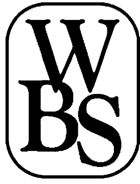

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Alimentary Tract

Genetic and serological markers to identify phenotypic subgroups in a Dutch Crohn's disease population

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Abstract

Background and aims. Both genetic and microbial factors seem to play a pivotal role in the aetiopathogenesis of Crohn's disease. The *CARD15* frameshift mutation might link host genetic factors and the indigenous microbial flora, since *CARD15* expression is stimulated by peptidoglycan, thereby activating NF-κB. It is hypothesised that *CARD15* mutation carriers have defective anti-microbial reactions, resulting in more penetrating lesions and antibody responses, which are now being used as highly specific markers for Crohn's disease. The serological marker anti-*Saccharomyces cerevisiae* antibody directed against cell wall oligomannosidic epitopes has high specificity for Crohn's disease. Perinuclear anti-neutrophil cytoplasmic antibodies have been found in a subgroup of Crohn's disease patients, mostly with colonic involvement.

Methods. We investigated the incidence of two *CARD15* mutations (3020insC and 2722G > C), anti-*S. cerevisiae* antibody, and perinuclear anti-neutrophil cytoplasmic antibody in 108 (73F/35M) patients with Crohn's disease with a mean duration of disease since diagnosis of 16 (1–41) years in relation to their phenotype, according to the Vienna classification.

Results. The prevalence of *CARD15* frameshift mutation was 21%. Of all patients, 62% were anti-*S. cerevisiae* antibody positive, and 9% had perinuclear anti-neutrophil cytoplasmic antibodies. The prevalence of both anti-*S. cerevisiae* antibodies and perinuclear anti-neutrophil cytoplasmic antibodies was higher in the mutation carriers compared to non-carriers. Remarkably, all patients with a *CARD15* mutation and positive anti-*S. cerevisiae* antibody had ileal disease. Carriership of the mutation was significantly associated with penetrating behaviour of the disease and weakly associated with stricturing behaviour. Furthermore, anti-*S. cerevisiae* antibody was associated with ileal disease involvement. Finally, most perinuclear anti-neutrophil cytoplasmic antibody positive patients showed ulcerative-like behaviour of disease (by means of colonic localisation).

Conclusions. Genetic and serologic markers might be useful in defining patient subgroups. This may result in a more accurate prediction of disease behaviour, prognosis and therapeutic approach.
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Keywords: ASCA; *CARD15*; Crohn's disease; Vienna classification

1. Introduction

The aetiology of Crohn's disease (CD), a non-specific chronic transmural inflammatory disease of the gastrointestinal tract, is still unknown. It is suggested that a genetic predisposition leads to an unregulated intestinal immune response to environmental factors.

Linkage analyses identified a susceptibility locus for CD (called IBD1) at the pericentromeric region of chromosome 16 [1]. Recently, an insertion mutation at nucleotide 3020 (3020insC) in exon 11 of the *CARD15* gene, originally reported as NOD2, was shown to be strongly associated with CD [2–4]. The 3020insC frameshift mutation is found in approximately 15% of the patients with CD in The Netherlands and in 2% of the general population [5]. The mutation is thought to result in an altered monocytic response to bacterial components by regulating apoptosis and/or NF-κB activation [6]. NOD2 has been shown to mediate

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responsiveness to preparations of lipopolysaccharide (LPS) and peptidoglycan (PGN). Recent analysis identified muramyl dipeptide derived from PGN as the bacterial structure recognised by NOD2. However, the mutant NOD2 response to PGN from a variety of Gram positive and Gram negative bacteria was significantly reduced compared with wild type patients, which confirms a functional consequence of the polymorphism and also the other two common variants (2104C > T in exon 4 (Arg702Trp) and 2722G > C in exon 8 (Gly908Arg) mutations) [3,7–9].

The role of bacteria in the pathogenesis of CD has been the subject of remarkable speculation and debate since the characterisation of this disorder. Although early studies focusing on specific mycobacteria and viral agents yielded conflicting results, the search for pathogenic organisms has more recently been supplanted by the hypothesis that abnormal mucosal immune responses to normal intestinal flora underlie the intestinal inflammation seen in both patients and animal models of colitis [10]. The identification of NOD2 as a susceptibility gene for CD offers additional support for this hypothesis by providing a demonstrable link between enteric bacteria and the innate immune system.

Antibodies to baker's yeast and brewer's yeast (anti-*Saccharomyces cerevisiae* antibodies, ASCA) have been described in up to 65% of patients with CD [11–15]. Recently, a higher occurence of ASCA in familial cases of CD was found [16,17]. Furthermore, ASCA was present in unaffected relatives as well, which supports the hypothesis of a relationship with a genetic predisposition. It has been demonstrated that the specific antigen is a mannan localised in the yeast cell wall [18]. It has been suggested that mannans are the major antigenic component of yeast cell walls and are also an important antigenic constituent of mycobacteria and other micro-organisms [12,19]. Both the origin as well as the clinical significance of ASCA in CD is uncertain. One hypothesis links the aetiology to increased intestinal permeability. Due to a presumed break of the epithelial barrier, increased exposure of the epithelium to common food antigens such as yeasts may result in an exaggerated antibody response, although an earlier study showed no association with ASCA and increased intestinal barrier [20]. The incidence of perinuclear anti-neutrophil cytoplasmic antibodies (pANCAs) in CD patients is low (10–15%) [21], but when present, it has been associated with ulcerative colitis-like behaviour [22]. It is hypothesised that pANCAs are due to cross-reactivity with bacterial antigens [23].

It has been suggested that CD patients with the *CARD15* mutation have an abnormal response to the indigenous bacterial flora [24]. Therefore, we hypothesise that this defect may result in an increased exposure to yeasts and bacterial antigens, eventually leading to the production of ASCA and pANCA. There are many variations in clinical presentation within the CD population and effort has been made to subdivide this heterogeneous group of patients in subgroups. The *CARD15* mutation has been described as a susceptibility factor for CD and only very recently some information was published in relation to clinical characteristics [5,25–29]. Very few data are available studying combined genetic, serological and clinical characteristics in CD patients [30].

We studied the presence of the *CARD15* mutation, ASCA and pANCA in relation to the Vienna classification in a group of Dutch CD patients with a long follow up.

2. Materials and methods

2.1. Study population

Participants of this study were recruited from the Outpatient Clinic of the Department of Gastroenterology of the VU University Medical Center, Amsterdam, The Netherlands. The study group comprised 108 Dutch subjects with CD. Patients' characteristics are shown in Table 1. Diagnosis of the disease was based on clinical, histopathologic and endoscopic findings according to the classification of Lennard-Jones [31]. Patients were categorised using the Vienna classification [32], which subdivides patients with CD according to age of onset (below 40 years or from 40 years on), localisation of disease (terminal ileum, colon, ileocolonic, or upper gastrointestinal tract) and disease behaviour (penetrating, stricturing, non-penetrating/non-stricturing) in the disease period from diagnosis to the first surgical procedure. The local ethical committee approved the study; all the patients gave written informed consent.

Table 1
Patients' characteristics and clinical data ($n = 108$)

Sex, females	73 (68%)
Age (mean (range))	41 (19–80)
Duration of disease (means(range))	16 (1–41)
Prior surgery	74 (69%)
ASCA	67 (62%)
pANCA	10 (9%)
CARD15 genotype	
3020incC and 2722G > C mutation	
Non-carriers	85 (79%)
Heterozygote	20 (18%)
Homozygote	3 (3%)
Vienna classification of patients	
Age	
<40	93 (86%)
>40	15 (14%)
Location	
Ileal	35 (32%)
Colonic	23 (21%)
Ileocolonic	49 (46%)
Upper GI	1 (1%)
Behaviour	
NSNP	38 (35%)
Stricturing	48 (45%)
Penetrating	22 (20%)

NSNP: non-stricturing non-penetrating.

2.2. CARD15 gene polymorphism typing

Blood was collected in EDTA-tubes and stored at room temperature until the genomic DNA was extracted from peripheral blood leukocytes according to a conventional proteinase K digestion and phenol/chloroform procedure. The 3020insC frameshift mutation in exon 11 and the 2722G > C mutation in exon 8 of the *CARD15* gene were detected with allele specific multiplex polymerase chain reaction (PCR) according to Ogura et al. [3].

2.3. ASCA ELISA

ASCA IgA and IgG were evaluated in commercially available ELISA kits, kindly provided by Inova Diagnostics, San Diego, CA [33]. The antigen consisted of phosphopeptidomannan (PPM) extracted from *S. cerevisiae*. ASCA ELISAs were performed according to the manufacturer's instructions. Results were expressed as arbitrary units with a cut off for positivity of 25 U/ml. Sera were considered positive if either IgA or IgG or both were positive. Sera were considered negative if both IgA and IgG ASCA were negative.

2.4. ANCA indirect immunofluorescence assay

The ANCA indirect immunofluorescence assay was performed semi-quantitatively by a standard Indirect Immunofluorescence Technique on ethanol fixed human leukocytes according to the First International Workshop on ANCA [34]. In short, human peripheral blood neutrophils were smeared on 12-well Nutacon slides and fixed in 96% ethanol (10 min at −20 °C). Slides were incubated with 1:20 diluted patient's serum (in phosphate buffered saline) and stained with rabbit anti-human IgG-FITC conjugate (Dako A/S, Denmark). The slides were evaluated by fluorescence microscopy. All sera were tested in duplicate and scored by two well-trained observers who were unaware of the patient's diagnosis. Depending on the brightness of immunofluorescence staining pattern, the reactions were graded into negative (−), weak positive (+), positive (++) and strong positive (+++). We only report positive versus negative results since grading of the staining has proved to be of no extra value in clinical studies on IBD. ANA positive sera were earlier excluded from the study.

2.5. Statistical analysis

χ^2 statistics and Fisher's exact test were used to compare the test frequencies. Patients who had at least one copy of the mutated allele were defined as carriers for a *CARD15* mutation. Patients who did not have any mutation were defined as reference. Binary logistic and multinominal logistic regression analysis was applied to fit statistical models to estimate the relative risk (RR) and corresponding 95% confidence interval (95% CI). The statistical models were adjusted for the age and gender of the subjects. The models that were fitted for the disease behaviour were also adjusted for the location of involvement in the intestine. A two-tailed *P* value of less than 0.05 was considered statistically significant. Statistical analysis was performed using Stata Version 7.0 for windows.

3. Results

In our group of 108 patients, we found 14 patients with the *CARD15* frameshift mutation, of whom 11 were heterozygous (10.2%) and 3 were homozygous (2.8%). Furthermore, nine other patients were carrier of the 2722 mutation (all heterozygous). Of these 108 patients, 53 were ASCA IgA positive, 64 were ASCA IgG positive and 67 were ASCA IgA and/or IgG positive (62%), whereas 48 were both ASCA IgA and IgG positive. Absolute values for ASCA varied between 2 and >300. We found pANCA antibodies in 10 patients (9%). The prevalence of ASCA and pANCA in patients with the mutation was significantly higher (16/23) as compared to the non-carriers (52/85). Of the 11 patients with heterozygous *CARD15* frameshift mutation, 8 had ASCA antibodies (IgA and/or IgG), whereas 2 out of 3 homozygous patients had ASCA antibodies. pANCA antibodies were found in six non-carrier patients and in four patients with the *CARD15* frameshift mutation (two homozygous, two heterozygous). Seven out of 10 patients with pANCA antibodies had colonic involvement of CD.

The relation between *CARD15* mutations, ASCA and Vienna classification is shown in Table 2. ASCA was significantly associated with ileal disease involvement (RR: 7.12; $P < 0.001$; 95% CI: 2.04–20.13). Moreover, carriership of the mutation seemed related with stricturing behaviour of the disease (RR: 3.88; 95% CI: 0.98–15.6) and ileal localisation of the disease (RR: 2.07; 95% CI: 0.47–9.08), although not statistically significant.

The relation of *CARD15* mutation and ASCA and the location of the disease are presented in Table 3. We found that nearly all patients with the *CARD15* frameshift mutation (15/16) and positive ASCA had ileal disease. Also non-carriers of the mutation with ASCA antibodies (34%) had ileal involvement (RR: 6.90; 95% CI: 1.73–27.3, $P < 0.001$). These patients also had a significant higher risk of ileocolonic involvement (RR: 6.90; 95% CI: 1.92–24.8, $P < 0.001$). Only one out of the 16 patients with a *CARD15* mutation and ASCA antibodies had colonic or upper gastrointestinal involvement.

Furthermore, we did not find decreased ASCA titers toward normal values in postoperative patients. Neither *CARD15* mutation nor ASCA could discriminate patients who underwent surgery from those with a more favourable clinical outcome (data not shown). To interpret these results, we estimated the incidence of CD in The Netherlands at approximately 6.9 per 100.000, whereas the prevalence was estimated at 140 per 100,000 inhabitants [35].

Table 2
Relation between ASCA, pANCA and *CARD15* with the Vienna classification

Vienna classification	ASCA			pANCA			*CARD15* 3020 and 2722 mutations		
	Positive ($n = 67$)	Negative ($n = 41$)	RR (95% CI)	Positive ($n = 10$)	Negative ($n = 98$)	RR (95% CI)	Carriers	Non-carriers	RR (95%CI)
Age									
<40	58 (87)	35 (85)	Reference	7 (70)	86 (88)	Reference	19 (83)	73 (87)	Reference
>40	9 (13)	6 (15)	0.87 (0.28–2.68)	3 (30)	12 (12)	2.87 (0.64–12.7)	4 (17)	12 (13)	1.35 (0.38–4.77)
Location									
Ileal	24 (36)	11 (27)	7.12 (2.04–20.1)[a]	3 (30)	32 (33)	1.86 (0.15–23.2)	8 (35)	27 (32)	2.07 (0.47–9.08)
Colonic	7 (11)	16 (39)	Reference	1 (10)	22 (22)	Reference	3 (13)	20 (24)	Reference
Ileocolonic	35 (52)	14 (34)	6.40 (2.03–20.1)[a]	6 (60)	43 (44)	2.14 (0.22–21.0)	12 (52)	37 (43)	2.11 (0.52–8.58)
Upper GI	1 (1)	0	–	0	1 (1)	–	0	1 (1)	–
Behaviour									
NSNP	21 (32)	17 (41)	Reference	5 (50)	33 (34)	Reference	4 (17)	35 (40)	Reference
Stricturing	33 (49)	15 (37)	1.73 (0.66–4.56)	4 (40)	44 (45)	0.87 (0.16–4.65)	13 (57)	35 (42)	3.88 (0.98–15.6)
Penetrating	13 (19)	9 (22)	1.28 (0.41–4.04)	1 (10)	21 (21)	0.63 (0.05–6.79)	6 (26)	15 (18)	4.94 (1.05–23.3)[b]

RR: relative risk adjusted for age at presentation and gender (for disease behaviour also adjusted for location of the disease); figures in parenthesis are percentages.
[a] $P < 0.001$.
[b] $P < 0.05$.

4. Discussion

In this study, the prevalence of two *CARD15* mutations, ASCA and pANCA were determined in a group of Dutch patients with CD. The frequency of these mutations was 21%, whereas the prevalence of ASCA and pANCA in our patient group were 62 and 9%, respectively, as has been described in the literature. We searched for a relation between both genetic and serological markers, and tried to link them with clinical features of disease.

We could confirm the relationship between the *CARD15* mutation and ileal localisation of the disease, consistent with other studies [25–27,30], although not statistically significant. The association of the *CARD15* mutation with ileal disease suggests that the *CARD15* genotype has an effect on the clinical presentation of CD. We found that carriers of the *CARD15* mutation had a higher risk for stricturing behaviour of disease, but this did not reach significance. However, carriership was significantly related with penetrating behaviour of the disease. In a recent report, we failed to demonstrate significant associations between the *CARD15* frameshift mutation and the major subgroups of the Vienna classification almost certainly due to the sample size, because the same trend was present [5]. Recently, Abreu et al. [36] described an association between the presence of NOD2 mutations and small bowel stricturing CD. We could confirm the association of ASCA and ileal localisation of the disease [37]. Furthermore, we found the incidence of ASCA to be higher in patients with stricturing and penetrating behaviour of the disease, although not significant. Others reported that higher ASCA levels were associated with early age of disease onset as well as both fibrostenosing and internal penetrating disease behaviours [19]. In contrast with an earlier report [38], we did not find decreased ASCA titers toward normal values in postoperative patients. The relation of ASCA and ileal disease has been described frequently in the literature [39], but to our knowledge except for the very recent publication by Abreu et al. [36], no reports have

Table 3
Relation between ASCA and *CARD15* polymorphism with Vienna classification

CARD15 3020 carriership	ASCA IgG or IgA	n	Location				
			Colonic	Ileal	RR (95%CI)	Ileocolonic	RR (95% CI)
−	−	37	15 (41%)	9 (24%)	Reference	13 (35%)	Reference
−	+	57	7 (12%)	23 (40%)	7.45 (2.03–27.4)*	27 (47%)	4.89* (1.50–15.9)
+	−	4	1 (25%)	2 (50%)	3.08 (0.22–42.2)*	1 (25%)	0.95 (0.05–17.2)
+	+	10	0	2 (20%)	-	8 (80%)	–
CARD15 3020 or 2722 carriership							
−	−	33	14 (43%)	9 (27%)	Reference	10 (30%)	Reference
−	+	52	8 (16%)	18 (34%)	6.90 (1.73–27.3) *	26 (50%)	6.90 (1.92–24.8)*
+	−	7	2 (29%)	2 (29%)	1.48 (0.16–13.6)	3 (42%)	1.81 (0.24–13.4)*
+	+	16	1 (6%)	6 (38%)	14.41 (1.34–154)*	9 (56%)	13.90 (1.42–136)*

RR: relative risk adjusted for age at presentation and gender.
* $P < 0.001$.

been published studying both genetic and serological markers in association with clinical phenotypes. Interestingly, all patients with the combination of carriership of the mutation and presence of ASCA had ileal involvement of the disease. Moreover, in patients with the mutation, the incidence of ASCA and pANCA were significantly higher compared with non-carriers. This could support our hypothesis that *CARD15* mutation carriers have defective anti-microbial reactions, resulting in antibody responses, such as ASCA and pANCA. Neither *CARD15* mutations nor ASCA could discriminate patients who underwent surgery from those with a more favourable clinical outcome. Finally, pANCA positivity was associated with ulcerative-like behaviour in CD patients as has been previously reported [19]. The reported associations of *CARD15* mutation and ASCA with ileal disease and pANCA with colonic involvement also accords with the hypothetical role of the intestinal flora in the pathogenesis of CD.

Although there is an increasing evidence that IBD results from the combined effects of environmental agents and host genetic factors, there is still a long way to define other genes and the environmental triggers that initiate the disease. A central role for specific immune responses to discrete antigens has been presumed, and circumstantial evidence in humans and direct studies of mutant murine models implicate luminal bacteria as necessary cofactors for initiating and perpetuating inflammation. Bacteria contain toxic compounds, which are potent stimuli of innate immune responses, as exemplified by bacterial cell wall components. However, the mechanistic basis of the interaction between the luminal flora and the intestinal flora remains to be fully defined. Possibly, bacterial products penetrate the epithelial barrier, either due to damage or via paracellular pathways to directly stimulate the underlying constituents of the mucosal immune system. Alternatively, products may interact at the apical surface and induce responses in the intestinal epithelial cell, which in turn produces cytokines, chemokines and other mediators inducing inflammatory activation of the mucosal immune system [40]. The surface epithelium serves a critical function as the defensive front line of the mucosal innate immune system in the gastrointestinal tract. Intestinal epithelial cell lines constitutively express several functional Toll-like receptors (TRLs) which appear to be key regulators of the innate response system [41]. Individual TLRs activate specialised anti-fungal or anti-bacterial genes through the activation of NF-κB family members. Yeasts for instance activate TLR2 [42–45]. Both Hugot and Ogura have emphasised the possibility of a functional connection between the leucine rich regions (LRR) in the NOD2 protein and the TLRs [46]. These TLRs themselves do not seem to always respond directly to micro-organisms but instead recognise conserved molecular patterns (LPS, LTA, mannans and glycans) [47].

The relation among genetic and serological markers of CD and their possible link with clinical features of disease are interesting, but further genotype, serology and clinical phenotype studies are warranted to clarify the findings in our study.

Conflict of interest statement

None declared.

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