Polymorphisms in the sodium-dependent ascorbate transporter gene *SLC23A1* are associated with susceptibility to Crohn's disease

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Abbreviations: IBD, Inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; ROS, reactive oxygen species; RNS, reactive nitrogen species; SNPs, single nucleotide polymorphisms

Keywords: Polymorphisms; Antioxidants; Vitamin C; Crohn's disease; Inflammatory bowel disease

ABSTRACT

Background: Crohn's disease (CD) and ulcerative colitis (UC) are two common inflammatory bowel diseases (IBD) associated with intestinal inflammation and tissue damage. Oxidative stress is suggested to play a major role in the initiation and progression of IBD. Supplementation of Vitamin C (ascorbate, ascorbic acid) has reduced oxidative stress in persons with IBD. The role of ascorbate transporters in IBD remains to be determined. *SLC23A1* is a major ascorbate transporter in the intestinal tract and some of its genetic variants have been associated with severely decreased ascorbate transport and lowered systemic levels. **Objective:** This study aimed to determine if common genetic variants in vitamin C transporter *SLC23A1* are associated with risk of IBD. **Design:** Genomic DNA samples from patients with CD (n=162) and UC (n=149) from the Manitoba IBD Cohort Study and ethnically matched controls (n=142) were genotyped for three *SLC23A1* polymorphisms (rs6596473, rs33972313, rs10063949) using TaqMan Assays. **Results:** variation at rs10063949 (G allele for heterozygote and homozygote) was associated with increased susceptibility to CD (OR=2.54, 95% CI 1.38, 4.66; OR=4.72, 95% CI 2.53, 8.81, $p<0.0001$; respectively). A strong linkage disequilibrium (LD) was observed across the *SLC23A1*region (variation rs6596473 with rs10063949) for CD and UC (D´=0.94, D´=0.96; respectively). The risk alleles confirmed a haplotype (CGG) which is carried more in CD patients (65.3%, $p<0.0001$) compared to controls (43.5%). **Conclusions:** A genetic variant (rs10063949-G) in the *SLC23A1* ascorbate transporter locus was identified which is associated with an increased risk of CD in a Caucasian Canadian IBD cohort. The presented evidence that *SLC23A1* variations can modulate the risk of CD has implications for understanding ascorbate transport in CD patients and provides a novel opportunity toward individualized nutritional therapy for the patients carrying the disease associated genotype.

INTRODUCTION

Inflammatory bowel disease (IBD) includes Crohn's disease (CD) and ulcerative colitis (UC), and results from the interface of environmental factors with an aberrant immune response in genetically susceptible individuals. IBD is accompanied by excessive production of reactive oxygen species which plays an important role in the pathogenesis of the disease through oxidative tissue damage (1). The antioxidant defense system of the intestinal mucosa is impaired in IBD patients which results in increased oxidative injury and eventually delayed recovery of the inflamed mucosa (2, 3). Intestinal biopsies from IBD patients have shown deficiencies and imbalances in the levels of different antioxidants, including Vitamin C (ascorbate) in inflamed mucosa compared with normal mucosa (2, 4).

Ascorbate is the primary essential water-soluble antioxidant from the diet which acts as direct scavenger of reactive oxygen species and contributes to prevention of oxidative damage. Ascorbate is also a redox cofactor for enzymes required for the synthesis of collagen, carnitine, and neurotransmitters (5-7). The antioxidant property of ascorbate is thought to prevent chronic diseases involving inflammatory events, including atherosclerosis (8-10), cancer (11), and type 2 diabetes (12, 13). In IBD patients, a loss of 35-73% total and reduced ascorbate has been observed in inflamed mucosa which contributes to the overall loss of its antioxidant capacity (4). Plasma deficiencies of ascorbate have also been observed in IBD patients (14-16) and has been attributed to extensive depletion of ascorbate as an antioxidant or to inadequate dietary uptake (17). Whether reduction in ascorbate levels in inflammatory tissue in IBD is more cause or effect is unknown.

The sodium-dependent ascorbate transporter 1 (SLC23A1) is the major ascorbate transporter in the intestinal epithelium (18, 19) and is also found in cells involved in the immune defense (20, 21). Cellular ascorbic acid uptake is enhanced when SLC23A1 is present (19, 22, 23). Global elimination of *slc23a1* in the mouse results in a dramatic decrease of ascorbic acid levels in cells and organs expressing *slc23a1* in the wild type (24). A non-synonymous genetic variation has been shown to reduce the transporters capacity by approximately 90% *in vitro* (24). We hypothesized that variation in the human *SLC23A1* gene down regulates transporter activity and reduces intracellular antioxidant capacity resulting in impeding the enterocytes barrier function and/or modulating some intestinal immune cells capability to respond to oxidative stress. Therefore, the objective of this study was to examine if genetic variation in *SLC23A1* gene could modulate the susceptibility to IBD or severity of its complications.

SUBJECTS AND METHODS

Study design and population

The study design has previously been described (25). Briefly, clinical data were collected from case records of participants in the Manitoba IBD Cohort Study, initiated in 2002. At enrollment in the Cohort Study, participants were at least 18 yr of age (18-80 yr) and diagnosed within the previous 7 yr (median 4.3 years). Controls included healthy individuals with no chronic immune diseases or first degree relatives with chronic immune diseases. A total of 311 persons with IBD (CD n=162, UC n=149) and 142 healthy controls were studied. All the study population (cases and controls) were Caucasian. The diagnosis and extent of IBD was determined based on surgical, endoscopic, radiologic, and histologic data. Phenotype was assigned according to the Montreal Classification (26). Every subject signed an informed consent, and this study was approved by Biomedical Research Ethics Board at University of Manitoba.

SNP selection and genotyping methods

Extensive sequencing analysis of the pattern of common genetic variation of *SLC23A1* gene has previously been performed defining haplotype tagging SNPs for the gene (27). Subsequently, a haplotype-based approach was implemented and three SNPs in the *SLC23A1* gene were identified (rs6596473C>G, rs33972313 A>G, and rs10063949 G>A). Variants were selected because they are located in the 5', middle and 3' region of *SLC23A1* and based on potential functional affect (e.g. rs33972313 is non-synonymous and had been shown to influence plasma ascorbic acid levels).

Genomic DNA was isolated from peripheral white blood cells as previously described (29). Genotyping was performed for all subjects for three SNPs in *SLC23A1* using TaqMan Real-Time PCR Assays (Applied Biosystems, Foster City, CA, USA), the assay condition for each optimized assay are shown in **Table 1**. Approximately 8% blinded quality control samples (36 individuals) were assayed 4 times, which showed 100% concordance.

Statistical analysis

Statistical analyses were performed using Statistical Analysis Systems software (SAS), version 9.3 (SAS Inc., Cary, NC, USA). All tests were two-sided and significant *p* values was set at $P<0.05$. Each of the three SNPs was analyzed individually using allele frequencies and carriage rates for association with CD and UC. Binary logistic regression was used to estimate odds ratios (ORs) and 95% confidence interval (CIs) for association between genotypes and CD and UC risk, adjusted for age and gender. Allelic frequencies and genotype frequencies were determined in UC, CD and controls. The homozygous common genotype was considered as reference group. In alternative analyses, a dominant model of inheritance was used to compare risk in the combined group of homozygous rare and heterozygotes genotypes to risk among the common homozygous genotype. Based on phenotype, comparisons of genetic frequencies and allelic frequencies were determined between UC, CD and controls by means of a 2x2 contingency table and chi-squared test. Haplotypes were calculated using Haploview 4.2 (Broad Institute, Cambridge, MA, USA). Multinomial logistic regression was used for association between genotypes and phenotype for CD and UC patients.

RESULTS

One hundred sixty two patients with CD, 149 patients with UC, and 142 ethnically matched healthy controls (n=142) were genotyped for three *SLC23A1* polymorphisms. Baseline characteristics of the study population are shown in **Table 2**. Genotype frequencies are summarized in **Table 3** (CD) and **Table 4** (UC). Genotype frequencies for all of the polymorphisms were in Hardy-Weinberg equilibrium within CD and UC groups.

Among the 3 variants in *SLC23A1* examined, the rs10063949-G allele is associated with increased CD risk (**Table 3**). Carriage of one (heterozygote) or two (homozygote) copies of the minor rs10063949 allele (G) was associated with increased risk of CD [OR=2.54, 95% CI 1.38, 4.66; OR=4.72, 95% CI 2.53, 8.81, $p<0.001$; respectively). None of the SNPs were associated with UC risk.

A strong linkage was observed across the *SLC23A1* gene (D' = 0.94 between rs6596473 and rs10063949) in persons with CD **(Figure 1A)** and the haplotype CGG which is carried in persons with CD (OR=2.40, 95% CI: 1.54, 3.88, $P<0.0001$) (**Table 3**).

Strong linkage (D'= 0.96) in the SLC23A1 locus was also observed in UC patients for the two SNPs (rs6596473 and rs10063949) (**Figure 1B**) and a major haplotype (frequency ≥5%) was inferred for UC patients (CAG: 41%). No association was found between *SLC23A1* genotype and haplotype with UC risk (**Table 4**). No correlation was found between the presence of any genotype and specific phenotypes for CD and UC (data not shown).

DISCUSSION

Here, for the first time, we identified a variation in the sodium-dependent ascorbic acid transporter gene *SLC23A1* that is associated with CD, but not with UC. This finding confirms our hypothesis that a disturbance in the antioxidant balance could contribute to the development and severity of IBD. The rs10063949-G allele in *SLC23A1* gene is associated with an increased CD risk. Of persons with CD, two thirds carried the G allele, corresponding to a CD risk which is elevated by 2.5 times compared to the rs10063949-A carriers. The rs10063949-GG genotype was significantly over transmitted in the CD patients (47.5%) compared to controls (25.4%). An allele dosage effect resembling haploinsufficiency is evident. Compared to rs10063949-AA homozygotes the 10063949-AG heterozygotes have a 2.5 fold elevated risk for CD and the 10063949-GG homozygotes have a 4.7 fold elevated CD risk. No relation was observed between genetic variants in *SLC23A1* and UC.

The SNP rs10063949 is located within the promoter region of the *SLC23A1* gene, which contains a variety of regulatory elements such as the hepatocyte nuclear factor 1 (HNF1), required for tissue specific transcription (30). Assuming that SNP rs10063949 contributes to intestinal inflammation, it would most likely act through differential regulation of *SLC23A1* expression, which consequently would result in reduced cellular accumulation of ascorbate. In the current study, finding a genetic alteration in *SLC23A1* transporter gene in CD suggests that the observation of low ascorbate levels in inflamed mucosa in the study by Head and colleagues (2) may be as much cause of inflammation as effect.

The implication of the association between SNP rs10063949 and CD but not UC is undetermined. We here suggest three possible cell-type specific mechanisms which could by themselves or in combination with each other cause the differential association. If one or all of them gets validated in future biological and clinical studies, SNP rs10063949 could be utilized as predictive biomarker for CD. Further it should be determined if it could also serve as a diagnostic biomarker for dietary intervention with ascorbate. Firstly, cell type specific transcription factors regulating *SLC23A1* expression in immune regulatory cells involved in the development of CD but not UC might be affected by SNP rs10063949. *SLC23A1* is expressed in lymphocytes, however, the specific expression during hematopoiesis and therefore in the different types of lymphocytes, and leukocytes in general, is not defined. Future genomic and functional studies should determine *SLC23A1* expression patterns in all lymphocyte types (including natural killer cells), to define if indeed ascorbate accumulates differently in carriers of the SNP 10063949 genotypes. The levels of ascorbate will determine the antioxidative capacity (11) and as a consequence would contribute to the differential regulation of gene expression influencing CD but not UC risk.

A second possible mechanism addresses a potential dysregulation of macrophage function through reduction in ascorbate uptake of macrophages in epithelioid granulomas. A granuloma is a collection of macrophages and other inflammatory cells. Epithelioid granulomas are among the most specific microscopic features of CD, distinguishing it from UC (31). Intracellular ascorbic acid has a regulatory role in the granulocyte macrophage-colony-stimulating (GM-CSF) signaling response (32), which is involved in proinflammatory processes, and therefore could determine the severity of CD.

A third possible explanation is related to the fact that the intestinal epithelium constitutes a physical barrier which contains immunogenic bacteria within the intestinal lumen and elevated oxidative stress can impede this barrier function (33, 34). *SLC23A1* is highly expressed in intestinal epithelial enterocytes, where its down-regulation may lead to decreased intracellular ascorbic acid levels to compromise the physical barrier function against immunogenic bacteria and/or the ability to withstand prolonged intrinsic macrophage challenges. This by itself may not explain the causality of a genetic variation associated to CD but not UC, however, it could enhance the severity of any immune dysregulations specific for CD. This might be even more potentiated by the fact that oxidative stress in epithelial cells and macrophages leads to the activation of the nuclear factor kappa B, an activator of pro-oxidative genes such as lipoxygenase, cycloxygenase-2, and inducible-nitric oxide synthase which leads to further elevated oxidative stress and reduced function of the intestinal barrier (35, 36).

Previously, the three SNPs examined in this study were shown to be associated with circulating plasma ascorbic acid, however the results are inconsistent (5, 37, 38). The inconsistency and/or heterogeneity among the results from different studies may be largely accounted for sensitivity of analyses in measuring plasma ascorbate, different assay protocols, and many confounding factors that often make the interpretation of observational data difficult (37). Therefore, there is a need for additional studies to confirm a possible relationship between *SLC23A1* genetic variations and circulating plasma ascorbic acid. In the current study, due to sampling collection methods, we were not able to include measurements of plasma ascorbic acid as a biomarker for ascorbate status. While this is a limitation of this study, we speculate that the association of the

229 genetic variation to CD may not influence plasma ascorbic acid levels, which are determined
230 through the renal re-absorption rather than intestinal absorption (19).
231
232 Compared to current genome wide association studies (GWAS) our Manitoban population-based
233 case-control cohort could be considered suboptimal in terms of sample size (39). However, a
234 previous study of the Manitoba IBD Cohort (40) found significant associations for some of the
235 previously described IBD-associated SNPs identified by GWAS in other large population based
236 cohorts . Therefore, these results from a well phenotyped cohort of moderate size are
237 noteworthy, but nonetheless should be reproduced in other cohorts.
238
239 If, as we hypothesize, intracellular ascorbate levels of specific intestinal cell types will be
240 decrease by the action of SNPs in *SLC23A1*, a supplementation with dehydroascorbate would be
241 worthy of study as the therapy of choice to compensate for this shortfall. Dehydroascorbate, the
242 oxidized form of Vitamin C, does not exist under physiologic conditions (41, 42). However, if
243 supplemented externally, dehydroascorbate enters the cell through facilitated glucose transporter
244 of the GLUT family, not SLC23A1 (23), and intracellular dehydroascorbate is immediately
245 reduced to ascorbate, the active form of vitamin C (41-43). As currently dehydroascorbate is a
246 minor component of some dietary supplements, the proposed gene specific personalized
247 nutritional therapy would boost intracellular vitamin C levels and be considered safe.
248 Confirmation of our findings in independent association studies is warranted before
249 implementing any nutritional intervention.
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Table 1: Primers used to examine the three single nucleotide polymorphism in SLC23A1 gene involved in this study[1]

dbSNP	CGF assay ID	Position	Alleles	TaqMan primers	TaqMan probes
rs6596473	A-007155	138738475bp	C/G	F: CATTGAGGCTGCCACTTGAC R: TGCCCATTTAGAGGATGCTAGACT	FAM: CCTATGGGCCTGAGACA VIC: CCTATGGGCGTGAGACA
rs33972313	001-1224	138743401bp	A/G	F: AGACCTCCAGTGCCTTCAGT R: GCAGCACGTCTGTCAAGGT	FAM: TCATGACCGTGTGGCT VIC:CATCATGACCATGTGGCT
rs10063949	A-006670	138747425bp	G/A	F: TTTGACCCAAGCCATGCAGATA R: GGCAGCTCAGACCAACCT	FAM: TTCTGCAAACTTGC VIC: TCTGCGAACTTGC

[1] Adopted from reference (28); CGF, Core genotyping facility; F, forward primer; R, reverse primer

Table2: General characteristics of the study subjects

Parameters	**Crohn´s Disease (n=162)**	**Ulcerative Colitis (n= 149)**	**Controls (n=142)**
Gender			
Female	97 (59.9%)	87 (58.4%)	80 (56.3%)
Male	65 (40.1%)	62 (41.6%)	62 (43.7%)
Age at diagnosis			
A1(<17 yr)	17 (10.5%)	12 (8.1%)	-
A2 (17-40 yr)	101 (62.3%)	78 (52.3%)	-
A3 (>40 yr)	44 (27.2%)	59 (39.6%)	-
Location			
L1 (Ileal)	69 (42.6%)	-	-
L2 (Colonic)	37 (22.8%)	-	-
L3 (Ileocolonic)	51 (31.5%)	-	-
L4 (isolated upper disease)	5 (3.1%)	-	-
E1(Denotes proctitis)	-	11 (7.4%)	-
E2 (Left-sided)	-	68 (45.6%)	-
E3 (Extensive colitis)	-	70 (47.0%)	-
Behaviour			
B1(Inflammatory)	69 (42.6%)	-	-
B2 (Stricturing)	54 (33.3%)	-	-
B3 (Penetrating/fistulizing)	39 (24.1%)	-	-

Note: No significance difference was found between the base line characteristic for study populations.

Table 3: Genotype, allele, and haplotype frequencies of SLC23A1 gene variants in Crohn's disease patients and control subjects

	Crohn´s disease n=162	**Controls n=142**	**OR (95% CI)**	***P***
rs6596473				
GG	77 (47.5%)	67 (47.2%)	REF	
CG	65 (40.1%)	64 (45.1%)	0.88 (0.55, 1.42)	0.61
CC	20 (12.3%)	11 (7.7%)	1.58 (0.71, 3.54)	0.26
C-carrier	105 (32.4%)	87 (30.6%)	1.09 (0.77, 1.53)	0.64
rs33972313				
GG	156 (96.3%)	138 (97.2%)	REF	
GA	6 (3.7%)	4 (2.8%)	1.33 (0.37, 4.08)	0.67
AA	0	0	ND	ND
A-carrier	6 (1.9%)	4 (1.4%)	1.32 (0.37, 4.73)	0.66
rs10063949				
AA	24 (14.8%)	53 (37.3%)	REF	
GA	61 (37.7%)	53 (37.3%)	2.54 (1.38, 4.66)	0.001
GG	77 (47.5%)	36 (25.4%)	4.72 (2.53, 8.81)	0.001
G-carrier	216 (66.7%)	125 (44%)	2.54 (1.83, 3.53)	0.001
Haplotype				
CGG	106 (65.3%)	62 (43.5%)	2.44 (1.54, 3.88)	0.0001

Odds ratios are adjusted for age and gender

Per allele effects are derived from binary logistic regression

ND = not determined

The haplotypes were formed by the SNPs rs6596473, rs33972313, and rs10063949

Table 4: Genotype, allele, and haplotype frequencies of SLC23A1 gene variants in ulcerative colitis patients and control subjects

	Ulcerative Colitis n=149	**Controls n=142**	**OR (95% CI)**	***P***
rs6596473				
GG	66 (44.3%)	67 (47.2%)	REF	
CG	68 (45.6%)	65 (45.8%)	1.05 (0.65, 1.69)	0.85
CC	15 (10.1%)	11 (7.7%)	1.36 (0.58, 3.19)	0.47
C-carrier	98 (32.9%)	87 (30.6%)	1.11 (0.78, 1.57)	0.56
rs33972313				
GG	142 (95.3%)	138 (97.2%)	REF	
GA	7 (4.7%)	4 (2.8%)	1.70 (0.49, 5.94)	0.40
AA	0	0	ND	ND
A-carrier	7 (2.3%)	5 (1.8%)	1.34 (0.42, 4.27)	0.62
rs10063949				
AA	58 (38.9%)	53 (37.3%)	REF	
GA	52 (34.9%)	53 (37.3%)	0.90 (0.53, 1.53)	0.69
GG	39 (26.2%)	36 (25.4%)	0.99 (0.55, 1.78)	0.97
G-carrier	131 (43.9%)	125 (44%)	1.01 (0.72, 1.38)	0.98
Haplotype				
CAG	63 (42.6%)	60 (42.5%)	1.00 (0.63, 1.59)	0.93

Odds ratios are adjusted for age and gender

Per allele effects are derived from Binary logistic regression

ND = not determined

The haplotypes were formed by the SNPs rs6596473, rs33972313, and rs10063949

Figure 1: Linkage observed across the *SLC23A1* gene locus in individuals with Crohn's disease **(A)** and Ulcerative colitis **(B)**. The degree of linkage disequilibrium (LD) is given in percentage, represented in the triangles. Red indicates a high degree of LD, while blue indicates uncertain results.