

CLINICAL RESEARCH

Influence of a nucleotide oligomerization domain 1 (*NOD1*) polymorphism and *NOD2* mutant alleles on Crohn's disease phenotype

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Supported by a grant of Ministerio Educacion y Ciencia (BFU 2006-15063); E.C. is participant of the Program "Contratos de apoyo a la Investigacion del Sistema Nacional de Salud". S.V. was supported by "Fondo Investigaciones Sanitarias" and participant of the Program for Stabilization of Investigators of "Direccio d'Estrategia i Coordinacio del Departament Salut de la Generalitat de Catalunya"

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Received: July 4, 2007 Revised: August 17, 2007

conferred the highest risk for severity of disease (26.3% with penetrating disease *vs* 3.8% with non-penetrating or stricturing behavior presented L1007finsC, $P = 0.01$ and 21.0% with penetrating disease *vs* 2.5% with non-penetrating or stricturing behavior carried double *NOD2* mutation, $P = 0.007$). Exclusion of patients with *NOD2* mutations from phenotype/*NOD1*-genotype analysis revealed higher prevalence of *1*1 genotype in groups of younger age at onset and colonic location.

CONCLUSION: This study suggests population differences in the inheritance of risk *NOD1* polymorphism and *NOD2* mutations. Although no interaction between *NOD1*-*NOD2* was noticed, a relationship between disease location and Nod-like receptor molecules was established.

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Key words: Crohn's disease; Nucleotide oligomerization domain 1; Nucleotide oligomerization domain 2

Cantó E, Ricart E, Busquets D, Monfort D, García-Planella E, González D, Balanzó J, Rodríguez-Sánchez JL, Vidal S. Influence of a Nucleotide oligomerization domain 1 (*NOD1*) polymorphism and *NOD2* mutant alleles on Crohn's disease phenotype. *World J Gastroenterol* 2007; 13(41): 5446-5453

<http://www.wjgnet.com/1007-9327/13/5446.asp>

Abstract

AIM: To examine genetic variation of nucleotide oligomerization domain 1 (*NOD1*) and *NOD2*, their respective influences on Crohn's disease phenotype and gene-gene interactions.

METHODS: (*ND1*+32656*1) *NOD1* polymorphism and *SNP8*, *SNP12* and *SNP13* of *NOD2* were analyzed in 97 patients and 50 controls. *NOD2* variants were determined by reaction restriction fragment length polymorphism analysis. *NOD1* genotyping and *NOD2* variant confirmation were performed by specific amplification and sequencing.

RESULTS: The distribution of *NOD1* polymorphism in patients was different from controls ($P = 0.045$) and not altered by existence of *NOD2* mutations. In this cohort, 30.92% patients and 6% controls carried at least one *NOD2* variant ($P < 0.001$) with R702W being the most frequent variant. Presence of at least one *NOD2* mutation was inversely associated with colon involvement (9.09% with colon *vs* 36.4% with ileal or ileocolonic involvement, $P = 0.04$) and indicative of risk of penetrating disease (52.63% with penetrating *vs* 25.64% with non-penetrating or stricturing behavior, $P = 0.02$). L1007finsC and double *NOD2* mutation

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. Although the etiopathogenesis of this disease remains poorly understood, both genetic and environmental factors have been suggested to predispose to CD. Various disease phenotypes, including age at diagnosis, sex, family history, location of disease, response to medical therapies and behavior of the disease may be genetically determined.

Experimental and observational data suggest that intestinal inflammation arises from abnormal immune reactivity to bacterial flora in the intestine of individuals who are genetically predisposed^[1]. The analysis of the molecules that participate in the response of commensal organisms revealed that gastric and intestinal cells are

largely deficient in TLR signaling and must rely on alternative systems, such as Nod-like receptors (NLRs) for the detection of pathogens. The mammalian NLR family is composed of more than 20 members that share a modular domain organization of a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding site domain and a N-terminal protein-protein-interaction domain composed of a CARD (caspase activation and recruitment domain), pyrin domain or Bir domain^[2].

The first NLRs reported to have a direct function as intracellular pattern recognition molecules were Nucleotide oligomerization domain 1 (*NOD1*) (*CARD4*) and *NOD2* (*CARD15*); both proteins detect distinct substructures from bacterial peptidoglycan. *NOD1* detects a unique tripeptide motif found in Gram-negative bacterial peptidoglycan and also in specific Gram-positive bacteria such as *Listeria* and *Bacillus* spp^[3]. *NOD2* detects muramyl dipeptide, the largest molecular motif common to Gram-negative and Gram-positive bacteria^[4]. It is expressed in intestinal epithelial cells, with high expression in Paneth cells in the small intestine, intestinal myofibroblasts, granulocytes, endothelial, and monocyte-derived cells^[5,6].

Identification of *NOD2* as the first susceptibility gene for CD was a breakthrough in understanding inflammatory bowel disease (IBD) pathogenesis. *NOD2* gene is located at the CD susceptibility locus (*IBD1*) on chromosome 16q12^[7,8] and it has more than 60 sequence variants. Although, disease-associated *NOD2* mutations linked to Blau syndrome and early onset of sarcoidosis have been found in the region encoding the nucleotide-binding site domain^[9,10], the three common genetic mutations linked to CD are mapped within or adjacent to the LRR region of *NOD2* (leading to protein changes at R702W, G908R, L1007fsC)^[7,8]. These mutations are associated with an altered NF- κ B activation and the linkage is particularly strong with ileal and ileocolonic CD^[11,12]. *NOD2* variants are associated with early surgery due to stenosis, postsurgical recurrence, familial CD^[13] and stricturing and penetrating forms of CD^[14].

CD association with *NOD2* has been widely replicated. However, investigations into the inheritance of the three risk alleles in *NOD2* associated with susceptibility to CD have demonstrated a remarkable heterogeneity across ethnicities and populations with regional variation across Europe^[15,16].

The discovery of *NOD2*-related innate immune defects in certain CD cases has led to speculation about defects in other pattern recognition receptors and downstream signaling molecules. The gene encoding *NOD1* (*CARD4*) is located within the chromosome 7p14 IBD locus, a region that contains an IBD susceptibility locus in British families^[17]. An association between a complex insertion/deletion polymorphism (*ND1+32656*1*) in *NOD1* and susceptibility to IBD has been described. Particularly, this polymorphism has been associated to age at diagnosis and to the presence of IBD extraintestinal manifestations^[18]. This *NOD1* polymorphism has also been associated to increased susceptibility to asthma^[19,20]. In both diseases, the mutation has been found to be an insertion/deletion polymorphism in an intron of *NOD1*. Convincing replication of these findings is pending, since no evidence

of association between *ND1+32656*1* and IBD was found in two recent well-powered data sets^[21,22].

The present study examines the genetic variation in *NOD1* and *NOD2* and their respective influences on the CD phenotype (age at diagnosis, disease location and behavior) in a cohort of well-characterized CD patients. Since *NOD1* and *NOD2* share structure and functions, a potential interaction between *NOD1* and *NOD2* variants in CD phenotype was analyzed. After stratifying patients by their *NOD2* genotype, the distribution of *NOD1* polymorphism was determined and the contribution of each genotype was studied in regard to the disease phenotype.

MATERIALS AND METHODS

Patients

Ninety-seven CD patients attending the IBD outpatient clinic of Hospital Sant Pau (Barcelona, Spain) were prospectively included in the study. Fifty healthy controls matched for age, sex and geography were also evaluated. CD diagnoses were based on clinical, radiologic, endoscopic and pathologic bases. Patients with CD were classified according to Montreal classification for age at onset, disease location and behavior^[23]. All patients and healthy controls gave informed consent and the study was approved by the local ethics committee.

Genotyping

Analysis of *NOD2* variants was performed as previously described, using genomic DNA extracted from blood samples by Qiagen kit (Qiagen, Heiden, Germany). A panel of 3 single nucleotide polymorphisms (*SNP8*, 12 and 13) was detected by a polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis (PCR-RFLP)^[7]. Each *NOD2* variant was initially amplified by PCR using specific primers (Table 1). The PCR products were subsequently analyzed by restriction enzyme cleavage and gel electrophoresis. For assay of the *SNP8*, the PCR product (185 bp) was digested with *MspI*, resulting in the following fragments: 20, 35, 54 and 76 bp in R702 homozygous; 20, 35 and 130 bp in 702W homozygous and 20, 35, 54, 76, and 130 bp in heterozygous. For assay of the *SNP12*, the PCR product (163 bp) was digested with *HhaI*, resulting in the following fragments: 163 bp in G908 homozygous; 27 and 136 bp in 908R homozygous and 27, 136 and 163 in heterozygous. In order to detect the *SNP13*, the PCR product (151 bp) was digested with *ApaI*, resulting in the following fragments: 151 bp for Leu1007 homozygous; 20 and 131 bp in 1007Pro homozygous and 20, 131 and 151 bp in heterozygous.

Genotyping of *NOD1* (*ND1+32656*) polymorphism and confirmation of the three *NOD2* mutations were performed by specific amplification with the primers described in Table 1 and the subsequent sequencing of the amplified products. Sequencing reaction was performed using ABI PRISM BigDye terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed by Genescan analysis on an ABI Prism 3100 Genetic Analyser according to the manufacturer's protocol (Applied Biosystem).

Table 1 Primers for *NOD2* and *NOD1* genotyping

	Forward	Reverse	Size (bp)
<i>NOD2</i>			
R702W	5'-AGATCACAGCAGCCTTCCTG-3'	5'-CACGCTCTTGGCCTCACC-3'	185
G908R	5'-CTCTTTTGGCCTTTTCAGATTCTG-3'	5'-CAGCTCCTCCCTCTTCACCT-3'	163
L1007finsC	5'-GGCAGAAGCCCTCCTGCAGGGCC-3'	5'-CCTCAAAATTCTGCCATTCC-3'	151
<i>NOD1</i>			
ND1+32656	5'-TGACTGTGTGTGACTCTCTCTGC-3'	5'-TGGTGAAAGCTCTCCACTATCTC-3'	250

Table 2 Genotype at *NOD2* polymorphisms in CD cases and healthy controls

Mutation	Group	Genotype count, <i>n</i> (%)			OR (95% CI) ¹	<i>P</i> ²
		WT/WT	Heterozygous	Homozygous		
R702W	CD	75 (77.32)	21 (21.65)	1 (1.03)	7.04 (1.58-31.30)	0.004
	Controls	48 (96.00)	2 (4.00)	0		
G908R	CD	92 (94.85)	5 (5.15)	0		0.166
	Controls	50 (100)	0	0		
L1007finsC	CD	89 (91.75)	8 (8.25)	0	4.40 (0.53-36.25)	0.167
	Controls	49 (98.00)	1 (2.00)	0		

¹ORs and probability values for disease status associated with carriage of at least 1 mutant allele (heterozygous, compound heterozygous and homozygous were grouped together). ²*P*-values were calculated with the Fisher's Exact test when comparing controls and CD patients.

Statistical analysis

Genotype and allele frequencies of the patients and controls were compared by the χ^2 test or Fisher exact test in 2×2 contingency tables with at least 1 expected value < 5 . Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to estimate relative risks. A two-tailed *P* value ≤ 0.05 was considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 14.0 for Windows (SPSS Inc., Chicago, Ill).

RESULTS

Frequencies of three *NOD2* mutant alleles and one *NOD1* polymorphism in CD patients and healthy controls

NOD2 gene mutations (R702W, G908R and L1007finsC) were determined in 97 CD patients and 50 healthy controls. Frequencies are summarized in Table 2. The distribution of genotypes at each mutation was significantly different in CD patients versus controls. R702W was the most frequent variant in CD and controls (21.65% and 4%, respectively, *P* = 0.004), and the only homozygous mutant patient in this cohort was found for this SNP8. Carriage of R702W was associated to the highest risk for CD in our cohort of patients (OR = 7.04; 95% CI: 1.58-31.30). Genotype frequency of the L1007finsC variant was lower than R702W, but showed a tendency to be higher in CD patients than in controls (8.25% *vs* 2%, *P* = 0.167; OR 4.40, 95% CI: 0.53-36.25). The *NOD2* variant with the lowest frequency in CD patients and in controls was G908R (5.15% *vs* 0%, *P* = 0.166). No homozygous *NOD2* mutant was found for L1007finsC or G908R. In this CD cohort, 30.92% of patients carried at least one variant of *NOD2* compared

with 6% of healthy controls (*P* < 0.001) (Table 3), conferring a high risk for CD (OR 7.01; 95% CI: 2.02-24.30). Six CD patients but no controls carried two *NOD2* variant alleles.

NOD1 complex insertion/deletion polymorphism (ND1+32656) was examined in the same cohort of patients and controls (Table 4). Fifty-two percent of controls were *1*1, 34% were *1*2 and 14% were *2*2, whereas 59.79% of CD patients were *1*1, 37.11% were *1*2 and only 3.09% were *2*2. The distribution of *NOD1* genotype according to the ND1+32656 polymorphism in CD patients and controls was statistically different (*P* = 0.045). Frequency of CD patients carrying *1 allele was 96.8% whereas in controls it was 86%, conferring a significant risk to develop the disease (OR 5.10; 95% CI: 1.25-20.68, *P* = 0.032).

Distribution of *NOD1* genotype according to WT or mutant *NOD2* was analyzed in CD patients to assess potential interactions between *NOD1* and *NOD2* (Table 4). Among those patients carrying at least one *NOD2* mutant allele, 60% of the patients were *1*1, 36.66% were *1*2 and 3.33% were *2*2. Similarly, 59.70% of *NOD2* WT/WT were *1*1, 37.31% were *1*2 and 2.98% were *2*2. The presence of *NOD2* mutant alleles had therefore no influence on the *NOD1* polymorphism distribution (*P* = 0.99), suggesting no gene-gene interactions.

Clinical characteristics of CD patients according to the *NOD2* genotype

CD patients were classified according to Montreal classification, with minor modifications as indicated in Table 5. The association of *NOD2* mutations to each CD phenotype was analyzed using each mutant genotype. The presence of at least one risk allele or the joint analysis of

Table 3 Distribution of *NOD2* mutations in CD patients and controls

<i>NOD2</i> genotype	CD Patients, <i>n</i> (%)	Controls, <i>n</i> (%)	OR (95% CI) ¹	<i>P</i> ²
At least one variant ³	30 (30.92)	3 (6)	7.01 (2.02-24.30)	< 0.001
Heterozygous	24 (24.74)	3 (6)	5.61 (1.59-19.0)	0.003
Compound heterozygous ⁴	5 (5.15)	0		
Homozygous	1 (1.03)	0		

¹ORs and probability values for disease status associated with *NOD2* genotype. ²*P*-values are calculated with the Fisher's Exact test when comparing controls and CD patients. ³At least one variant was considered if any subject had at least one copy of the variant allele. ⁴Compound heterozygous was defined as the presence of two different variants.

compound heterozygous and homozygous for *NOD2* mutations were considered as a single independent variable.

A high proportion of patients in this cohort were diagnosed under the age of 40 (A1 + A2, *n* = 2 + 78), whereas 17 patients were diagnosed over 40 years (A3). All 6 patients with 2 mutant *NOD2* alleles were diagnosed before 40 years of age.

To study the association between genotype and disease location, one L1 + L4 patient was included in the L1 group (*n* = 34, 35.05%), and another L2 + L4 patient was included in the L2 group (*n* = 23, 23.71%). *NOD2* WT/WT patients were similarly distributed in L1, L2 and L3 groups: in 21.65% patients disease location was terminal ileum (L1), in 20.62%, it was colonic (L2), and in 26.80%, it was ileocolonic (L3). However, location of disease in *NOD2* mutant patients was not identical to *NOD2* WT/WT patients (*P* = 0.08). Despite the fact that R702W polymorphism was the most frequent in this cohort, only one patient with colon location carried this mutation (the patient had a double *NOD2* mutation). The presence of at least one mutant *NOD2* gene was inversely associated with exclusively colonic involvement (L2) (*P* = 0.04, OR 0.26; 95% CI: 0.07-0.96). When analyzing compound heterozygous and homozygous *NOD2* mutations, 2.06% of patients were L1, 1.03% of patients were L2 and 3.09% of patients were L3, indicating a comparable distribution.

Similarly to location, behavior groups in CD patients were simplified as follows: B1 group included 4 patients B1p (*n* = 37, 38%), B2 included 4 patients B2p (*n* = 41, 42%) and B3 included 4 patients B3p (*n* = 19, 19.5%). Distribution of *NOD2* mutations was different depending on disease behavior (*P* = 0.003). The presence of at least one mutant *NOD2* allele was indicative of risk of penetrating disease (B3) (*P* = 0.02, OR 3.22, 95% CI: 1.14-9.06) with the allele L1007finsC being indicative of the highest risk (*P* = 0.007, OR 8.92; 95% CI: 1.91-41.68). The frequency of double *NOD2* mutants was significantly higher in the B3 group than in the B2 and B1 groups (66% of the double *NOD2* mutants were B3, 33.3% were B2 and non-double *NOD2* mutants were B1). Presence of two *NOD2* mutant alleles was therefore indicative of risk for severity of disease (*P* = 0.01, OR 10.13; 95% CI: 1.70-60.40). The exam of the behavior through the course

Table 4 Genotype frequencies of *ND1+32656* in CD patients and controls

Group	<i>NOD2</i>	<i>n</i> ¹	<i>NOD1</i> genotype, <i>n</i> (%)		
			*1*1	*1*2	*2*2
CD		97	58 (59.79) ²	36 (37.11)	3 (3.09)
	WT/WT	67	40 (59.70)	25 (37.31)	2 (2.98)
	Mutant ³	30	18 (60.00)	11 (36.66)	1 (3.33)
Controls		50	26 (52.00)	17 (34.00)	7 (14.00)
	WT/WT	47	23 (48.93)	17 (36.17)	7 (14.89)
	Mutant	3	3 (100)	0	0

CD: Crohn's disease; WT: Wildtype. ¹Number of CD patients or controls;

²Results are expressed as number of CD patients (% of CD patients); ³*NOD2* mutant was considered any subject that inherited at least one copy of the variant allele.

of disease showed an expected changing pattern^[24]. There was a progressive reduction in the proportion of patients in the group B1 (51.6% patients with 5 years of disease, 34.9% patients with 6-9 years of disease and 21.7% patients with 10-15 years of disease). Inversely, there was a progressive increase in the proportion of patients in the groups of more complicated forms, B2 (34.5% patients with 5 years of disease, 44.2 patients with 6-9 years of disease and 52.2% patients with 10-15 years of disease) and B3 (13.8% patients with 5 years of disease, 20.9% patients with 6-9 years of disease and 26.1% patients with 10-15 years of disease). Selecting the group of patients with 6-9 years of disease (*n* = 43), the presence of at least one *NOD2* mutation was indicative of risk of penetrating disease (the B3 group) (*P* = 0.046, OR = 5.55, 95% CI: 1.14-27.01) and 66.7% of the double *NOD2* mutants were included in the B3 group. Twelve patients with perianal disease (B1p, B2p, and B3p) were analyzed separately. Four of these patients presented one *NOD2* mutation, one was a compound heterozygous and the rest were *NOD2* WT/WT.

Clinical characteristics of CD patients according to the *NOD1* genotype

Phenotype of CD patients was analyzed according to the *ND1+32656* polymorphism of *NOD1* gene. Distribution of *NOD1* genotype according to older age at diagnosis (A3 *P* = 0.64), location (L1 *P* = 0.28, L2 *P* = 0.56 and L3 *P* = 0.26) and behavior (B1 *P* = 0.55, B2 *P* = 0.99 and B3 *P* = 0.99) of the disease were not different from healthy controls. Similarly to a previous report, *ND1+32656* genotype distribution in the group of early-onset CD (A1 + A2) was different from that observed in healthy controls. Only 2.5% of CD patients in the early-onset group had *2*2 genotype compared to 14% of healthy controls (*P* = 0.04).

Since *NOD2* mutations have a strong association with some CD clinical characteristics, and in particular with the ileal location, 30 CD patients that presented at least one *NOD2* mutation were excluded from the phenotype/genotype study to prevent any influence of *NOD2* (Table 6). When comparing the distribution of *NOD1*

Table 5 Clinical characteristics of CD patients according to *NOD2* genotype

Clinical features	<i>n</i> ¹	<i>NOD2</i> genotype, <i>n</i> (%)					<i>P</i> ² OR (95% CI)
		WT/WT	R702W/WT	G908R/WT	L1007fsinsC/WT	Heter.compound & homozygous	
Age at diagnosis							
< 40 yr (A1 + A2)	80	54 (55.67) ³	13 (13.40)	3 (3.09)	4 (4.12)	6 (6.18)	0.57 1.56 (0.46-5.27)
> 40 yr (A3)	17	13 (13.40)	3 (3.09)	0	1 (1.03)	0	
Location							
Ileal (L1)	34	21 (21.65)	8 (8.25)	1 (1.03)	2 (2.06)	2 (2.06)	0.26 1.67 (0.69-4.06)
Colonic (L2)	23	20 (20.62)	0	1 (1.03)	1 (1.03)	1 (1.03)	0.04 0.26 (0.07-0.96)
Ileocolonic (L3)	40	26 (26.80)	8 (8.25)	1 (1.03)	2 (2.06)	3 (3.09)	0.5 1.38 (0.57-3.29)
Behavior							
Non-stricturing, non-penetrating (B1)	37	24 (24.74)	8 (8.25)	3 (3.09)	2 (2.06)	0	0.5 1.37 (0.56-3.29)
Stricturing (B2)	41	34 (35.05)	5 (5.15)	0	0	2 (2.06)	0.01 0.29 (0.11-0.78)
Penetrating (B3)	19	9 (9.28)	3 (3.09)	0	3 (3.09)	4 (4.12)	0.02 3.22 (1.14-9.06)

CD: Crohn's disease; WT: Wildtype. ¹Number of CD patients in each subgroup; ²*P*-values, odds ratios and confidence intervals refer to the comparison of presence versus absence of the at least one mutant *NOD2* allele; ³Results are expressed as number of CD patients (% of CD patients).

polymorphism in each phenotype group with healthy controls, a higher prevalence of *1*1 was observed in the group A1 + A2 (*P* = 0.04). Interestingly, there was a clear tendency of the colonic group (L2) to have a higher frequency of *1*1 and lower frequency of *1*2 and *2*2 than controls, but the distribution of the *ND1+32656* polymorphism was not statistically different because the low number of cases decreased the power of the test. Distribution of this *NOD1* polymorphism in the other clinical subgroups of CD patients was comparable to healthy controls. Seven of the 12 patients with perianal disease (B1p + B2p + B3p) were *NOD2* WT/WT. In this subgroup of patients, *NOD1* polymorphism analysis showed that three of them were *1*1 and four were *1*2.

DISCUSSION

The frequency of *NOD2* mutant alleles associated to CD in our cohort of patients was within the European range, but deviated somewhat from populations of nearby geographic regions^[25,26]. The frequency of R702W was one of the highest described in Caucasian populations, whereas the frequency of L1007fsinsC was lower than in other studies^[27]. This observation is consistent with marked racial and regional differences described in the inheritance of the three risk *NOD2* alleles^[16].

As expected, carriage of *NOD2* mutations conferred a high risk for developing CD, but this was neither necessary nor sufficient for CD development. The three *NOD2* mutations were not equally involved in CD susceptibility. The presence of R702W showed the strongest risk for CD in our cohort of patients. However, this mutation was not associated to CD in Galician, Finnish or Scottish populations^[26]. The mutation with the strongest CD association in several familial and non-familial studies was L1007fsinsC^[14], but this was not so in our cohort. Although the frequency of L1007fsinsC was noticeably elevated in CD patients, the absence of controls with this genotype precluded a statistical comparison.

We found an association between the polymorphism

Table 6 Clinical characteristics of *NOD2* WT/WT CD patients according to *NOD1* genotype

Clinical features	<i>n</i> ¹	<i>NOD2</i> WT/WT			
		<i>NOD1</i> :	*1*1	*1*2	*2*2
Age at diagnosis					
< 40 yr (A1 + A2)	54		62.96 ²	35.18	1.85
> 40 yr (A3)	13		46.15	46.15	7.69
Location					
Ileal (L1)	21		57.14	38.09	4.76
Colonic (L2)	20		70	25	5
Ileocolonic (L3)	26		53.84	46.15	0
Behavior					
Non-stricturing, non-penetrating (B1)	24		62.5	33.33	4.16
Stricturing (B2)	34		61.76	35.29	2.94
Penetrating (B3)	9		44.44	55.55	0
Controls	47		48.93	36.17	14.89

WT: Wildtype. ¹Number of CD patients or controls in each subgroup; ²Values are expressed as the percent of patients in each clinical subgroup.

located at the intron IX-exon IX boundary of *NOD1* and susceptibility to CD in our cohort of patients. These results confirm a previous report associating this *NOD1* polymorphism with early IBD-onset and extraintestinal manifestations^[18]. Although one recent study did not show a significant association with IBD^[21], this *NOD1* non-coding polymorphism showed a strong association with asthma and the presence of elevated IgE levels in three independent panels of subjects^[20]. Other *NOD1* polymorphisms in the coding sequence have been previously examined and showed no influence in CD susceptibility^[28]. Mutations with phenotypic effects should be predominantly found at the coding sequence but complex disease susceptibility is often mediated through regulatory polymorphisms. In this case, *ND1+32656* may affect the binding of an unknown nuclear factor^[20]. The involvement of *NOD1* gene is not surprising, since *NOD1*, similarly to *NOD2*, is involved in the recognition of intracellular bacterial pathogen-associated molecular patterns^[29] and the two molecules share structure and functional similarities. Certain polymorphisms and

mutations in these molecules may, therefore, result in abnormalities during bacterial recognition with direct implications for CD pathogenesis. Given the importance of these results, further confirmatory studies are warranted in more and larger IBD populations. In order to maximize the opportunities to compare clinical subgroups, location was kept simple and genotyping was specifically blinded to clinical status. Mutations of the *NOD2* gene were rare among our patients with disease limited to the colon (L2). This is in accordance with recent studies showing that *NOD2* mutations (particularly L1007fsinsC) are strongly related to an increased risk of developing ileal CD. In our cohort of patients we only found this association after combining ileal and ileocolonic patients. This could be the consequence of the low rates of limited ileal CD in our cohort of patients compared to other studies (ranging from 40% to 50% in CD patients)^[25,26]. Since location remains relatively stable during the course of the disease^[24], the low rates of ileal CD seen in our patients could be attributable to the impact of interobserver disagreement^[30], variation of disease location among different backgrounds^[31] and even differences in diagnostic techniques. The present study suggests a relationship between disease location and different Nod-like receptor molecules, with relevant clinical implications. Distinctive subcellular location, trafficking, and expression of each Nod-like receptors could be confining the association of *NOD1* and *NOD2* with location of the disease at different parts of the gastrointestinal tract. In healthy humans, *NOD2* is expressed in Paneth cells within the crypts of the small intestine but not in colonic epithelium^[6]. On the other hand, colon intestinal epithelial cells constitutively express *NOD1*^[32]. *NOD1* or *NOD2* prevalence in colon or ileum could also be due to the predominance of different intracellular organisms or enteroinvasive bacteria for which they are receptors^[33]. Further studies are needed to better clarify this subject.

A higher genetic load of *NOD2* mutations increased the susceptibility to CD and determined an aggressive course of the disease. Although CD behavior is a dynamic process progressing towards complicated forms in 80% of patients^[24], the presence of *NOD2* variants could predict a stricturing and penetrating disease^[14]. In addition, *NOD2* variants have been associated with early surgery due to stenosis and with CD recurrence after surgery^[13]. No association was established between the *NOD1* polymorphism and disease behavior.

When comparing these results with other published genotype/phenotype associations, potential confounding factors should be taken into account to understand the differences. Agreement in Montreal classification, modification of the phenotype during follow-up, as well as the mixture of populations in some studies could be masking the particularities of each population. Our study adds two novel approaches to previous studies. First, two functionally related genes were analyzed for the first time in the same population, and second, the association phenotype/*NOD1* genotype was established after ruling out the strong influence of *NOD2*. Although this work emphasized the importance of *NOD1* and *NOD2* on CD disease phenotype, the complexity of IBD genetics

should not be ignored. Individual combinations of genetic risk factors from other molecules such as OCTN, DLG5, TUCAN, MDR1, TNF and TLRs^[34-40] would depict a specific clinical picture for each CD patient.

ACKNOWLEDGMENTS

We thank Carolyn Newey for editorial assistance and Ignasi J Gich for advice in the statistical analysis.

COMMENTS

Background

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. Genetic and environmental factors have been suggested to predispose to CD. CD association with *NOD2* mutations has been widely replicated but with remarkable heterogeneity across populations. Similarly to *NOD2*, *NOD1* has a direct function as intracellular pattern recognition molecules but detecting different substructures from bacterial peptidoglycan. The present study examines the genetic variation in *NOD1* and *NOD2* and their respective influences on the CD phenotype in a cohort of well-characterized CD patients.

Research frontiers

Individual combinations of genetic risk factors from *NOD2*, *NOD1* and other molecules, such as OCTN, TNF and TLRs, would depict a specific clinical picture for each CD patient.

Innovations and breakthroughs

This study adds two novel approaches to previous studies. First, *NOD2* and *NOD1* were analyzed for the first time in the same population and second, the association phenotype/*NOD1* genotype was established after ruling out the strong influence of *NOD2*. The present results suggest a relationship between disease location and different Nod-like receptor molecules.

Applications

This is an association study that compares the allele or genotype frequencies of two genes between affected and unaffected individuals of Crohn's disease. Exploring new gene variants associated with inflammatory bowel disease would make possible the identification of proteins located in certain pathophysiological pathways.

Terminology

NODs are cytosolic proteins that contain a nucleotide-binding oligomerization domain (NOD). As sensors of bacterial components, *NOD1* and *NOD2* are triggered by host recognition of specific motifs in bacterial peptidoglycan and, upon activation, induce the production of proinflammatory mediators.

Peer review

This is a very well written paper. Authors examined genetic variation of *NOD1* and *NOD2*, their respective influences on Crohn's disease phenotype and gene-gene interactions. This study suggests population differences in the inheritance of risk *NOD1* polymorphism and *NOD2* mutations.

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S- Editor Zhu LH L- Editor Alpini GD E- Editor Lu W