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CLINICAL RESEARCH

Role of CARD15, DLG5 and OCTN genes polymorphisms in children with inflammatory bowel diseases

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Abstract

AIM: To investigate the contribution of variants of CARD15, OCTN1/2 and DLG5 genes in disease predisposition and phenotypes in a large Italian cohort of pediatric patients with inflammatory bowel diseases (IBD).

METHODS: Two hundred patients with Crohn's disease (CD), 186 ulcerative colitis (UC) patients, 434 parents (217 trios), and 347 healthy controls (HC) were studied. Polymorphisms of the three major variants of CARD15, 1672C/T and -207G/C SNPs for OCTN genes, IGR2096a_1 and IGR2198a_1 SNPs for the IBD5 locus, and 113G/A variant of the DLG5 gene were evaluated. Potential correlations with clinical sub-phenotypes were investigated.

RESULTS: Polymorphisms of CARD15 were significantly associated with CD, and at least one variant was found in 38% of patients (15% in HC, OR = 2.7, $P < 0.001$). Homozygosis for both OCTN1/2 variants was more common in CD patients (1672TT 24%, -207CC 29%) than in HC (16% and 21%, respectively; $P = 0.03$), with an increased frequency of the TC haplotype (44.8% *vs* 38.3% in HC, $P = 0.04$). No association with the DLG5 variant was found. CD carriers of OCTN1/2 and DLG5 variants more frequently had penetrating disease ($P = 0.04$ and $P = 0.01$), while carriers of CARD15 more frequently had ileal localization ($P = 0.03$). No gene-gene interaction was found. In UC patients, the TC haplotype was more frequent (45.4%, $P = 0.03$), but no genotype/phenotype correlation was observed.

CONCLUSION: Polymorphisms of CARD15 and OCTN genes, but not DLG5 are associated with pediatric onset of CD. Polymorphisms of CARD15, OCTN, and DLG5 genes exert a weak influence on CD phenotype.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; CARD15; DLG5; Carnitine/organic cation transporter gene

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INTRODUCTION

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) have become increasingly common causes of morbidity in children[1,2]. Their prevalence has been on rise with children and adolescent currently accounting for approximately 30% of IBD patients[3,4]. Although the precise etiology of IBD remains elusive, both animal models and human studies point towards a strong genetic susceptibility.

The characterization of the NOD2/CARD15 gene at the IBD1 locus (16q12) in 2001 as the first gene conferring susceptibility to CD represented a milestone observation[5-7]. Although the role of NOD2 protein in CD remains under evaluation[8], it is surely involved in the innate immune response to bacterial pathogens[9,10]. CARD15 major variants are mostly associated with ileal disease and stricturing behaviour[7]. In pediatric series, a correlation with ileal localization[11-14], stricturing behaviour[12,13,15] early surgery[12,13] growth delay[13,14], and higher disease activity[13,16] has been reported, although with conflicting findings[17-19].

Functional polymorphisms in the carnitine organic cation transporter cluster (OCTN1/2)[20] on chromosome 5q31 and mutations in disc large gene 5 (DLG5)[21] on

the long arm of chromosome 10 (10q23) have also been reported to be associated with CD. Linkage on the 5q31 genomic area, the so-called IBD5 risk haplotype, was first reported by Rioux *et al*[22]. This 250-kilobase region contains 5 genes and multiple genetic variants with strong linkage disequilibrium[22]. A number of studies have confirmed the association of the IBD5 risk haplotype with CD[23-28] and UC[28,29]. Peltekova *et al*[20] found two novel single nucleotide polymorphisms (SNPs) with functional mutations, generating a 2-alleles risk haplotype (TC) predisposing to CD. Replication studies have found inconsistent genotype/phenotype correlation[30-34] with some concordance for presence of perianal fistulae[31,33], which is similar to that observed for the IBD5 risk haplotype[25,28]. More importantly, although Peltekova *et al*[20] in preliminary functional studies demonstrated that the OCTN1/2 variants resulted in impaired transport function of various organic cations and carnitine, but the precise link with IBD pathogenesis remains unexplained. All subsequent replication studies were unable to confirm the association of TC haplotype in the absence of the IBD5 risk haplotype[30-33], making the assumption of OCTN variants as causal genes still disputable[35].

Concerning the DLG5 gene, Hampe *et al*[36] initially described a susceptibility locus on chromosome 10 in a genome linkage study. Stoll *et al*[21] further narrowed down this risk region and identified two distinct haplotypes associated with IBD and CD. More specifically, the "risk haplotype D" was defined by a single non-synonymous SNP 113G→A; this nucleotide change resulting in the amino acid substitution R30Q, probably impedes scaffolding of the protein evaluated in silico analysis. A collaborative study[37], investigating two independent case-control cohorts and one family-based collection confirmed the association in 2 of the 3 cohorts, although with a modest effect on the relative risk to IBD (OR = 1.25). Other groups, however, reported negative findings[30,31,38]. More recently, a more thorough evaluation of the previously reported cohorts with other large control populations, has demonstrated that the DLG5 gene is a risk factor for CD only for men (OR = 2.49, CI 1.5-4), but not for women (OR = 1.01)[39]. Intriguingly, this difference is driven by a gender-dependent transmission ratio distortion among healthy controls (frequency of A allele: men 5.2%, women 11.3%).

In young patients, IBD might offer the opportunity to understand the pathopysiology of the diseases in a form that is closer to their underlying cause and mechanisms than in adult, because of a lower influence of environmental risk factor (i.e. smoking). Paradoxically, in contrast to the data in adults, there are studies mainly looking at CARD15 polymorphisms[11-19,40], while being scarce and conflicting on OCTN1/2 and DLG5 genes[41-44]. Moreover, many pediatric series are flawed by a small sample size[11,12,15,16,18,19,43,44], little information on UC patients[13,15,41], and lack of control populations[11,12,16,17,19].

In this study we have investigated the contribution of variants of CARD15, OCTN1/2 and DLG5 genes in IBD predisposition in a large Italian pediatric cohort. We also examined the genotype/phenotype relationships and gene-gene interactions.

MATERIALS AND METHODS

Study population

Patients under age of 18 at diagnosis were recruited into this study from 16 tertiary pediatric and gastroenterologic centres in Italy, in cooperation with a multicenter study endorsed by the SIGENP (Italian Society for Pediatric Gastroenterology, Hepatology and Nutrition). Ethical approval was obtained at all participating centres. All study participants were Caucasian and their parents gave a written informed consent.

The diagnosis of CD or UC was established by conventional clinical, radiological, endoscopic and histological criteria[45]. Indeterminate colitis was excluded from the study.

The recruited population was comprised of 200 patients with CD and 186 patients with UC. Blood samples were also taken from 434 parents, making 217 complete family trios (108 CD and 109 UC, respectively) available for analysis.

A total of 347 adult healthy unrelated blood donors (184 male, mean age 32 years, range 20-45) who served as controls were randomly recruited from their sites to minimize the potential geographic differences among San Giovanni Rotondo (Southern Italy, $n = 147$), Rome (Centre, $n = 100$) and Padova (Northern Italy, $n = 100$).

Data collection

Retrospective data were collected on patients using a standardized questionnaire obtaining information on patient and parental smoking details (at least one cigarette/d), ethnicity, and IBD family history. Additional clinical data were collected on patient demographics, age at IBD diagnosis, medications, extra-intestinal manifestations and need for resective surgery. Standard investigations employed in these patients were upper GI endoscopy, ileo-colonoscopy, and barium examination. CD disease location and behavior were categorized according to the Vienna classification[46]. Presence of perianal fistulae was analysed separately according to the Montreal's proposal[47].

In all patients, weight and height percentiles were calculated at diagnosis. Presence of growth retardation was defined as a reduction below the 5th percentile for weight, height or both.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols[48], and genotyped in the laboratory of San Giovanni Rotondo Hospital.

Genotyping for Arg702Trp and Leu1007fsinsC common CARD15 variants was performed by DHPLC (denaturing high performance liquid chromatography, Wave System, Transgenomic Ltd, UK) and restriction fragment length polymorphisms (RFLP) assay was used for Gly908Arg (G/C). The 380 base pairs PCR product was digested with Hha I (New England Biolabs, Ipswich, MA), yielding 2 fragments of 138 and 242 base pairs in the presence of C allele and visualized on 2% (w/v) agarose gel. One hundred random samples were also confirmed by sequencing on ABI 310 DNA sequencer (Applied

Table 1 Primers sequences, methodology, and restriction enzymes used for genotyping

Gene locus	SNPs		Sequence	Methods/enzyme
CARD15	R702W	For	5′ACCTTCAGATCACAGCAGCC3′	DHPLC
		Rev	5′GCTCCCCCATACCTGAAC3′	
	G908R	For	5′AAGTCTGTAATGTAAAGCCAC3′	RFLP/Hha Ⅰ
		Rev	5′CCCAGCTCCTCCCTCTTC3′	
	L1007fsinsC	For	5′CTCACCATTGTATCTTCTTTTC3′	DHPLC
		Rev	5′GAATGTCAGAATCAGAAGGG3′	
IBD5	IGR2198a_1	For	5′GGGGCAATTCTATGAGGACA3′	RFLP/Nla Ⅲ
		Rev	5′CCAGAGACACTGGGACATCA3′	
	IGR2096a_1	For	5′GTAGCGAGAGGCTCCACAGT3′	RFLP/Dra Ⅰ
		Rev	5′TCCTCCATGCTACTGCTCTG3′	
OCTN1	C1672T	For	5′GGGTAGTCTGACTGTCCTGATTG3′	TaqMan
SLC22A4		Rev	5′TCTGGAAGAGTCATTCCCAAACTTTC3′	
		VIC	5′AAGGGTGAGGATTC3′	
		FAM	5′AAGGGTGAAGATTC3′	
OCTN2	G207C	For	5′CCGCTCTGCCTGCCA3′	TaqMan
SLC22A5		Rev	5′GCGGCTGGCCTTACATAGG3′	
		VIC	5′CAGGCCCGGAACC3′	
		FAM	5′CAGGCCCGCAACC3′	
DLG5	R30Q		C_7432738_10	TaqMan

DHPLC: Denaturing high performance liquid chromatography; RFLP: Restriction fragment length polymorphisms; TaqMan: ABI Prism 7700 sequencer detector.

Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations.

For OCTN1/2 genotyping, the SLC22A4 1672C/T and SLC22A 5-207G/C primers were designed using Primer Express V 1.5 (Applied Biosystems). SNPs were genotyped using the Taqman system ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA) as previously described[33].

For the IBD5 locus, the IGR2096a_1 and IGR2198a_1 SPNs were selected from the haplotype originally described by J. Rioux[22] (http://www.genome.wi.mit.edu/IBD5). Genotyping was performed by restriction fragment analysis as previously described[28]. Results were confirmed by direct sequencing of representative samples for each genotype, using ABI cycle sequencing kit V 1.1 and the ABI 310 genetic analyzer.

For the DLG5 gene, we genotyped the 113 G→A variant (rs1248696) tagging the haplotype D, the over-transmitted haplotype in the study by Stoll *et al*[21], with 7700 TaqMan bi-allelic discrimination system. PCR reactions (15 mL) were performed in 1 × TaqMan Universal PCR Master Mix, 1 × Genotyping Assay Mix, and 50 ng of genomic DNA. After 2 min at 50℃, and 10 min at 94℃ initial denaturation, reaction was amplified for 40 cycles: 15 s at 94℃, and 60 s at 60℃.

A summary of primer sequences and methods is depicted in Table 1.

Detection of antibodies

Whenever serum samples were available, anti-nuclear cytoplasmatic antibodies (ANCA) were tested by a standard immuno-fluorescence technique[49]. In addition, presence of anti-saccharomyces cerevisiae antibodies (ASCA) was investigated by means of a commercial ELISA assay (Quanta Lite™ ASCA, Inova Diagnostics Inc, San Diego, USA)[50].

Data analysis

Comparison of allele and genotype frequencies was performed by Chi-square and Fisher exact tests when appropriate. Student's *t* test was used to compare means of continuous variables with the SPSS software ver. 11.5. Tests for Hardy-Weinberg equilibrium, linkage disequilibrium, haplotype frequency analysis and transmission disequilibrium were performed by the Haploview Software ver. 3.2 (http://www.broad.mit.edu/personal/jvbarret/haploview).

Power calculation was performed using the PS software (http://biostat.mc. vanderbilt. edu/twiki/bin/view/Main/PowerSampleSize).

Genotype-phenotype associations were analysed by means of univariate and multivariate stepwise logistic regression with the SPSS software. This approach allowed to take into account a dose-response effect (heterozygote or homozygote), the possible interactions between genes, and the effect of potential confounding variables (duration of follow-up, disease localization, etc.). The obtained *P* values were corrected (exact *P* value) for multiple comparisons. A possible interaction between CARD15, OCTN1/2, and DLG5 genes was also investigated.

RESULTS

Clinical findings

Main clinical characteristics of patients included in the study are depicted in Table 2.

The 200 CD patients had a mean age of 21 ± 8 years (range 1-18) with a slight male predominance (male/female = 115/85). The 186 UC patients had a mean age of 19 ± 9 years (range 1-18) with a female predominance (73/113). The mean age at diagnosis and the mean duration of follow-up (8-9 years) were similar between the two groups of patients. Eighteen percent

Table 2 Clinical and demographic characteristics of patients with CD and UC as evaluated in last follow-up (mean ± SD)

	Crohn's disease (n = 200)		Ulcerative colitis (n = 186)
Age (yr)	21 ± 8		19 ± 9
Age at diagnosis (yr)	12 ± 4		11 ± 5
Duration of follow-up (yr)	9 ± 7		8 ± 8
Sex (male/female)	115/85		73/113
Localization CD: n (%)			
- Ileum	38 (19)		
- Ileo-colon	116 (58)		
- Colon	45 (23)		
- Upper G-I tract	35 (18)[1]		
Localization UC: n (%)			
- Rectum-sigmoid			44 (24)
- Left colon			54 (29)
- Pancolitis			88 (47)
Disease type CD: n (%)	(Vienna)	(Montreal)	
- Inflammatory	104 (52)	135 (67)	
- Stricturing	40 (20)	45 (23)	
- Penetrating	56 (28)	20 (10)	
Growth delay: y/n (%)	60/84 (42)		21/126 (14)
Surgery: y/n (%)	51/149 (26)		13/137 (7)
EIM: y/n (%)	79/121 (40)		46/140 (25)
Perianal fistulae: y/n (%)	36/164 (18)		1/185 (0.5)
Family history: y/n (%)	18/182 (9)		22/164 (12)
ASCA: y/n (%)	77/32 (71)		18/57 (24)
ANCA: y/n (%)	18/86 (17)		65/50 (57)
Smoking y/n (%)	44/156 (22)		40/146 (21)

EIM: Extra-intestinal manifestations. [1]One patient had upper G-I involvement only.

of CD had an upper G-I involvement. The incidence of resective surgery (26% *vs* 7%, $P < 0.01$), extra-intestinal manifestations (40% *vs* 25%, $P = 0.002$), growth delay (42% *vs* 14%, $P < 0.001$) and perianal fistulae (18% *vs* 0.5%, $P < 0.001$) was significantly higher in CD than in UC patients. In the latter group, almost half of the patients had a pancolitis (47%). The incidence of ASCA and ANCA was 71% and 17% in CD patients, and 24% and 57% in UC patients, respectively, with a significant difference ($P < 0.0001$ and $P < 0.0001$, respectively).

Genotyping findings

The error rate of genotyping (defined as the percentages of disagreement in genotypes among 200 random samples tested in duplicate) was < 1% for all SNPs.

All genotypes in patients and healthy controls were in Hardy-Weinberg equilibrium. A strong linkage disequilibrium (D' > 91) was identified between the two OCTN1/2 variants, and between each OCTN1/2 variant and the IBD5 risk haplotype markers as well ($r^2 > 0.62$, $P < 0.001$) (Figure 1).

Table 3 summarizes the distribution of genotypes for the CARD15 variants (Arg702Trp, Gly908Arg and Leu1007fsinsC), OCTN1/2 variants (SLC22A4 and SLC22A5), the IBD5 risk haplotype SNPs (IGR2096a_1 and IGR2198a_1), and DLG5 gene polymorphism (113G→A).

Regarding the CARD15 gene, all three variants were frequently seen in CD patients as compared to controls ($P < 0.001$). By combining the three variants,

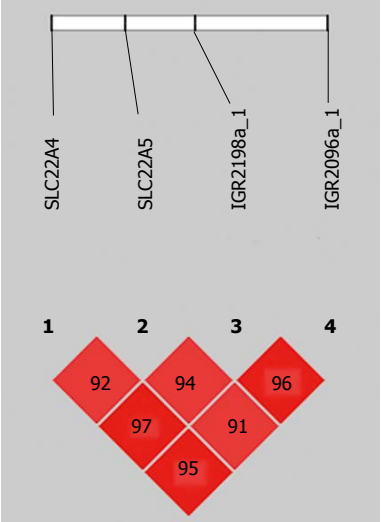

Figure 1 Evaluation of linkage disequilibrium in control subjects between IBD5 risk haplotype SNPs and OCTN1/2 variants ($r^2 > 0.62$; $P < 0.001$).

38% of CD patients had at least one mutation, and 15% had two mutations (homozygosis or compound heterozygosis). Respective figures in healthy controls were 15% and 1%. In carriers of one mutation, the odd risk was 2.7 (CI = 1.7-4.2; $P < 0.001$) while that for two mutations was 17 (CI = 5-58; $P < 0.001$).

As for the OCTN1 and OCTN2 variants, they were both significantly increased in CD patients for the homozygous genotype (1672TT: 24% *vs* 16% in controls, $P = 0.03$) (-207CC: 29% *vs* 21% in controls, $P = 0.03$). Moreover, the two-point haplotype, consisting of the 1672T and -207C alleles (OCTN-TC), was associated with CD (44.8% *vs* 38.3% in controls, $P = 0.043$). The 1672TT (22% *vs* 16% in controls) and -207CC (29% *vs* 21% in controls) genotypes were also increased in UC patients, the difference being statistically significant ($P = 0.10$ and $P = 0.05$, respectively). Accordingly, the frequency of TC haplotype was significantly increased also in UC patients (45.4% *vs* 38.3 in controls, $P = 0.03$).

The markers of IBD5 (IGR2096a_1 and IGR2198a_1) risk haplotype were both increased for the homozygous genotype in CD patients compared to controls, although with this sample size, only the AA genotype of the SNP IGR2096a_1 was significantly increased (23% *vs* 14% of controls, $P = 0.01$). Among individuals lacking the IBD5 risk haplotype (homozygous with respect to the non-risk associated allele of IGR2096a_1), only 7% of patients with CD and 5% of controls carried the TC haplotype, while in presence of the IBD5 risk haplotype, the TC haplotype was found in 61.9% of CD patients and 58.7% of controls. Similar data were obtained in UC patients (data not shown, available on request).

The distribution of 113G→A polymorphism of DLG5 was similar in patients and controls. No significant differences in allele or genotypes frequencies were noted. We also attempted to analyse the data based on gender of cases and controls based on the recent findings of male predominance[39], but results did not differ.

TDT analysis

TDT analysis of UC trios did not show significant transmission distortion of any genetic markers. As expected, a significant over-transmission towards affected

Table 3 Genotype distributions of CARD15, OCTN1-2, DLG5 genes, and IBD5 locus polymorphisms

SNPs	Genotype	Crohn's disease	*P* value OR 95% IC	Controls	*P* value OR 95% IC	Ulcerative colitis
CARD15			TT and CT *vs* CC			
R702W	TT	5 (3)	$P < 0.001$	1 (0)	NS	0 (0)
	CT	38 (19)	3.47 (2.06-5.86)	25 (7)		17 (9)
	CC	153 (78)		321 (93)		167 (91)
G908R	CC	2 (1)	CC and CG *vs* GG	0 (0)	NS	1 (1)
	CG	25 (13)	$P < 0.001$	18 (5)		12 (7)
	GG	170 (86)	2.90 (1.55-5.42)	329 (95)		171 (93)
1007fsInsC	CC	1 (1)	CC and CG *vs* GG	0 (0)	NS	0 (0)
	CG	25 (13)	$P = 0.0005$	11 (3)		10 (5)
	GG	172 (87)	4.62 (2.23-9.57)	336 (97)		176 (95)
OCTN1/2						
C1672T	TT	42 (24)	TT *vs* CT and CC	57 (16)	$P = 0.10$	36 (22)
	CT	79 (46)	$P = 0.03$	178 (51)		76 (47)
	CC	52 (30)	1.63 (1.04-2.56)	112 (32)		49 (30)
-G207C	CC	50 (29)	CC *vs* CG and GG	72 (21)	$P = 0.05$	46 (29)
	CG	84 (49)	$P = 0.035$	183 (53)		77 (48)
	GG	38 (22)	1.57 (1.03-2.38)	92 (27)		38 (24)
IBD5						
IGR2198a_1	GG	40 (21)	GG *vs* CG and CC	55 (16)	$P = 0.09$	39 (22)
	CG	93 (49)	$P = 0.13$	175 (50)		86 (48)
	CC	58 (30)		117 (34)		54 (30)
IGR2096a_1	AA	44 (23)	AA *vs* AC and CC	50 (14)	NS	32 (18)
	AC	89 (47)	$P = 0.01$	176 (51)		91 (51)
	CC	58 (30)	1.78 (1.13-2.79)	121 (35)		56 (31)
DLG5						
113G/A	AA	1 (1)	NS	2 (1)	NS	1 (1)
	AG	16 (10)		57 (16)		26 (15)
	GG	148 (90)		288 (83)		144 (84)

Table 4 Analysis of possible interaction between OCTN TC-haplotype and DLG5 variant with presence (or absence) of at least one CARD15 variant

		CD		UC		Controls	
CARD15		DLG5	OCTN TC haplotype (%)	DLG5	OCTN TC haplotype (%)	DLG5	OCTN TC haplotype (%)
CARD15+	DLG5+	5 (3)	42.6	7 (4)	35.2	11 (3)	44.8
	DLG5-	56 (34)		27 (16)		38 (12)	
CARD15-	DLG5+	12 (7)	46.2	20 (12)	48.7	44 (13)	39.1
	DLG5-	92 (56)		116 (68)		241 (72)	

DLG5-positive was defined as the AA or AG genotypes. Data expressed as absolute values or percentages (between brackets). All comparison had a *P* value > 0.05.

offspring was observed for CARD15 variants in CD trios: the Arg702Trp (transmitted/untrasmitted [T/U], 24/8, $P = 0.004$) and Leu1007fsinsC variants (T/U, 14/2, $P = 0.027$) were significantly over-transmitted. We did not observe distortion of transmission towards affected offspring for the Gly908Arg CARD15 variant, OCTN1/2 variants, and for the two IBD5 risk-haplotype-tagging SNPs IGR2198a_1 and IRG2096a_1. Regarding the DLG5 variant, an over-transmission of the wild-type allele of the 113G→A SNP towards affected offspring was found (T/U, 21/9, $P = 0.02$).

Gene-gene interaction

For the evaluation of gene-gene interactions, all subjects were stratified according to their CARD15 genotype. Genotypes and allele frequencies for the investigated variants of OCTN1/2 and TC haplotype, and for G113A variant of DLG5 were not different between CARD15-positive (carrying at least 1 CARD15 variant) and CARD15-negative subjects (Table 4). Similarly, no interactions between OCTN1/2 and DLG5 variants were found (data not shown, available on request).

Genotye-phenotype analysis

For the analysis of possible correlation between genotype and phenotype, patients were classified on the basis of presence or absence of at least one CARD15 variant, TC haplotype, and DLG5 risk genotype (carriers of the A allele). Data are shown in Table 5 and Table 6. CD patients carrying the CARD15 variants had more frequently ileal localization and less frequently a colonic localization (OR = 2.8, $P = 0.029$). Carriers of TC haplotype of OCTN1/2 variants had more occurrence of a penetrating disease (48.7% *vs* 43.5%) as compared with inflammatory/stricturing behaviour; this difference was statistically significant when perianal fistulae were not taken into account (62% *vs* 43%; $P = 0.038$) according to Montreal's classification. Similarly, a more frequent penetrating disease

Table 5 Genotype-phenotype correlation in CD patients evaluated by comparisons of carriers and non-carriers of at least one CARD15 variant (CARD15+), AA/AG genotypes of DLG5 variant (DLG5+) and presence of TC haplotype of OCTN1/2 variants (TC+)

CD *n* = 200	CARD15+ (*n* = 75)	CARD15- (*n* = 121)	DLG5+ (*n* = 17)	DLG5- (*n* = 148)	OCTN TC haplotype (%) +	-
Localization CD, *n* (%)						
Ileum	17 (23)	21 (17)	1 (6)	33 (22)	46	54
Ileo-colon	48 (64)	64 (53)	13 (76)	80 (54)	47	53
Colon	10 (13)	35 (29)	3 (18)	34 (23)	39	61
Upper G-I tract	16 (21)	18 (15)	3 (18)	29 (20)	53	47
Ileo *vs* colon	*P* = 0.029 OR 2.83 (1.10-7.33)					
Disease type CD, *n* (%)	Vienna-Montreal	Vienna-Montreal	Vienna-Montreal	Vienna-Montreal	V-M	V-M
- Inflammatory	37 (50) 48 (64)	66 (54) 86 (71)	7 41) 9 (52)	87 (59) 109 (74)	45/44	55/56
- Stenosing	19 (25) 21 (28)	19 (16) 22 (18)	4 (24) 4 (24)	26 (17) 30 (20)	40/41	60/59
- Penetrating	19 (25) 6 (8)	36 (30) 13 (11)	6 (35) 4 (24)	35 (24) 9 (6)	49/62	51/38
F *vs* S+ I (according to Montreal)			*P* = 0.011 OR 4.75 (1.29-17.57)		*P* = 0.038 OR 2.13 (1.03-4.4)	
Growth delay y/n, (%)	24/35 (41)	36/47 (43)	4/5 (44)	54/62 (47)	49/44	51/56
Resective surgery y/n, (%)	23/52 (31)	27/94 (22)	5/12 (29)	26/122 (18)	42/46	58/54
EIM y/n, (%)	27/48 (36)	49/72 (41)	9/8 (53)	58/90 (39)	40/48	60/52
Perianal fistulae y/n, (%)	13/62 (17)	23/98 (19)	2/15 (12)	26/122 (18)	45/45	55/55
ASCA (pos/neg), (%)	31/8 (79)	46/24 (66)	8/3 (73)	63/29 (68)	46/58	54/42
ANCA (pos/neg), (%)	5/26 (16)	13/59 (18)	1/11 (8)	15/66 (19)	44/49	56/51
Steroids need, y/n (%)	48/27 (64)	86/35 (71)	12/5(71)	96/52 (65)	43/48	57/52

P values are corrected for multiple comparisons.

Table 6 Genotype-phenotype correlation in UC patients evaluated by comparison of carriers vs non-carriers of at least one CARD15 variant (CARD15+), the AA/AG genotypes of DLG5 variant (DLG5+), and presence of TC haplotype of OCTN1/2 variants (TC+)

UC *n* = 186	CARD15+ (*n* = 36)	CARD15- (*n* = 148)	DLG5+ (*n* = 27)	DLG5- (*n* = 144)	OCTN TC haplotype (%) +	-
Localization UC, *n* (%)						
Rectum-sigmoid	7 (19)	36 (24)	9 (33)	28 (19)	45	55
Left colon	11 (31)	43 (29)	6 (22)	44 (31)	44	56
Pancolitis	18 (50)	69 (47)	12 (45)	72 (50)	47	53
Growth delay y/n, (%)	3/26 (10)	18/98 (16)	5/14 (26)	16/101 (14)	43/46	57/54
Resective surgery y/n, (%)	4/32 (11)	9/139 (6)	1/26 (4)	9/135 (6)	31/46	69/54
EIM y/n, (%)	9/27 (25)	37/111 (25)	4/23 (15)	40/104 (28)	51/44	49/56
ASCA (pos/neg), (%)	4/10 (29)	14/46 (23)	5/10 (33)	13/46 (22)	42/46	58/54
ANCA (pos/neg), (%)	13/6 (68)	52/43 (55)	11/8 (58)	52/40 (57)	43/40	57/60
Steroids need, y/n, (%)	26/10 (72)	109/39 (74)	21/6 (78)	105/39 (73)	46/46	54/54

(not including perianal fistulae) was found in CD patients with DLG5 risk variant (24% *vs* 6%; $P = 0.011$). No other significant differences were found between CD and UC patients (Table 6). No further correlation at this sample size could be observed when considering patients with two CARD15 variants, homozygous for TC haplotype or DLG5 variant. Similarly, no correlation could be found when evaluating subjects with all three concomitant gene variants (at least one CARD15 variant, OCTN TC-haplotype and DLG5 113G/A).

The correlations between at least one CARD15 variant with ileal localization (OR = 2.8, CI = 1.1-7.5; $P = 0.031$), OCTN TC-haplotype and DLG5 113G/A variant (OR = 4.05, CI = 1.05-15.5; $P = 0.041$) with penetrating disease were also confirmed at the stepwise logistic regression, after correction for potential confounders (duration of follow-up).

DISCUSSION

This study reports in the largest available cohort of pediatric IBD patients the contribution of variants of CARD15, OCTN1/2 and DLG5 genes on disease predisposition and correlation with phenotype. Reports of pediatric series to date available have been limited to mainly CARD15 variants[11-19], small number of subjects[11,12,15,16,18,19], and scarce information on UC patients[13,15,41].

The carriage rate of CARD15 variants in our pediatric population was 38% for CD patients and 20% for UC patients, not significantly different from figures in adult Italian population (36% for CD and 15% for UC, respectively)[51]. This finding is in agreement with previously reported frequencies in CD pediatric series ranging form 8.6% up to 60%, with lowest incidence in Swedish and Scottish population, similarly to the rates reported in corresponding adult population. Accordingly, this figure does not support the hypothesis of a stronger effect of CARD15 variants in early onset of CD. The relative contribution of each of the three common variants in our data is consistent with data from adult populations of IBD patients: 1007finsC confers the greatest risk (OR = 4.6, CI = 2.2-9.5) and G908R the least

(OR = 2.9, CI = 1.5-5.4). More specifically, the 1007finsC and R702W variants were associated with susceptibility to CD both in case control and family-based analysis, while the G908R variant was associated only in case control studies.

Homozygous carriers of OCTN1/2 variants were significantly more frequent in CD patients as compared to controls (OR = 1.6, $P = 0.03$). The TC haplotype was found in 44.8% of CD patients and 38.3% of controls ($P = 0.04$). No evidence of interaction with CARD15 gene was found since the frequency of TC haplotype was similar in CARD15-positive and CARD15-negative patients. Furthermore, a trend towards an increase of the homozygous carriers of OCTN1/2 variants was noticed in UC patients ($P = 0.05$), with a significant increase of TC haplotype (45.4%; $P = 0.03$), with similar finding in Italian adult population[33] and two other pediatric cohorts[41,42]. In contrast, no correlation of OCTN1/2 variants has been found in other pediatric series[43,44], perhaps for the smaller sample size of patients and controls. Our study confirmed the strong linkage disequilibrium between OCTN1/2 variants and SNPs tagging the IBD5 risk haplotype. Contrary to the Peltekova's study[20], the large majority of homozygous carriers of OCTN1/2 variants and TC haplotype were found in the background of IBD5-positive risk allele[35]. Although the possible causal role of the OCTN1/2 variants could not be excluded, further functional studies are needed to confirm and explain the role of these genes in IBD.

The DLG5 variant (G113A) was not correlated with pediatric IBD, even after stratifying patients and controls for gender in both the association study and family-based analysis. In addition, the A (risk) allele was significantly under-transmitted ($P = 0.02$), similarly to another adult series[31]. This might reflect a reduced effect of this variant in pediatric onset of IBD (5% *vs* > 10% in corresponding adult population)[39] and/or an insufficient power of the study sample. Based on the observed frequency of this variant in the control population (9%), our study sample could detect a ± 4.5% difference with a power of 90% and significance of 0.01.

No interaction between DLG5 variant and CARD15 or OCTN1/2 variants was found.

When the genotype-phenotype correlation was explored, there was only a weak correlation with ileal disease for CARD15 variants ($P < 0.03$) and penetrating disease for OCTN1/2 and DLG5 ($P < 0.04$, and $P < 0.02$, respectively). All these correlations were confirmed in the stepwise logistic regression analysis.

The correlation of CARD15 variants with ileal disease has been widely reported in most studies of adult[7] and child[11-14,41,44] Caucasian populations. Although some explanations for this consistent association have been put forward (e.g. presence of NOD2 in Paneth cells in terminal ileum, impaired regulation of Paneth-cell mediated antimicrobial response, perhaps through impaired production of α-defensin)[52], the clinical implications are unclear yet. Less than 50% of CD patients with ileal localization in this and other studies carry one CARD15 variant; moreover, carriers of this variant do not have a disease course and response to therapy, being different from non-carriers[53].

The association of OCTN1/2 and DLG5 variants with penetrating disease is intriguing since both genes are involved in maintaining mucosal integrity and permeability. A correlation between OCTN1/2 variants and penetrating disease has been reported in adult population[31-33], but not in the only two available studies in children with CD, in which no correlation[44] or only a correlation with growth delay was found[41]. Obviously, difference in selection of patients and duration of follow-up may explain this discrepancy. In our series, patients with penetrating disease were more than double than in the above-mentioned studies[41,44]. Moreover, our definition of growth delay (below 5th percentile) was more stringent than that of the Scottish study[41] (below 9th percentile). Nevertheless, when considering patients with both height and weight delay below the 5th percentile, a trend towards a correlation with TC-haplotype was found in our study ($P = 0.07$).

Regarding the correlation of DLG5 variant with penetrating disease, no reports are available so far in children with IBD. Moreover, data in adult population are very conflicting with no clear genotype-phenotype correlation demonstrated.

Finally, no correlation with age at diagnosis, family history, active and passive smoking, previous appendectomy, presence of ANCA and ASCA, growth delay, use of steroids, or need for surgery could be demonstrated in our series. Moreover, despite a significant increase of TC-haplotype of OCTN1/2 variants in UC patients, no correlation with any clinical phenotype was found. A potential limitation of this study lies in the large number of centres involved and the possible lack of reproducibility of clinical classification (disease behaviour). This choice allowed, however, to accomplish the study in the largest population of pediatric IBD patients so far for genetic purpose. Moreover, all patients were enrolled at academic referral centres and sorting of clinical features was obtained through a previously validated standardized questionnaire.

In conclusion, this study has demonstrated that the three common CARD15 variants and the recently described OCTN1/2 variants, but not the 113G/A DLG5 variant, are associated with susceptibility to CD in children. Furthermore, all three investigated genes exert a weak influence on clinical expression of CD; CARD15 variants (Arg702Trp, Gly908Arg and Leu1007fsinsC) are slightly more frequent in subjects with ileal localization, while OCTN (1672C/T and -207G/C) and DLG5 (113G/A) polymorphisms correlate with presence of penetrating behaviour.

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