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Risk Factors for Perianal Crohn's Disease: The Role of Genotype, Phenotype, and Ethnicity

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OBJECTIVES: Perianal disease (PD) is a frequent complication of Crohn's disease (CD). The lack of association between PD and development of intestinal penetrating disease may suggest that PD is a distinct phenotype with specific genetic or clinical risk factors. This study was undertaken to evaluate the role of genotype, clinical, and demographic characteristics with PD.

METHODS: Phenotypic data on 121 CD patients with PD and 179 patients without PD were carefully characterized. The patients were genotyped for disease-associated OCTN1/2 and NOD2/CARD15 variants and the TNF-α promoter polymorphisms. Analysis was performed to evaluate the differences in phenotype and genotype frequencies between the PD group and the non-PD group.

RESULTS: PD was associated with rectal involvement (odds ratio [OR] 2.27, 95% CI 1.32–3.91) and with Sephardic (non-Ashkenazi) Jewish ethnicity (OR 1.71, 95% CI 1.02–2.9). No association was found among the studied OCTN, NOD2, TNF-α variants and the risk for PD.

CONCLUSIONS: The strongest factor associated with PD is rectal inflammation. OCTN1/2, NOD2/CARD15, and TNF-α promoter variants do not play a role in the risk to PD in the Jewish Israeli population. The association of ethnicity with PD may suggest that there are as yet unknown genetic variants that are associated with PD.

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disease that may manifest itself throughout the gastrointestinal tract, or by extraintestinal symptoms. Unlike many other inflammatory conditions of the gastrointestinal tract, CD may cause perianal disease (PD). This presentation is not infrequent. The prevalence of PD in CD ranges between 3.8% and 80% (1, 2). The large discrepancies in prevalence data may be because of the variable defining criteria. In 20–36% of patients PD precedes the overt intestinal disease. In the majority of patients it occurs either concurrently or after the diagnosis of intestinal disease (1, 3).

The spectrum of PD includes anal fissures, skin tags, and hemorrhoids as milder manifestations, whereas perianal abscesses, external fistulas, rectovaginal fistulas, or anorectal strictures define the more severe end of this spectrum. Anal fistulas, deep cavitating ulcers, and dense anorectal strictures usually carry a poor prognosis, with many of the affected patients ultimately requiring a proctectomy and permanent stoma (4).

For many years, PD was considered to be a variant of the penetrating CD. According to the Vienna Classification of CD (5), perianal fistulas, inflammatory masses, and/or abscesses at any time in the course of the disease were defined as penetrating disease behavior. This categorization has been challenged.

Recent studies have shown that perianal CD may be a distinct phenotype and not related to the "classic" penetrating disease (6–9). This is based on the lack of association between PD and development of intestinal penetrating disease in the majority of patients, as well as serological (9), genetic (10), and clinical distinctions between cases with or without PD, irrespective of the presence or absence of internal fistulization. These observations may suggest that perianal CD is a distinct phenotype with possible specific susceptibility genes or environmental factors.

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The discovery of the first susceptibility gene, NOD2/CARD15, has helped us to understand many aspects of this diverse disease, and to serve as the first reproducible association between a genotype and a phenotype in CD (ileal disease and stricturing behavior). These findings have helped us to understand that many aspects of disease phenotype (including disease behavior) may be related to genotype.

The three NOD2/CARD15 mutations were not found to be associated with PD (6). However, two other genes or loci have been reported to be associated with PD (10–14). There are conflicting results concerning the *IBD5* haplotype on chromosome 5q31. The latter is a 250-kb region of the human genome that contains multiple variants that are associated with CD. More recently, data have suggested that mutations of two genes within the *IBD5* region, the organic cation transporters OCTN1 and OCTN2, may be independently associated with CD. The construction of a 2-allele risk haplotype OCTN1/2-TC (OCTN1 exon 9 1672C/T and OCTN2 promoter -207G/C) was reported to be associated with CD (10).

Two recent studies have reported a significant association between the *IBD5/OCTN* haplotypes and PD, whereas two others have not (10–13). A single report showed that the frequency of the −238A allele of TNF-α promoter gene was significantly higher in CD patients with PD than those without PD (14).

These conflicting results raise the possibility of problems in methodology, or ethnic diversity, in understanding the role of genotype in PD. We undertook a focused evaluation of the role of genotype, clinical, and demographic characteristics with PD in order to define specific parameters associated with this phenotype.

METHODS

Human Subjects and Phenotypic Analysis

The study population comprised 375 patients with CD. The patients were recruited from the Rambam Medical Center (Haifa, Israel), the Tel-Hashomer Medical Center (Ramat-Gan, Israel), and the Wolfson Medical Center (Holon, Israel). All study participants were Israelis of Jewish ethnicity and gave their written informed consent. The study protocol was approved by the local hospital and the Ministry of Health ethics boards.

Confirmation of CD required a first-hand review of endoscopic, pathology, and radiology reports, and operative notes. Confirmation also required that confounding diagnoses (ameobiasis, diverticulitis, intestinal tuberculosis, ulcerative colitis) were ruled out when there was a concern.

CD patients without perianal involvement with disease duration of less than 2 yr were excluded from the study.

The phenotypic evaluation of the patients was carried out by reviewing the patients' medical records as well as filling in a clinical questionnaire by the referring gastroenterologist.

Age at diagnosis of CD, tobacco consumption, and ethnicity were determined from medical records, questionnaires, and interviews. Patients were considered smokers if they smoked a minimum of seven cigarettes per week for at least 1-yr anytime lifelong. Family history of inflammatory bowel disease (IBD) was considered in a first cousin or a closer relative (*e.g.*, grandparent, avuncular or first degree relative). Confirmation of the IBD diagnosis in relatives was not complete, and in some cases was based only on the patient's interview. Ashkenazi and Sephardic ethnicities were carefully assigned on the basis of the birthplace of the four grandparents or the birthplace of the older generations.

The disease site was determined using the Vienna Classification (5). Disease behavior was not included in our study because it does not identify PD as a distinct phenotype. Disease location was classified as terminal ileal, colonic, ileocolonic, and upper gastrointestinal (disease limited proximal to the ileum) on the basis of endoscopic, radiological, or pathologic studies. Both small bowel and large bowel examinations were required for classification.

PD was defined as the presence of perianal abscess, fistula, or multiple deep fissures outside the midline. Skin tags or midline fissures were not considered adequate for definition of PD.

In addition to the phenotype determination according to the Vienna classification, rectal involvement was also determined for every patient.

DNA Extraction

DNA was extracted from peripheral whole blood using the Qiagen Genomic DNA Purification Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol.

Genotyping

NOD2/CARD15 MUTATIONS ANALYSIS. Patients were genotyped for Arg702Trp and Leu1007fsinsC mutations using single tube allele specific PCR and for Gly908Arg using restriction enzyme digestion assay as described before (15).

OCTN1/OCTN2 GENOTYPING (12). The polymorphisms 1672C→T in OCTN1 (*SLC22A4),* −207G→C in OCTN2 (*SLC22A5)* were investigated by restriction fragment length polymorphism analysis (12). Results were confirmed by sequencing representative samples for each genotype.

TNF-α PROMOTER POLYMORPHISMS. The TNF-α polymorphisms examined included TNF 208G/A, 308 G/A, 857 C/T, and 863 C/A using the pyrosequencing technology (16). The primers' sequences for the PCR are described elsewhere (17). PCR products were prepared for pyrosequencing analysis using the PSQ sample preparation kit according to the standard protocol from pyrosequencing AB and analyzed for the various SNPs on a PSQ 96MA pyrosequencer, using the 0.2-μM sequencing primers and the SNP reagent kit according to standard protocols. The order of nucleotide dispensation was decided based on suggestions provided by the PSQ HS 96 SNP software 1.0 (pyrosequencing AB), which was also used for automatic assay evaluation and genotype scoring.

Statistical Analysis

The association between every SNP and PD was evaluated by a permutation test (18) as follows: we used the Pearson χ^2 score as the test statistic. The contingency table for the Pearson χ^2 score was built based on chromosome counting (and not on individuals). This statistic assumes the multiplicative model for penetrance. The same statistic was calculated for 10^7 data sets with the same genotypes and randomly permuted labels of the phenotype (corrected for subpopulations, see below). The fraction of times that this value exceeded the original score was used as the *P* value.

The association between PD and discrete phenotypes (rectal involvement, smoking, etc.) was evaluated with the same permutation test while treating PD as an artificial locus. For continuous traits (age at diagnosis and duration of disease), the analysis of variance score was used instead of the Pearson χ^2 score, and a permutation test was performed in a similar way.

Because there are two different population groups in the study (Ashkenazim and Sephardim), all permutation tests were corrected as follows: The statistic score was calculated for each population separately, and the test statistic was defined to be the weighted average of these scores, where the weight of each score is the size of its corresponding population. In calculating the *P* value, permutations were generated by 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. Patients with mixed or unknown ethnicity were excluded from the cohort when applying the population correction.

Power Calculations

The power of the permutation test for the association between one SNP and PD was calculated as follows. Two simulation setups were done, one for an SNP with a high minor allele frequency (40%, similar to OCTN) and another with a low frequency (10%, similar to CARD15/NOD2). In each setup, several differences in allele frequency between PD patients and non-PD patients were tested. For each of these differences, the following test was performed: 1,000 data sets of 121 PD patients and 179 non-PD patients were simulated, where alleles were drawn according to the defined allele frequency. The permutation test described above was applied to each of these data sets. The power of the test was calculated as the fraction of the data sets that received a *P* value lower than 0.05. For single nucleotide polymorphisms (SNPs) with minor allele frequency of about 10%, when the difference in the minor allele frequency between PD and non-PD patients is 7%, the power of the test reaches 80%. For SNPs with minor allele frequency of about 40% (*e.g.*, OCTN SNPs), a difference of 12% is needed in order to reach 80% power.

RESULTS

Patient Characteristics

Data were obtained from 300 CD patients with adequate follow-up: 121 patients with PD and 179 patients without PD. Data regarding ethnicity, age at onset, duration of disease, disease location, extraintestinal manifestations, and rectal involvement are presented in Table 1. The significant differences (excluding genotype) comparing CD patients with and without perianal involvement were ethnicity and rectal involvement. Among the Sephardic patients, a significantly higher percentage had PD (47% *vs* 34% in Ashkenazi patients, $P = 0.0457$, odds ratio [OR] 1.71, 95% CI 1.02–2.9). The frequency of rectal involvement was significantly higher in the perianal group (34.9% *vs* 19.1%, OR 2.27, 95% CI 1.32–3.91). This association was significant with and without stratifying by populations ($P = 0.01$ and 0.004, respectively), suggesting that the two associations found are independent. The frequency of rectal involvement was higher in the perianal group in each population separately (Ashkenazi Jews—39.1% *vs* 18.9%, Sephardic Jews—31.6% *vs* 21.2%).

Table 1. Demographic and Clinical Characteristics in CD Patients With or Without Perianal Disease

	All CD (N = 300)	Perianal CD (N = 121)	Nonperianal CD (N = 179)	*P* Value
Male patients	167 (55.6%)	70 (57.8%)	97 (54.2%)	0.26
Smoking: currently	68/296 (23%)	32/119 (26.9%)	36/177 (20.3%)	0.44
Smoking: past	28/296 (9.5%)	11/119 (9.2%)	17/177 (9.6%)	0.73*
Age at diagnosis (yr)	23.48 ± 12.2	22.12 ± 9.5	24.35 ± 13.5	0.17
Family Hx (first degree)	55/297 (18.5%)	22/118 (18.6%)	33/179 (18.4%)	0.51
Duration of disease	9.2 ± 6.7	9.95 ± 6.7	8.73 ± 6.7	0.33
Urban	226/262 (86.2%)	70/84 (83.3%)	156/178 (87.6%)	0.35
Ashkenazi Jews†	144/296 (48.6%)	49/119 (41.2%)	95/177 (53.7%)	0.0457
Sephardic Jews†	98/296 (33.1%)	46/119 (38.7%)	52/177 (29.4%)	
Extent: terminal ileum	104/300 (34.7%)	37/121 (30.6%)	67/179 (37.4%)	0.17
Extent: colon only	40/300 (13.3%)	21/121 (17.4%)	19/179 (10.6%)	0.41
Extent: ileocolon	126/300 (42%)	53/121 (43.8%)	73/179 (40.8%)	0.15
Extent: upper GI	30/300 (10%)	10/121 (8.2%)	20/179 (11.2%)	0.41
Extraintestinal disease	77/300 (25.6%)	33/121 (27.3%)	44/179 (24.6%)	0.97
Rectal involvement	71/284 (25%)	37/106 (34.9%)	34/178 (19.1%)	0.01

*Patients were divided into three smoking categories: never, currently, and past.
†Fifty-four patients have mixed Ashkenazi–Sephardic ethnicity.

Table 2. Genotype Frequencies of NOD2/CARD15 and OCTN1/2 Polymorphisms in Patients With or Without PD

	OCTN1 C1672T (%)	OCTN2G-207C (%)	OCTN1/2 TC-Haplotype	Gly908A (%)	Arg702Trp (%)	fs1007insC (%)
CD (N = 300)			51 (17%)			
Wild	89 (29.7)	48 (16.1)		238 (79.9)	270 (90.6)	263 (88.3)
Hetero	158 (52.7)	150 (50.1)		59 (19.8)	28 (9.4)	34 (11.4)
Homo	53 (17.7)	101 (33.8)		1 (0.3)	0	1 (0.3)
PD (N = 121)			21 (17.4%)			
Wild	40 (33.1)	21 (17.4)		98 (82.3)	112 (94.1)	104 (87.4)
Hetero	58 (47.9)	65 (53.7)		21 (17.7)	7 (5.9)	15 (12.6)
Homo	23 (19)	35 (28.9)		0	0	0
NPD (N = 179)			30 (16.8%)			
Wild	49 (27.4)	27 (15.2)		140 (78.2)	158 (88.3)	159 (88.8)
Hetero	100 (55.9)	85 (47.8)		38 (21.2)	21 (11.7)	19 (10.6)
Homo	30 (16.7)	66 (37)		1 (0.6)	0	1 (0.6)

Wild = wild-type; hetero = heterzygote; homo = homozygote, PD = perianal disease; NPD = nonperianal disease.

We did not find an association between rectal involvement and ethnicity ($P = 0.97$).

No significant differences were noted for all other parameters (age at diagnosis, family history of IBD, urban place of birth, extent of disease, and extraintestinal manifestations). The percentage of smokers among the PD patients was higher, but not statistically significant after population correction.

Analysis of OCTN1/2 and NOD2/CARD15 Variants

Table 2 presents the genotype frequencies of NOD2/CARD15 and OCTN1/2 polymorphisms and the OCTN1/2 TC-haplotype in patients with or without PD. No association was found between these polymorphisms and PD. Stratifying patients with PD to the different perianal manifestations (anal fissure, fistulae, or abscess) did not reveal any differences either (data are not shown). Because PD was associated with ethnicity and rectal involvement we tested for associations between genotype and these variables. Only the NOD2/Card15 polymorphism fs1007insC was associated with ethnicity ($P = 0.0006$, $P = 0.002$ after correction to multiple testing by a permutation test). Its minor allele frequency among Ashkenazi Jews was 9% compared with only 1.5% in Sephardic Jews. However, as mentioned above, the SNP is not associated with PD ($P = 0.86$). The lack of association might be caused by the low minor allele frequency of the SNP and the small number of patients (only three Sephardic Jews carry the mutation). No association was found between the polymorphisms and rectal involvement (with and without stratifying by populations).

Analysis of TNF-α Promoter Polymorphisms

The genotype frequencies of TNF-α promoter polymorphisms are presented in Table 3. For technical reasons (lack of source DNA and procedure difficulties) some samples are missing in this analysis. No association was found among these polymorphisms and PD, ethnicity, or rectal involvement.

DISCUSSION

Recent observations suggest that the presence of perianal and enteric fistula describes two different phenotypes. The Working Party of the 2005 Montreal World Congress of Gastroenterology suggested a revised classification of CD (19). In the Montreal classification, perianal fistulas and abscesses are not included in the definition of penetrating disease.

In our rigorously phenotyped cohort of Israeli Jewish CD patients, we demonstrated that the CARD15/NOD2, OCTN1/2, and TNF-α promoter polymorphisms are not associated with PD. In our cohort, the predictors for PD after population correction were ethnicity and rectal involvement.

An eventual goal in the genetic studies of IBD is to identify biologically relevant genotype–phenotype associations and to

Table 3. TNF-α Polymorphisms in Patients With PD or Without PD (NPD)

	TNF-238 G/A	TNF-308 G/A	TNF-857 C/T	TNF-863 C/A
CD (N = 259)				
Wild	209/248 (84.3%)	216/248 (87.1%)	157/258 (60.9%)	174/259 (67.2%)
Hetero	37/248 (14.9%)	31/248 (12.5%)	89/258 (34.5%)	78/259 (30.1%)
Homo	2/248 (0.8%)	1/248 (0.4%)	12/258 (4.6)	7/259 (2.7%)
PD (N = 114)				
Wild	88/106 (83%)	93/108 (86.1%)	72/114 (63.2%)	76/114 (66.7%)
Hetero	17/106 (16%)	14/108 (13%)	37/114 (32.4%)	34/114 (29.8%)
Homo	1/106 (1%)	1/108 (0.9%)	5/114 (4.4%)	4/114 (3.5%)
NPD (N = 145)				
Wild	121/142 (85.2%)	123/140 (87.9%)	85/144 (59%)	98/145 (67.6%)
Hetero	20/142 (14.1%)	17/140 (12.1%)	52/144 (36.1%)	44/145 (30.3%)
Homo	1/142 (0.7%)	0/140 (0%)	7/144 (4.9%)	3/145 (2.1%)

apply them to the clinical practice. To date, we have good reason to believe that both susceptibility and clinical phenotype of PD may be genetically determined. Although many phenotypic associations with CD-associated genetic variants have been reported, these findings have been variable. The most established phenotypic associations have been for CARD15. Many studies reported an association of the CD-associated CARD15 variants with ileal disease (20–25) and possibly stricturing disease (22, 25). No significant correlation was found with PD. Our results support the lack of association of CARD15 mutations and PD. This finding may be consistent with the role of rectal involvement in PD, because CARD15 mutations are positively correlated with ileal disease and inversely correlated with colitis.

Recently, the *IBD5* region has been studied for phenotypic associations.

Armuzzi *et al.* (11) reported an association of the *IBD5* haplotype with both perianal and ileal CD. Newman *et al.* (10) reported an association of the OCTN-TC haplotype with ileal CD, independent of PD involvement. In contrast Torok *et al.* (12) reported novel phenotypic associations with the *IBD5*/OTCN-TC haplotype and colonic CD, as well as with nonfistulizing and nonstricturing behavior. Vermeire *et al.* (13) observed that both *IBD5* and OCTN-TC haplotype were associated with PD and penetrating behavior. Our study, which specifically examined genotype interaction with PD, did not replicate Vermeire's findings.

There are a number of possible explanations for these differences, including both genetic and disease heterogeneity among populations. The *IBD5* locus was not shown yet to be associated with CD among the Jewish population. Other difficulties in this phenotype–genotype correlation to date have been small sample size and differences in phenotypic classification systems.

Polymorphisms in the TNF promoter gene have been found to be either associated with IBD and/or to alter disease phenotype or activity in various studies (26–31). Data regarding the biological effects of some of these polymorphisms appear to be conflicting. Certain polymorphisms appear to increase TNF production (−308A, −238A, −857C), whereas others (−863A) may decrease TNF production, raising the possibility of differing phenotypes based on variable levels of potential inflammation (26–35). As mentioned, there is only one study that showed that the frequency of −238A allele of TNF-α was significantly higher in CD patients with perianal lesion than in those without perianal lesion (14). This observation was not replicated in our study. Of note, Fidder *et al.* (36) investigated the frequency of TNF-857 polymorphism in Israeli Jewish IBD patients and concluded that this polymorphism does not contribute to susceptibility to IBD; neither does it define the phenotype of CD in this population.

In our study we observed that Sephardic Jewish CD patients are more likely than Ashkenazi patients to develop PD (OR 1.71).

We have previously shown (37) that all three NOD2/CARD15 mutations are independently associated with CD in the Israeli Jewish population. However, the Ashkenazi Jewish CD patients have a higher carrier rate of CARD15 mutations compared with Sephardic/non-Ashkenazi Jews (47.4% *vs* 27.45%, $P = 0.034$). The disease phenotype was similar among the Ashkenazi and Sephardic patients except for a higher prevalence of extraintestinal manifestations in the Sephardic patients. This may indicate that there are racial differences in disease location and behavior that may reflect underlying genetic variations and have important implications for diagnosis and management of disease. Interestingly, studying the phenotypic characterizations of African-American and Hispanic CD patients revealed that both populations have a higher prevalence of PD (38). It is also known that the CARD15 mutation frequency is lower in the African-American CD population (39). These observations may suggest that PD is not associated with the susceptibility genes that are relevant for the Caucasian and the Ashkenazi Jewish populations where the prevalence of IBD is higher.

Our results show a strong association between PD and rectal inflammation (OR 2.27). It has been previously shown that PD is more common with colonic inflammation, but we have reinforced this observation by showing that the incidence of PD increases with more distal involvement of the colon. This was also shown by Hellers *et al.* (40), who found that 92% of patients with rectal CD developed perianal fistula.

In conclusion, our study failed to replicate the genetic association of OCTN1/OCTN2 risk haplotype with perianal CD. The only predictors that we have found for PD among the Jewish population in Israel are rectal involvement and Sephardic Jewish ethnicity. The effect of ethnicity may suggest that there are as yet unknown genetic variants that are associated with PD.

Interpretation of genotype–phenotype associations in studies such as ours and by previous groups may be confounded by the small sample size of individual stratified subgroups, differences in phenotypic classification, duration of disease, and ethnic differences. Future collaborative efforts to incorporate large data sets using standardized and rigorously defined phenotypic classification schemes will be critical in clarifying these conflicting observations. Further genotype–phenotype correlation studies using the Montreal classification where PD is defined as a distinct phenotype may help to find the genetic predictors for this phenotype.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Perianal Crohn's disease (CD) is a distinct phenotype and not related to "classic" penetrating disease.
- Recent studies have reported a significant association between the *IBD5/OCTN* haplotypes and perianal disease (PD).
- Colonic disease is associated with PD.

What Is New Here

- CARD15/NOD2, OCTN1/2, and TNF-α promoter polymorphisms are not associated with PD in a cohort of Israeli Jewish CD patients.
- The incidence of PD increases with more distal involvement of the colon.
- Ethnicity is a predictor for PD. Sephardic Jews have a higher risk for PD than Ashkenazi Jews.

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CONFLICT OF INTEREST

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