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NOD2 Status and Human Ileal Gene Expression

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Abstract

Background—NOD2 single nucleotide polymorphisms have been associated with increased risk of ileal Crohn's disease. This exploratory study was conducted to compare ileal mucosal gene expression in Crohn's disease (CD) patients with and without NOD2 risk alleles.

Methods—Ileal samples were prospectively collected from eighteen non-smoking CD patients not treated with anti-TNFα biologics and nine non-smoking control patients without inflammatory bowel disease undergoing initial resection, and genotyped for the three major NOD2 risk alleles (Arg702Trp, Gly908Arg, Leu1007fs). Microarray analysis was performed in samples from four $NOD2^{R}$ (at least one risk allele) CD patients, four $NOD2^{NR}$ (no risk alleles) CD patients and four $NOD2^{NR}$ controls. Candidate genes selected by significance analysis of microarrays (SAM) were confirmed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays of all the samples.

Results—SAM detected upregulation of 18 genes in *affected* ileum in $NOD2^{R}$ compared to $NOD2^{NR}$ CD patients, including genes related to lymphocyte activation. SAM also detected altered ileal gene expression in *unaffected* $NOD2^{NR}$ ileal mucosal CD samples compared to $NOD2^{NR}$ control samples. QRT-PCR conducted on all the samples confirmed that increased *CD3D* expression in *affected* samples was associated with $NOD2^{R}$ status, and that increased

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MUC1, *DUOX2*, *DMBT1* and decreased *C4orf7* expression in *unaffected* samples was associated with CD, independent of NOD2 status.

Conclusions—The results support the concept that NOD2 risk alleles contribute to impaired regulation of inflammation in the ileum. Furthermore, altered ileal gene expression, independent of NOD2 status, is detected in the *unaffected* proximal margin of resected ileum from CD patients.

Keywords

NOD2; Crohn's disease; ileum; microarray

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory disorder that can affect any part of the GI tract, but most commonly involves the terminal ileum. The majority of patients with ileal CD eventually undergo resection of diseased ileum [1]. Unfortunately, surgery is not curative. Endoscopic evidence of recurrent disease in the formerly unaffected neo-terminal ileum is often observed within months after resection [2]. Nucleotide-binding oligomerization domain 2 or *NOD2* genetic polymorphisms were the first genetic risk alleles identified for Crohn's disease [3–7]. Three single nucleotide polymorphisms (SNPs), Arg702Trp, Gly908Arg and Leu1007fs, account for about 80% of the NOD2 variants associated with Crohn's disease. The frequency of these risk alleles is <5% in Caucasian control subjects but approximately 30% in CD patients. *NOD2* risk alleles are associated with ileal disease location, younger age of onset and earlier initial surgery [6–8].

NOD2 is an intracellular receptor of bacteria that recognizes muramyl dipeptide (MDP), a component of the bacterial cell wall. NOD2 is expressed in monocytes, macrophages and epithelial cells, particularly in Paneth cells [9, 10]. Expression of α-defensins (*DEFA5* and *DEFA6*), which are anti-microbial peptides secreted by Paneth cells, is reported to be decreased in ileal tissues collected from CD patients harboring *NOD2* risk alleles ($NOD2^{R}$ patients) compared to CD patients with only notno risk alleles ($NOD2^{NR}$) in one study [11], but a subsequent study reports that expression is decreased with inflammation and is not related to NOD2 genotype [12]. While one study detected no significant difference in colonic gene expression between $NOD2^{R}$ and $NOD2^{NR}$ CD patients [13], another study reported that expression of the deleted in malignant brain tumors 1 (*DMBT1*) gene, is decreased in disease *affected* colon biopsies from $NOD2^{R}$ compared to $NOD2^{NR}$ CD patients [14]. This gene encodes a member of the scavenger receptor cysteine-rich superfamily that binds bacteria and is implicated in mucosal protection [15]. Increased expression of *DMBT1* has been reported to correlate with disease activity in inflamed Crohn's disease tissues and DMBT1 knockout mice exhibit increased susceptibility to dextran sodium sulfate-induced colitis [16, 17].

To further examine the functional consequences of the NOD2 risk alleles, ileal mucosal gene expression was compared in tissue samples that were prospectively collected from CD patients with and without NOD2 risk alleles undergoing initial ileocolic resection.

MATERIALS AND METHODS

Patients and acquisition of ileal tissue samples

This study was approved by the Washington University-St. Louis Human Research Protection Office. CD patients undergoing initial ileocolic resection (ICR) were prospectively enrolled in a consecutive fashion by the Washington University Digestive Diseases Research Core Center (DDRCC) Tissue Procurement Facility to donate surgically resected tissue samples between April 2005 and October 2007. The indications for surgery included failure of medical management, small bowel obstruction, fistula, abscess or perforation. The diagnosis of Crohn's disease was established according to conventional clinical and pathological criteria [18]. Patients who were unwilling or unable to give informed consent were excluded. Gender and race were recorded as reported by the patients. The patients were phenotyped using the Montreal classification with respect to age of diagnosis, disease location and disease behavior at the time of surgery as previously described [19, 20]. A detailed smoking history and medication history was obtained by reviewing the medical records and interviewing the patients. Samples from patients who smoked (≥ 7 cigarettes a week) or received anti-TNFα therapy (e.g. infliximab or adalimumab) within a year of surgery were excluded from this study. Control ileal mucosal biopsies were collected from non-smoking patients without inflammatory bowel disease undergoing initial surgery.

Biopsies of macroscopically disease *affected* and *unaffected* areas of ileal mucosa were collected from fresh pathologic specimens using Radial Jaw^{TM}4 large capacity biopsy forceps (Boston Scientific Corp., Natick, MA). Biopsies of ileal mucosa were also collected from fresh pathologic specimens of patients without inflammatory bowel disease undergoing either a right hemicolectomy or subtotal colectomy for colonic neoplasm or colonic inertia. A minimum of 4 biopsies were taken within a 2 × 2 cm^2 area from each region, immediately placed in an RNA stabilization solution (RNAlater, Applied Biosystems/Ambion, Austin, TX), and archived at −80°C. The samples were de-identified and linked to a detailed clinical database by a patient study code.

Genotyping of NOD2 SNPs

Each subject was genotyped for the Leu1007fsInsC (rs2066847, SNP13), R702W (rs2066844, SNP8) and G908R (rs2066845, SNP12) SNPs by direct sequencing or by a Taqman MGB (Applied Biosystems, Foster City, CA) genotyping platform [21] using genomic DNA prepared from peripheral venous blood or tissue. $NOD2^{R}$ patients (R = risk alleles) were defined as patients who carried at least one of the three risk alleles ($NOD2^{R/R}$ and $NOD2^{R/NR}$). $NOD2^{NR}$ patients (non-risk allele) were defined as patients who carried none of the three risk alleles ($NOD2^{NR/NR}$). In addition, the patients were genotyped for the ATG16L1T300A (rs2241880) and IL23R381N (rs11109026) risk alleles by the Sequenom Technology Core within the Washington University Division of Human Genetics (http://hg.wustl.edu/info/Sequenom_description.html).

RNA isolation and Microarray Analysis

Total RNA was prepared from ileal biopsies stored in RNAlater using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. RNA quality was assessed using an Agilent 2100 Bioanalyzer. The test ileal RNA and a common reference ileal RNA ($NOD2^{NR}$, non-IBD) were labeled using the Agilent low RNA input linear amplification kit. The resulting probes were hybridized to Agilent Whole Human Genome Arrays (Agilent No.G4410A), using established protocols. Each array was globally normalized such that the overall ratio of medians of the test and reference probes was made equal to one. The arrays have been deposited in Gene Expression Omnibus (GEO accession no. GSE17594). A primary statistical analysis of the array data was performed using the significance analysis of microarrays (SAM) procedure [22]. These analyses were performed on log-transformed mean signal intensities. SAM assigns a gene-specific *t*-test (q-value) based on changes in gene expression relative to the standard deviation of repeated measurements for that gene. The criteria set for differences between groups in the SAM analysis were $q < 0.05$, fold change < 0.5 or > 2.

Quantitative real time RT-PCR

One μg total RNA was used for first strand cDNA synthesis using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. cDNA was then diluted and amplified using SYBR Green Master Mix 2× reagent (Applied Biosystems, Foster City, CA) in an Applied Biosystems 7500 Real-time analyzer in a total of 25 μl per reaction, per the manufacturer's instructions. The target gene *X* was expressed relative to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene as $-\Delta C_T$ (C_T of *GAPDH* – C_T of gene *X*), and represents a logarithmic transformation of the mRNA levels [23]. Each measurement was carried out in triplicate. The primers (see Table 1) were selected from the Primer Bank database (http://pga.mgh.harvard.edu/primerbank/). In addition to the candidate genes identified by SAM, interleukin-8 (IL-8) mRNA expression was also measured by RT-PCR as a marker of intestinal inflammation. Fold change between categorical groups was calculated as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ was the difference between the median ΔC_T values for the two groups.

Statistical analysis

Statistical analyses were carried out with the aid of Graphpad Prism 3.0. Differences in the clinical characteristics between two patient groups were compared by the nonparametric Mann-Whitney test. Differences in median levels of mRNA expression between more than two categorical groups were compared by the Kruskal Wallis test, a nonparametric alternative to the one-way analysis of variance test. The Dunn's post-test was calculated to compare pairs of categorical groups when the Kruskal Wallis test was significant. A *P* value of <0.05 was considered statistically significant. All P-values were two-tailed.

An empirical procedure was applied to estimate the power in the sample for every gene that is on the microarray and passed data quality control. The power calculation was based on a two-sample t-test with pooled variance similar to the SAM statistic. We assumed that for each gene the test statistic based on the log-transformed expression levels followed an asymptotic t-distribution. The observed variation in the control samples was used to

approximate the standard deviation; and various effect sizes were consider for a realistic power analysis. To minimize false positive rates, Bonferroni correction was used to derive a conservative estimate adjusting for multiple testing.

RESULTS

Clinical characteristics of patients

The ileal CD mucosal RNA samples used in this study were prospectively collected from 18 CD patients (median age 36 years, range 18–74 years, 43% female), who underwent an initial ileocolic resection between April 1, 2005 and October 1, 2007 and had never smoked or received anti-TNFα therapy within one year of surgery. The clinical characteristics of the CD patients as well as the 9 $NOD2^{NR}$ control subjects are summarized in Table 2. Seven of the 18 CD patients harbored at least one of the three major NOD2 risk alleles ($NOD2^{R}$) and eleven patients had no risk alleles ($NOD2^{NR}$). The median age of diagnosis in the $NOD2^{R}$ group (21 years, range 17–27 years) was significantly younger than that of the $NOD2^{NR}$ group (33 years, range 18–71 years, P <0.01). The median age of surgery in the $NOD2^{R}$ group (28 years, range 24–37 years) was also younger than the $NOD2^{NR}$ group (40 years, range 18–74 years, $P < 0.05$). However, there was no significant difference in the median duration of disease prior to surgery between $NOD2^{NR}$ and $NOD2^{R}$ CD patients. These observations are consistent with previous reports that $NOD2^{R}$ alleles are associated with younger age of disease onset and initial surgery [6–8]. The CD patients were homogeneous with respect to disease location, since all of the patients had disease located in the ileum with or without disease limited to the cecum (Montreal classification L1). With the exception of one patient, all patients exhibited either striucturing disease behavior (Montreal classification B2) or penetrating disease behavior (Montreal classification B3) at the time of surgery (see Table 2).

Comparison of gene expression by microarray

In order to identify candidate genes that exhibited altered expression due to NOD2 risk variants, whole human genome microanalyses were performed using RNA samples isolated from regions of macroscopically *unaffected* and *affected* ileal mucosa collected from the first four $NOD2^{R}$ CD patients recruited for the study (see Table 2). Because all the $NOD2^{R}$ CD patients were Caucasian, the four $NOD2^{NR}$ CD patients selected for microarray analysis were also Caucasian. The microarrays probed with RNA prepared from *unaffected* regions of the resected ileum were analyzed separately from those probed with RNA from *affected* regions. Significance analysis of microarrays (SAM) detected 18 genes ($q < 0.05$, fold change >2) that were upregulated in *affected* regions of the ileum in $NOD2^{R}$ CD patients compared to $NOD2^{NR}$ CD patients (see Table 3). As shown in Table 4, the mean log ratios of the intensities for the test probe relative to the reference control probe for the α-defensin genes, *hDEFA5* and *hDEFA6*, were not significantly decreased in the $NOD2^{R}$ CD patients compared to $NOD2^{NR}$ CD patients.

To identify candidate genes that reflect early pathogenic changes prior to the development of macroscopic disease, we performed SAM analysis to compare four microarrays probed with $NOD2^{NR}$ CD *unaffected* mucosal RNA with four microarrays probed with $NOD2^{NR}$ control

mucosal RNA. SAM detected upregulation of 165 genes ($q < 0.05$, fold change > 2) and downregulation of 196 genes ($q < 0.05$, fold change <0.5) in *unaffected* ileum from $NOD2^{NR}$ CD patients compared to $NOD2^{NR}$ control patients (see supplementary Tables 1 and 2).

Confirmation of candidate genes by qRT-PCR

Altered expression of selected candidate genes were then confirmed by qRT-PCR analysis of samples from all the patients listed in Table 2 (7 $NOD2^{R}$ CD patients, 11 $NOD2^{NR}$ CD patients and 9 $NOD2^{NR}$ control patients). We focused on candidate genes with ($q < 0.001$ and fold change either <0.5 or >2). The median *CD3D* mRNA expression was 3-fold higher ($P = 0.004$) in *affected* ileal samples from 7 $NOD2^{R}$ CD patients compared to 11 $NOD2^{NR}$ CD patients. While there was a trend towards increased expression of the *CD48* and *CYBB* genes in *affected* ileal samples from $NOD2^{R}$ CD patients compared to $NOD2^{NR}$ patients, the differences did not reach statistical significance. There was no significant difference in *hDEFA5* or *hDEFA6* expression between $NOD2^{R}$ and $NOD2^{NR}$ patients detected by qRT-PCR assays (data not shown).

Because of the patchy nature of the inflammatory changes in Crohn's disease, we measured IL-8 mRNA expression as an internal marker of inflammation rather than by grading the histopathology of adjacent biopsies. While the median IL-8 mRNA expression levels in *affected* ileum was significantly elevated compared to control ileal samples, both the *affected* and *unaffected* ileum from CD patients exhibited a considerable range of IL-8 mRNA expression levels (see Figure 1). Upregulation of *MUC1 DUOX2* and *DMBT1* expression and downregulation of *C4orf7* gene (encodes follicular dendritic cell secreted protein) expression was confirmed by qRT-PCR analysis of a larger set of disease *unaffected* mucosal RNA from 17 CD patients (both $NOD2^{R}$ and $NOD2^{NR}$ CD patients) and 9 $NOD2^{NR}$ control subjects.The altered expression of these genes could potentially be linked to upregulation of pro-inflammatory genes such as IL-8, even in samples of macroscopically disease *unaffected* ileal samples. We therefore recalculated the differences in the expression of these genes after excluding CD ileal samples with elevated IL-8 mRNA expression (defined as exceeding the mean control IL-8/GAPDH ΔC_T level by greater than one standard deviation or 1.4). Upregulation of the *MUC1* and *DMBT1* genes, and downregulation of the *C4orf7* gene, remained significant at a level of $P = 0.03$ (uncorrected for multiple comparisons).

DISCUSSION

The major aim of this exploratory study was to identify candidate ileal genes that were affected by $NOD2^{R}$ status by comparing separately, whole human genome expression profiling of disease *unaffected* (proximal margin) and *affected* regions of the resected ileum in CD patients undergoing initial ileocolic resection. A secondary aim was to identify candidate genes that were altered in the disease *unaffected* proximal margin in CD patients compared to control non-IBD patients. In this study, the CD patients were relatively homogeneous with respect to disease location and had advanced disease requiring surgical intervention. Since smoking was linked to ileal disease location [24] and anti-TNFα (e.g.

Remicade or Humira) therapy often resulted mucosal healing [25], these patients were excluded from this analysis. However, the patients were still heterogeneous with respect to exposure to other medications (e.g. steroids, immunomodulators). Because of the small sample size, patients with different NOD2 SNPs were combined into a single categorical group, regardless of gene dosage. In addition, the patients were potentially genetically heterogeneous at other CD susceptibility loci [26]. Genotyping at the ATG16L1T300A (rs2241880) revealed, however, that all of the CD patients carried at least one risk allele. Genotyping at the IL23R (rs11209026) revealed that all of the CD patients were homozygous for the risk allele except for two that were heterozygous for the risk allele.

A post-hoc power analysis estimates that 28 and 14 microarrays are were required in each categorical group to reach a median power of 80% for respectively a 2-fold and 3-fold change in expression for the >40, 000 genes present on the microarray. Hence the study is was underpowered to determine whether lack of expression differences in genes (e.g. *hDEFA5* or *hDEFA6*) is was significant. In addition, the sample is was heterogeneous with respect to the three NOD2 risk alleles, which may not have differing effects on gene expression.

Nonetheless, a limited number of candidate genes were confirmed by qRT-PCR on samples in addition to the original set used for the microarrays. Upregulation of *CD3D*, in the *affected* ileum of $NOD2^R$ CD patients was confirmed by qRT-PCR assays. Upregulation of this gene as well as others [27–32] identified by SAM analysis, supported the concept that *NOD2* risk alleles contributed to impaired regulation of inflammation in ileal CD. Both reduced tolerance to bacterial products [33, 34] and impaired clearance of invasive bacteria in $NOD2^R$ CD patients [35] were previously proposed to drive deregulated inflammation.

MUC1, *DUOX2* and *DMBT1* gene expression are were confirmed by qRT-PCR to be significantly upregulated in the disease *unaffected* proximal margin of CD compared to control non-IBD ileal resection specimens. *MUC1* encodes mucin 1, an epithelial glycoprotein that is overexpressed in human adenocarcinoma and inflammatory bowel diseases [36]. This protein is implicated in host defense and in exacerbation of colitis in animal models [37]. *DUOX2* encodes dual oxidase 2, which is implicated in the generation of hydrogen peroxide and is expressed along the length of the GI tract [38]. Upregulation of *DUOX2* has been previously reported in disease *unaffected* regions of the colon from CD patients compared to control non-IBD patients [39]. *DMBT1* encodes a member of the scavenger receptor cysteine-rich superfamily that binds bacteria and is implicated in mucosal protection [15]. Some of the observed changes could reflect inflammation as detected by elevated IL-8 mRNA expression in samples collected from the CD patients. However, increased expression of *MUC1* and *DMBT1* was still observed after exclusion of CD samples with elevated IL-8 mRNA expression levels. Because both of these genes are implicated in mucosal defense against bacteria, these results suggest that there are abnormal mucosal-microbial interactions in the region of the ileocolic anastomosis at the time of surgery. Down-regulation of *C4orf7* gene expression, which encodes a follicular dendritic cell secreted peptide [40, 41], was also observed after exclusion of CD samples with elevated IL-8 mRNA expression levels. SAM also detected down-regulation of other genes (*CCL23, BACH2, LRMP, FCRL4*, see Supplementary Table 2) associated with organized

lymphoid structures and/or B-cell function in the disease *unaffected* proximal margin of the resected ileum in the CD patients [42–45]. These changes in gene expression support the hypothesis that changes in lymphoid follicles and the associated epithelium play a role in the pathogenesis of Crohn's disease [46].

Increased expression of a number of genes related to neuronal differentiation in *unaffected* regions of the ileum, such as *Olig1, DRD4, NPAS3, LBX1 and IRX 1* (see supplementary Table 1), could reflect previously described neural proliferation in mildly affected areas or areas adjacent to severely diseased areas of the intestine in CD [47]. Isolation of a pure cell population by methods such as laser capture microdissection would be required in order to distinguish between changes in gene expression within a cell population from changes reflecting architectural alterations or compositional changes (e.g. influx of inflammatory cells) in the mucosal samples.

Further analysis of the contribution of individual NOD2 risk alleles and other risk alleles to CD pathogenesis will require collection of additional patient samples with detailed annotation of disease phenotype, genetic susceptibility loci and environmental exposures. Nonetheless, in this limited dataset, our results support the concept that NOD2 risk alleles contribute to impaired regulation of inflammation in the ileum. Furthermore, altered ileal gene expression, independent of NOD2 status, is was detected in the disease *unaffected* proximal margin of the ileocolic resection specimen of CD patients compared to control patients.

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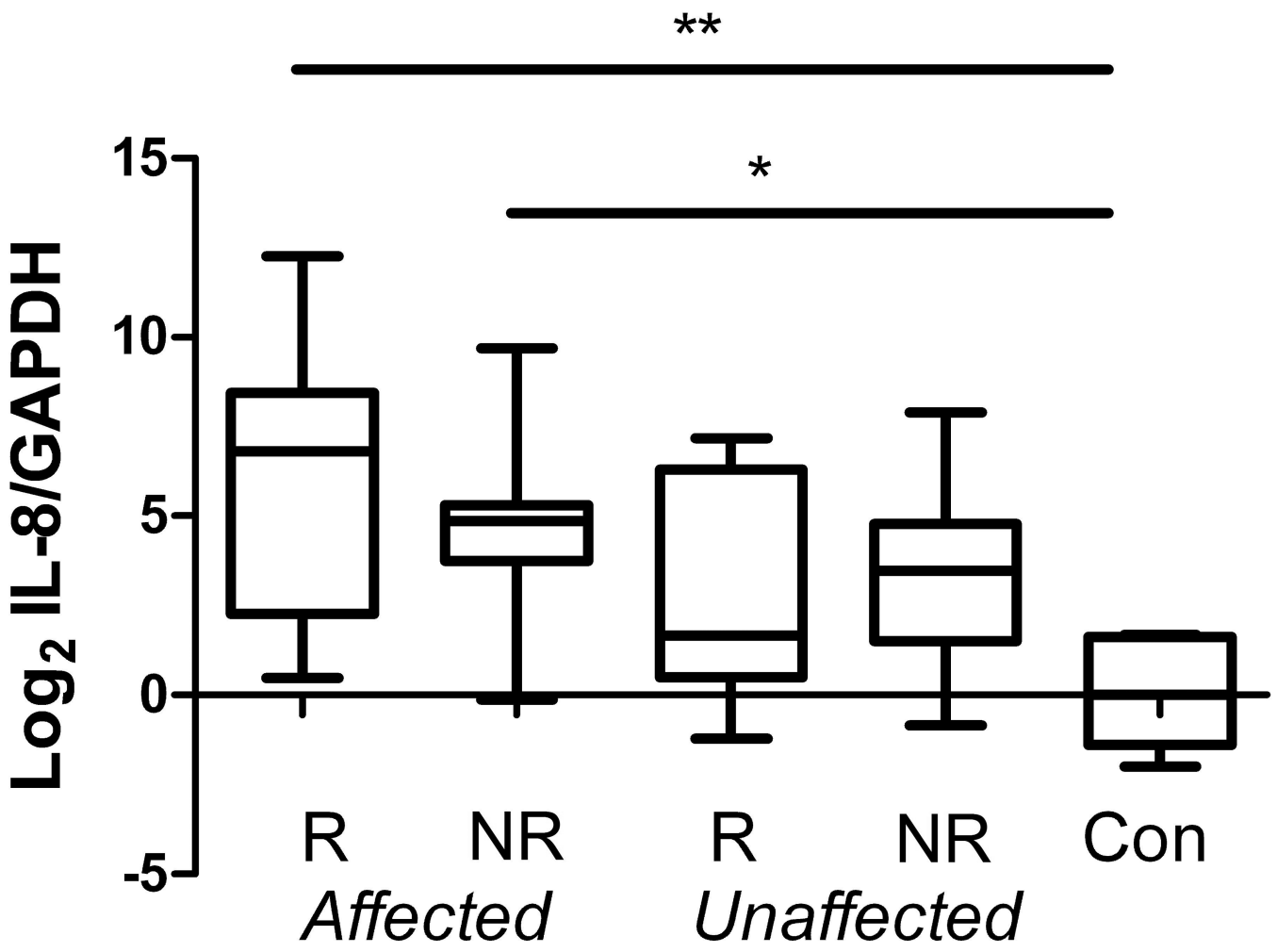

Figure 1.

IL-8 mRNA expression is increased in *affected* ileum from NOD2^{R}(R) CD and NOD2^{NR}(NR) CD patients compared to control subjects (Kruskal Wallis test $P = 0.004$, * and ** denotes significance in Dunn's multiple comparison tests between categorical groups $P < 0.05$). The ends of the box represent the 25th and 75th percentiles respectively. The line in the box represents the median value. The whiskers represent the minimum and maximum values. The results are expressed relative to the median $-\Delta C_T$ values from control subjects.

Table 1

Primer sequences for qRT-PCR assays.

Primer Name	Primer Sequence	Accession Number
hIL-8 Forward	5’-ACT GAG AGT GAT TGA GAG TGG AC-3'	NM_000584
hIL-8 Reverse	5’-AAC CCT CTG CAC CCA GTT TTC-3'	NM_000584
hGAPDH Forward	5’-CAT GAG AAG TAT GAC AAC AGC CT-3'	NM_002046
hGAPDH Reverse	5’-AGT CCT TCC ACG ATA CCA AAG T-3'	NM_002046
hCD3D Forward	5’-TAC CGT GCA AGT TCA TTA TCG AA-3’	NM_000732
hCD3D Reverse	5’-GCC TTC CAG TCT CAT GTC CAG-3’	NM_000732
hMUC1 Forward	5’ –AAG CAG CCT CTC GAT ATA ACC T -3’	NM_002456
hMUC1 Reverse	5’ –GGT ACT CGC TCA TAG GAT GGT -3’	NM_002456
hDMBT1 Forward	5’-TGC ACC TGG TCA TAC GGA GTT GAA-3'	NM_004406
hDMBT1 Reverse	5’-TGG ATT CTG GGA CGT CAG GTC TAT-3'	NM_004406
hC4orf7 Forward	5’-AGT GGC TGT TGG TTT CCC AG-3’	NM_152997
hC4orf7 Reverse	5’-GGC GAA ATG GAT ATG GGT AAG G-3’	NM_152997

Table 2

a. Clinical characteristics of NOD2^{R} CD patients.

Patient	NOD2 Genotype	Age at Surgery (years)	Disease Duration[1] (years)	Disease Behavior[2]	Sex	Race[3]	Medication[4]
15*	R702W/WT	37	10	B2	F	W	C,D
196*	InsC/R702W	24	7	B3	F	W	B
235*	R702W/WT	28	11	B3	M	W	B,D
312*	InsC/InsC	28	1	B3	F	W	B
382	InsC/G908R	24	3	B3	M	W	A,B,C
404	R702W/WT	24	0	B3	F	W	A,B,C,D
442	InsC/WT	33	13	B1	M	W	C,D

b. Clinical characteristics of NOD2^{NR} CD patients.

Patient	NOD2 Genotype	Age at Surgery (years)	Disease Duration[1] (years)	Disease Behavior[2]	Sex	Race[3]	Medication[4]
16	WT/WT	40	4	B2	F	A	
19*	WT/WT	47	19	B3	M	W	B,C
22*	WT/WT	74	3	B3	M	W	A, D
124*	WT/WT	36	7	B2	M	W	A
178	WT/WT	20	0	B3	F	W	B
204	WT/WT	48	2	B2	M	B	A,C,D
163	WT/WT	35	1	B3	F	B	A,C
218	WT/WT	38	5	B3	M	W	B,C,D
256*	WT/WT	46	16	B3	M	W	A,D
233	WT/WT	69	5	B2	F	W	A, C, D
576	WT/WT	18	0	B2	M	W	B,C

C. Clinical characteristics of NOD2^{NR} non-IBD control subjects.

Sample	NOD2 Genotype	Age at Surgery (years)	Sex	Race[1]	Diagnosis
N13*	WT/WT	61	M	W	Colonic inertia
N14	WT/WT	72	M	W	Colon cancer
N15*	WT/WT	71	M	W	Colon cancer
N17*	WT/WT	56	F	W	Ileal nodule
N19	WT/WT	47	F	W	Fistula, adenomatous polyp
N21	WT/WT	51	M	B	Colonic inertia
N33*	WT/WT	55	F	W	Colonic inertia
N34*	WT/WT	41	F	W	Colonic inertia
N39	WT/WT	40	F	W	Colonic inertia

[1] Disease duration is defined as Age at Surgery – Age of Diagnosis

[2] Disease behavior at the time of surgery is defined using the Montreal Classification: B1 inflammatory (not stenosing or penetrating); B2, stenosing; B3, penetrating.

[3] Race: W, white; B, black; A, asian.

[4] Medication: A, 5-aminosalicylic acid; B, antibiotics (in addition to perioperative cefoxitin); C steroids; D immunomodulators (azathioprine, 6-mercaptopurine or methotrexate).

* Samples selected for microarray analysis

[1] Disease duration is defined as Age at Surgery – Age of Diagnosis

[2] Disease behavior at the time of surgery is defined using the Montreal Classification: B1 inflammatory (not stenosing or penetrating); B2, stenosing; B3, penetrating.

[3] Race: W, white; B, black; A, asian.

[4] Medication: A, 5-aminosalicylic acid; B, antibiotics (in addition to perioperative cefoxitin); C steroids; D immunomodulators (azathioprine, 6-mercaptopurine or methotrexate).

* Samples selected for microarray analysis

[1] Race: W, white; B, black; A, asian.

* Samples selected for microarray analysis

Table 3

Upregulated genes in *affected* ileum from $NOD2^{R}$ vs. $NOD2^{NR}$ CD patients.

Gene Accession	Gene Description	Fold change	q-value
NM_000732*	T-cell surface glycoprotein CD3 delta chain precursor (CD3D)	3.6	<0.001
THC2437822	Unknown	2.9	<0.001
THC2437476	Unknown	3.6	<0.001
NM_001778	CD 48 antigen (CD48)	6.6	<0.001
NM_002514	Nephroblastoma overexpressed gene (NOV)	3.3	<0.001
NM_000397	Cytochrome b -245, beta polypeptide (CYBB)	2.4	<0.001
NM_002828	Protein tyrosine phosphatase, receptor type C (PTPRC)	3.9	<0.001
NM_013351	T-box expressed in T-cells (TBX21)	2.9	<0.001
NM_001037332	Cytoplasmic FMR1 interacting protein 2 (CYFIP2)	3.0	<0.001
NM_004951	Epstein-Barr virus induced gene 2 (EBI2)	5.2	<0.001
ENST00000381961	Unknown	3.0	<0.001
NM_004510	SP110 nuclear body protein (SP110)	2.3	<0.001
NM_002988	Chemokine (C-C motif) ligand 18 (CCL18)	4.2	0.04
NM_002738	Protein kinase C, beta 1 (PRKCB1)	4.7	0.04
ENST00000371030	Unknown	4.1	0.04
NM_024315	C7 open reading frame 23	2.1	0.04
A_24_P289043	Unknown	2.3	0.04
THC2337832	Unknown	2.9	0.04

Verified by real-time RT PCR

Table 4

Mean *hDEFA5* and *hDEFA6* $\log_2$ ratios of intensities (relative to a control $NOD2^{NR}$ RNA) from $NOD2^{R}$ and $NOD2^{NR}$ CD microarrays

Gene ID	Tissue	$NOD2^{R}$ log ratio Mean ± std dev	$NOD2^{NR}$ log ratio Mean ± std dev	*P* (t-test)
hDEFA5	*Unaffected* ileum	−1.6 ± 2.4	1.1 ± 0.6	0.1
	Affected ileum	−1.3 ± 0.6	−0.6 ± 0.8	0.4
hDEFA6	*Unaffected* ileum	−1.3 ± 2.7	1.1 ± 0.4	0.1
	Affected ileum	−1.2 ± 1.2	0.0 ± 0.8	0.2

Table 5

qRT-PCR of selected genes with altered regulation in *unaffected* ileum from CD patients regardless of NOD2 genotype.

Gene Accession	Gene Symbol	CD (n=16) vs. Control (n=9) Fold-change	*P* -value	CD (n=7)* vs. Control (n=9) Fold-change	*P*-value
NM_002456	MUC1	12	0.0007	5	0.03
NM_014080	DUOX2	10	0.008	3	0.3
NM_00739	DMBT1	4	0.004	3	0.03
NM_152997	C4orf7	0.05	0.008	0.01	0.02

* Excluded samples with elevated IL-8 mRNA expression