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Combination of Innate and Adaptive Immune Alterations Increased the Likelihood of Fibrostenosis in Crohn's Disease

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Abstract

Background—Mutations in the nucleotide oligomerization domain-2 (*NOD2*) gene and positive antibodies to microbial antigens have been found to be associated with the Crohn's disease (CD) phenotype, fibrostenosis. The aim of this study was to confirm these relationships in a large cohort of CD patients and to determine the correlation between the presence of *NOD2* variants and antibodies to oligomannan, CBir, outer membrane porin-C (OmpC), and I2 in CD patients with fibrostenosis.

Methods—Sera and DNA from 731 unrelated CD patients were tested for *NOD2* variants (SNP 8, 12, and 13) and the antibodies. The results were correlated with CD phenotypes, fibrostenosis, internal penetrating, perianal penetrating, and ulcerative colitis (UC)-like as well as other clinical features.

Results—The presence of *NOD2* allelic variants was primarily associated with fibrostenosis, secondarily with small bowel disease and small bowel surgery, and was inversely associated with UC-like disease. This association was present in patients with a fibrostenosis only (Vienna B2) and those with both stricturing and penetrating disease. The presence and level of antibodies to microbial antigens was also associated with the fibrostenosis phenotype. In the 316 patients with fibrostenosis the prevalence of *NOD2* variants was significantly correlated with the antibody titer by quartile sum score. Further, when these patients with fibrostenosis were clustered by quartile sum score, the odds ratio for fibrostenosis was significantly higher in the patients with *NOD2* variant alleles within each cluster, indicating synergy.

Conclusions—Defects of innate (*NOD2* variants) and adaptive (antibodies to microbial antigens) immunity act synergistically to increase the risk of the fibrostenosis phenotype.

Keywords

NOD2; Crohn's disease; fibrostenosis; phenotype

Crohn's disease (CD) is a heterogeneous disorder with variability in disease presentation as well as the response to medical and surgical therapies. The natural history of the disease is influenced by site of involvement (intestinal versus colonic), and disease behavior, uncomplicated (inflammatory), or complicated by stricture (fibrostenosis), or by abscess, mass, or fistula (penetrating). The prevailing pathogenic hypothesis of CD is hyperresponsiveness of the gut mucosal immune system to normal luminal bacteria. This aberrant response results from a combination of defects in innate and acquired immunity. The relationship between markers of altered immunity and the clinical features and phenotypes of CD has been extensively studied.

NOD2 (nucleotide oligomerization domain 2) was the first gene to be linked to CD.^{1,2} From a meta-analysis of 42 studies, the odds ratio (OR) for a single allelic variant was 2.2–4.1 for non-Jewish Caucasians and 1.7–2.5 for Jewish patients.³ Three major *NOD2* polymorphisms have been recognized in CD, referred to initially as single nucleotide polymorphisms 8, 12, and 13 (SNP8, SNP12, SNP13), more properly referred to as R702W, G908R, and 1007fs. Carriage of 2 alleles increased the OR to 17.1.³ All 3 mutations of the *NOD2* gene, in vitro, are associated with impairment of muramyl dipeptide (MDP)-driven nuclear factor-kappa B activation (NF- κ B).⁴ In mononuclear cells from homozygote CD subjects impaired secretion of MDP-induced interleukin 1- α and interleukin 8 was observed, indicating a defect in innate immunity in some CD patients.⁵

NOD2 has been primarily linked to 2 clinical features of CD, small bowel involvement and the fibrostenosis phenotype. The association with small bowel disease location has been found by most investigators; the overall OR was 2.5.⁶ However, there was some variability in the association of *NOD2* expression and the fibrostenosis phenotype, overall OR of 1.9.³ In a recent review 10 studies examined the genotype/phenotype interaction; 6 reported a significant association.⁶ The sample size, allelic frequency, and ethnicity of the reported patients were comparable between the studies that found a significant association and those that did not. In 2 prospective studies, no association was found between the *NOD2* genotype and progression from the inflammatory to the fibrostenosing phenotype.^{7,8} Ahmed et al⁹ reported that the fibrostenosis and *NOD2* association in their study was not independent by logistic regression of the association with ileal disease.

Many CD patients have serologic evidence of a loss of tolerance to luminal bacteria. Duchmann et al¹⁰ were the first to show that CD patients have reactivity to multiple bacterial antigens. Specific associations between antibody responses to oligomannan (anti-*Saccharomyces cerevisiae* [ASCA], *Escherichia coli* outer membrane protein C [anti-OmpC], a CD-related protein from *Pseudomonas fluorescens* anti-CD-related bacterial sequence [I2], and Cbir1 flagellin [anti-Cbir1]) and CD phenotypes have been described.^{10–12} The presence of antibody positivity and elevated antibody titer has been directly associated with small bowel disease, the fibrostenosis and internal penetrating phenotypes, and small bowel surgery, and is inversely correlated with colonic disease.^{11,12} These associations were strongest when the individual antibody responses were combined either as the number of positive antibodies or as the total antibody level. In the prospective study of Scottish patients the presence and the magnitude of antibody responses to ASCA, anti-I2, and anti-OmpC was associated with progression of disease type.⁷

The in vitro evidence of defective MDP recognition and NF- κ B activation found with *NOD2* mutations provides a theory for a defect in innate immunity. We have recently shown that the defect in innate immunity is associated with a presumed compensatory hyperresponse in adaptive immunity.¹⁴ CD phenotypes are likely associated with specific genetic defects as well as markers of loss of tolerance to luminal bacteria. The purpose of this study was to

clarify the relationship between the presence of allelic variants to the *NOD2* gene and the presence and titer of antibodies to microbial antigens, Cbir, I2, OmpC, and oligomannan and the fibrostenosis phenotype. Further, in those CD patients with fibrostenosis, we proposed that an association would be found between innate (*NOD2* mutations) and adaptive (microbial antigens) immune alterations. Our findings indicate that the presence of *NOD2* variants and elevated titers of antibody to microbial antigens both contribute individually and, further, act synergistically to increase the likelihood of fibrostenosis in CD patients.

MATERIALS AND METHODS

Patient Population

Serologic, genetic, and phenotypic data were analyzed in 731 unrelated patients from the Cedars-Sinai IBD Database. Two hundred and ninety-four of these patients were previously reported¹¹; all patients were enrolled into the database between 1987 and 2005. Phenotypes and other clinical data were determined by chart review by pairs of physician-observers blinded to serologic and genetic data. The reviews were done in late 2005 in order to have current clinical status at the time of data analysis. The phenotypes were based on previously published criteria,¹¹ and included fibrostenosis, internal penetrating, perianal perforating, and ulcerative colitis (UC)-like. Since progression from 1 phenotype to another occurs in CD, our practice has been to record all phenotypes for a patient rather than a single dominant phenotype. However, in order to permit comparison to other studies, patient grouping by the Vienna Classification is also included.¹⁵ Other clinical data included disease location and history of small bowel surgery. All questions were resolved by an independent review by 2 physicians (A.I., S.D.).

Serologic Evaluations

All blood samples were taken at the time of consent and enrollment. Sera were analyzed for expression of ASCA, anti-I2, anti-OmpC, in a blinded fashion by enzyme-linked immunosorbent assay (ELISA) as previously described.^{11,12} Antibody levels were determined and results expressed as ELISA units (EU/mL) that are relative to a Cedars-Sinai Laboratory (IgA-I2, IgA-OmpC) or a Prometheus Laboratory standard (San Diego, CA; IgA and IgG ASCA) derived from a pool of patient sera with well-characterized disease found to have reactivity to these antigens. Quantization of IgG anti-CBir1 reactivity was expressed in ELISA units derived based on a proportion of reactivity relative to a standardized positive control.¹² As ASCA can be expressed in both an IgA and IgG class, positivity to ASCA was determined if either class of antibody was above the reference range. In determining a quantitative measure of ASCA, the reactivity was first log-transformed and standardized. The higher of 2 standardized units was then used to determine the quartile of reactivity. With the exception of determining variance (see Statistical Analysis), the magnitude of reactivity to the other 3 antigens was not standardized as each is represented by a single class of antibody. The magnitude of the serologic response to each antigen was divided into 4 equal quartiles in CD patients to determine quartile sum scores as previously described.^{11,12} By adding individual quartile scores for each microbial antigen, a quartile sum (QS) (range, 4–16) was derived that represents the cumulative semiquantitative immune response toward all 4 antigens.

Genetic Analysis

Three *NOD2* variants that have been previously associated with CD (R702W, G908R, and 1007fs) were adapted to the TaqMan MGB (Applied Biosystems, Foster City, CA) genotyping platform as previously described.^{11,16}

Statistical Analysis

To determine the associations between antibody responses toward microbial antigens, autoantigens, and NOD2 genotype status and disease phenotype characteristics, univariate analyses with χ^2 tests were performed using SAS (v. 8.02; SAS Institute, Cary, NC). Odds ratios (OR) and 95% confidence intervals (CI) were calculated to compare the odds of positive serum reactivity toward the microbial antigens (I2, OmpC, ASCA, and CBir) in the group of patients with a certain disease characteristic (e.g., fibrostenosing CD) with these odds in the group of patients without such a characteristic. The Cochran–Armitage test for trend was used to test whether there was a linear trend in the proportion of patients with a disease phenotype characteristic as the level of antibody responses increased by quartiles. A P value (P trend) < 0.05 suggests that the linear trend is statistically significant. Multivariate analysis with logistic regression modeling was also performed to determine the primary associations among qualitative serological responses and NOD2 with disease phenotypes. Zelen's exact test was used to compare the homogeneity of ORs and when significant indicated a synergistic effect.

RESULTS

Patient Demographics

The clinical, serologic, and genetic characteristics of the 731 CD patients are listed in Table 1. This cohort is characterized by complicated small bowel disease; not only do 81% have small bowel involvement but in that group 69% have a history of fibrostenosis ($n = 178$), internal penetrating ($n = 94$), or both phenotypes ($n = 138$), and 57% have had surgery. The differences in classification systems is illustrated in that 43% of patients had a history of fibrostenosis, but only 17% had fibrostenosis without penetrating disease (Vienna B2). At least 1 positive serology was present in 87% of patients. Sixty-eight percent were wildtype for the *NOD2* gene, while a single variant allele was found in 27%, and 5% were heterozygous for 2 SNPs or homozygous for a single SNP.

NOD2 Variants and Fibrostenosis

The presence of allelic variants to the *NOD2* gene was associated directly with small bowel disease involvement ($P < 0.001$), the fibrostenosing phenotype ($P < 0.0001$), a history of small bowel surgery ($P < 0.05$), and inversely with the UC-like phenotype ($P < 0.01$) (Table 2). Forty-one percent of the 316 patients with a history of this complication were variant positive as compared to 25% of those patients without fibrostenosis. The presence of 2 allelic variants, either homozygote or compound heterozygote, increased the likelihood of the fibrostenosis phenotype (Table 3). The prevalence of fibrostenosis was 39%, 56%, and 61% for 0, 1, or 2 variants, respectively (P for trend < 0.0001).

Is there a general association between the presence of *NOD2* variants and small bowel CD, or is it specific for the fibrostenosis phenotype? To further analyze this relationship the prevalence of *NOD2* allelic variants was also determined in the phenotypic subgroups using the Vienna Classification. In this system Vienna B2 are patients with only fibrostenosis and B3 are those with internal penetrating disease with or without other complications. First, there is a significant positive correlation between *NOD2* variants and fibrostenosis in patients with only that phenotype, Vienna Classification B2, OR = 2.2 (confidence limits 1.5, 3.3, $P < 0.0001$), as well as patients with fibrostenosis plus other phenotypes, OR = 2.1 (1.5, 2.9, $P < 0.0001$). Second, there was no association between *NOD2* variants and internal perforating disease in either ours or the Vienna Classification, B3 (Table 2). In fact, among patients with only internal penetrating disease (i.e., without fibrostenosis or perianal complications, $n = 64$) there was a significant inverse association with *NOD2* variants, OR = 0.5 (0.3, 0.98, $P = 0.04$). Since patients with small bowel disease are most likely to have

fibrostenosis, internal penetrating, or both phenotypes (30%, 14%, 23%, respectively), it follows that the association of *NOD2* with fibrostenosis leads to the correlation with small bowel disease. Similarly, small bowel surgery is associated with the presence of *NOD2* variants since 70% of these patients have the fibrostenosis phenotype. Finally, since fibrostenosis is rarely found in patients with UC-like phenotype, 12%, there is a negative association between *NOD2* variants and UC-like disease.

These results indicate that the presence of variant alleles of the *NOD2* gene is primarily associated with the fibrostenosis phenotype. The links to small bowel disease location and small bowel surgery are secondary to the fibrostenosis association. The inverse correlation between *NOD2* variants and the penetrating phenotype, another small bowel complication, further supports the unique relationship between fibrostenosis and this defect in innate immunity, mutations of the *NOD2* gene. Next, the relationship between fibrostenosis and defects of adaptive immunity was analyzed.

Relationship Between Serologic Responses to Microbial Antigens and Fibrostenosis

In prior publications a significant association between the presence and level of antibodies to oligomannan, Cbir, OmpC, and I2 and the CD phenotypes, fibrostenosis and internal penetrating has been demonstrated.^{9,10,11,15} Similarly, in this cohort the prevalence of fibrostenosis is significantly correlated with the number of positive antibodies (Fig. 1). The range is from 12% prevalence in those patients without any positive antibodies to 64% in those with all 4 antibodies ($P < 0.0001$ for trend). The magnitude of the serologic response to each antigen was ranked lowest to highest and then divided into quartiles (1 to 4). Individual antibody quartile scores were summed, the lowest possible score was 4, and the highest was 16. Figure 2 summarizes the prevalence of fibrostenosis by quartile score and ranges from 7% at a score of 4 to 72% at a score of 16 ($P < 0.001$ for trend).

Relationship of *NOD2* Combined with Serologic Responses and Fibrostenosis

We reported that the presence of *NOD2* variant alleles was very strongly associated with the number and level of combinations of antibodies to oligomannan, Cbir, OmpC, and I2.¹² In this cohort, the 316 patients with fibrostenosis, the association with the number of positive antibodies and *NOD2* mutations did not achieve statistical significance ($P = 0.059$). Even though there was an increase in the prevalence of patients with variants comparing 0, 1, and 2 positive antibodies, the percents from 2 to 4 were comparable. But, there was a significant correlation between the prevalence of *NOD2* variant alleles and the quartile sum score for antibody level ($P < 0.001$ for trend) (Fig. 3). When the quartile sums are clustered, 4–6, 7–9, 10–13, and 14–16 allelic variants were found in only 5% of the patients at the lowest cluster versus 51% at the highest cluster. Finally, there was no significant association between the prevalence of individual antibodies, oligomannan, Cbir, OmpC, or I2, with *NOD2* variants (data not shown).

Thus, we observed: 1) an association between innate immunity and phenotypes of CD, since there is a significant association between *NOD2* allelic variants and the presence of fibrostenosis, and, 2) confirming earlier work that associates adaptive immunity with CD phenotypes,⁹ we found a significant relationship between fibrostenosis and antibody positivity and antibody level. Thus, it appears that fibrostenosis may be related to alterations in both innate and adaptive immunity. One possibility is that such defects act independently. Since we have shown that the presence of *NOD2* variants and the quantitative antibody response are significantly associated, an alternative possibility is that they have a synergistic effect that leads to fibrostenosis. In order to determine the nature of that association the following analysis was performed.

We grouped patients by antibody level into 4 strata, quartile sum scores 4–6, 7–9, 10–13, and 14–16. Within each stratum the subjects are subdivided by the presence or absence of allelic variants. The ORs for developing fibrostenosis were calculated using the lowest strata and the absence of allelic variants as the reference. By using Zelen's exact test to compare the homogeneity of ORs, a synergistic effect can be detected when the test is significant. As shown in Table 4, there is a significant difference between the ORs in each strata, i.e., the ORs for a given quartile sum are increased by the presence of *NOD2* variants, and the ORs for *NOD2* variants are increased by a higher quartile sum. The presence of variants to the *NOD2* gene acts synergistically with the quantitative response to microbial antigens in patients with CD to increase the likelihood of fibrostenosis.

DISCUSSION

This study has demonstrated that the fibrostenosis phenotype of CD is significantly associated both with mutations of the *NOD2* gene and with the presence and high titer of combinations of antibodies to oligomannan, OmpC, I2, and Cbir. Furthermore, this combination of altered innate and adaptive immune responses acts synergistically to increase the development of fibrostenosis in a given patient. For example, in the highest strata of the quartile sum scores, 14–16, where the prevalence of fibrostenosis is 64%, there is still a significantly greater likelihood for fibrostenosis (OR 9.6 versus 4.8) in those patients with *NOD2* genetic loss of function as compared to those with a wildtype genotype.

In prior studies, the strongest association between *NOD2* variants and clinical features of CD was the small bowel disease location. However, in this study the dominant relationship was found to be with the fibrostenosis phenotype. The 3 possible phenotypes of small bowel disease are inflammatory, stricturing, or penetrating. Among patients with any history of fibrostenosis ($n = 316$) or the subgroup with fibrostenosis only ($n = 125$, Vienna B2) there was a significant association with *NOD2* genetic variant alleles ($P < 0.0001$). Patients with the inflammatory phenotype, included in Vienna B1 group, demonstrated a significant inverse association with fibrostenosis ($P < 0.01$). A significant inverse association was also found between *NOD2* variant alleles and patients with the penetrating phenotype alone ($P < 0.04$). The prevalence of the fibrostenosis phenotype in patients with small bowel disease involvement (53%) and those with a history of small bowel surgery (70%) led to a significant association with the presence of *NOD2* variant alleles. Finally, the confirmation of a significant association between the *NOD2* gene and fibrostenosis in this study was related to the prevalence of both variables in our patient population. The sample size of this study is larger than prior reports, including 2 earlier from this institution.^{11,16}

In a previous study, the correlation of genetic defects and abnormal responses to combinations of microbial antigens was shown.¹⁴ The presence of *NOD2* variant alleles was correlated with increasing number of positive antibodies and increasing antibody level, as reflected in the quartile sum score. This was found in CD patients and in unaffected relatives. This relationship hinged on the interaction between mutations of a single gene with the immunologic response to multiple antigens. There was no significant correlation between individual antibodies and *NOD2* variants, which is similar to prior findings.^{11,14} There is variability to the antibody response to microbial antigens among CD patients, even within a specific phenotype. The ability to measure 4 CD-related antibodies permitted the detection of an association with the presence and level of the multiple antibody combinations.

This study is the first to demonstrate that correlation within a specific CD phenotype. Further, it has been shown that loss of *NOD2* function, innate immune response, coupled with loss of tolerance to microbial antigens, adaptive immune response, increases the

likelihood of a specific CD complication, fibrostenosis. As depicted in Figure 4, innate and adaptive immune responses are associated among individuals with CD. In those with *NOD2* gene mutations and an increased antibody responsiveness, the clinical phenotype of fibrostenosis is highly prevalent.

These findings suggest that associations with other phenotypes, treatment responses, and clinical characteristics will be based on the discovery of other genetic defects in innate immunity coupled with the loss of tolerance to microbial antigens. There are a number of candidate genes in CD whose presence in patients with evidence of adaptive immune defects would emphasize the importance of altered immunity in the pathogenesis of inflammatory bowel disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

The prevalence of the fibrostenosis in patients with 0, 1, 2, 3, or 4 positive antibodies to oligomannan, CBir, OmpC, or I2 in the 731 CD patients. The probability was significant for trend, $P < 0.0001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 2.

The prevalence of fibrostenosis in patients grouped according to the quartile sum score for antibodies to oligomannan, CBir, OmpC, or I2 in the 731 CD patients. The probability was significant for trend, $P < 0.0001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 3.

The prevalence of *NOD2* variant alleles in the 316 patients with fibrostenosis is described. (A) The prevalence for *NOD2* variants is compared with the number of positive antibodies (antibody sums) (P for trend = 0.059). (B) The prevalence is compared with the quartile sum scores, clustered 4–6, 7–9, 10–13, 14–16 (P for trend < 0.001). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Immune Alterations in Crohn's Disease Patients with the Fibrostenosis Phenotype

FIGURE 4.

This schematic diagram depicts the association between innate and adaptive immune responses in CD in general and their synergistic effect in fibrostenosis.

TABLE 1

Clinical Characteristics of the CD Cohort

Clinical characteristic	Cohort (n = 731)
Sex (M/F%)	51/49
Mean age (range)	38.3 yrs (8–91 yrs)
Mean age at onset (range)	26.7 yrs (2–78 yrs)
Disease location (%)	
Small bowel only	22.4
Colon only	18.7
Small bowel and colon	59.0
Disease behavior (%)	
Perianal perforating	30.6
Internal penetrating	31.7
Fibrostenosing disease	43.2
UC-like	22.6
Small bowel surgery	46.4
Vienna B1, (nonstricturing, nonpenetrating)	30.8
Vienna B2, (stricturing)	17.1
Vienna B3, (penetrating)	52.1
Serologic profile (%)	
ASCA positive	50.3
Anti-I2	58.1
Anti-OmpC	37.2
Anti-Cbir	56.4
<i>NOD2</i> genotype for SNP 8,12, and 13 (%)	
No mutations	68.3
Heterozygotes	26.5
Compound heterozygotes or homozygotes	5.2

TABLE 2Disease Behavior and *NOD2* Variant Associations

Phenotype		<i>N</i>	Allelic Variants	Probability
Perianal perforating	Yes	224	31%	0.7
	No	507	32%	
Small bowel disease	Yes	595	35%	<0.001
	No	136	19%	
Internal perforating	Yes	232	30%	0.4
	No	499	33%	
Fibro-stenosis	Yes	316	41%	<0.0001
	No	415	25%	
Small bowel surgery	Yes	339	36%	<0.05
	No	392	28%	
UC-like	Yes	165	23%	<0.01
	No	566	34%	
Vienna B1	Yes	225	25%	<0.01
	No	506	35%	
Vienna B2	Yes	125	47%	<0.0001
	No	606	29%	
Vienna B3	Yes	381	31%	0.5
	No	350	33%	

TABLE 3Prevalence of Fibrostenosis by *NOD2* Allelic Variants

	0 Variants	1 Variant	2 Variants
Percent	39% (187/499)	55% (106/194)	61% (23/38)
Odds Ratio		1.37	1.57

P for trend < .0001.

TABLE 4Demonstration of Synergism Between *NOD2* Variants and Antibody Levels in Fibrostenosis

Quartile	Variant	Odds Ratio (CL)	Probability
4–6	No	Reference	Reference
	Yes	0.2 (0–1.2)	
7–9	No	1.2 (0.6–2.2)	0.004
	Yes	2.7 (1.3–5.5)	
10–13	No	3.3 (1.8–6.0)	0.003
	Yes	7.3 (3.7–14.4)	
14–16	No	4.8 (2.3–10.1)	0.01
	Yes	9.6 (4.2–21.8)	