Is Age of Onset of Crohn's Disease Governed by Mutations in NOD2/Caspase Recruitment Domains 15 and Toll-Like Receptor 4? Evaluation of a Pediatric Cohort

ESTHER LESHINSKY-SILVER, AMIR KARBAN, ERENA BUZHAKOR, MARCELO FRIDLANDER, BENJAMIN YAKIR, RAMI ELIAKIM, SHIMON REIF, RONI SHAUL, MONA BOAZ, DORIT LEV, AND ARIE LEVINE

Molecular Genetics Laboratory [E.L.-S., E.B.], Department of Epidemiology [M.B.], Genetics Institute [D.L.], and Pediatric Gastroenterology Unit [A.L.], the E. Wolfson Medical Center Holon, Holon, Israel, 58100; Division of Gastroenterology [A.K., R.E.], Rambam Medical Center Haifa, Haifa, Israel, 31096; Danyel Biotech [M.F.], Science Park, Rehovot, Israel, 78124; Department of Statistics [B.Y.], the Hebrew University of Jerusalem, Jerusalem, Israel, 90000; Pediatric Gastroenterology Unit [S.R.], Dana Children's Hospital, Tel Aviv, Israel, 64239; Pediatric Gastroenterology [R.S.], Bnei Zion Medical Center, Haifa, Israel, 31048; and Tel Aviv University Sackler School of Medicine [A.L.], Tel Aviv, Israel, 60000

ABSTRACT

Crohn's disease (CD) is caused by a combination of environmental and genetic factors. It is not clear at present whether age of onset (AOO) is a random event or dictated by genotype or environmental factors. Mutations in the NOD2/caspase recruitment domains 15 (CARD15) and in the Toll-like receptor 4 (TLR4) gene have been associated with increased susceptibility for CD. We sought to determine whether single or multiple mutations in these genes are linked to earlier susceptibility for CD. A cohort of 189 patients with CD (82 pediatric onset, 107 adult onset) were genotyped for three disease-associated single-nucleotide polymorphisms (SNPs), one haplotype association (JW1-SNP5), and one background polymorphism (P268S) of the NOD2/CARD15 gene and for two SNPs of TLR4. Analysis of heterozygosity, homozygosity, alleles, and haplotypes of cohort on age or pediatric onset was performed. AOO ranged from 8 mo to 68 y. The presence of the three NOD2/CARD15 and two TLR4 mutations, the NOD2/CARD15 JW haplotype, compound heterozygosity, and homozygosity were not associated with AOO. Presence of P268S in the absence of known NOD2/

CARD15 mutations was correlated with increasing age and adult onset of CD, whereas pediatric-onset disease was associated with male gender and the wild-type NOD2/CARD15 haplotype. Mutations in NOD2/CARD15 and TLR4 are not significantly associated with AOO in our population. Mutations that are not in linkage disequilibrium with the background mutation P268S of the NOD2/CARD15 gene probably play a more significant role in pediatric-onset disease. (*Pediatr Res* 58: 499–504, 2005)

Abbreviations

AOO, age of onset
CARD, caspase recruitment domain
CD, Crohn's disease
IBD, inflammatory bowel disease
LLR, leucine-rich repeat
LPS, lipopolysaccharide
SNP, single-nucleotide polymorphism
TLR4, Toll-like receptor 4

Crohn's disease (CD) is thought to result from combined effects of environmental agents and a genetically susceptible host. A central role for the innate immune responses to discrete antigens has been postulated. A perturbation in the immune response to luminal bacteria leads to inflammation

in the gastrointestinal tract (1–4). The disease can affect both children and adults. Early presentation of the disease in the first decades of life carries significant morbidity and mortality (5–7). A high risk for complications associated with early onset of the disease is thought to reflect longer lifetime duration of disease and medication use, effects of the disease during periods of growth and development, and possibly a more severe phenotype of the disease as a result of a specific genotype-causing predisposition to early onset of the disease.

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Correspondence: Esther Leshinsky-Silver, Ph.D., Molecular Genetics Lab, E. Wolfson Medical Center, POB 5, Holon, Israel, 58100; e-mail: leshsil2@inter.net.il or leshinsky@wolfson.health.gov.il.

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The first susceptibility gene that has been identified in patients with CD is the NOD2/caspase recruitment domains 15 (CARD15) gene on chromosome 16 (8–12). NOD2/CARD15 is a protein composed of 2 N-terminal CARD, a nucleotide-binding domain, and C-terminal leucine-rich repeats (LLRs). Functional studies have shown that NOD2/CARD15 confers responsiveness to bacterial peptidoglycans through LLRs. It has been suggested that NOD2/CARD15 activates the nuclear factor- κ B pathway in response to bacterial luminal exposure through the specific recognition of bacterial muramyl dipeptides (13–15).

Three main mutations (R 702W, G908 R, and 1007 frame-shift) have been described in CD and have been linked to the phenotype of the disease (8–10), whereas other studies have not found such a correlation (16–18). Carriage of single nucleotide polymorphisms (SNPs) in the NOD2/CARD15 gene has been linked to ileal disease. A haplotype association was linked to development of CD in the Ashkenazi Jewish population (P268S and JW1), although this is controversial (19,20). Recently, five novel changes in the NOD2 gene were revealed, one of which occurred only in Ashkenazi Jews (20). These have not been evaluated in childhood-onset disease.

Genetic susceptibility, leading to early presentation of the disease, theoretically could be due to the type of mutation or to a combination of mutations in the same or different genes. Studies involving the three early NOD2/CARD15 mutations in fact found earlier adult onset with compound heterozygotes, although this was associated with familial disease. However, the three early mutations in NOD2/CARD15 do not account for the majority of patients and led to a mean onset that was only 3 y younger (16,21). Another study found that NOD2/CARD15 mutations confer an odds ratio for early onset that was borderline at best (22).

The human Toll-like receptors are a type I transmembrane protein with an extracellular domain consisting of a LLR region and an intracellular domain homologous to that of the human IL-1 receptor (23). Toll-like receptor 4 (TLR4) seems to be the major receptor for lipopolysaccharide (LPS) isolated from most bacterial organisms (24). Downstream, LPS-induced signaling through TLRs rapidly leads to nuclear factor- κ B activation and cytokine expression in monocytes (23).

Recent studies suggest that the overlying epithelial mucous surface may be severely impaired in patients with inflammatory bowel disease (IBD). Moreover, in active ulcerative colitis (UC) and CD, TLR4 was strongly up-regulated, whereas TLR2 and TLR5 remained unchanged (25). This shows a selective TLR expression in intestinal epithelium, suggesting that alternation in the response system contributes to the pathogenesis of the disease.

Two common co-segregating missense mutations were described in TLR4 gene: Thr399Ile and Asp299Gly, affecting the extracellular domain of the TLR4 receptor and associated with a blunted response to inhaled LPS in humans (specifically the Asp299Gly) (26). The relatively high frequency of these mutations in the white population may reflect modified responses of carriers to alternative TLR4 agonists (27). Spontaneous IBD-like disease occurs in mice with mutations in the TLR4 gene (28,29). Recently, an association between the Asp299Gly mutation and CD was described, strengthening a possible link between mutations in TLR4 and susceptibility to CD (30). The aim of this study was to elucidate the role of mutations in the NOD2/CARD15 and TLR4 genes in pediatric CD cohort and to find a possible age-related phenotype–genotype correlation in an attempt to explain the diversity in age of onset (AOO) of CD.

METHODS

Study population. Patients were eligible when CD was confirmed by established criteria on the basis of clinical, radiologic, endoscopic, and histopathologic findings. Radiologic criteria included strictures or cobblestone pattern or linear ulcerations on a barium swallow with small bowel follow-through. All patients in the study had both a radiologic and an endoscopic work-up with biopsies. Patients with IBD-like disease in the presence of a known immune deficiency state, such as glycogen storage disease or chronic granulomatous disease, were excluded. The study consisted of two consecutively recruited cohorts of single-family members, through four pediatric gastroenterology and two adult gastroenterology programs distributed throughout central and northern Israel. The first cohort included patients with clinical onset of disease <18 y of age (pediatric cohort), and the second cohort included patients with clinical onset $\geq$ 18 y of age. The Wolfson Medical Center Review Board and the Israel Ministry of Health Genetic Research Review Board approved the recruitment protocol. Informed consent was obtained from all parents and patients. Data regarding the AOO and family history was obtained from the patients and the referring gastroenterologist and registered before genotyping. All genotyping was performed by a single investigator who was blinded to phenotypic data (E.L.-S.). The control group consisted of 159 healthy control subjects analyzed by our centers and previously described (31).

Genetic analysis. Genomic DNA was extracted from whole peripheral venous blood, using a commercially available kit (Gentra, Minneapolis, MN) in accordance with the manufacturer's instructions. The NOD2 mutations were analyzed by the Pyrosequencing technology (32). PCR was performed with the corresponding oligonucleotides to amplify the desired DNA fragment (Table 1). SNP was analyzed with a PSQ sample preparation kit from Pyrosequencing AB (Uppsala, Sweden). Analysis of the various SNPs was performed on a PSQ 96MA pyrosequencer, using 0.2- μ M sequencing primers according to standard protocols. The order of nucleotide dispensation was decided on the basis of suggestions provided by the PSQ HS 96 SNP software 1.0 (Pyrosequencing AB), which was also used for automatic assay evaluation and genotype scoring.

The two mutations of the TLR4 gene (Asp299Gly and Thr399Ile) were analyzed by PCR amplification using mismatched primers to generate a restriction site in the mutant allele according to Lorenz *et al.* (33). Briefly, for the Asp299Gly mutation, the following primers were used: forward primer 5'-GATTAGCATACTTAGACTACTACCTCCATG-3' and reverse primer 5'-GATCAACTTCTGAAAAAGCATTCCAC-3'. For the Thr399Ile, forward primer 5'-GGTTGCTGTCTCAAAGTGATTTGGGAGAA-3' and reverse

Table 1. Primers used for NOD2 genotyping by the pyrosequencing method

SNP	Primer forward	Primer reverse	Sequence primer	Amplicon (bp)
P268S	Biotin-ACACAGAGAATGTCCTGGAGGT	AGCTGAATGGGAAGACAAGAG	GGCTCTTCTGCGGG	244
R702W	CACAACCTTCAGATCACAGCAG	Biotin-GGATGGAGTGGAAGTGCTTG	TCTGAGAAGGCCCTG	165
G908R	cactgtctgttgactcttttg	Biotin cctgatctcccaagaaaactg	gccttttcagATTCTGG	160
1007fs	Biotin-atctcttttccagGTTGTCCA	gatcctcaaaattctgccattc	TTCCTTTCAAGGG	198
JW1	ctaccacaatgttaggcaat	Biotin-attccaagtccaccagaaga	gattcaagaggagacact	90

Table 2. Entry data and presence of single or multiple NOD2 and TLR4 mutations

Parameter	Group 1 (n = 82)	Group 2 (n = 107)	Total (n = 189)
Age	12.8	30.0	22.3 ± 12.6
% male*	*64%	*49%	56%
Double NOD2	8.5%	8.4%	8.4%
Any NOD2	31.7%	37.4%	35%
No NOD2	68.3%	62.6%	65%
Any TLR4	10.6%	10.3%	10.4%
No TLR4	89.4%	89.7%	89.6%

* $p < 0.05$.

primer 5'-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3' were used. PCR was performed in a 50- μ L volume that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 250 μ M dNTPs, 1 μ M of each primer, 200 ng of genomic DNA, and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystem, Roche Diagnostic System, Alameda, CA) with an initial denaturation step of 10 min at 95°C to activate the polymerase followed by 35 cycles of 94°C for 15 s, 55°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 10 min. Predicted sizes were confirmed by agarose gel electrophoresis. PCR product was digested overnight with *Nco*I (for the Asp299Gly mutation) and *Hinf*I (for the Thr399Ile mutation). Digests were run on a 3% Nusieve agarose to determine the TLR4 alleles.

Statistics. The statistical analysis was conducted with the R statistical programming environment (<http://cran.r-project.org>). AOO was treated as a continuous response. In the genotype test (Table 2), the genotypes of each of the SNPs was entered as a three-level factor in an ANOVA test via the application of the *aov* function. A linear regression was used for testing the additive effect of the mutated alleles. The number of mutated alleles served as an explanatory variable, and the significance of the slope was tested using the *t* test (implemented by the *lm* function). All analyses were corrected for an additive gender-by-origin effect. For that sake, we introduced a factor that identifies all male individuals who are either Ashkenazi or of mixed origin. It should be noted that this covariate turned out to be the most significant in all analyses. However, it had only a small effect on the significance of the genetic covariates.

Associations between haplotypes and the AOO were examined with the *haplo.score* function. The most frequent haplotypes involving each gene are presented in Table 3. This function examines the additive effect of haplotype alleles and is parallel to the investigation of the additive effect of alleles of a single SNP. We used the options *trait.type* = "gaussian" and *skip.haplo* = 0.05. The analysis was adjusted for an Ashkenazi-male effect. The per-haplotype *p* values and the *p* value of the most significant haplotype (corrected for the multiple testing over all haplotypes) were computer based on 10,000 simulations. The study has 85% power to detect a 4.4-y difference in age for the presence of a mutation.

RESULTS

Population data. Data were obtained from 189 patients. There were 82 patients in the early-onset cohort (mean age of onset 12.77 ± 4.48 ; range 8 mo to 18 y) and 107 patients in the adult-onset cohort (mean age of onset 29.98 ± 12.29 ; range 19–68 y). Our population consisted of 96% Jews and 4% non-Jews. Nearly 51% of our cohort was of pure Jewish-Ashkenazi decent, 33% were of Jewish-Sephardic decent, 12% were of a mixed Ashkenazi-Sephardic decent, and 4% were non-Jewish white individuals. No association was found between ethnicity and AOO ($p = 0.61$), and there was no difference in ethnic breakdown between age groups. Data regarding age, gender, and NOD2 carriage are presented in Table 2.

There were 106 men and 83 women. Mean age for men was 20.7 ± 1.0 versus 24.7 ± 1.7 y for women ($p = 0.032$). Men tended to be younger when they were of Ashkenazi or mixed origin, in comparison with Sephardic origin. The mean age for

Table 3. Association among NOD2, TLR4 haplotypes, and CD AOO

	Frequency (%)	Additive effect	<i>p</i> value
Frequent NOD2 haplotypes: all SNPs			
8-12-13-JW1-5*			
1-1-1-1-1	59.2	−1.81	0.07
1-1-2-2-2	5.7	−0.56	NS
1-1-1-2-2	8.9	−0.67	NS
1-2-1-1-2	10.8	1.52	NS
1-1-1-1-2	9.2	2.55	0.011 (0.05)
Frequent TLR4 haplotypes: all SNPs			
299–399			
1-1	94.24	NS	NS
1-2	1.4	NS	NS
2-2	4.67	NS	NS

Additive effect denotes increase in age if result is positive, decrease in age if negative.

O Corrected for multiple testing.

* SNP8 = R702W, SNP12 = G908R, SNP13 = 1007fs, SNP5 = P268S; 1 = wild type, 2 = mutated.

Ashkenazi men was 19.3 ± 1.3 y versus 22.3 ± 1.7 y for Sephardic men and for Ashkenazi women was 23.44 ± 1.6 versus 24.9 ± 2.2 for Sephardic women. There was a trend for a decrease in the male-to-female ratio as AOO increased. The effect of ethnicity on male gender and AOO is portrayed in Figure 1.

NOD2/CARD15 disease-associated mutations and JW1 haplotype. Disease-associated mutations were found in 35% of the cohort, in comparison with 10.7% of a control population. The mutations R702W(SNP8), G908R(SNP12), and 1007FS(SNP13) were found in 18 (9.5%), 40(21%), and 21 (11.1%) of patients, respectively. The sum of these mutations is higher than the 35% prevalence rate because of compound heterozygosity.

Figure 1. Frequency of male individuals within CD-AOO subgroups (by ethnicity). Note that male individuals tend to have earlier AOO when they were of Ashkenazi or mixed origin (0,10 and 10,20 columns).

Table 4. Testing genotypic association between SNPs in NOD2 and TLR4 genes and AOO of CD

NOD2 SNPs	Wild-type [average (SE)]	Heterozygotes [average (SE)]	Double dose [average (SE)]	<i>p</i> value
R702W (SNPs)	22.49 (0.98)	23.07 (2.58)	13.00 (1.53)	0.49
G908R (SNP12)	21.98 (1.02)	24.77 (2.18)	25.00 (NA)	0.36
1007fs (SNP13)	22.54 (0.98)	22.47 (2.78)	15.00 (3.00)†	0.89
JW1	22.84 (1.29)	22.21 (1.26)	16.43 (2.11)	0.66
P268S (SNP5)	20.87 (1.35)	22.71 (1.49)	25.34 (2.10)‡	0.18
Any TLR4 299 or 399	21.3 (1.07)	20.8 (2.08)	21.3 (NA)	0.9

* Only three patients in this group.

† Only two patients in this group.

‡ Only one patient in this group.

In the <10 y group, NOD2/CARD15 mutations excluding JW1 were found in 27% of patients. The JW1 haplotype was identified in 26% of patients, followed by SNP 13 (fs1007; 17.4%) and SNP 12 (R702W; 8.7%). Only one of six of the patients in the <5 y of age group had a NOD2/CARD15 mutation.

The effect of genotype by cohort is presented in Table 2, and the effect of homozygosity and compound heterozygosity for NOD2 and TLR4 SNPs on AOO are presented in Table 4. These had no effect on AOO or gender (NS), although the number of homozygotes for R702W and fs1007 in our population was exceedingly small [five patients, (2.6%)]. No homozygotes or compound heterozygotes were found in the control population. The JW1 haplotype was more commonly found than the NOD2/CARD15 mutations but was not associated with AOO or gender.

The effect of each SNP or the presence of any mutation on mean AOO is presented in Tables 4 and 5. No association was found between alleles that were associated with disease susceptibility and AOO. The distribution of NOD2-positive patients as a percentage of the patient population by decade appears in Figure 2. A trend to older AOO was found for the background polymorphism P268S ($p = 0.07$), which was strengthened by haplotype analysis.

Probabilist haplotype analysis. We included only the most frequent SNPs of the NOD2 gene (>5%). The two most common haplotypes that carry the SNP8 mutation in our sample are estimated to have frequencies of <4% and <1%, respectively. Other haplotypes are even rarer. Analysis of haplotypes, presented in Table 3, shows an association between

AAO and two haplotypes. The wild-type haplotype (no NOD2-associated mutations and no P268S), which occurred in 60% of patients, showed a tendency for association with pediatric onset ($p < 0.07$). A haplotype characterized by presence of P268S in patients who were naïve for other mutations significantly linked to older AOO ($p = 0.01$). No other haplotype was associated with AOO.

Mutations in TLR4. Mutation analysis was performed in 144 patients (66 with childhood onset and 78 with adult onset in TLR4). Mutations and haplotype analysis are presented in Tables 2–5. TLR4 mutations were not correlated with AOO.

DISCUSSION

The factors that determine AOO of CD are at present unknown. Because presence of multiple alleles of NOD2/CARD15 seems to have a modest effect on AOO, we hypothesized that an additional hit in a disease-associated gene along the same PRR pathway may contribute to AOO (16,22). In our study, which compared pediatric-onset with adult-onset disease and probabilist haplotypes *versus* AOO (independent of an age cutoff), we did not find a correlation between NOD2 and TLR4 genotype and earlier onset of disease. This was also true for homozygotes and compound heterozygotes. In fact, we found that there was a trend to later onset disease with more of the classical (R702W, G908R, and fs1007) NOD2/CARD15 mutated alleles (Fig. 2), although this did not reach statistical significance, possibly because of our sample size. JW1 was unrelated to age. We surmise that the differences noted in our study are due to the inclusion of a pediatric cohort and use of single-family members, although differences in ethnicity might also influence AOO. Jewish Ashkenazi ethnicity seems to cause younger onset in male individuals irrespective of NOD2/CARD15. Carriage of fs1007 in our population was uncommon, and homozygotes for fs1007 or R702W were exceedingly rare in our population. This has also been noted in previous studies from Israel from our group and others (31,34,35), so in any case, these mutations or double-dose NOD2/CARD15 mutations do not explain early AOO in the vast majority of cases in our population.

Presence or absence of the background polymorphism P268S was found to be correlated with onset of disease. Absence of a mutation was significantly associated with decreasing age of CD onset. Although this could be perceived to be a protective effect of the mutation, this concept seems

Table 5. Testing additive association between SNPs in NOD2 and TLR4 genes and CD AOO

NOD2 SNPs	Wild-type [average (SE)]	Additive effect* [years (SE)]	<i>p</i> value
R702W (SNP8)	24.29 (1.12)	−0.78 (2.52)	0.76
G908R (SNP12)	23.81 (1.19)	3.03 (2.12)	0.15
1007fs (SNP13)	24.25 (1.13)	−0.19 (2.59)	0.94
JW1	24.69 (1.26)	−1.18 (1.60)	0.46
P268S (SNP5)	22.60 (1.46)	2.27 (1.25)	0.07
Any mutation†	23.99 (1.23)	1.21 (1.43)	0.40
Any TLR4 299 or 399	22.9 (0.96)	−1.3 (3.2)	0.7

* Additive effect is in years: positive results indicate increasing age; negative results indicate decreasing age.

† Any mutation refers only to mutated alleles on R702W, G908R, and 1007fs.

Figure 2. Frequency of any NOD2/CARD15 mutation (SNPs 8, 12, and 13) by AOO (portrayed by decades).

unlikely given that this mutation has not been shown to alter NOD2 function. A more plausible explanation is related to the presence of linkage disequilibrium between P268S and mutations in NOD2/CARD15. All of the previously described disease-associated mutations and haplotypes occur on a common background that contains P268S (8,16,19,20). Thus, P268S to date seems to function as a marker for mutations in the NOD2/CARD15 gene and possibly the *IBD1* locus. This finding is consistent with the findings of Sugimura *et al.* (19) and Tukel *et al.* (20), who used variant P268S as a marker for unknown mutations.

Because the well-recognized mutations do not account for all of the linkage between this site and CD (19–21,30), other as-yet-unidentified mutations in this region in linkage with P268S may be responsible for disease in adults. Conversely, the lower prevalence of P268S in the juvenile-onset cohort without recognized NOD2/CARD15 mutations may indicate that mutations on the *IBD1* locus in or outside the NOD2/CARD15 gene or genes from other loci could be primarily responsible for pediatric onset of CD. This hypothesis was reinforced further by haplotype analysis in patients with or without recognized NOD2/CARD15 mutations. We found that P268S mutations were associated with increasing age and adult-onset disease primarily in patients without other mutations in NOD2/CARD15, whereas absence of P268S was correlated with pediatric onset in patients without other mutations in NOD2/CARD15. The JW1 haplotype was not responsible for this difference, because haplotypes with P268S but without JW1 showed an even stronger association with increasing age. We did not perform NOD2 and TLR4 analysis in a control population in this study because these have been performed by our centers on 159 healthy control subjects (31) and previously published (36) and are not relevant to AOO in established disease.

Finally, the significantly higher percentage of male individuals with juvenile onset may also raise the possibility that a gender-specific locus may lead to altered AOO. Fisher *et al.* (37) reported that gender-specific linkage for male disease was present for the major histocompatibility region on chromosome 6p, a region referred to as the IBD3 Locus. Gender linkage was not found for disease related to the IBD1 locus carrying NOD2/CARD15.

In conclusion, the common disease-associated mutations in the NOD2/CARD15 and TLR4 genes, homozygosity, and compound heterozygosity do not seem to play a role in AOO of disease in our population. Haplotypes without these mutations are associated with AOO of disease. Haplotypes in linkage disequilibrium with the P268S polymorphism in the NOD2/CARD15 gene seem to be associated with increasing age and adult-onset disease. Conversely, predominantly male gender in the juvenile group and absence of the P268S background polymorphism might indicate that mutations located outside NOD2/CARD15 may play a more significant role in juvenile- or pediatric-onset disease.

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