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## A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease

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Crohn's disease is a chronic inflammatory disorder of the gastrointestinal tract, which is thought to result from the effect of environmental factors in a genetically predisposed host. A gene location in the pericentromeric region of chromosome 16, *IBD1*, that contributes to susceptibility to Crohn's disease has been established through multiple linkage studies<sup>1-6</sup>, but the specific gene(s) has not been identified. *NOD2*, a gene that encodes a protein with homology to plant disease resistance gene products is located in the peak region of linkage on chromosome 16 (ref. 7). Here we show, by using the transmission disequilibrium test and case-control analysis, that a frameshift mutation caused by a cytosine insertion, 3020insC, which is expected to encode a truncated *NOD2* protein, is associated with Crohn's disease. Wild-type *NOD2* activates nuclear factor NF- $\kappa$ B, making it responsive to bacterial lipopolysaccharides; however, this induction was deficient in mutant *NOD2*. These results implicate *NOD2* in susceptibility to Crohn's disease, and suggest a link between an

innate immune response to bacterial components and development of disease.

The idiopathic inflammatory bowel diseases (IBDs), which include Crohn's disease (CD) and ulcerative colitis, are chronic disorders of the gastrointestinal tract with unknown aetiology, and with a combined prevalence of about 150–200 cases per 100,000 in western countries<sup>8,9</sup>. Although the aetiology of IBD is unknown, an abnormal inflammatory response directed against enteric microflora in a genetically susceptible host has been proposed<sup>10</sup>. Familial clustering of disease and studies of twins strongly suggest that IBD, and in particular CD, is a genetic disorder<sup>11</sup>. Genome-wide searches for IBD-susceptibility genes have resulted in the identification of several loci for CD and/or ulcerative colitis, most notably for CD, in the pericentromeric region of chromosome 16 (*IBD1*)<sup>1–6</sup>.

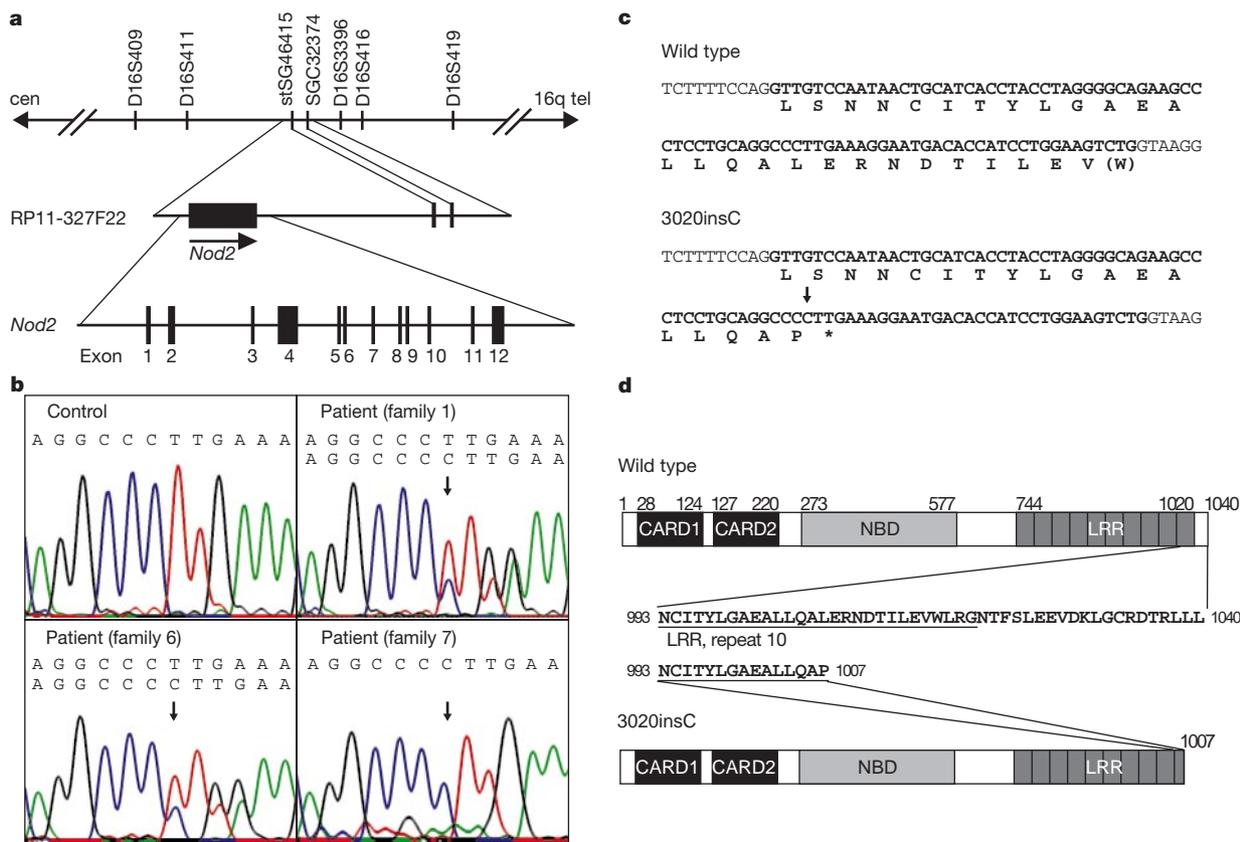
*NOD2* has structural homology with both the apoptosis regulators Apaf-1/Ced-4 and a class of plant disease resistant (R) gene products<sup>7</sup>. Like the latter gene products, *NOD2* comprises an amino-terminal effector domain, a nucleotide-binding domain and leucine-rich repeats (LRRs) (ref. 7). *NOD2* has been mapped to chromosome 16q12 (ref. 7) and is tightly linked to markers D16S3396, D16S416 and D16S419 (Fig. 1a)—a site that precisely overlaps with *IBD1* (ref. 1). Given the genomic localization and the role of *NOD* proteins in recognizing bacterial components<sup>12</sup>, we thought that *NOD2* might function as a susceptibility gene for CD.

The 12-exon genomic organization of the *NOD2* gene was determined by aligning the complementary DNA sequence

(AF178930) with one genomic bacterial artificial chromosome (BAC) clone, RP11-327F22 (AC007728) (Fig. 1a). All coding exons and flanking introns were sequenced in 12 affected individuals from pure CD families with increased linkage scores at D16S3396, as well as in 4 case controls. In three CD patients, a cytosine insertion was observed in exon 11 at nucleotide 3020 (3020insC) (Fig. 1b). 3020insC resulted in a frameshift at the second nucleotide of codon 1007 (Fig. 1b), and a Leu1007→Pro substitution in the tenth LRR, followed by a premature stop codon (Fig. 1c). The predicted truncated *NOD2* protein contained 1,007 amino acids instead of the 1,040 amino acids of the wild-type *NOD2* protein (Fig. 1d).

We used an allele-specific polymerase chain reaction (PCR) assay (Fig. 2) to type 3020insC in IBD families and case controls. Analysing only one CD patient per independent family, we observed preferential transmission (Table 1) from heterozygous parents to affected children of 3020insC (39 transmissions and 17 non-transmissions;  $P = 0.0046$ ). Analysing all independent nuclear families by sib-TDT ([ftp://lahmed.stanford.edu/pub/aspex/index.html](http://lahmed.stanford.edu/pub/aspex/index.html)), the empirical  $P$  value was similar,  $P = 0.0007$ . As expected from linkage studies<sup>2,5</sup>, no preferential transmission of 3020insC was observed among families with ulcerative colitis (data not shown). As 365 of the 416 independent CD families have several affected individuals, the applicability of these associations to the more common, sporadic cases requires further study.

Additional support for association to CD was provided by case-



**Figure 1** Identification of a frameshift *NOD2* mutation in affected individuals from CD families. **a**, Physical map of the region of interest at 16q12. Approximate positions of chromosomal and genetic markers are based on ref. 22. The human genomic BAC clone RP11-327F22 contains the *NOD2* gene and markers stSG46415 and SGC32374. The exon–intron organization of the human *NOD2* gene is shown underneath. **b**, DNA sequence electropherograms (exon 11) from control and three affected individuals from CD families. Patients from families 1 and 6 are heterozygous, whereas the patient from family 7 is homozygous for 3020insC. The cytosine insertion is indicated by an arrow.

**c**, Nucleotide and predicted amino-acid sequence of exon 11 and flanking introns from wild-type control and patients with 3020insC. The exon sequence is shown in bold. The site of 3020insC is indicated by an arrow. Residue (W) indicates that a nucleotide from exon 12 contributes to the codon. **d**, Domain structure of *NOD2*, illustrating the site of protein truncation. Caspase-recruitment domains (CARDs), the nucleotide-binding domain (NBD) and ten LRRs are shown. Residues of the tenth LRR are underlined. Numbers indicate residue positions.

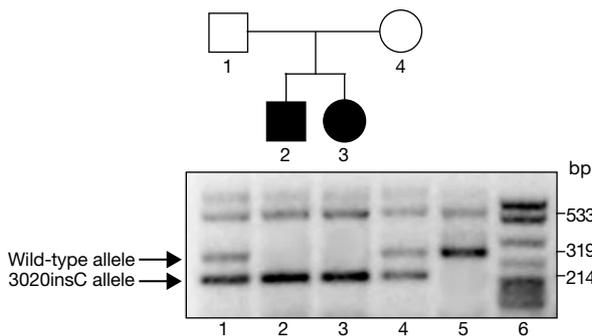
**Table 1 TDT demonstrates preferential transmission of the 3020insC to CD patients**

Source	One CD patient per family			All CD patients	
	Transmitted	Not transmitted	P value	Transmitted	Not transmitted
Univ. of Chicago	21	10	0.0046	32	16
Johns Hopkins	4	4		10	8
Univ. of Pittsburgh	14	3		26	9
Total	39	17		68	33

control analysis, in which, using one CD individual per independent family, the 3020insC allele frequency among all CD groups was 8.2% (Table 2). The allele frequencies of 3020insC were comparable among Jewish (8.4%) and non-Jewish Caucasians (8.1%). Among case controls (Table 2), the allele frequency in four separate Caucasian cohorts of 4.0% was significantly lower than in CD patients ( $P = 0.0018$ , by large-sample approximations to a two-sample binomial test). The allele frequency of the 3020insC among 182 unrelated ulcerative colitis patients was 3.0%, and was significantly lower than the frequency among CD patients ( $P = 0.0010$ ). The genotype frequencies of 3020insC in unrelated CD individuals were 11 homozygotes, 46 heterozygotes and 359 wild-type homozygotes.

Among case controls, there were 23 heterozygous individuals, with the remaining being wild-type homozygotes. The genotype-relative risk (GRR) for heterozygous and homozygous 3020insC was 1.5 and 17.6, respectively, as compared with wild-type controls. Given its frequency, 3020insC is unlikely to account completely for the observed evidence of linkage at *IBD1*, and other variants of *NOD2* may confer additional disease risk. For example, two single-nucleotide polymorphisms in *NOD2* have been identified, 2722G→C (Gly908→Arg) and 2104C→T (Arg702Trp), which are highly associated with CD by the transmission disequilibrium test (data not shown). Furthermore, other susceptibility genes might also be present in this broad region<sup>1-6</sup> of linkage on chromosome 16.

*NOD2* has been shown to activate NF-κB and to confer responsiveness to bacterial lipopolysaccharides<sup>7,12</sup>. To test the ability of wild-type and mutant *NOD2* to activate NF-κB, human embryonic kidney (HEK) 293T cells were transiently co-transfected with wild-type or 3020insC plasmids and an NF-κB reporter construct. In the absence of lipopolysaccharide (LPS), expression of both wild-type and mutant *NOD2* induced activation of NF-κB (Fig. 3a). Notably, equivalent levels of wild-type and mutant *NOD2* protein expression (as assessed by immunoblotting of total lysates) resulted in similar levels of NF-κB activation (Fig. 3a).



**Figure 2** Determination of transmission of the 3020insC mutation in a CD family by allele-specific PCR. Multiplex PCR was used to generate a nonspecific 533-bp product, along with allele-specific amplicons: a 319-bp fragment (wild type) and a 214-bp fragment (3020insC). In this family, both parents (lanes 1 and 4) are heterozygous for 3020insC, whereas both children (lanes 2 and 3) have CD and are homozygous for 3020insC. Lane 5, wild-type control; lane 6, pBR322 DNA MspI markers. Numbers on the right indicate the size of fragments.

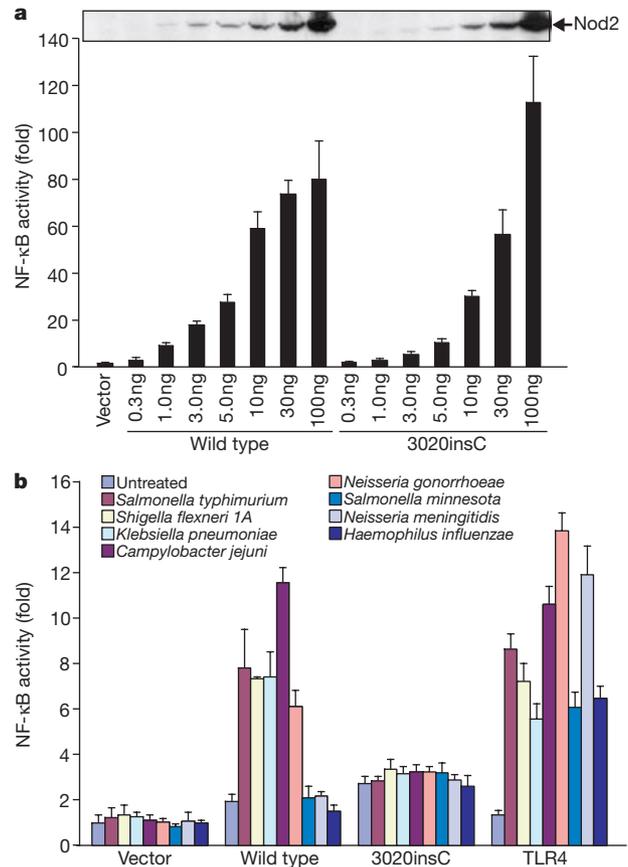
**Table 2 Allele frequency of 3020insC in unrelated Crohn's disease patients and controls**

Source	Crohn's disease			Case controls		
	Sample size	3020insC*		Source	Sample size	3020insC*
Univ. of Chicago	212	7.3	0.0018	Chicago	65	3.8
Johns Hopkins	88	6.8		Baltimore	46	3.2
Univ. of Pittsburgh	116	10.8		San Francisco	81	3.1
				Germany	94	5.3
Total	416	8.2		287	4.0	

For analysis, one CD patient was selected from 416 independent families. The difference in allelic frequency between CD patient and control was significant ( $P = 0.0018$ , by the large sample approximations to a two sample binomial test).

\* Per cent allele frequency.

Like *NOD2*, cytosolic plant disease resistant proteins have carboxy-terminal LRRs that are critical for the recognition of pathogen components and induction of pathogen-specific responses<sup>13-15</sup>. We therefore compared the ability of wild-type and mutant *NOD2* proteins to induce NF-κB activity in response to LPS. Because overexpression of *NOD2* induces potent NF-κB activation (Fig. 3a), we transfected the cells with low amounts of wild-type and mutant



**Figure 3** Differential responsiveness of wild-type and mutant *NOD2* to LPS. **a**, HEK293T cells were co-transfected in triplicate with the indicated amounts of pcDNA3 (vector), wild-type pcDNA3-*NOD2*, or pcDNA3-*NOD2* 3020insC and pEF-BOS-β-gal and pBVI-luc reporter plasmids. Values represent means ± s.d. Expression of wild-type and mutant *NOD2* proteins in cell extracts is shown on top. **b**, HEK293T cells were co-transfected in triplicate with 0.3 ng of pcDNA3-*NOD2*, 3 ng of pcDNA3-*NOD2* 3020insC, 3 ng of pcDNA3-TLR4 plus 3 ng of pcDNA3-MD-2 (indicated by TLR4) or pcDNA3 (vector) and pEF-BOS-β-gal and pBXIV-luc. Under these conditions, both wild-type and mutant *NOD2* constructs induced similar levels of basal NF-κB activity. Eight hours after transfection, cells were treated with 10 μg ml<sup>-1</sup> of LPS from indicated bacteria. Values represent means ± s.d. Results are representative of at least five independent experiments.

NOD2 plasmids to induce similar levels of protein expression and basal NF- $\kappa$ B activity (Fig. 3a). LPS from various bacteria induced NF- $\kappa$ B activation in cells expressing wild-type NOD2, but not in cells transfected with control plasmid (Fig. 3b).

Significantly, the ability of mutant NOD2 to confer responsiveness to LPS was greatly diminished when compared with wild-type NOD2 (Fig. 3b). Differential regulation of NOD2 function by LPS from different bacteria was observed (Fig. 3b), whereas all LPS preparations induced NF- $\kappa$ B activation comparably in cells transfected with Toll-like receptor-4 (TLR-4), a cell-surface receptor for LPS<sup>16</sup>.

The innate immune system regulates the immediate response to microbial pathogens and is initiated by recognition of specific pathogen components by receptors in host immune cells<sup>16</sup>. NOD1 and NOD2 seem to function as intracellular receptors for LPS with the LRRs required for responsiveness<sup>12</sup>. We have shown here that truncation of the tenth LRR of NOD2 is associated with CD. Consistent with earlier linkage studies<sup>2,5</sup>, this variant is associated solely with CD, and not with ulcerative colitis. Functional analyses indicate that the disease-associated NOD2 variant is significantly less active than the wild-type protein in conferring responsiveness to bacterial LPS. In plant NOD2 homologues, the LRRs determine the specificity for pathogen products and alterations in LRRs can result in unresponsiveness to particular pathogens<sup>13–15</sup>. Similarly, genetic variation in the LRRs of TLR4 account for inter-individual differences in bronchial responsiveness to aerosolized LPS<sup>17</sup>.

Several mechanisms can be envisaged to account for susceptibility to CD in individuals carrying this variant. NOD2 is a cytosolic protein whose expression is restricted to monocytes, with no expression detected in lymphocytes<sup>7</sup>. A deficit in sensing bacteria in monocytes/macrophages might result in an exaggerated inflammatory response by the adaptive immune system. A related possibility is that wild-type NOD2 may mediate the induction of cytokines such as interleukin-10 that can downregulate the inflammatory response<sup>18,19</sup>. Finally, variation in the LRRs of plant NOD2 homologues can result in recognition of new specificities for pathogen components<sup>13,14</sup>. Thus, it is also possible that NOD2 variants might act as gain-of-function alleles for unknown pathogens. Our studies implicate NOD2 in susceptibility to CD, and suggest a link between an innate response to bacterial components and development of disease. □

## Methods

### IBD families

IBD families were ascertained for linkage and association studies (affected child with both parents) through the University of Chicago, the Johns Hopkins Hospital and the University of Pittsburgh. In all cases informed consent for a molecular genetic study was obtained, and the study protocol was approved by the individual institutional review boards.

### Allele-specific PCR

We used primers framing a 533-base-pair region surrounding the 3020insC allele to amplify genomic DNA isolated from controls and patients by PCR (sense, 5'-CTGAGCCTTGTGATGAGC-3'; antisense, 5'-TCTTCAACCACATCCCCATT-3'). In addition, each PCR reaction contained two additional primers designed to detect the wild-type allele (sense, 5'-CAGAAGCCCTCCTGAGCCCT-3') and another primer designed to detect the 3020insC allele (antisense, 5'-CGCGTGTCAATTCCTTTCATGGGGC-3'). The 3020insC was confirmed by DNA sequencing. We performed multiplex PCR with all four primers in one tube. PCR products were isolated on 2% agarose gels and visualized with ethidium bromide.

### Data analysis

The *P* values for the TDT test<sup>20</sup> were calculated using a binomial exact test. Simulations (500,000 replicates) were done using the sib-TDT software (<ftp://lahmed.stanford.edu/pub/aspex/index.html>) to calculate empirical probabilities for the TDT  $\chi^2$  statistic when all independent nuclear families were counted. This calculation was done by permuting parent alleles while fixing the IBD status of siblings within a family. We estimated the frequency of the genotypes in the affected individuals from 416 unrelated CD patients. The GRRs<sup>21</sup> are defined as the ratio of the marginal penetrance of the 3020insC homozygote and heterozygote genotypes to the wild-type homozygotes. Using Bayes rule, the GRRs can be expressed as a function of the allele frequencies in the case and control groups. For the

control group, we assumed that the alleles are in Hardy–Weinberg equilibrium.

## Expression plasmids and immunoblotting

The expression plasmids pcDNA3-NOD2, pcDNA3-TLR4 and pcDNA3-MD-2 have been described<sup>7,12</sup>. The expression plasmid producing the NOD2Δ33 mutant (3020insC) was generated by PCR and cloned into pcDNA3 (Invitrogen), and confirmed by DNA sequencing. Expression of untagged NOD2 proteins in transfected cells was determined by immunoblotting using affinity-purified rabbit anti-NOD2 antibody, as described<sup>7</sup>. To raise the antibody, we overexpressed recombinant NOD2 protein (residues 28–301) in *Escherichia coli* strain BL21(DE3) using the pET-30a vector (Novagen). Recombinant NOD2 protein containing a C-terminal histidine tag was purified using a nickel column (Novagen) and injected into rabbits.

## NF- $\kappa$ B activation assay

We carried out NF- $\kappa$ B activation assays as described<sup>7,12</sup>. Briefly, HEK293T cells were co-transfected with 12 ng of the reporter construct pBVI-Luc, the indicated amounts of each expression plasmid and 120 ng of pEF-BOS- $\beta$ -gal in triplicate in the presence or absence of LPS<sup>7,12</sup>. LPS from various sources were obtained from Sigma or from several investigators. Twenty-four hours after transfection, cell extracts were prepared and the relative luciferase activity was measured as described<sup>7,12</sup>. Results were normalized for transfection efficiency with values obtained with pEF-BOS- $\beta$ -gal.

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