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ORIGINAL ARTICLE

Lack of association of the 3′-UTR polymorphism in the NFKBIA gene with Crohn's disease in an Israeli cohort

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Abstract
Background Crohn's disease (CD) is a chronic inflammatory disease of the gastrointestinal tract associated with dysregulation of the immune response. It is caused by a combination of environmental and genetic factors. Patients with CD have a TH1-type inflammatory response characterized by nuclear factor kappa B (NFκB) activation. Mutations in the bacterial pattern recognition receptors NOD2/CARD15 and Toll-like receptor 4 (TLR4) genes, which lead to activation of NFκB under normal circumstances, have been associated with increased susceptibility for CD. NFκB plays a critical role in the immune response and is down-regulated by NFκB inhibitor α (NFKBIA). NFKBIA was found to be a susceptibility gene for German CD patients lacking NOD2/CARD15 mutations.

Materials and methods A cohort of 231 Israeli CD patients previously genotyped for the single nucleotide polymorphisms (SNPs) in the CARD15, TLR4 susceptibility genes for CD, was analyzed for the 3′-untranslated region (UTR) SNP of the NFKBIA gene in comparison to 100 healthy ethnically matched controls. We evaluated the contribution of the 3′-UTR SNP in NFKBIA in patients with or without other SNPs in CARD15 to age of onset, disease location, and disease behavior (Vienna classification).
Results We did not identify a significant difference in allele and genotype frequencies between either groups or an effect on phenotype. No interactions were found between NFKBIA and any NOD2.
Conclusions The contribution of population diversity to susceptibility genes for CD plays an important role in disease-associated variants and is important for better understanding of the pathologic mechanisms of the polymorphism.

Keywords Crohn's · Genotype · NOD2 · CARD15 · NFKBIA

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Introduction

Crohn's disease (CD) is caused by a combination of environmental and genetic factors. A dysregulation of the immune response to luminal microbiological or nutritional antigens seems, at the level of the innate immune system, to play a major role in the pathogenesis of CD [1–4]. The genetic predisposition is well established by epidemiological and genome wide linkage analysis [5, 6]. Mutations in the leucine-rich repeat region of the pattern recognition receptor (PRR) NOD2/CARD15 gene, leading to loss of function, are associated with increased susceptibility for CD

[7–10]. A loss of function polymorphism in another PRR gene, Toll-like receptor 4 (TLR4) [11–13], which shares a similar bacterial sensing region, is also associated with CD [12].Both PRR products lead to downstream activation of nuclear factor kappa B (NFκB), which leads to transcription and activation of cytokines such as tumor necrosis factor (TNF) α and interferon-γ, key players in the pathogenesis and inflammation associated with CD. Polymorphisms in the TNFα promoter [14, 15] may also cause loss of NFκB function. Therefore, genes associated with activation or modulation of NFκB may contribute to the genetic predisposition for CD or disease behavior [10–15]. NFκB can be activated by exposure of cells to bacterial or viral products, inflammatory cytokines such as TNF or IL-1, or lymphocyte activation. NFκB activation has been implicated in many biological processes, including inflammation, apoptosis, and immunoregulation.

NFκB activates the transcription of a number of genes by binding as dimmer to specific target sequences in their promoters. The most abundant NFκB heteromer consists of p65 and p50 subunits. The NFκB inhibitor alpha (NFKBIA) regulates its activity by association–dissociation to NFκ B and exposing–protecting from proteosomes [16–20] Activation of NFκ B may play a major role in the pathogenesis of CD, and indeed it was shown that high levels of p65 are present in the mucosa of CD patients [19]. Therefore, factors affecting the activation of NFκB may be good candidate genes for the predisposition to CD, especially in CARD15-negative patients.

The NFKBIA gene is located on chromosome 14q13, which is close to the IBD4 region found in genome wide linkage analysis [21]. NFKBIA was found to be a susceptibility gene in German CD patients lacking CARD15 mutations [22].

A cohort of 231 CD patients genotyped previously to NOD2, TLR4, and TNFα was analyzed for the polymorphism G/A in the 3′-untranslated region (UTR) of the NFKBIA gene (SNPrs696) [23].

Materials and methods

Patients were eligible if CD was confirmed by established criteria based on clinical, radiological, endoscopic, and histopathological findings. All patients in the study had both a radiological and an endoscopic work-up with biopsies. The study cohort consisted of single-family members, from pediatric and adult gastroenterology programs distributed throughout central and northern Israel 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 age of onset (AOO), gender, disease location, disease behavior (inflammatory, structuring, or penetrating), and family history were obtained from the patients and the referring gastroenterologist and registered before genotyping. We did not analyze disease behavior in this study, as we did not require prolonged follow-up over time as an entry requirement. All genotyping was performed by a single investigator blinded to phenotypic data (Leshinsky-Silver). The control group consisted of 100 healthy human DNA controls, with the same ethnicity distribution, from the National Laboratory for the Genetics of Israeli Populations at the Tel-Aviv University.

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.

NOD2 genotyping was performed as previously described [14, 15].

The G/A polymorphism in the 3′-UTR of the NFKBIA was genotyped by amplifying a 424-bp fragment with the primer pair: F;GGCTGAAAGAACATGGACTTG, R;GTACACCATTTACAGGAGGG in 50 μl reaction mixture containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphates, 1 μM of each primer, 100 ng of genomic DNA, and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystem, Roche Diagnostic System, California) with an initial denaturation step of 10 min at 95°C to activate the polymerase followed by 35 cycles of 94°C; 15 s, 55°C; 45 s, 72°C; 45 s, and a final extension of 10 min at 72°C. Predicted sizes were confirmed by agarose gel electrophoresis. Polymerase chain reaction product was digested overnight with *Hae*III .Digests were run on a 3% agarose to determine the G/A alleles: 424 bp for A and 306 and 118 bp for G.

Statistical analysis

Haplotype reconstruction and association analyses

The association between NFKBIA mutation and Crone's disease was evaluated by a permutation test [24] as follows: the Pearson χ^2 score is calculated and declared as the test statistic. Then, the same statistic is calculated for many data sets with the same genotypes and randomly permuted labels of the case and control individuals. The fraction of times that this value exceeds the original value is used as the p value. This test has the advantage of not assuming a specific distribution function. This test was applied to cases vs controls and also to the following phenotypes: ileal

disease, Vienna, and AOO. For testing association to AOO, two different scores were used. In the first test, every individual was labeled as AOO≤18 or AOO>18, and the Pearson χ^2 score was used as the test statistic. In the second test, the Kolmogorov–Smirnoff score was used as the test statistic.

As there are different population groups in the study, the test was corrected as follows: the Pearson score was calculated for every population separately, and the test statistic was defined to be the weighted average of these scores (the weight of each score was proportional to the size of its population group). The p value was calculated by a permutation test as mentioned above, with the difference of randomly permuting the labels within each population independently. This statistic avoids the bias in the p value that might occur because of the mixture of different populations. There were four population groups: Ashkenazim, Sefaradim, mixed (Ashkenazim and Sephardim), and other (only eight individuals). As the mixed group is not a distinct population group, the same test was also done using only the Ashkenazim and Sephardim population groups. All three p values (without the population correction and the two types of population correction) were calculated and considered.

Testing interactions between NFKBIA and NOD mutations

The permutation test can be readily generalized to handle association between haplotypes and the disease, e.g., by adding block haplotypes as artificial loci with states corresponding to common haplotypes. Similarly, one can add loci interactions as artificial loci, whose states are the allele combinations. First, the NOD genotypes were phased by using the software GERBIL [25]. Then, the artificial loci, called Any NOD, was built as follows: a haplotype that contained at least one NOD mutation was considered as an Any NOD mutation, and a haplotype with all three wild-type NOD alleles was considered as an Any NOD wild-type allele. Interactions between Any NOD and NFKBIA were tested for the same phenotypes mentioned above.

Table 1 Entry data for cohort 1 (231 individuals)

Percent	Parameters
53.5	Age (<18)
58.5	Gender (Male)
38.5	Ileal
18.5	Colitis
43.0	Ileocolonic disease
55.5	Inflammatory behavior (Vienna classification 1)
26.0	Stricturing disease (2)
18.5	Penetrating disease (3)
28	Family history
64.5 (49% heterozygote and 15.5% homozygote)	NFKBIA (mutant allele)
39 (30% heterozygote and 9% homozygote)	Any NOD2 (mutant haplotype)

Table 2 Entry data for cohort 2—individuals without any NOD mutations (139 individuals)

Percent	Parameters
55	Age (<18)
54	Gender (Male)
36.5	Ileal
24.5	Colitis
39.0	Ileocolonic disease
58.5	Inflammatory behavior (Vienna classification 1)
22.0	Stricturing disease (2)
19.5	Penetrating disease (3)
31.5	Family history
67 (51% heterozygote and 16% homozygote)	NFKBIA (mutant allele)

Results

Population data

Data were obtained from 231 patients. There were 53.5% under 18 years of age, 58.5% men, and 28% with a family history of CD. Our population consisted of 96% Jews and 4% non-Jews. Fifty one percent were of pure Ashkenazi decent , 33% of Jewish–Sephardic decent, 12% of mixed Ashkenazi–Sephardic decent, and 4% of non-Jewish Caucasians. Data regarding age, gender, and disease location are presented in Table 1.

No correlation between NFKBIA and disease can be seen by looking at the mutant allele frequency (case=0.41 and control=0.37; p=0.38 or 0.54 with population correction) or with phenotype.

Table 3 Association study of cohort 2

Test	p Value without population correction	p Value after population correction 2
AOO	0.13	0.56
Ileal involvement	0.65	0.53
Colonic involvement	0.19	0.11
Inflammatory	0.61	0.7

Table 4 Power calculations

Simulated minor allele frequency in control population (%)	Simulated minor allele frequency in cases population (%)	Difference in minor allele frequency between cases and controls (%)	Power of the test (%)
37.5	42.5	5	21.8
35	45	10	65
32.5	47.5	15	95.1
30	50	20	99.8

The Hardy Weinberg equilibrium was found in all subpopulations (Ashkenazim—control, Ashkenazim—case, Sephardim—control, and Sephardim—case).

No interactions were found between NFKBIA as well as the presence of single or multiple NOD2/CARD15 variant alleles.

As NOD2/CARD15 disease-associated mutations were found in 23.5% of our cohort and might mask the effect of NFKBIA on disease susceptibility or disease behavior, we created a second cohort (cohort 2) by removing individuals with NOD/CARD15 mutations. This cohort contained 139 individuals (subpopulations: Ashkenazim [56], Sefaradim [50], Mixed [18], other [7], and unknown [8]).

Table 2 describes the entry data of cohort 2.

We did not identify an association of the NFKBIA mutation with any phenotype in cohort 2 (Table 3). Power calculation is presented in Table 4.

Discussion

Present theories regarding the pathogenesis of CD implicate a central role for the intestinal flora, the innate immune system, and a TH1 response. The innate immune system responds to intestinal bacteria at the epithelial barrier via PRR that initiate a cascade that leads to activation of NFκB and release of cytokines characteristic of the inflammatory process involved in CD.

CD phenotype varies in AOO, disease location, and disease behavior. Variability in different signaling pathways via loss of function or gain of function may play a crucial role in the pathogenesis of the phenotypic variants of CD. We and the others have shown that mutations in NOD2/CARD15 were significantly associated with ileal disease. With other phenotypes, such as AOO, there is a population-dependent association, as some cohorts demonstrated an association between multiple NOD2/CARD15 mutations with AOO, although we and the others failed to demonstrate such an association [26, 27].

NFκB plays a crucial role in diseases associated with a dysregulated immune response. NFKBIA down regulates the activity of NFκB by trapping it to the cytoplasm. The human NFKBIA gene contains six axons spanning approximately 3.5 kb. It harbors a relatively large number of variants: ten exonics, of which only one variant causes a change of S32I, described in autosomal dominant ectodermal dysplasia, and the rest are synonymous and do not affect gene coding. The other SNPs are either in the intones or in the 3′-UTR. The 3′-UTR SNPs, located downstream exon 6, may have a significance because there is accumulating evidence that the 3′-UTR of mRNA regulates gene expression and may be altered in various disease states [28].

We evaluated the effect of a single polymorphism in the 3′-UTR of NFKBIA on disease susceptibility and phenotype in our CD cohort and performed an additional analysis in NOD2/CARD15-negative individuals with CD.

We did not find an association between NFKBIA genotype and CD susceptibility. Case–control frequencies were similar for both cohorts (Table 4). This is in contrast to a previous study by Klein et al. [22] in a cohort of German CD patients, which claimed that the AA genotype of the single polymorphism in the 3′-UTR of NFKBIA was significantly increased in NOD2/CARD15-negative CD patients. These authors speculate that the polymorphism might decrease the inhibitory effect by the NFKBIA, which can lead to enhanced secretion of inflammatory cytokines such as TNFα by macrophages. It is unclear at present if this discrepancy between our results is due to cohort bias in one of the studies or to differences in susceptibility genes among different ethnic groups or populations. Ethnic diversity for credible susceptibility genes in CD, such as NOD2/CARD15, is well established [29, 30]. Data on ethnic differences in disease-associated variants is important for a better understanding of the pathologic mechanisms of the polymorphism. The NFKBIA A allele frequency is 0.37–0.41 in the European population and 0.60–0.70 in the African and Afro-American population (http://www.ncbi.nlm.nih.gov/SNP).

We've also looked at the contribution of the A allele in the NFKBIA gene to AOO, disease location, and disease behavior in our CD cohort. We did not find an association with AOO, ileal disease, and disease behavior in both cohorts (with and without a variant NOD2 allele).

In conclusion, we do not believe that the 3′-UTR polymorphism alters disease susceptibility or phenotype in our population.

References

1. Hugot JP, Zouali H, Lesage S, Thomas G (1999) Etiology of the inflammatory bowel diseases. Int J Colorectal Dis 14:2–9
2. Fiocchi C (1998) Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 115:182–205
3. Thompson NP, Driscoll R, Pounder RE, Wakefield AJ (1996) Genetics versus environment in inflammatory bowel disease; results of a British twin study. BMJ 312:95–96
4. Sartor JB (1997) Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. Am J Gastroenterol 92:5S–11S
5. Newman B, Siminovitch K (2003) Inflammatory bowel disease: Crohn's disease and the success of the NODern genetics. Clin Invest Med 26:303–312
6. Mathew C, Lewis CM (2004) Genetics of the inflammatory bowel disease: progress and prospects. Hum Mol Genet 13:161–168
7. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411:599–603
8. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 411:603–606
9. Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, van Deventer S, Forbes A, Nikolaus S, Lennard-Jones JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, Mathew CG (2001) Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet 357:1925–1928
10. Inohara N, Nunez G (2001) The NOD: a signaling module that regulates apoptosis and host defense against pathogens. Oncogene 20:6473–6481
11. Aderem A, Ulevitch RJ (2000) Toll-like receptors in the induction of the innate immune response. Nature 406:782–787
12. Cario E, Podolsky DK (2000) Differential alternation in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 68:7010
13. Franchimont D, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T, Quertinmont E, Abramowicz M, Van Gossum A, Deviere J, Rutgeerts P (2004) Deficient host–bacteria interactions in inflammatory bowel disease: the toll-like receptor(TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. Gut 53:987–992
14. Levine A, Karban A, Eliakim R, Shaoul R, Reif S, Pacht A, Wardi J, Yakir B, Leshinsky-Silver E (2005) A polymorphism in the TNF-α promotor gene is associated with pediatric onset and colonic location of Crohn's disease. Am J Gastroenterol 100:407–413
15. Levine A, Raanan A, Wine E, Weiss B, Karban A, Shaoul R, Eliakim R, Reif S, Pacht A, Yakir B, Friedlander M, Kaniel Y, Leshinsky-Silver E (2005) TNF promotor polymorphism and modulation of growth retardation and disease severity in pediatric Crohn's disease. Am J Gastroenterol 100:1–7
16. Schreiber S, Nikolaus S, Hampe J (1998) Activation of nuclear factor kappa B inflammatory bowel disease. Gut 42:477–484
17. Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Scholmerich J, Gross V (1998) Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 15:357–369
18. Neurath MF, Fuss I, Schurmann G, Pettersson S, Arnold K, Muller-Lobeck H, Strober W, Herfarth C, Buschenfelde KH (1998) Cytokine gene transcription by NF-kappa B family members in patients with inflammatory bowel disease. Ann NY Acad Sci 859:149–159
19. Neurath MF, Pettersson S (1997) Predominant role of NF-kappa B p65 in the pathogenesis of chronic intestinal inflammation. Immunobiology 198:91–98
20. Miterski B, Bohringer S, Klein W, Sindern E, Haupts M, Schimrigk S, Epplen JT (2002) Inhibitors in the NFkappaB cascade comprise prime candidate genes predisposing to multiple sclerosis, especially in selected combinations. Genes Immun 3:211–219
21. Duerr RH, Barmada MM, Zhang L, Pfutzer R, Weeks DE (2000) High-density genome scan in Crohn disease shows confirmed linkage to chromosome 14q11-12. Am J Hum Genet 66:1857–1862
22. Klein W, Tromm A, Folwaczny C, Hagedorn M, Duerig N, Epplen JT, Wolff H, Schmiegel WH, Griga T (2004) A polymorphism of the NFKBIA gene is associated with Crohn's disease patients lacking a predisposing allele of the CARD15 gene. Int J Colorectal Dis 19:153–156
23. Glavac D, Ravnik-Glavac M, O'Brien SJ, Dean M (1994) Polymorphisms in the 3′ untranslated region of the I kappa B/MAD-3 (NFKBI) gene located on chromosome 14. Hum Genet 93:694–696
24. Zhang K, Deng M, Chen T, Waterman MS, Sun F (2002) A dynamic programming algorithm for haplotype block partitioning. Proc Natl Acad Sci USA 99:7335–7339
25. Kimmel G, Shamir R (2005) GERBIL: genotype resolution and block identification using likelihood. Proc Natl Acad Sci USA 102:158–162
26. Wine E, Reif S, Leshinsky-Silver E, Weiss B, Shaoul RR, Shamir R, Wasserman D, Lerner A, Boaz M, LevineA (2004) Pediatric Crohn's disease and growth retardation: the role of genotype, phenotype, and disease severity. Pediatrics 114:1281–1286
27. Leshinsky-Silver E, Karban A, Buzhakor E, Yakir B, Eliakim R, Reif S, Shaul R, Boaz M, Lev D, Levine A (2005) Is age of onset of Crohn's disease governed by mutations in NOD2/CARD15 and Toll-like receptor 4? Evaluation of a pediatric cohort. Pediatr Res 58:499–504
28. Conne B, Stutz A, Vassalli JD (2000) The 3′-untranslated region of messenger RNA: a molecular hotspot for pathology? Nat Med 6:637–641
29. Inoue N, Tamura K, Kinouchi Y, Fukuda Y, Takahashi S, Ogura Y, Inohara N, Nunez G, Kishi Y, Koike Y, Shimosegawa T, Shimoyama T, Hibi T (2002) Lack of common NOD2 variants in Japanese patients with Crohn's disease. Gastroenterology 123:86–91
30. Yamazaki K, Takazoe M, Tanaka T, Kazumori T, Nakamura Y (2002) Absence of mutation in the NOD2/CARD15 gene among 483 Japanese patients with Crohn's disease. J Hum Genet 47:469–472