INFLAMMATORY BOWEL DISEASE

Polymorphisms in the *DLG5* and OCTN cation transporter genes in Crohn's disease

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Background and aims: Recent data suggest identification of causal genetic variants for inflammatory bowel disease in the *DLG5* gene and in the organic cation transporter (OCTN) cluster, both situated in previously described linkage regions.
Patients and methods: The polymorphisms in *DLG5* (113 G→A, 4136 C→A, and DLG5_e26), *SLC22A4* (1672 C→T), and *SLC22A5* (−207 G→C) were assessed in 625 patients with Crohn's disease (CD), 363 patients with ulcerative colitis (UC), and 1012 healthy controls. Association with disease susceptibility, clinical phenotypes, and possible genetic interactions of these polymorphisms with disease associated *CARD15/NOD2* mutations was analysed.
Results: No significant association of *DLG5* polymorphisms with CD or UC was observed. Homozygosity for the OCTN-TC haplotype was associated with an increased CD risk (OR = 1.65), which was even greater in the presence of *CARD15* mutations. Genotype-phenotype analysis revealed that this association was particularly strong in patients with colonic disease. The TC haplotype was associated with non-fistulising non-fibrostenotic disease, an earlier age of disease onset, and reduced need for surgery.
Conclusion: Our observations argue against a role of *DLG5* polymorphisms in the susceptibility for inflammatory bowel disease, whereas the OCTN polymorphisms are associated with CD. However, due to the comparable weak association observed herein, extended linkage disequilibrium analyses of these variants with the *IBD5* haplotype tagged single nucleotide polymorphims might be advisable before definitive conclusions about their causative role in CD can be drawn.

Recently, Stoll and colleagues[1] identified disease associated genetic variations responsible for the previously described linkage of Crohn's disease (CD (MIM 266600)) with chromosome 10q23.[2] The authors described two haplotypes in the *DLG5* gene which were associated with inflammatory bowel disease (IBD (MIM 601458)). One of them is distinguished by a non-synonymous single nucleotide polymorphism (SNP) (113G→A, resulting in the amino acid substitution R30Q) and was significantly overtransmitted in individuals with both IBD and CD in particular. The second haplotype, which is distinguished by the SNP DLG5_e26, was significantly undertransmitted in these groups. A third less common SNP in *DLG5* (4136C→A, resulting in the amino acid substitution P1371Q) also showed a significant association with CD. Based on observations from in silico analyses, the authors concluded that the variants R30Q and P1371Q probably impair the function of DLG5.[1] Genetic interactions between the variant R30Q and CD associated *CARD15* mutations were also suggested.

Simultaneously, a Canadian group reported the identification of functional mutations in the carnitine/organic cation transporter (OCTN) genes on chromosome 5q31, which were associated with CD.[3] The *IBD5* locus on chromosome 5q31, for which association with IBD has been repeatedly confirmed,[4–7] harbours the cytokine cluster and is therefore an attractive candidate region for IBD. However, identification of the causal genetic variants is difficult due to the strong linkage disequilibrium across this region. By resequencing the five genes in the *IBD5* interval, Peltekova and colleagues[3] identified 10 new SNPs, including two in the *SLC22A4* and *SLC22A5* genes coding for OCTN1 and OCTN2. These two mutations form a haplotype (TC) which was associated with CD, as shown by case control analysis. In line with the previously reported interaction between *IBD5* and *CARD15*,[7 8] the disease risk was enhanced in the presence of both the TC haplotype and *CARD15* mutations. Based on functional studies, the authors suggested that the 1672C→T missense substitution in *SLC22A4* and the −207G→C transversion in the *SLC22A5* promoter contribute to disease susceptibility by impairing OCTN activity or expression, respectively.

Albeit both studies are of paramount importance, these observations are yet unconfirmed and the contribution of these genetic variations to disease phenotype is unknown. Herein, we sought to reproduce the described genetic associations. In order to delineate the causative role of OCTN variants, the strength of their association was compared with that of another marker in the *IBD5* susceptibility locus, IGR2078a_1.[4] In addition, the associations of these polymorphisms with disease phenotype and their interaction with the CD specific *CARD15* mutations were determined.[9–11]

METHODS

Human subjects and phenotypic analysis

The study population comprised 625 patients with CD, 363 patients with ulcerative colitis (UC (MIM 191390)), and 1012 healthy controls from Southern Germany. Patients were recruited from three tertiary referral centres. Healthy unrelated blood donors served as controls. All study

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; SNP, single nucleotide polymorphism; OCTN, carnitine/organic cation transporter; OR, odds ratio

Table 1 Primer sequences and restriction enzymes used for SNP-genotyping

SNP	Primers	Restriction enzyme	Length of restriction fragments
SLC22A4 1672C→T (rs1050152)	F: CGTCATGGGTAGTCTGACTGTCCTGATTGGGATC R: TCCTACTTACCATTTCACTTTCTGCATCTGCTCTAAGG	*BamH I*	allel C: 30 bp+88 bp allel T: 118 bp
SLC22A5 −207G→C (rs2631367)	F: GCGCCGCTCTGCCTGCCAG R: AGGGTAGGCTCGCGAGCTGACACC	*Msp I*	allel G: 44 bp+83 bp allel C: 127 bp
DLG5 113G→A (rs1248696)	F: GGAAGGCGCAGTCCCCACCACCCCTCCTCAC R: AAGGCCAGGCGCTTGCGGAGCTCGTTTCTCTCCTGG	*Msp I*	allel G: 89 bp+35 bp allel A: 124 bp
DLG5 4136 C→A (rs2289310)	F: AGCTCACACCTGGACCCTGCCGGTAC R: TCACAGCAACGTCTGCTGACCTGGAGCTCCACTGC	*Xcm I*	allel C: 127 bp+36 bp allel A: 163 bp
DLG5_e26	F: CGACATCCTCTACGTGGATGACACCTTACC R: AGATAAGAAGCAGAATCCCTCCTCCACCAGC	*Mwo I*	insA: 156 bp+85 bp delA: 240 bp
IGR2078a_1	F: TCACTGAGCACAGCTTCTACAGTGCCA R: GTTCCTAATCTGAACACAGAAGCCAGGGAG	*Hae III*	allel A: 216 bp allel G: 122 bp+94 bp
CARD15 2104C→T (rs2066844)	F: TGGGGCCTGCTGGCTGAGTG R: GTGCAGCTGGCGGGATGGAG	*Msp I*	allel C: 76 bp+45 bp allel T: 121 bp
CARD15 2722G→C (rs2066845)	F: TCTGGCTGGGACTGCAGAGG R: CCCCTCGTCACCCACTCTGTCGC	*BstU I*	allel G: 131 bp allel C: 109 bp+22 bp
CARD15 3020insC	F: GGCTAACTCCTGCAGTCTCTTTAACTGG R: ACTTCCAGGATGGTGTCATTCCGCTCAAGG	*Mwo I*	non-ins C: 168 bp insC: 143 bp+26 bp

The underlined bases in the primers differ from the original sequences and served to introduce a restriction site or to disrupt a natural site within the primer sequence.

participants were Caucasian and gave their written informed consent. The study protocol was approved by the local ethics board. Diagnosis of CD or UC was established by conventional clinical, radiological, endoscopic, and histological criteria. Cases with indeterminate colitis were not enrolled. Extensive clinical characterisation was available in 412 patients with CD. Phenotypic details were obtained by standardised retrospective analysis of medical records. CD phenotype was classified according to age at disease onset, location, and behaviour of disease. Location was classified as current or past ileal disease only, colonic disease only, or both. Disease behaviour was classified by current or past behavioural types as fistulising, fibrostenotic or non-fistulising, non-fibrostenotic. Fibrostenotic disease was defined by occurrence of constant luminal narrowing demonstrated by radiological, endoscopic, or surgical-pathological methods. Fistulising disease was defined by the presence of abdominal or perianal fistulas at any time in the course of disease. Non-fistulising, non-fibrostenotic disease was characterised as CD, which was never complicated by occurrence of fistulae or stenosis. Because many patients had disease at both sites (ileal and colonic) and displayed fibrostenotic as well as fistulising disease behaviour, data were also analysed as any colonic, any ileal, or any fibrostenotic or fistulising disease, respectively (see tables 2, 4, 5). Additionally, need for surgery (ileocaecal resection) in the past medical history was evaluated.

Genotyping

The polymorphisms 113G→A, 4136C→A, and DLG5_e26 within the *DLG5* gene,[1] as well as 1672C→T in *SLC22A4*, −207G→C in *SLC22A5*,[3] and the marker IGR2078a_1G→A,[4] were investigated by restriction fragment length polymorphism analysis. Primer sequences and reaction conditions are depicted in table 1. Results were confirmed by sequencing representative samples for each genotype. Genotyping of CD patients for mutations in the *CARD15* gene was performed by restriction fragment length polymorphism analysis or DNA sequence analysis (Radlmayr and colleagues,[12] Klein and colleagues,[13] Török and colleagues, and Schnitzler and colleagues, unpublished data).

Data analysis

Each marker was tested for Hardy-Weinberg equilibrium in the control population. Association testing as well as reconstruction of haplotypes was performed using the EM algorithm (UNPHASED–module cocaphase)[14] and the MCMC algorithm (PHASE), as described elsewhere.[15 16] Both methods furnished virtually identical results. For estimation of individual haplotypes, the SNPHAP program (http://www-gene.cimr.cam.ac.uk/clayton/software) was used. Statistical analysis was performed using the χ^2 or Fisher's exact test and the *t* test, respectively. For multiple comparisons, the Bonferroni correction was applied. Corrected p values are indicated as p_c. The strength of association for the OCTN variants was compared with the association noted for the IGR2078a_1 marker by analysis of the reconstructed haplotypes, conditional testing (UNPHASED), and a genotype regression analysis, as described elsewhere.[17]

Table 2 Clinical characteristics of the 412 patients with Crohn's disease included in the phenotypic analyses

n	412
Age at disease onset* (y)	
Mean (median) [range]	27.8 (24) [7–71]
Age at disease onset* (n (%))	
<40 y	289 (84.8%)
⩾40 y	52 (15.2%)
Disease location (n (%))	
Ileal and no colonic	50 (12.2%)
Colonic and no ileal	62 (15.1%)
Both ileal and colonic	298 (72.7%)
Any ileal	348 (84.9%)
Any colonic	360 (87.8%)
Disease behaviour (n (%))	
Fistulising† and non-fibrostenotic	58 (14.1%)
Fibrostenotic and non-fistulising	97 (23.5%)
Fistulising and fibrostenotic	165 (40.1%)
Non-fistulising, non-fibrostenotic	92 (22.3%)
Any fistulising	223 (54.1%)
Any fibrostenotic	262 (63.6%)
Surgery (n (%))	
Ileocaecal resection	144 (34.9%)
No ileocaecal resection	268 (65.1%)

*Information about age at disease onset was available in 341 patients.
†Including perianal fistulae.

RESULTS

There were no significant differences between cases and controls with respect to age or sex. The clinical characteristics of the CD patients included in the extensive phenotypic analyses are presented in table 2.

Table 3 Genotype and haplotype distributions for the *DLG5* and *SLC22A4/SLC22A5* polymorphisms, respectively, and for the IGR2078a_1 marker

SNP marker	Genotype	Crohn's disease	p Value	Ulcerative colitis	p Value	Controls
DLG5*						
113G→A	GG	501 (81.5%)	NS	284 (80.0%)	NS	781 (80.3%)
	AG	107 (17.4%)		67 (18.9%)		172 (17.7%)
	AA	7 (1.1%)		4 (1.1%)		19 (2.0%)
4136C→A	CC	569 (92.5%)	NS	318 (89.6%)	NS	888 (91.4%)
	AC	44 (7.2%)		34 (9.6%)		82 (8.4%)
	AA	2 (0.3%)		3 (0.8%)		2 (0.2%)
DLG5_e26	insAinsA	260 (42.3%)	NS	151 (42.5%)	NS	415 (42.7%)
	insAdelA	285 (46.3%)		165 (46.5%)		430 (44.2%)
	delAdelA	70 (11.4%)		39 (11.0%)		127 (13.1%)
SLC22A4						
1672C→T	CC	189 (30.2%)		121 (33.3%)	NS	328 (32.4%)
	CT	283 (45.3%)		180 (49.6%)		518 (51.2%)
	TT	153 (24.5%)	0.0005‡	62 (17.1%)		166 (16.4%)
SLC22A5						
−207G→C	GG	163 (26.1%)		105 (28.9%)	NS	271 (26.8%)
	GC	282 (45.1%)		170 (46.8%)		533 (52.7%)
	CC	180 (28.8%)	0.0009‡	88 (24.3%)		208 (20.5%)
OCTN haplotype						
SLC22A4/	C/G	606 (48.5%)		378 (52.1%)	NS	1075 (53.1%)
SLC22A5	T/C	587 (47.0%)	0.0082	302 (41.6%)		850 (42.0%)
	C/C	55 (4.4%)		44 (6.1%)		99 (4.9%)
	T/G	2 (0.1%)		2 (0.2%)		0 (0.0%)
IGR2078a_1†						
	GG	189 (31.4%)		124 (34.7%)		340 (33.8%)
	GA	279 (46.3%)		179 (50.2%)		507 (50.5%)
	AA	134 (22.3%)	0.0013‡	54 (15.1%)	NS	158 (15.7%)

*615 patients with Crohn's disease (CD), 355 patients with ulcerative colitis (UC), and 972 controls were tested for polymorphisms in the *DLG5* gene.
†602 patients with CD, 357 patients with UC, and 1005 controls were tested for the IGR2078a_1 marker.
‡p values are given for the *SLC22A4* 1672TT, *SLC22A5* −207CC, and IGR2078a_1AA genotypes; significance levels for the allele frequencies were p = 0.0041 for the 1672C→T polymorphism in *SLC22A4*, p = 0.0128 for the −207G→C polymorphism in *SLC22A5*, and p = 0.0128 for the IGR2078a_1 A/G marker; no significant differences for the heterozygous and carrier status (combined frequencies for homozygous and heterozygous genotypes with respect to the risk associated allele) were noted; these values were also significant after performing Bonferroni's correction.

Role of DLG5 and OCTN variants in disease susceptibility

DLG5 polymorphisms and disease susceptibility

The distribution of the three polymorphisms in the *DLG5* gene was similar in patients and controls. No significant differences in allele or genotype frequencies were noted (table 3). Odds ratios (OR) for carriers of at least one *DLG5* 113A allele and for carriers of at least one *DLG5* 4136A allele were 0.93 (95% confidence interval 0.71–1.21) and 0.85 (0.57–1.26) for CD and 1.02 (0.75–1.40) and 1.23 (0.80–1.88) for UC, respectively. Inverse ORs for the postulated[1] under-transmitted allele DLG5_e26 delA (genotypes DLG5_e26delAdelA and DLG5_e26delAinsA combined) were 0.98 (0.80–1.21) for CD and 0.99 (0.77–1.28) for UC.

OCTN polymorphisms and disease susceptibility

The strongest association with CD was noted for the polymorphism *SLC22A4* 1672C→T (allele frequencies for the T allele 47.1% in CD compared with 42.0% in controls; p = 0.0041) whereas for the promoter polymorphism −207G→C in *SLC22A5* frequencies for the C allele were 51.4% in CD compared with 46.9% in controls (p = 0.0128) (table 3). The two point haplotype consisting of the alleles 1672T and −207C (OCTN-TC) was associated with CD (47.0% frequency in CD patients *v* 42.0% in controls; p = 0.0060, $p_c = 0.0240$; OR 1.22 (1.06–1.41)) (table 3). The risk associated with the TC haplotype was only observed in homozygotes, which were more frequent in CD compared with controls (24.5% *v* 16.4%; p = 0.00008, $p_c = 0.0006$; OR

Table 4 Frequency of the OCTN-TC haplotype and of the IGR2078a_1A risk associated allele stratified by clinical phenotype of Crohn's disease

Subgroup	No of individuals (n = 412)	Frequency of the TC haplotype (%)	Significance *v* controls (p value*; OR (95% CI))	Frequency of IGR2078a_1A (%)	Significance *v* controls (p value*; OR (95% CI))
Disease location					
Ileal and no colonic	50	40.0	NS	37.0	NS
Colonic and no ileal	62	50.8	NS	49.1	NS
Any colonic disease	360	48.6	0.0025; 1.31 (1.10–1.55)	47.5	0.0089; 1.29 (1.26–1.57)
Disease behaviour					
Any fistulising†	223	46.9	NS	45.7	NS
Any fibrostenotic†	262	46.4	NS	45.0	NS
Non-fistulising non-fibrostenotic	92	53.3	0.0040; 1.57 (1.15–2.15)	52.2	0.0056; 1.56 (1.13–2.14)
Need for surgery					
Ileocaecal resection	144	43.1	NS	43.1	NS
No ileocaecal resection	268	50.0	0.0011; 1.38 (1.14–1.68)	48.3	0.0048; 1.33 (1.09–1.63)

*p values given were significant (p<0.05), also after application of Bonferroni's correction. †Data were also analysed for isolated fistulising or fibrostenotic disease behaviour but no further significant association was noted.

Table 5 Frequency of the homozygous OCTN-TC haplotype and of the homozygous IGR2078a_1AA genotype stratified by clinical phenotype of Crohn's disease

Subgroup	No of individuals (n = 412)	Frequency of the TC haplotype (%)	Significance *v* controls (p value*; OR (95% CI)	Frequency of IGR2078a_1A (%)	Significance *v* controls (p value*; OR (95% CI)
Disease location					
Ileal and no colonic	50	10.0	NS	8.0	NS
Colonic and no ileal	62	33.9	0.0008; 2.61 (1.45–4.68)	31.7	0.0032; 2.45 (1.32–4.50)
Any colonic disease	360	26.1	<0.0001; 1.80 (1.34–2.43)	23.9	0.0016; 1.66 (1.20–2.28)
Disease behaviour					
Any fistulising†	223	23.8	NS	21.5	NS
Any fibrostenotic†	262	24.4	NS	21.4	NS
Non-fistulising non-fibrostenotic	92	27.2	NS	26.1	NS
Need for surgery					
Ileocaecal resection	144	22.9	NS	21.5	NS
No ileocaecal resection	268	25.0	0.0016; 1.70 (1.21–2.37)	22.4	NS

*p values given were significant ($p<0.05$), also after application of Bonferroni's correction. †Data were also analysed for isolated fistulising or fibrostenotic disease behaviour but no further significant association was noted.

1.65 (1.28–2.13)). The frequency of heterozygotes, in contrast, was lower in CD patients than in controls (45.1% *v* 51.2%; OR 0.78 (0.64–0.96)). No differences in haplotype frequencies were found between patients with UC and controls (table 3).

Linkage disequilibrium analysis of OCTN variants with the IGR2078a_1 marker

The OCTN polymorphisms were in strong linkage disequilibrium with the marker IGR2078a_1 and among themselves (values of D' ranging from 0.96–1.00, r^2 from 0.74 to 0.87). The frequency of the risk associated allele IGR2078a_1A was higher in patients with CD compared with controls (45.4% *v* 40.9%; $p = 0.0128$). Consistent with previous observations,[18] in the present study a significant association with CD was obtained only for the homozygous genotype of the *IBD5* risk haplotype (IGR2078a_1AA) which was present in 22.0% of patients with CD compared with 15.7% of controls ($p = 0.0013$, $p_c = 0.0039$; OR = 1.53 (1.18–2.00)).

Among individuals lacking *IBD5* risk haplotypes (homozygous with respect to the non-risk associated allele of IGR2078a_1), only 19.1% of patients with CD but 27.8% of controls carried at least one 1672T or −207C allele ($p = 0.0344$, OR 0.62 (0.39–0.97)). Thus in the absence of the IGR2078a_1 risk allele, the OCTN-TC haplotype occurred less frequently in patients than in controls, which contrasts with the data of Peltekova *et al*.[3] However, statistical analysis of reconstructed haplotypes as well as conditional logistic analysis (UNPHASED) performed to evaluate the relative importance of the three markers showed the strongest effect in case of the polymorphisms *SLC22A4* 1672C→T. A stronger effect for this variant, which designated it as the most important factor in this region among the three factors studied, was confirmed by genotype logistic regression analysis.[17]

CD phenotypes

Although no association of the three polymorphisms in the *DLG5* gene with IBD was observed, we further tested for possible associations with CD phenotypes. However, no significant associations with respect to disease location, disease behaviour, need for surgery, or age at disease onset were obtained (data not shown).

The highest frequency of the OCTN-TC haplotype was found in patients with colonic disease only (50.8%) compared with 48.1% in both ileal and colonic disease and 40% in isolated ileal disease (table 4). Due to this observation and the small number of patients with disease confined to one site, we further analysed the association of the OCTN-TC haplotype with any colonic disease, irrespective of the presence of ileal disease. Notably, the association of the TC haplotype with CD was observed only in patients with colonic involvement, where the frequency of the TC haplotype was 48.6%, compared with 42% in controls ($p = 0.0025$, $p_c = 0.0196$; OR = 1.31 (1.10–1.56)). In patients with ileal disease only, such an association was not noted (40% in patients *v* 42% in controls; NS). Similar to the overall disease association, a dose effect for the homozygous OCTN-TC haplotype was observed for colonic disease (26.1% in patients with colonic involvement *v* 16.4% in controls; $p = 0.00008$, $p_c = 0.0013$; OR 1.80 (1.34–2.42)) while patients with isolated ileal disease displayed a frequency of 10 % (table 5).

In the presence of at least one OCTN-TC haplotype (homozygous or heterozygous for the TC haplotype), the non-fistulising, non-fibrostenotic phenotype was more prevalent than in patients without an OCTN-TC haplotype (25% *v* 15%) but this difference was not statistically significant. Compared with controls, the frequency of the TC haplotype was significantly increased in patients with the non-fistulising, non-fibrostenotic phenotype (53.3% *v* 42% in controls; $p = 0.0040$, $p_c = 0.0320$; OR 1.57 (1.15–2.15)) whereas in patients with fistulising or fibrostenotic disease this difference was not significant (45.9% *v* 42% in controls; NS) (table 4). No dose effect for the homozygous TC haplotype was noted (table 5).

The need for ileocaecal resection was lower in patients with at least one OCTN-TC haplotype compared with patients lacking this haplotype (32.2% *v* 44.2%; $p = 0.016$, OR 1.7 (1.1–2.7)) (tables 4, 5). In the vast majority of patients, ileocaecal resection had been performed in cases with both ileal and colonic involvement. Only a few patients with disease confined to the ileum and none of the patients with exclusive colonic CD had undergone ileocaecal resection.

We also evaluated the effect of the OCTN-TC haplotype on age at disease onset in 341 patients with CD. A significant earlier age at disease onset was observed in patients homozygous for the OCTN-TC haplotype (median age of 22 years (mean 24.8)) than in patients with one or no OCTN-TC haplotype (median age of 25 years (mean 28.6)) ($p = 0.011$).

Due to the strong linkage disequilibrium between the OCTN polymorphisms and the IGR2078a_1 marker, similar associations with colonic involvement, non-fistulising, non-fibrostenotic disease behaviour, and the need for surgery were observed for the *IBD5* susceptibility marker IGR2078a_1 (tables 4, 5), and an association of the IGR2078a_1AA genotype with earlier age at disease onset was also noted ($p = 0.023$).

Table 6 Interaction of *DLG* genotypes and OCTN haplotypes with risk associated *CARD15* mutations in Crohn's disease

		CARD15 mutant (risk associated)	*CARD15* wild-type (non-risk associated)	p Value
DLG5 genotype				
113G→A	GG	193 (81.4%)	308 (81.5%)	NS
	AG	40 (16.9%)	67 (17.7%)	
	AA	4 (1.7%)	3 (0.8%)	
4136C→A	CC	221 (93.2%)	348 (92.1%)	NS
	AC	16 (6.8%)	28 (7.4%)	
	AA	0 (0.0%)	2 (0.5%)	
DLG5_e26	insAinsA	98 (41.3%)	162 (42.8%)	NS
	insAdelA	109 (46.0%)	176 (46.6%)	
	delAdelA	30 (12.7%)	40 (10.6%)	
SLC22A4				
1672C→T	CC	59 (24.7%)	130 (33.7%)	0.047
	CT	114 (47.7%)	169 (43.8%)	
	TT	66 (27.6%)	87 (22.5%)	
SLC22A5				
−207G→C	GG	110 (27.2%)	53 (22.2%)	NS
	GC	171 (44.3%)	111 (46.4%)	
	CC	105 (28.5%)	75 (31.4%)	
OCTN haplotype *SLC22A4/SLC22A5*				
	C/G	215 (45.0%)	391 (50.7%)	
	T/C	244 (51.0%)	343 (44.4%)	0.0203
	C/C	17 (3.6%)	38 (4.9%)	
	T/G	2 (0.4%)	0 (0.0%)	

Interaction of *DLG5* and OCTN mutations with the *CARD15/NOD2* genotype

In our population, 38.2% of the patients with CD carried at least one *CARD15* mutant (R702W, G908R, or 1007 fs). We tested for epistasis between *DLG5* variants and disease associated *CARD15* mutations (table 6). The frequency of the *DLG5* 113A allele in CD patients carrying risk associated *CARD15* alleles was comparable with that observed in patients lacking these variants (10% *v* 9.7%; NS). Further significant interactions between risk associated variants of *CARD15* and the *DLG5* polymorphisms were also not observed.

Significant interactions regarding CARD15 status were obtained for the *SLC22A4* 1672C→T polymorphism and the two point OCTN-TC haplotype ($p = 0.047$ and $p = 0.0203$, respectively) (table 6). In patients with CD carrying at least one risk associated *CARD15* mutation, the frequency of the OCTN-TC haplotype was increased compared with patients with non-risk associated variants of *CARD15* (51% *v* 44.4%). The association of this haplotype with CD was stronger in individuals with risk associated *CARD15* mutations (51 *v* 42% in controls; $p = 0.0004$, $p_c = 0.0033$; OR 1.44 (1.17–1.77)) but was not significant in CD patients with non-risk associated variants of *CARD15* (44.4 *v* 42% in controls; NS). Again, a dose effect for the homozygous TC haplotype was noted, which was present in 27.2% of patients with and in 22.8% of patients without CD associated *CARD15* mutations.

DISCUSSION

In order to reproduce the previously described[1] association of IBD with variations in the *DLG5* gene, the two haplotype tagging SNPs 113G→A (R30Q) and DLG5_e26 and the SNP 4136 C→A (P1371Q) were studied in a population of comparable size and equal ethnic background. In our case control analysis, we could not confirm the association of these three SNPs with CD or UC. Not even a tendency towards an increased frequency of the *DLG5* variants R30Q and P1317Q in CD was noted and the frequencies of these variants among CD patients were lower than in controls (table 3). Differences in the frequencies for the DLG5_e26 alleles were minimal. Ethnic differences can be excluded as a reason for the divergent results because all participants in the preceding study and in our study were Western Europeans. The genotype distribution in the control population was in accordance with that predicted by the Hardy-Weinberg equilibrium, which makes methodological flaws unlikely. To rule out disease heterogeneity between the two populations as a possible cause, we further tested if the polymorphisms in the *DLG5* gene were associated with specific clinical subgroups of CD. However, significant differences in the distribution of the *DLG5* polymorphisms after stratification of CD patients with respect to disease location and behaviour or need for surgery were not found. Moreover, the frequency of mutations in the *CARD15* gene in our CD population was in accordance with that reported for CD in other European populations (data not shown).[19] Significant interactions between the *DLG5* polymorphisms and disease associated *CARD15* mutations were also not seen here. Taken together, our observations strongly argue against a role for the three *DLG5* polymorphisms in susceptibility to CD. Although the polymorphisms studied here showed no association with CD or UC, it cannot be ruled out that other variants encoded by this gene contribute to susceptibility to IBD. A recently published study reported the association of another variant in *DLG5* with CD in a Japanese population.[20] However, as demonstrated by the absence of disease associated *CARD15* mutations, significant ethnic differences between the Japanese and Caucasians exist.[21 22]

Peltekova and colleagues[3] reported identification of two new SNPs in the OCTN gene cluster at 5q31 (*IBD5*), with significant association to CD. The polymorphisms 1672C→T (L503F) in *SLC22A4* and −207G→C in *SLC22A5* create a two allele risk haplotype (OCTN-TC) which was found with increased frequency in patients with CD and showed significant interactions with CD associated *CARD15* mutations. The authors performed preliminary functional experiments and demonstrated that the two SNPs impair transcription and transporter function of the OCTNs, thereby reducing carnitine transport.[3] Based on these functional implications and on the observation that the OCTN variants were significantly associated with CD, also in the absence of *IBD5* risk haplotypes, the authors suggested that the *SLC22A4*

and *SLC22A5* 1672T and −207C variants per se rather than other alleles in the *IBD5* susceptibility locus were causative for CD.

In the present study, the association of the *SLC22A4* and *SLC22A5* 1672T and −207C variants and of the respective two allele haplotype (TC) with CD was confirmed. However, the frequency of the OCTN-TC haplotype in our CD patients was significantly lower than in the original Canadian CD population[3] (47% *v* 54%) whereas the frequency in both control populations was equal (42%). This observation is compatible with other studies reporting a lower risk conferred by the *IBD5* locus in Europeans.[5 7 18] In line with these observations, the risk associated with the OCTN-TC haplotype was only observed in homozygotes and not in heterozygotes. The OR for this haplotype was 1.65 in TC homozygotes, which is substantially lower than the 27% population risk in the study by Peltekova and colleagues[3] or the ORs obtained in the extended study of the same group[23] (ORs 3.73 and 2.25 for non-Jewish and Jewish populations, respectively). The Canadian population in the original studies[3 23] seems to partially superpose with the population used in the initial linkage disequilibrium study by Rioux and colleagues,[4] or at least it has the same provenance.[24] However, the *IBD5* related risk increase reported in this population was greater than that observed in the replication study from Quebec[5] and in the other European populations.[7 18] Significant phenotypic differences between our population and the population in the original studies were not observed.

We also tested for interactions between the OCTN-TC haplotype and the IGR2078a_1 marker. In contrast with the preceding studies,[3 23] in the absence of an IGR2078a_1 risk allele, the OCTN-TC haplotype and risk alleles occurred less frequently in patients than in controls (19.1% *v* 27.8%; $p = 0.0344$). However, statistical analysis performed to assess the relative importance of each of the three markers showed the strongest effect in case of the polymorphism *SLC22A4* 1672C→T. Due to the relative position of the IGR2078a_1 marker and the two SLC22 genes,[4] investigation of further markers in the vicinity of the SLC22A4 polymorphisms seems to be of interest.

We further investigated the association of the OCTN polymorphisms with CD phenotypes. A higher frequency of the OCTN-TC haplotype was observed in patients with colonic involvement compared with exclusive ileal disease. Carriage of the homozygous OCTN-TC haplotype was associated with a higher relative risk for colonic disease (OR 1.8; tables 4, 5). Importantly, in the absence of colonic involvement, the OCTN-TC haplotype was not associated with CD. This observation contrasts with previous observations which describe no association between *IBD5*[5] and OCTN variants[23] with subgroups of patients with CD, respectively. However, in the study by Armuzzi and colleagues,[18] a lower frequency for the *IBD5* risk haplotype in disease restricted to the ileum compared with colonic disease only was reported. We found a moderate increase in the frequency of the TC haplotype among patients without fistulas or stenosis. Compatible with this observation and the negative association with ileal involvement, a tendency towards a lower frequency of ileocaecal resection in the presence of at least one OCTN-TC haplotype was noted (table 4). A significantly younger age at disease onset was observed in patients homozygous for the OCTN-TC haplotype compared with patients carrying only one or no OCTN-TC haplotype ($p = 0.011$). This is in agreement with the reported younger age of disease onset in patients with the homozygous *IBD5* risk haplotype.[4 7]

In the present study, an association with the OCTN-TC haplotype was only seen in patients with one or two mutated *CARD15* alleles and not in the case of the wild-type. This is in accordance with the analysis by Peltekova and colleagues,[3] and with preceding observations reporting epistasis between genetic variants at the 5q31 locus and *CARD15*.[7] However, the data regarding interactions between *IBD5* and *CARD15* are conflicting. Some studies reported an independent action in conferring risk to CD[5 6 18] but epistasis with respect to development of UC.[5 8] Here, we found no association of the OCTN polymorphisms with UC.

In summary, we were unable to reproduce the previously described association of the *DLG5* polymorphisms 113G→A, 4136C→A, and DLG5_e26 with CD in a cohort of comparable size and equal ethnic background. Thus our data argue against a role for these polymorphisms in susceptibility to CD. On the other hand, the reported association of the *SLC22A4* 1672T and *SLC22A5* −207C alleles with CD and the interaction of the respective two allele haplotype TC with risk associated *CARD15* mutations was confirmed. Additionally, we provided evidence for an association of this risk haplotype with colonic involvement in CD, earlier age at disease onset, decreased prevalence of the fistulising and fibrostenotic phenotype, and reduced need for surgery. However, given the overall weak association of the polymorphisms in the *SLC22A* genes observed here and the strong linkage disequilibrium in the 5q31 region, extended linkage disequilibrium analyses of these OCTN1 and OCTN2 variants with the *IBD5* haplotype tagged SNPs are needed before definitive conclusions about *SLC22A4* and *SLC22A5* as causative genes for CD can be drawn.

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