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BRIEF ARTICLE

NOD2/CARD15, *ATG16L1* and *IL23R* gene polymorphisms and childhood-onset of Crohn's disease

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Abstract

AIM: To assess whether the polymorphisms of *NOD2/CARD15*, autophagy-related 16-like 1 (*ATG16L1*), and interleukin-23 receptor (*IL23R*) genes play a more critical role in the susceptibility of childhood-onset than in adult-onset Crohn's disease (CD).

METHODS: Polymorphisms R702W, G908R, and 3020insC of *NOD2/CARD15*; rs2241880 A/G of *ATG16L1*, and rs11209026 (R381Q) of *IL23R* gene were assessed in 110 childhood-onset CD, 364 adult-onset CD, and 539 healthy individuals. Analysis of polymorphisms R702W, G908R, and 3020insC of *NOD2/CARD15* genotyping was performed by allele specific polymerase chain reaction (PCR) or by PCR-restriction fragment length polymorphism analysis. The polymorphisms rs2241880 A/G of the *ATG16L1*, and rs11209026 (R381Q) of the *IL23R* gene in the children's cohort were genotyped by PCR and melting curve analysis whereas adult group genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 5.0 (500K).

RESULTS: The 3020insC allele in *NOD2/CARD15* was significantly higher in childhood than in adult-onset CD (P = 0.0067). Association with at least 1 *NOD2/CARD15* variant was specific for ileal disease (with or without colonic involvement). Even if the frequency of G allele of the rs2241880 *ATG16L1* polymorphism was increased in both paediatric and adult CD patients compared to controls (P = 0.017 and P = 0.001, respectively), no difference was observed between the childhood and the adult cohort. The rare Q allele of *IL23R* rs11209026 polymorphism was underrepresented in both paediatric and adult CD cases (P = 0.0018 and P = 0.04, respectively) and no difference was observed between the childhood and the adult cohort. The presence of the rs2241880 *ATG16L1* and rs11209026 *IL23R* polymorphisms did not influence disease phenotype.

CONCLUSION: Polymorphism 3020insC in *NOD2/CARD15* occurs statistically significantly more often in patients with childhood-onset CD than in patients with adult-onset CD. The *ATG16L1* and *IL23R* variants are associated with susceptibility to CD, but not early-onset disease.

Key words: Genetics; Childhood-onset; Inflammatory bowel disease; Crohn's disease; Genetic susceptibility; *NOD2/CARD15*; *ATG16L1*; *IL23R*; Polymorphisms

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INTRODUCTION

Inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are characterized by chronic relapsing inflammation of the digestive tract. As a multifactorial disorder, IBD is caused by a complex interaction of genetic, microbial, and immunological factors. Approximately 10%-20% of all IBD will present either in childhood or adolescence[1].

Although age of onset seems to be a random event, recent data have shown that a subgroup of patients with early-onset IBD may have specific phenotypes that differs from adult onset IBD, suggesting that the pathogenesis of pediatric IBD and adult IBD may differ[2-4]. A compelling speculation is that pediatric-onset IBD is more likely to be influenced by genetics compared to late onset, as there is less time for environmental modifiers to influence the onset of the disease. Adult-onset IBD is more likely to be confounded by abundant environmental exposure compared to childhood-onset IBD populations[5].

Genetic risk factors for IBD have been extensively studied during the last year. *NOD2/CARD15* polymorphisms R702W, G908R, and 3020insC are independently associated with an increased risk of developing CD[6-8]. Existing data remain conflicting as to whether *NOD2/CARD15* polymorphisms are associated with the age of onset of IBD, with some studies showing an effect toward a younger age of onset[9,10], and others showing no effect[11,12].

Recently, genome wide association studies (GWAS), in addition to offering further confirmation of the importance of the *NOD2/CARD15* gene, provided evidence for several determinants, including genes encoding autophagy-related 16-like 1 (*ATG16L1*) and interleukin-23 receptor (*IL23R*)[13]. Moreover, Kugathasan *et al*[14], by employing GWAS in a cohort of individuals with pediatric-onset IBD, provide further insights into disease pathogenesis.

Hampe *et al*[15] were the first group to implicate the autophagy pathway in CD. The association of the Ala-197Thr (rs2241880 A/G) variant of the *ATG16L1* gene with susceptibility to CD, has now been replicated in several independent cohorts[16,17]. Recent studies have explored genotype associations in adult-onset IBD, and to a more limited extent in pediatric disease. Prescott *et al*[18] suggested an association between the Ala197Thr variant allele and early-onset CD, as well as an effect of *ATG16L1* genotype on age at diagnosis. Baldassano *et al*[19] replicated this association in a pediatric cohort. In contrast, Van Limbergen *et al*[20] reported that the *ATG16L1* variant is not associated with early-onset IBD in a pediatric population in Scotland.

The *IL23R* gene is located on chromosome 1p31 and its corresponding ligand IL23 is a key component of the immunoregulatory pathway. The identification of an association between the R381Q variant of *IL23R* and CD is thus an important step toward the delineation of pathways related to inflammation to chronic inflammatory cascade characteristics of CD. Recent studies suggest that the R381Q variant in *IL23R* is associated with pediatric-onset CD[21,22].

In view of these discrepant data regarding the association of key regulatory genes with CD susceptibility, the purpose of our study was investigate whether the known DNA polymorphisms in the *NOD2/CARD15*, *ATG16L1*, and *IL23R* genes determine susceptibility for CD in Greek children, and to compare these data with the frequency of these gene polymorphisms in adult-onset CD.

MATERIALS AND METHODS

Patients and controls

We examined 110 Greek children with CD, diagnosed before the age of 17, who attended the First Department of Pediatrics of Athens University, "Aghia Sophia" Children's Hospital between January 2007 and December 2008. The diagnosis of CD was based on standard clinical, endoscopic, radiologic and histopathologic criteria[23]. Cases of UC and indeterminate colitis were excluded. Also excluded were children who had concomitant immune-mediated diseases such as asthma, diabetes type 1, juvenile diabetes, or juvenile arthritis. Blood samples from 364 adult CD patients were collected at the Inflammatory Bowel Disease (IBD) Outpatient Clinic of the Evagelismos Hospital. Most of them had already been used for genotype studies on *NOD2/CARD15*[24]. The main clinical characteristics of IBD patients are detailed in Table 1. This cohort was compared to 539 healthy controls (94 children and 445 adults). Before commencement of the study, the Ethics Committee at the participating centers approved the recruitment protocols. All participants were informed of the study.

Genotyping

DNA was isolated from blood with the NucleoSpin blood kit (Macherey-Nagel, Germany). Patients were genotyped for the 3 common *NOD2/CARD15* polymorphisms i.e. R702W, G908R, and 3020insC using previously described methods[8,24]. Polymorphisms rs2241880 A/G of the *ATG16L1*, and rs11209026 (R381Q) of the *IL23R* gene in the children's cohort were genotyped by PCR and melting curve analysis, using a pair of fluorescence resonance energy transfer (FRET) probes in LightCycler® 2.0 Instrument (Roche Diagnostics, Manheim, Germany) as previously described[25,26]. The adult group genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 5.0 (500K)[27].

Statistical analysis

The sample size and the power of the present sample size

Table 1 Demographics and clinical features of patients with CD (mean ± SD) *n* (%)

	Childhood-onset CD (*n* = 110)	Adult-onset CD (*n* = 364)
Sex (male/female)	60/50	178/186
Age of diagnosis (yr)	11.5 ± 4.8	28.99 ± 14.22
Family history in first-degree relative	8 (7.27)	12 (3.29)
Smoking habit		
Never		146 (40.11)
Ex-smoker		32 (8.79)
Current		186 (51.10)
Localization of disease		
Ileitis	21 (19.09)	121 (33.24)
Colitis	29 (26.36)	38 (10.50)
Ileocolitis	48 (43.64)	187 (51.38)
Upper gastroenteric	12 (10.91)	18 (4.94)
Disease features		
Inflammatory	78 (70.91)	223 (61.26)
Stricturing	20 (18.18)	99 (27.20)
Fistulizing	12 (10.91)	42 (11.54)
Extraintestinal manifestations	21 (19.09)	58 (15.93)

CD: Crohn's disease.

(90%) were calculated using the http://sampsize.sourceforge.net/iface/s3.html#cc software. Statistical analysis was done using the software package GraphPad v. 3.00 (GraphPad Software, San Diego, CA). The Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotypes in 2 × 3 chi-square tables. All the samples were in Hardy-Weinberg equilibrium ($P > 0.1$). For comparison between categorical variables, the Fisher's exact test or χ^2 test was used where appropriate. Single marker allelic tests were performed with Fisher's exact test. Odds ratios (OR) were calculated for the minor allele at each polymorphisms.

RESULTS

Genotype and allele frequencies of the *NOD2/CARD15* polymorphisms R702W, G908R, and 3020insC are detailed in Table 2.

The R702W polymorphism frequency did not differ significantly between child- and adult-onset CD. However, a statistically significant association was found between the R702W polymorphism and adult-onset CD only. The T allele frequency, leading to heterozygous and homozygous R702W polymorphisms, was increased in the adult CD population (9.1%) compared to controls (5.47%) ($P = 0.004$). In pediatric-onset CD, T allele frequency was also increased (8.18%), but no statistical significant difference was observed when compared to controls.

The C allele frequency, encoding the G908R variant, was also found significantly elevated (10%) in adult patients ($P = 0.014$), but was slightly reduced (9.54%) in childhood CD patients and in controls (6.77%).

For the 3020insC polymorphic allele, a significantly increased prevalence was found both in paediatric (16.36%), and in adult (9.47%) CD patients as compared, with the controls ($P < 0.0001$ in both cases). The

Table 2 Genotype and allele frequencies of *NOD2/CARD15* polymorphisms in childhood-onset CD patients, adult-onset CD, and controls *n* (%)

	Childhood-onset CD (*n* = 110)	Adult-onset CD (*n* = 364)	Controls (*n* = 539)
R702W			
Genotype			
CC	94 (85.45)	300 (82.40)	482 (89.40)
CT	14 (12.72)	62 (17.00)	55 (10.20)
TT	2 (1.81)	2 (0.55)	2 (0.37)
P value[1]	NS		
P value[2]	NS		
P value[3]		0.0260	
T allele	18 (8.18)	66 (9.10)	59 (5.47)
P value[1], OR (95% CI)	NS		
P value[2], OR (95% CI)	NS		
P value[3], OR (95% CI)		0.0040, 1.72 (1.19-2.48)	
G908R			
Genotype			
GG	91 (82.73)	295 (81.00)	468 (86.83)
GC	17 (15.45)	65 (18.00)	69 (12.80)
CC	2 (1.81)	4 (1.00)	2 (0.37)
P value[1]	NS		
P value[2]	NS		
P value[3]		0.0420	
C allele	21 (9.54)	73 (10.00)	73 (6.77)
P value[1], OR (95% CI)	NS		
P value[2], OR (95% CI)	NS		
P value[3], OR (95% CI)		0.0140, 1.53 (1.09-2.15)	
3020insC			
Genotype			
-	78 (70.90)	301 (82.69)	503 (93.32)
insC/-	28 (25.45)	57 (15.66)	35 (6.49)
insC/insC	4 (3.64)	6 (1.65)	1 (0.18)
P value[1]	< 0.0001		
P value[2]	0.0200		
P value[3]		< 0.0001	
insC allele	36 (16.36)	69 (9.47)	37 (3.43)
P value[1], OR (95% CI)	< 0.0001, 5.5 (3.39-8.94)		
P value[2], OR (95% CI)	0.0067, 1.87 (1.21-2.88)		
P value[3], OR (95% CI)		< 0.0001, 2.95 (2.04-4.44)	

[1]Childhood-onset *vs* controls; [2]Childhood-onset *vs* adult onset; [3]Adult onset *vs* controls. NS: Not significant.

frequency of 3020insC polymorphism was significantly higher in the paediatric cohort that in the adult-onset cohort ($P = 0.0067$).

Concerning the genotype-phenotype correlation ileal involvement was more frequent in individuals with at least one *NOD2/CARD15* polymorphism (78.25%) than in wild-type carriers (59%), in both cases of child- and adult-onset CD (OR = 2.46, 95% CI: 1.33-4.57, $P = 0.006$). The examined variants did not influence CD behavior in the present study.

Concerning the rs2241880 A/G polymorphism of the *ATG16L1* gene, the frequency of the G allele was increased in both pediatric and adult CD patients compared to controls ($P = 0.017$ and $P = 0.001$, respectively) as shown in Table 3. No association of the *ATG16L1*

Table 3 Genotype and allele frequencies of *ATG16L1* polymorphism rs2241880 and *IL23R* polymorphism rs11209026 in childhood-onset CD patients, adult-onset CD, and controls *n* (%)

	Childhood-onset CD (*n* = 110)	Adult-onset CD (*n* = 364)	Controls (*n* = 539)
rs2241880			
Genotype			
AA	17 (15.45)	46 (12.64)	104 (19.30)
AG	45 (40.91)	177 (48.63)	274 (50.83)
GG	48 (43.64)	141 (38.74)	161 (29.90)
P value[1]	0.0190		
P value[2]	NS		
P value[3]		0.0040	
G allele	141 (64.09)	459 (63.05)	596 (55.29)
P value[1], OR (95% CI)	0.0170, 1.44 (1.07-1.95)		
P value[2], OR (95% CI)	NS		
P value[3], OR (95% CI)		0.0010, 1.38 (1.14-1.67)	
rs11209026			
Genotype			
RR	105 (95.45)	329 (90.38)	458 (84.97)
RQ	5 (4.54)	32 (8.79)	79 (14.66)
QQ	0	3 (0.82)	2 (0.37)
P value[1]	0.0120		
P value[2]	NS		
P value[3]		0.0220	
Q allele	5 (2.27)	38 (5.22)	83 (7.69)
P value[1], OR (95% CI)	0.0018, 0.28 (0.11-0.69)		
P value[2], OR (95% CI)	NS		
P value[3], OR (95% CI)		0.0400, 0.66 (0.44-0.98)	

[1]Childhood-onset *vs* controls; [2]Childhood-onset *vs* adult onset; [3]Adult onset *vs* controls.

polymorphism with early-onset CD was seen in our childhood-onset CD case-control analysis *vs* adult-onset CD analysis (Table 3). Furthermore, *ATG16L1* polymorphism did not influence the disease location and behaviour in the population studied.

The minor allele (Q) of the rs11209026 (R381Q) polymorphism of the *IL23R* gene was underrepresented in both childhood-onset and adult-onset CD, compared to controls ($P = 0.0018$ and $P = 0.04$, respectively) as shown in Table 3. No genotype-phenotype correlations were found among the CD patients studied with *IL23R* rs11209026 (R381Q) polymorphism.

DISCUSSION

Our present survey represents the first Greek study to document the frequency of the *NOD2/CARD15*, *ATG16L1*, and *IL23R* gene polymorphisms in childhood-onset CD, and compare them to those in an adult-onset CD cohort.

Our results confirm the previously reported association of *NOD2/CARD15* 3020insC mutation with early-onset CD[9,28,29]. In our study, only the *NOD2/CARD15* 3020insC mutation was strongly associated with childhood-CD susceptibility, and its frequency was significantly higher in the childhood cohort than in the adult-onset cohort, whereas in previous studies of early-onset CD patients, significantly higher carrier rates were found either for all the 3 *NOD2/CARD15* mutations[10] or for G908R and/or 3020insC only[12,30]. Others did not find any differences in the frequency of the 3 major *NOD2/CARD15* mutations between a childhood-onset and an adult-onset CD cohort[31]. Both ileitis and ileocolitis were more frequent in carriers of *NOD2/CARD15* polymorphisms, indicating an association of *NOD2/CARD15* polymorphisms with ileal involvement. This confirms previous findings in both pediatric and adult patients[8,10,12,30,32]. In contrast to other studies indicating an association between *NOD2/CARD15* polymorphisms and stricturing behaviour[12] we did not find any significant association between *NOD2/CARD15* polymorphisms and CD phenotype. These conflicting results can be explained by the regional and ethnic differences in genotypes, and the relatively small numbers of patients included in these studies.

Recent studies have reported *ATG16L1* rs2144880 variant genotype association with adult-pediatric onset CD. So far, reports in the literature have been conflicting. Specifically, Prescott *et al*[18] and Baldassano *et al*[19] demonstrated an association of this variant with diagnosis at an earlier age. Van Limbergen *et al*[20] and Latiano *et al*[31] have suggested that the *ATG16L1* rs2144880 variant is associated with susceptibility to adult CD in Scotland, but not to early-onset disease. In our study in the Greek population, we were able to demonstrate an effect of this *ATG16L1* polymorphism on both paediatric and adult CD susceptibility. However, the allele and genotype frequencies in childhood-onset CD were comparable to that seen in adults and therefore, we can not support an association of *ATG16L1* with early-onset CD in Greece. In agreement with previous studies, in the genotype-phenotype analysis, no association was detected in the cases tested[25,33].

Regarding the rs11209026 (R381Q) polymorphism of the *IL23R* gene, our study confirms the recently described associations between variants in the *IL23R* gene in both pediatric and adult-onset CD[19-21,31]. Recently Yamazaki *et al*[34] did not find any positive association of the *IL23R* gene polymorphism with CD in the Japanese population. Furthermore, in agreement with previous studies we did not observe any association of the rs11209026 (R381Q) polymorphism of the *IL23R* gene with the disease location and phenotype[33,35]. This finding can be attributed to the distinct ethnic difference of genetic backgrounds of CD that has been reported previously for other genes between Japanese and Caucasian populations. It should be noted that the different results in allele frequencies between the studies can be explained by large regional and ethnic differences in genotypes, by the broad spectrum of clinical phenotypes of patients with CD, and by the relatively small numbers of cases included in most studies.

In conclusion, this study demonstrates that the 3020insC mutation in *NOD2/CARD15* gene is associated with CD in a Greek childhood-onset CD cohort. Moreover, the 3020insC mutation occurred significantly more often in childhood onset patients with CD than in

adult-onset CD patients. Our results provide an independent confirmation of the association of the *ATG16L1* rs2144880 and the *IL23R* rs11209026 (R381Q) polymorphisms with susceptibility to CD without supporting their implication in early-onset disease. Therefore, further studies are needed to specifically identify gene variants that predispose children to early paediatric onset disease.

COMMENTS

Background

As a multifactorial disorder, inflammatory bowel disease (IBD) is caused by a complex interaction of genetic, microbial, and immunological factors. Approximately 10%-20% of all IBD will present either in childhood or adolescence. Recent data have shown that a subgroup of patients with early-onset IBD may have specific phenotypes that differ from adult onset IBD, suggesting that the pathogenesis of paediatric IBD and adult IBD may differ. The study assesses whether the polymorphisms of *NOD2/CARD15*, autophagy-related 16-like 1 (*ATG16L1*), and interleukin-23 receptor (*IL23R*) genes play a more critical role in the susceptibility of childhood-onset than adult-onset Crohn's disease (CD).

Research frontiers

Although several gene loci have been associated with susceptibility to CD in adults, the aetiology of childhood CD is still unknown. The current study is one of the first studies assessing the impact of candidate gene's polymorphisms and disease susceptibility in childhood CD in a Greek cohort.

Innovations and breakthroughs

It is important to investigate the genetic variation in susceptibility to CD and identify markers that will facilitate identification of individuals at risk of developing this disease. The results suggest that the polymorphism 3020insC in *NOD2/CARD15* occurs statistically significantly more often in patients with childhood-onset CD than in patients with adult-onset CD. The *ATG16L1* and *IL23R* variants are associated with susceptibility to CD, but not early-onset disease.

Applications

The results of this study will help us to further understand the genetic determinants of childhood CD.

Peer review

The present study demonstrates that the 3020insC mutation in *NOD2/CARD15* gene is associated with CD in a Greek childhood-onset CD cohort. Moreover, the 3020insC mutation occurred significantly more often in childhood onset patients with CD than in adult-onset CD patients.

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