colonic beta-defensin induction is intact, as described elsewhere.^{21–23} We suggest that the antibacterial barrier in the colonic mucosa is weakened because of relative defensin deficiency in colonic CD. This finding complements our previous report of a diminished alpha-defensin synthesis in ileal CD, which was further pronounced with a concomitant *NOD2* mutation.^{19,45} This differential compromise of the complex defensin system may explain the different phenotypes of colonic versus ileal CD^{20,49} as well as the observation that bacteria of the normal flora adhere and sometimes invade the epithelium in CD.^{50–52} Possibly, this barrier problem triggers the chronic inflammation known as CD.

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Web Resources

The URLs for data presented herein are as follows:

Clone Registry, <http://www.ncbi.nlm.nih.gov/genome/clone/>
Ensembl Genome Browser, <http://dec2005.archive.ensembl.org/>
(for release 36.35i [December 2005])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for CD, *NOD2* [*CARD15*], *DLG5*, *OCTN*, HD-5, HD-6, *HBD-2*, *HBD-3*, *HBD-4*, UC, *albumin*, *IL-8*, *IGHG1*, *amylase*, *alpha-synuclein*, Parkinson disease, *PMP22*, Charcot-Marie-Tooth disease, early-onset Alzheimer disease, *APP*, *CCL3L1*, HIV/AIDS, *CYP2D6*, cystic fibrosis, *DEFA1*, *DEFA3*, *DEFA5*, and *DEFA6*)

dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/>

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