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Variations in the *IBD5* locus confer the risk of inflammatory bowel disease in a Manitoban Caucasian Cohort

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Abstract

Background: Crohn´s disease (CD) and ulcerative colitis (UC) are two distinct manifestations of inflammatory bowel disease (IBD). Polymorphisms in the *SLC22A4* and *SLC22A5* genes were associated within the IBD5 locus, but their contribution to the pathology remains unclear.

Objective: This study investigated the association to IBD of common and rare variations within the *SLC22A4* and *SLC22A5* genes in the Manitoban IBD cohort.

Design: DNA samples from 160 CD patients, 149 UC patients and 142 age and gender matched healthy controls were genotyped for selected single nucleotide polymorphisms (SNPs) tagging both genes.

Results: The *SLC22A5* genotypes rs11739135-CC and rs17622208-AA associated with increased susceptibility for CD (OR=7.84, 95% CI 2.84-21.6, p=0.000; OR=2.26, 95% CI 1.14-4.44, p=0.019, respectively). Moreover, rs11739135-CC homozygosity was associated with UC (OR=4.18, 95% CI 1.48-11.78, p=0.007). None of the common polymorphisms tested in *SLC22A4* were associated with either CD or UC. Two rarer genotypes in *SLC22A4*, rs11568500-A and rs11568510-G, were not detected.

Conclusion: Variations in the proximal part of the *SLC22A5* gene associated with IBD distinct from other variations in the IBD5 locus, including those of *SLC22A4*. Therefore, disturbed carnitine transport might be involved in IBD etiology in a small percentage of individuals.

Introduction

Crohn´s disease (CD) and ulcerative colitis (UC) are the main manifestations of inflammatory bowel disease (IBD). The disease arises from a complex interplay of environmental, host immune dysregulations and genetic factors [1].

The IBD5 locus in chromosome *5q31* was first identified to confer CD risk in a Canadian population [1], and in further analysis a 250 kb *IBD5* haplotype was associated to CD [2]. Both, the susceptibility to CD and UC were located to *IBD5* in a German cohort [3]. The IBD5 genomic region contains immune related genes: interleukin-4 (IL4), IL13, IL5 and interferon regulatory factor-1 (IRF1), but it also contains two organic cation/carnitine transporters *SLC22A4* and *SLC22A5*.

It was suggested by Peltekova et al. [4] that the non-synonymous single nucleotide polymorphism (SNP) rs1050152, located in *SLC22A4* exon 9, and the SNP rs2631367, located in the *SLC22A5* 5´UTR, were true functional polymorphisms determining CD risk in the *IBD5* locus in a haplotype-independent manner. The associations in the *IBD5* locus have been replicated [5-12], but not independently from linked SNPs in the *IBD5* haplotype.

Moreover, the *IBD5* locus including *SLC22A4* and *SLC22A5* has been associated with UC [3,11,13,14], but associations were not replicated in a Canadian [7], a Belgian [15], and other cohorts of Asian ancestry [16, 17]. Meta-analyses suggest that SNPs rs12521868 (IGR2096), rs11739135 (IGR2198) and rs17622208 (IGR2230) tag the IBD5 locus and are in linkage with *SLC22A4*-rs1050152 and *SLC22A5*-rs2631367 and associated with CD and UC in Caucasian cohorts [18,19].

Taken together, there is possible evidence that functional genetic variations in the *SLC22A4* and *SLC22A5* genes, encoding organic cation transporter proteins OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*), contribute to IBD risk. However, it remains undetermined if the variants act independent, or together with each other or nearby variations in immunity-related genes. Therefore, we investigated associations of both common and rarer variations in the *SLC22A4* gene and common variations in the *SLC22A5* gene in a cohort of Caucasian individuals in Manitoba, Canada. We included rarer variations in *SLC22A4* known to abrogate the protein's function since we hypothesize that they would be observed in the disease cohort if elimination of the gene would have a role in disease development.

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Subjects and methods

Study population

The study population included 311 IBD patients from the Manitoba Inflammatory Bowel Disease Cohort Study that has been described previously [20]. We included Caucasian age and gender matched CD (n=162), and UC (n= 149) patients as well as healthy controls (n=142). The diagnosis and classification of CD and UC was determined based on radiologic, endoscopic and histological data as established based on the Montreal classification [21]. The phenotypic characteristics of CD and UC patients are shown in Table 1.

Genotyping

All protocols were approved by the University of Manitoba Research Ethics Committee. Genomic DNA was isolated from peripheral blood as described previously [22]. The common SNPs rs1050152 (*SLC22A4*), rs17622208 (*SLC22A5*), rs11739135, (3' of *SLC22A5*) and rs12521868 (*C5orf56*), previous reported to tag the *IBD5* locus and the rare functional SNPs rs11568500, rs11568510 in *SLC22A4* were genotyped by PCR-RFLP analysis.

The PCR amplifications were performed in the NEB Taq Polymerase 5X Master Mix (New England BioLabs) following the manufacturer's protocol under the following cycling conditions and the primers listed in Table 2, initial denaturation at 95°C for 30s, followed by 35 cycles of denaturation at 95°C for 15s, annealing at 50°C for 15s, extension at 68°C for 2 min and final extension at 68°C for 5 min.

The amplicons were digested by allele-specific restriction endonucleases (New England BioLabs) according to manufacturer's protocols as listed in Table 2. Restriction patterns were analyzed by gel electrophoresis in a 2% Ultrapure agarose gel (Invitrogen) after ethidium bromide staining under UV light (Gel Doc, BIO-RAD). Amplicons of known genotype for every SNP were sub cloned using polyAA cloning (TOPO®TA Cloning, Invitrogen) and used as positive and negative controls for further PCR and restriction analysis.

Statistical Analyses

SNPs were tested for Hardy-Weinberg equilibrium. The case-control associations of genotype and allele frequencies of each SNP were tested using binary logistic regression. Odds ratios (OR) were calculated with 95% confidence interval (CI) using 2x2 contingency tables and x^2 test. The analysis was carried out using SPSS 18.0. The linkage disequilibrium (LD) and haplotype analysis in the *IBD5* region was performed with Haploview 4.2 [23].

Table 1. Phenotypic characteristics of the Caucasian IBD cohort

	Crohn´s Disease cohort (n=154)	Ulcerative Colitis cohort (n= 143)
Gender		
Female	91 (59.1%)	87 (60.8%)
Male	63 (40.9%)	56 (39.2%)
Age at diagnosis		
A1(<16 years)	14 (9.1%)	12 (8.4%)
A2 (16-40 years)	101 (65.6%)	78 (54.5%)
A3 (>40 years)	39 (25.3%)	53 (37.1%)
Location		
L1 (Ileal)	69 (44.8%)	-
L2 (Colonic)	33 (21.4%)	-
L3 (Ileocolonic)	51 (33.1%)	-
L4 (isolated upper disease)	1 (0.6%)	-
E1 (UP limited to rectum)	-	10 (7%)
E2 (Left sided, distal)	-	66 (46.2%)
E3 (extensive, pancolitis)	-	67 (46.9%)
Behaviour		
B1(Inflammatory)	66 (42.9%)	-
B2 (Stricturing)	51 (33.1%)	-
B3 (Penetrating/fistulizing)	37 (24%)	-

Table 2. Primer sequence, restriction enzymes and cutting pattern for RFLP genotyping

Gene	dbSNP	Primer Sequence 5´- 3´	Endonuclease	Cutting pattern (bp)
SLC22A4	rs1050152	Forward: TTGATGTTCTTATGTCCCGG	MnlI	C: 212+97 bp
		Reverse: TGTGCCCAGCCAACAATATG		T: 309 bp
SLC22A4	rs11568500	Forward: ACCTTGGCAACCTACACATC	Sau96I	G:168 bp
		Reverse: TTCAGAGGGTTAGAGGGA		A:85bp
SLC22A4	rs11568510	Forward: TTCCTTGGCAGTGGAATCTG	BspmI	A:312 bp
		Reverse: GAACAAAAGTGTGTCCAGGT		G:203+109 bp
IGR2096	rs12521868	Forward: ATCCTCCATGCTACTGCT	DraI	G: 308 bp
		Reverse: TGGTGTAGCCAGAGTAGA		T:159 + 149 bp
IGR2198	rs11739135	Forward: ACTGGCTCTTTACCTGGGAA	SfaNI	G: 369 bp
		Reverse: AACTAGTCCCAACGAGATGA		C:245 + 124 bp
SLC22A5 IGR2230	rs17622208	Forward: AGGTCTATTCCCAGGGAA	DdeI	G: 164 + 119 bp
		Reverse: ACTCAGAAGCTGTCCATC		A:283 bp

Results

Common SNPs and haplotypes in the *SLC22A5* gene are associated with Crohn's Disease

Two SNPs in the *SLC22A5* gene associated with the risk of CD, and as a likely consequence several haplotypes inferred of these SNPs associated also with CD (Table 3). Specifically, carriers of the SNP rs17622208 A-allele showed an 40% elevated risk for CD (OR= 1.4; 95% CI 1.01-1.92; p=0.04), consistently elevating the risk for the combined genotypes AA/GA (OR= 2.76; 95% CI 1.54-4.95; p=0.001) (Table 3).

Similarly, carriers of the SNP rs11739135 C-allele showed an 80% elevated risk for CD (OR=1.8; 95% CI 1.28-2.5; p= 0.000). Significantly, the disease risk for rs11739135-CC homozygotes is strongly elevated with an OR of 7.84 (95% CI 2.84-21.6; p=0.000) through the fact that 20.6% of CD patients, but only 3.5% of healthy controls carried that genotype (Table 3).

Neither of the other two common tag-SNPs in the *IBD5* locus associated to CD, but the haplotype TACT [inferred from SNPs rs1050152, rs17622208, rs11739135, and rs12521868, respectively]

Table 3. Genotype and allele frequencies in Crohn´s disease and control subjects

	Crohn´s disease (*n*= 160)	**Controls (*n*=142)**	**OR (95% CI)**	***p***
SLC22A4 rs11568510				
Exon 2				
AA	160 (100%)	142 (100%)	ND	
GG	0 (0%)	0 (0%)	ND	
SLC22A4 rs11568500				
Exon 3				
GG	160 (100%)	142 (100%)	ND	
AA	0 (0%)	0 (0%)	ND	
SLC22A4 rs1050152				
Exon 9	*n*= 160	*n*=142		
CC	42 (26.3%)	51 (35.9%)	Ref.	
CT	79 (49.4%)	61 (43%)	1.57 (0.93-2.66)	0.09
TT	39 (24.4%)	30 (21.2%)	1.58 (0.84-2.95)	0.15
CT + TT	118 (73.8%)	91 (64.1%)	1.60 (0.96-2.57)	0.07
C allele	163 (51%)	163 (57%)	0.77 (0.56- 1.06)	0.11
T allele	157 (49%)	121 (43%)	1.3 (0-94-1.8)	0.11
***SLC22A5* rs17622208**				
Intron 2	*n*= 159	*n*=142		
GG	21 (13.2%)	42 (29.6%)	Ref.	
GA	94 (59.1%)	61 (43%)	3.08(1.67-5.70)	0.000
AA	44 (27.7%)	39 (27.5%)	2.26 (1.14-4.44)	0.019
GA + AA	138 (86.8%)	100(70.4%)	2.76 (1.54-4.95)	0.001
G allele	136 (43%)	145 (51%)	0.72 (0.52-0.98)	0.04
A allele	182 (57%)	139 (49%)	1.4 (1.01-1.92)	0.04
***SLC22A5* rs11739135**				
Intergenic near 3'	*n*= 160	*n*=142		
GG	48 (30%)	57 (40.1%)	Ref.	
GC	79 (49.4%)	80 (56.3%)	1.17 (0.72-1.92)	0.53
CC	33 (20.6%)	5 (3.5%)	7.84 (2.84-21.6)	0.000
GC + CC	112 (70%)	85 (59.8%)	1.56 (0.97-2.52)	0.06
G allele	175 (55%)	194 (68%)	0.56 (0.40-0.78)	0.000
C allele	145 (45%)	90 (32%)	1.8 (1.28-2.5)	0.000
***C5orf56* rs12521868**				
Intron 2	*n*= 159	*n*=142		
GG	43 (27%)	53 (37.3%)	Ref.	
GT	83 (52.2%)	62 (43.7%)	1.65 (0.98-2.77)	0.06
TT	33 (20.8%)	27 (19%)	1.51 (0.78-2.88)	0.22
GT + TT	116 (72.9%)	89 (62.7%)	1.61 (0.98-2.62)	0.06
G allele	169 (53%)	168 (59%)	0.78 (0.57-1.08)	0.13
T allele	149 (47%)	116 (41%)	1.28 (0.92-1.76)	0.13
IBD5 Haplotype[a]				
CGGG	59 (36.4%)	71 (50%)	0.55 (0.33-0.93)	0.02
TACT	63 (38.8%)	42 (29.8%)	1.8 (1.07-3.04)	0.02
CAGG	21 (12.9%	9 (6.3%)	2.8 (1.87-3.84)	0.00
TGCT	7 (4.2%)	1 (0.3%)	4.8 (2.97-6.45)	0.00
TAGT	6 (3.2%)	14 (9.7%)	0.45 (0.33-0.65)	0.00

[a] Haplotypes were formed by the SNPs rs1050152, rs17622208, rs11739135, rs12521868, respectively.

reflected an elevated risk for CD (OR=1.8; 95% CI 1.07-3.04; p=0.02). Other haplotypes also associated with CD, such as haplotype CAGG, which was found in 12.9% of CD patients as compared to 6.3% in healthy controls (p=0.00). Moreover, haplotype TGCT was carried by 4.2% of CD patients compared to 0.3% in healthy controls (p=0.00). The haplotype CGGG conferred protection from CD (OR=0.55; 95% CI 0.33-0.93; p=0.02) (Table 3).

CC-homozygosity for SNP rs11739135 in the *SLC22A5* gene elevated the risk for Ulcerative Colitis

SNP rs11739135-CC homozygotes showed an almost 3-fold elevated risk for UC (OR=4.18; 95% CI 1.48-11.78; p=0.07), due to overrepresentation in 14.8% of CD patients compared to 3.5% in healthy controls (Table 4). No other tested SNP or haplotype had an impact on the risk for UC.

The rare genotypes rs11568500-A and rs11568510-G in the *SLC22A4* gene were not present in the patient cohort

The rare functional polymorphisms rs11568500-A and rs11568510-G in *SLC22A4* were not present in the CD and UC patients or in the control groups (Tables 3,4). This Caucasian population carried only the homozygous ancestral genotype.

Discussion

None of the common SNPs in the *IBD5* locus except in the *SLC22A5* gene associated with IBD, where the alleles rs17622208-A and rs11739135-C elevated the risk for CD, and rs11739135-C for UC. This confers with previously reported associations for the *SLC22A5* gene. However, does not replicate previous associations in the flanking genes *SLC22A4* and *C5orf56*.

Table 4. Genotype and allele frequencies in ulcerative colitis and control subjects

	Ulcerative colitis (*n*= 149)	Controls (*n*=142)	OR (95% CI)	*p*
***SLC22A4* rs11568510** *Exon 2*				
AA	149 (100%)	142 (100%)	ND	
GG	0 (0%)	0 (0%)	ND	
***SLC22A4* rs11568500** *Exon 3*				
GG	149 (100%)	142 (100%)	ND	
AA	0 (0%)	0 (0%)	ND	
***SLC22A4* rs1050152** *Exon 9*				
CC	54 (36.2%)	51 (35.9%)	Ref.	
CT	69 (46.3%)	61 (43%)	1.07 (0.64-1.79)	0.80
TT	26 (17.4%)	30 (21.2%)	0.82 (0.43-1.57)	0.55
CT + TT	95 (63.8%)	91 (64.1%)	0.99 (0.61-1.59)	0.95
C allele	177 (59%)	163 (57%)	1.09 (0.78-1.51)	0.62
T allele	121 (41%)	121 (43%)	0.92 (0.66-1.28)	0.62
***SLC22A5* rs17622208** *Intron 2*				
GG	44 (29.5%)	42 (29.6%)	Ref.	
GA	76 (51%)	61 (43%)	1.19 (0.69-2.04)	0.53
AA	29 (19.5%)	39 (27.5%)	0.71 (0.37-1.35)	0.29
GA + AA	105 (70.5%)	100(70.4%)	1.00 (0.61-1.66)	0.99
G allele	164 (55%)	145 (51%)	1.17 (0.85-1.62)	0.33
A allele	134 (45%)	139 (49%)	0.85 (0.61-1.18)	0.33
***SLC22A5* rs11739135** *Intergenic near 3'*				
GG	60 (40.3%)	57 (40.1%)	Ref.	
GC	67 (45%)	80 (56.3%)	0.79 (0.49-1.29)	0.36
CC	22 (14.8%)	5 (3.5%)	4.18(1.48-11.78)	0.007
GC + CC	89 (59.7%)	85 (59.8%)	0.99 (0.62-1.59)	0.98
G allele	187 (63%)	194 (68%)	0.78 (0.55-1.10)	0.15
C allele	111 (37%)	90 (32%)	1.28 (0.91-1.80)	0.15
***C5orf56* rs12521868** *Intron 2*				
GG	58 (38.9%)	53 (37.3%)	Ref.	
GT	67 (45%)	62 (43.7%)	0.99 (0.59-1.64)	0.96
TT	24 (16.1%)	27 (19%)	0.81 (0.42-1.58)	0.54
GT + TT	91 (61.1%)	89 (62.7%)	0.93 (0.58-1.50)	0.78
G allele	183 (61%)	168 (59%)	1.10 (0.78-1.53)	0.58
T allele	115 (39%)	116 (41%)	0.91 (0.65-1.27)	0.58
***IBD5* Haplotype[a]**				
CGGG	74 (49.4%)	72 (50.2%)	0.96 (0.57-1.63)	0.88
TACT	46 (30.9%)	43 (29.7%)	1.04 (0.61-1.76)	0.88

[a]Haplotypes were formed by the SNPs rs1050152, rs17622208, rs11739135, rs12521868. All SNPs conferred to the Hardy-Weinberg equilibrium.

The common nonsynonymous SNP rs1050152-T in *SLC22A4* which encodes amino acid 503F was previously reported by Peltekova et al. [4] to be present in 53% of CD cases but only 23% of healthy control, indicating a strong disease association, and these findings had been replicated in different cohorts [9,24,25]. However, this association could not be replicated by others (5,6,14,15,26). For example, Waller *et al.* [14] found that 27% of CD cases and 22% of controls carried rs1050152-T. Similarly, we did not find the rs1050152-T associated with IBD in our Manitoba Caucasian cohort were 49% of CD patients, 41% of UC patients, and 43% of controls carried the risk genotype. These findings support the recently formulated hypothesis that the increased rs1050152-T frequency in IBD cases is related to recent positive selection in the IBD5 locus and that other linked disease-causing variants have hitchhiked to relatively high frequency to determine the risk haplotype [27]. They postulated that a recombination breakpoint exists telomeric of *SLC22A4* and that the causative variations are located in the genetic region after that breakpoint, which includes *SLC22A5*. Our data are consistent with this hypothesis, since we did not see disease associations in *SLC22A4*. Moreover, haplotype analysis by Waller *et al.* [14] and Silverberg *et al.* [11] does indicated that *SLC22A4* and *SLC22A5* lie in distinct linkage blocks, therefore variations in both genes could independently modify the disease risk. This could explain that *SLC22A4* is not involved in disease etiology in our cohort, but in others.

The assumption that *SLC22A4* is not involved in disease etiology is also supported by the fact that we did not find the two rarer *SLC22A4* functional variations rs11568510-G and rs11568500-A, which abrogate transport activity for the organic cation TEA totally or by 50%, respectively [28]. We had chosen to genotype both rarer SNPs due to their proven impact on the proteins function to query the model of "genetic heterogeneity", which postulates that the genetic contribution to complex traits is determined by the abundance of rare genetic variants of high effect on the disease phenotype [29]. The absence of these detrimental variations also supports the assumption that *SLC22A4* variations do not determine the IBD risk in our cohort.

Our findings also differ from reports that both *SLC22A4* and *SLC22A5* SNPs are within a single genetic linkage block. This might be due to the fact that most studies reported associations for *SLC22A4/SLC22A5* haplotypes rs1050152/rs2631372 [9,24,25] and rs1050152/rs2631367 [4,14], where the tag-SNPs are located 5' of the *SLC22A5* gene, which could still be in a haplotype block with *SLC22A4*. Therefore, we assume that the previously reported eQTL-type [4,9,24] associations for *SLC22A5* with IBD are due to SNPs in the *SLC22A4* haplotype block. Considering the existing data for linkage breakage between the *SLC22A4* and *SLC22A5* genes just 5' of *SLC22A5* [11,14] we did choose tag-SNPs further located within *SLC22A5*. This explains why we could achieve distinct and independent associations for our cohort. These two SNPs in *SLC22A5* strongly elevated the risk for CD and UC are located in intron 2 (rs17622208) and distal to the 3'UTR (rs11739135), which makes both unlikely candidates to be the functional causal variation, which remain to be identified.

The *SLC22A5* gene encodes OCTN2, a carnitine transporter mediating cellular uptake, indicating a role of cellular carnitine deficiency in IBD. This is supported by the observation that intestinal levels of carnitine are reduced by 90% in *Slc22a5*$^{-/-}$ knockout mice, which develop spontaneous perforations, micro-abscess, necrotic villi leading to gut atrophy [30]. Neonate *Slc22a5*$^{-/-}$ mice showed increased enterocytes and lymphocytes apoptosis, which disturbs the epithelial barrier to initiate inflammatory processes [31]. Moreover, oral carnitine supplementation or local carnitine-liposomes administration reduced inflammation and histological damage in the murine trinitrobenzene sulphonic acid induced colitis [32,33]. Supplementation of propionyl-L-carnitine improved clinical and endoscopic response in UC [34]. Dietary carnitine might therefore be considered to be tested as a supplemental IBD treatment in individuals of the described *SLC22A5* genotypes.

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