Granulomas in Crohn's disease: Are newly discovered genetic variants involved?

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Abstract

Background: Non-caseating granulomas exist in a substantial portion of patients with Crohn's disease (CD). Several single nucleotide polymorphisms (SNPs) have been identified as having strong association with CD, including SNPs within the autophagy related 4 homolog A (ATG4A) gene and the neutrophil cytosolic factor 4 (NCF4) gene. We hypothesized a possible association between the presence of granulomas in CD patients and variants in the ATG4A and NCF4 genes.

Aims: To investigate whether variants in the NCF4 and ATG4A genes are associated with granuloma formation in a cohort of Israeli patients with CD, exploring demographic and clinical characteristics that differ between granuloma positive and granuloma negative patients.

Methods: 307 Israeli patients with CD were studied. Patients with CD who underwent biopsy or resection of the intestine were classified according to presence or absence of granulomas. Using PCR-RFLP we determined the allele frequency in SNP rs4821544 (NCF4 gene) and SNP rs807185 (ATG4A gene) for all patients.

Results: Granulomas were found in 85 out of 307 CD patients (27%). There were no significant differences between patients with or without granulomas in allele frequency in SNPs rs4821544 and rs807185. CD Patients with granuloma were younger at diagnosis than patients without granuloma (mean age 19 vs. 27, respectively, \(P < 0.0001\)) and were more likely to undergo surgery (55.3% vs. 34.8%, respectively, \(P = 0.002\)).

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1 Shared first authorship.
2 Shared senior authorship.
1. Background

Crohn’s disease (CD) and ulcerative colitis (UC) represent the two common forms of inflammatory bowel disease (IBD). CD usually becomes clinically evident during the second to third decade of life, with a second peak occurring during the 6th–8th decade. The frequency of CD varies among different geographic and ethnic backgrounds, being especially high among Ashkenazi Jews. The most commonly involved region of the gut in CD is the distal ileum, but any region from the mouth to anus can be affected.

Microscopic features of CD include focal ulceration, transmural inflammation and granuloma formation, and the presence of granulomas is diagnostic of CD. The frequency of granulomas varies between 15% in a single endoscopic biopsy and 70% in studies involving surgical resections or multiple biopsies. Granulomas are more prevalent in younger patients. Some studies have reported a higher frequency of granulomas along the distal portions of the gut, with the anus being the most common site. Studies have suggested that the presence of granulomas portends a poor prognostic sign, with more aggressive disease, more hospitalizations, and a need for surgery at a younger age. Other studies have found no such association. Historical reports demonstrated higher recurrence rates after surgery in patients without granulomas but later reports have not replicated these findings. No association was found between granuloma formation and fistulizing or perianal disease or extra-intestinal manifestations. Current or previous smokers were found to have a lower frequency of granulomas. Granulomas are found more frequently in untreated patients than in treated patients. A study with serial biopsies and surgical pathology suggested that granulomas may evolve or regress during the course of the disease.

In the last two decades progress has been made in defining some of the genetic factors that predispose patients to CD. Using linkage studies, the first susceptibility gene identified as a risk factor for CD was the NOD2/CARD15 gene, located on chromosome 16. Later on, linkage to a locus on chromosome 5q (IBD5) was also found. Several well replicated loci have been identified as having a strong association with CD, including the interleukin-23 receptor (IL23R) gene, the autophagy related 16-like 1 (ATG16L1) gene, the p47 GTPase family member immunity-related GTPase M (IRGM) gene on chromosome 5, and the neutrophil cytosolic factor 4 (NCF4) gene on chromosome 22. The NCF4 gene encodes a protein known as p40phox, one of the six sub-units of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase enzyme system. This system is responsible for the oxidative burst in which electrons are transported from NADPH to molecular oxygen, resulting in reactive oxidative intermediates. The importance of this system has been demonstrated in patients with chronic granulomatous disease (CGD), in which the inherited absence of one of the sub-units of the NADPH system results in recurrent infections and granuloma formation in multiple organs, including the gut.

Autophagy is an important component of the innate response against pathogenic invasion. NADPH oxidase activation has been shown to be an essential mechanism for pathogen degradation inside the phagosome, and as recently demonstrated, also inside the autophagosome. Recent hypothesis on the pathogenesis of CD have focused on autophagy, particularly in Paneth cells or macrophages, as possible culprits for disease development.

Recently published data has identified an association between a SNP in the autophagy related 4 homolog A (ATG4A) gene on chromosome X and granuloma positive CD. Atg4s are cysteine proteases that share several conserved cysteine residues and have a complex and important role in autophagy. Given that defects in the intracellular clearance of bacteria are related to granuloma formation, we hypothesized that polymorphisms in the NCF4 or the ATG4A genes might be associated with a granuloma positive CD.

2. Materials and methods

2.1. Study population

408 consecutive CD patients were recruited for a phenotype–genotype study in Israel. Out of these 408 patients, 307 patients who underwent an endoscopic biopsy or surgical resection, with a gastrointestinal mucosal specimen submitted for histopathological review, were selected. These 307 patients were recruited from six hospitals in Israel and included 270 (87%) Jewish patients and 27 (13%) non-Jewish patients. All patients were diagnosed as CD by an expert gastroenterologist using accepted criteria including clinical presentation, radiologic, endoscopic and histopathologic findings. All patients gave their informed consent and for pediatric patients consent of the patients and their legal guardian was acquired. The study was approved by the ethics committee at the Rambam Health Care Campus and by the Israel Ministry of Health genetic research review board. Our primary end point was to evaluate a possible association between the previously described SNPs in the NCF4 and ATG4A genes and the presence of granuloma in patients with CD.

Secondary end points were differences in NOD2 mutation prevalence, demographic, and clinical characterization between granuloma positive and granuloma negative patients.
2.2. Histology

We used either surgically resected specimens or biopsies from upper or lower endoscopy for granuloma confirmation. Histological records were reviewed for the presence of epithelioid granulomas. These were defined as discrete collections of at least five epithelioid cells (activated histiocytes with homogeneous eosinophilic cytoplasm) with or without accompanying multinucleate giant cells. Patients were classified as either granuloma negative or granuloma positive.

2.3. Phenotype data collection

For all recruited patients a physician guided questionnaire was filled including age, sex, ethnic background, family history of IBD and smoking status. Data regarding age of diagnosis and extra-intestinal manifestation were collected from patients and their electronic chart. Disease extent and type were classified according to the Montreal classification.

2.4. Genetic analysis

Genomic DNA was extracted from whole peripheral venous blood, using a commercially available kit (Genta, Minneapolis, MN).

For the three NOD2 mutations Arg702Trp, Gly908Arg, and Leu1007fsinsC, patients were genotyped using PCR-RFLP as previously described. A restriction enzyme digestion assay was used for the detection of SNP rs4821544. An amplification product of 235 kb was amplified from genomic DNA using the forward primer 5′-GAATTCCTTCCTCCCTCAC-3′ and the reverse primer 5′-CTCAAGGCCTCATGAAAAGC-3′.

Products were digested by the enzyme Hinfl (Fermentas) for 1 h at 37 °C — those with Thiamine in rs4821544 were digested to two separate products, 54 kb and 181 kb in length. Products with cytosine in rs4821544 were not digested. All products were analyzed on horizontal 2% agarose gel.

For the detection of SNP rs807185 in the ATG4A gene restriction enzyme digestion was also used. An amplification product of 222 kb was amplified from genomic DNA using the forward primer 5′-GAGCATGTGCTGCAAGA-3′ and the reverse primer 5′-GGGGGAATGATCCTCTCTCTG-3′. Products were digested by the enzyme Alul (Fermentas) for 1 h at 37 °C — products with adenosine in rs807185 were digested to two separate products, 88 kb and 134 kb in length. Products with Tyrosine in rs807185 were not digested. All products were analyzed on horizontal 2% agarose gel as restriction fragment length polymorphisms.

2.5. Statistical analysis

The data were evaluated by the SPSS software, version 16 (SPSS Inc. Chicago, IL, USA). Descriptive statistics (mean, median, standard deviation, minimum and maximum levels) were used for all variables in the study. Fisher’s Exact Test and Pearson chi-square were used for detection of differences in the prevalence of demographic variables (gender, ethnicity, family history, smoking status) and clinical variables (NOD2 mutations, SNP rs4821544 in NCF4 and SNP rs807185 in ATG4A) between patients with and without granulomas. A T-test was applied to compare differences in age at diagnosis between patients with and without granuloma. Multivariate analysis by the logistic regression was employed to study the parameters associated with the presence of granuloma (age at diagnosis, NCF4 mutations, gender, disease extent, smoking status) and to analyze the parameters that could influence the presence of granulomas. P < 0.05 was considered significant.

3. Results

Histopathology revealed granulomas in 85 out of 307 patients (27%). Of the 281 patients with available biopsies, 50 were positive for granulomas in at least one biopsy (18%), as compared to 41 out of 124 surgical specimens (33%). Six patients had granulomas in both biopsies and surgical specimens, three had granulomas in biopsies but not surgical specimens, and 31 had granulomas in their surgical specimens but not in their biopsies.

3.1. Granuloma and CD phenotype

Patients with granuloma positive CD were significantly younger at diagnosis than patients with granuloma negative CD (mean age 19 vs. 27, respectively, P < 0.0001) and were more likely to have undergone gastrointestinal surgery (55.3% vs. 34.8%, respectively, P = 0.002). Patients with granuloma negative CD were more likely to have smoked in the past as compared to patients with granuloma positive CD (15.3% vs. 3.7%, respectively, P = 0.0005). There were no other differences between the groups in all other clinical variables (Table 1).

3.2. Granuloma and genotype

In the granuloma positive group, 29 patients (34%) had at least one mutation in the three SNPs in NOD2, as compared to 70 patients (32%) in the granuloma negative group. There were no statistically significant differences comparing the allele frequencies of the three mutations in NOD2/CARD15 between granuloma positive and granuloma negative groups (Table 2).

Although there was a higher frequency of homozygotes for the minor allele (cytosine) of SNP in rs807185 in the granuloma positive patients compared to the granuloma negative patients (24.7% vs. 14.4% respectively, P = 0.08), this did not reach statistical significance.

There were no differences in the frequency for the allele in minority (adenosine) of SNP rs807185 in the ATG4A gene between granuloma positive and granuloma negative groups (Table 3). No statistically significant differences were shown in a multi-variant analysis which included all phenotype characterizations (data not shown).

4. Discussion

Epithelioid granulomas in the gastrointestinal tract are a highly specific, but relatively non-sensitive, histopathological
finding of Crohn’s disease.\textsuperscript{1} In our cohort of 307 Israeli patients with CD, 85 (27%) patients were found to have granulomas. This frequency is in concordance with some large studies\textsuperscript{8,12,17} but lower than the frequency reported in other studies.\textsuperscript{6,9} Even though the prevalence of granulomas was higher in our surgical specimens (33%), this does not fully account for the lower prevalence of granulomas in our cohort. One possible explanation is the dynamic nature of granulomas, as previously suggested, with a higher prevalence in untreated patients.\textsuperscript{6,13}

Regarding our primary endpoint, we did not find any association between granuloma formation in patients with CD and genetic variants in the autophagy related genes we investigated. This is in contrast to the previous finding of association between SNP in ATG4A and granuloma formation.\textsuperscript{41} Reasons for the lack of association could be a small cohort and a lack of statistical power, a heterogeneous ethnic population and a true insignificance of this specific SNP in the ATG4A gene in granuloma formation. To the best of our knowledge, our study is the first to investigate the

<table>
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<tr>
<th>Table 1</th>
<th>Phenotype characterization of granuloma positive as compared to granuloma negative CD patients.</th>
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<tbody>
<tr>
<td>Crohn’s disease, granuloma positive (n=85)</td>
<td>Crohn’s disease, granuloma negative (n=222)</td>
</tr>
<tr>
<td>Gender: male</td>
<td>52 (61%)</td>
</tr>
<tr>
<td>Age at diagnosis(year): mean</td>
<td>19.47 (±9.11)</td>
</tr>
</tbody>
</table>

| Ethnicity |  |
| Ashkenazi Jews | 41 (53.9%) | 100 (51.5%) | \(P=0.78\) |
| Sephardic Jews | 25 (32.9%) | 56 (28.9%) | \(P=0.55\) |
| Mixed Jews | 10 (13.2%) | 38 (19.6%) | \(P=0.28\) |
| Christian Arabs | 1 (20%) | 12 (55%) | \(P=0.32\) |
| Muslim Arabs | 4 (80%) | 6 (27%) | \(P=0.04\) |

| Family history of IBD |  |
| First degree relative | 14 (17.1%) | 34 (15.5%) | \(P=0.73\) |
| Secondary degree relative | 10 (12.2%) | 20 (9.1%) | \(P=0.52\) |
| No family history | 58 (70.7%) | 165 (75.3%) | \(P=0.46\) |

| Extra-intestinal manifestation | 50 (56%) | 122 (55%) | \(P=0.67\) |
| Surgery | 47 (55.3%) | 77 (34.8%) | \(P=0.002\) |

| Disease distribution |  |
| L1: Isolated terminal ileum | 21 (26.6%) | 79 (37.3%) | \(P=0.09\) |
| L2: Isolated colon | 12 (15.2%) | 35 (16.5%) | \(P=0.85\) |
| L3: Ileocolonic | 39 (49.4%) | 83 (39.2%) | \(P=0.14\) |
| L4: Upper gastrointestinal tract | 7 (8.9%) | 15 (7.1%) | \(P=0.62\) |

| Disease behavior |  |
| B1: Inflammatory | 40 (49.4%) | 121 (56.5%) | \(P=0.29\) |
| B2: Strictureing | 16 (19.8%) | 47 (22%) | \(P=0.75\) |
| B3: Penetrating | 25 (30.9%) | 46 (21.5%) | \(P=0.09\) |
| Peri-anal disease (yes) | 24 (29.6%) | 45 (21%) | \(P=0.12\) |

| Smoking status |  |
| Current smoker | 23 (28.4%) | 46 (21.4%) | \(P=0.22\) |
| Past smoker | 3 (3.7%) | 33 (15.3%) | \(P=0.0005\) |
| Never smoked | 55 (67.9%) | 136 (63.3%) | \(P=0.49\) |

<table>
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<tr>
<th>Table 2</th>
<th>NOD2/CARD15 status in patients with granuloma positive as compared to granuloma negative CD.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly908Arg</td>
<td>66 (77.6%)</td>
</tr>
<tr>
<td>Arg702Trp</td>
<td>80 (94.1%)</td>
</tr>
<tr>
<td>Leu1007fsinsC</td>
<td>74 (87.1%)</td>
</tr>
</tbody>
</table>
association between NCF4 and granuloma formation. Given the very appealing possible relationship between the NADPH system, autophagy, granuloma formation, and Crohn’s disease and the higher frequency (though not significant) of homozygotes for the minor allele (cytosine) of SNP in rs4821544 in the granuloma positive patients compared to the granuloma negative patients, we believe that further investigation into this aspect of the pathogenesis might be revealing. It is important to note that the association between the NCF4 variant and CD which was initially noted could not be reproduced in a subsequent European study.\textsuperscript{46} We also did not find any association between NOD2/CARD15 mutations and the presence of granulomas.

In our cohort, we found a significant association between the presence of granulomas and a younger age at diagnosis.\textsuperscript{47} This finding is consistently replicated in most large studies of granuloma positive patients,\textsuperscript{7–9} and strengthens the hypothesis of genetic influence on granuloma formation. We also found that patients with granuloma positive CD were more likely to undergo surgery of the gastrointestinal tract, supporting the view that granulomas predict a more aggressive disease.\textsuperscript{11,48} Limitations in our current investigation include the lack of a perspective follow-up and timeframe from diagnosis to surgery.

Patients with granuloma positive CD were less likely to be past, but not current, smokers. Leong et al. also found that current or previous smoking reduces the risk of granuloma formation.\textsuperscript{14} Nicotine has been shown to have immunomodulating effects on mucosal inflammation, though we would have expected to find a lower occurrence in current smokers as well.

In summary, we believe our study uniquely adds to the small yet growing body of information connecting phenotype and genotype in CD. In our cohort there was no association between granuloma formation and newly discovered SNPs in autophagy genes.

**Statement of authorship**

Y.M. recruited patients, helped in all laboratory work, collected archive information and histologic reports, wrote and edited the manuscript.

A.K. conceived of the study, recruited patients and serum collection, planned the PCR process and helped in the editing of the final manuscript.

S.N. was in charge of and carried out all aspects of the laboratory investigation.

B.W. recruited patients, including demographic details and serum collection.

E.L. recruited patients, including demographic details and serum collection.

R.E. and A.L. recruited patients and reviewed and edited final manuscript.

All authors read and approved the final manuscript.

**Acknowledgement**

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No source of funding was made for this study.

There were no study sponsors.

**References**


**Table 3** Allele frequency of SNPs rs4821544 and rs807185 in patients with granuloma positive as compared to granuloma negative CD.

<table>
<thead>
<tr>
<th>SNP</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Frequency for the allele in minority</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4821544</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease, granuloma positive</td>
<td>21 (24.7%)</td>
<td>36 (42.4%)</td>
<td>28 (32.9%)</td>
<td>0.45</td>
<td>P=0.08</td>
</tr>
<tr>
<td>Crohn disease, granuloma negative</td>
<td>32 (14.4%)</td>
<td>104 (46.8%)</td>
<td>86 (38.7%)</td>
<td>0.378</td>
<td></td>
</tr>
<tr>
<td>rs807185</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease, granuloma positive</td>
<td>42 (50%)</td>
<td>17 (20.2%)</td>
<td>25 (29.8%)</td>
<td>0.398</td>
<td>P=0.10</td>
</tr>
<tr>
<td>Crohn’s disease, granuloma negative</td>
<td>124 (56.6%)</td>
<td>47 (21.5%)</td>
<td>48 (21.9%)</td>
<td>0.326</td>
<td></td>
</tr>
</tbody>
</table>

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