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BRIEF ARTICLE

Analysis of single nucleotide polymorphisms in the region of *CLDN2-MORC4* in relation to inflammatory bowel disease

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Abstract

AIM: To investigate a possible genetic influence of claudin (*CLDN*)*1*, *CLDN2* and *CLDN4* in the etiology of inflammatory bowel disease.

METHODS: Allelic association between genetic regions of *CLDN1*, *CLDN2* or *CLDN4* and patients with inflammatory bowel disease, Crohn's disease (CD) or ulcerative colitis were investigated using both a case-control study approach (one case randomly selected from each of 191 Swedish inflammatory bowel disease families and 333 controls) and a family-based study (463 non-Swedish European inflammatory bowel disease -families). A nonsynonymous coding single nucleotide polymorphism in *MORC4*, located on the same linkage block as *CLDN2*, was investigated for association, as were two novel *CLDN2* single nucleotide polymorphism markers, identified by resequencing.

RESULTS: A single nucleotide polymorphism marker (rs12014762) located in the genetic region of *CLDN2*

was significantly associated to CD (case-control allelic OR = 1.98, 95%CI: 1.17-3.35, *P* = 0.007). *MORC4* was present on the same linkage block as this CD marker. Using the case-control approach, a significant association (case control allelic OR = 1.61, 95%CI: 1.08-2.41, *P* = 0.018) was found between CD and a nonsynonymous coding single nucleotide polymorphism (rs6622126) in *MORC4*. The association between the *CLDN2* marker and CD was not replicated in the family-based study. Ulcerative colitis was not associated to any of the single nucleotide polymorphism markers.

CONCLUSION: These findings suggest that a variant of the *CLDN2-MORC4* region predisposes to CD in a Swedish population.

Key words: Crohn's disease; Genetic predisposition; Inflammatory bowel disease; Single nucleotide polymorphism; Tight junctions

Core tip: Tight junction proteins are key components in the regulation of paracellular permeability and therefore we considered claudin genes as candidate genes in the study. Association was identified between a single nucleotide polymorphism marker (rs12014762) in the *CLDN2-MORC4* region and the occurrence of Crohn's disease (CD) in a Swedish population. Additionally, a nonsynonymous coding single nucleotide polymorphism (rs6622126) in *MORC4* was associated to CD. Our findings add further support for a genetically impaired intestinal epithelial barrier as one predisposing factor in the etiology of CD.

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INTRODUCTION

Chronic inflammatory bowel disease (IBD) encompasses Crohn's disease (CD), ulcerative colitis (UC) and, in the absence of a confident diagnosis, unclassified colitis (IBD-U). Susceptibility to IBD and the broad spectrum of phenotypic expressions depends on contributions from environmental factors and a genetic predisposition. Several etiological factors have been suggested, including the presence of specific strains of commensal enteric bacteria, defective bacterial killing, aberrant regulation of innate and adaptive immune responses and an impaired intestinal barrier[1]. Consistent with this, several genetic associations in IBD have been described[2-8].

Both CD and UC have been associated with an increase in intestinal permeability[9-11]. Based on findings of an increased intestinal permeability among healthy first-degree relatives to CD patients, a role for a tight junction (TJ) based genetic contribution to permeability changes has been suggested as a predisposing susceptibility factor for CD[12]. This suggestion has been contradicted by other studies[13-15]. However, by defining a normal range of intestinal permeability in healthy controls, a subgroup of healthy first-degree relatives to CD patients with increased intestinal permeability has been identified[16]. An increased permeability response to acetylsalicylic acid has been observed in CD patients and their relatives, indicating hereditary factors underlying this responsiveness[14]. An increased permeability may be primary or a consequence of subclinical intestinal inflammation present as an inherited abnormality in relatives of CD-patients[17]. It is still unknown whether this disturbed permeability is caused by genetic or environmental factors, but several studies provide support for a genetic rather than environmental induced increase[18,19].

The barrier of epithelial cells, with their apical TJ-structure, is critical for the permeability properties of the intestine. The TJ-structure is a multicomponent protein complex that serves to seal and regulate permeability across the paracellular space between adjacent epithelial cells, with the family of membrane-spanning claudin-proteins as the major determinants[20,21]. Claudin-1 and claudin-4 have been associated with a tight TJ-structure whereas claudin-2 expression results in a more leaky epithelial layer[22-24]. Expressions of claudins, and other TJ-proteins, are subject to regulation by different cytokines[20]. Claudin-4 seems to be preferentially expressed in M-cells[25] and the dome area of the follicle-associated epithelium[26], which has been suggested to be the site of initial inflammation in ileal CD[27].

Our aim was to elucidate a possible genetic influence of tight junction-components to IBD-susceptibility and therefore we conducted genetic association studies using single nucleotide polymorphism (SNP) markers of the genetic regions of claudin 1 (*CLDN1*, chromosome 3q28-q29), claudin 2 (*CLDN2*, chromosome Xq22.3-q23) and claudin 4 (*CLDN4*, chromosome 7q11.23). Furthermore, in order to identify putative functional sequence variants of *CLDN2*, the promoter region, exon-intron boundaries and exons harbouring the 5' untranslated region and protein coding region were amplified and resequenced.

MATERIALS AND METHODS

Study subjects

The IBD-families in this study originated from the large European collaboration that lead to the discovery of *NOD2/CARD15* as a CD susceptibility gene[5,28]. Swedish IBD-patients were selected for inclusion in a case-control study, whereas the remaining non-Swedish families were used in a family based genetic association study (Table 1). Samples from an anonymized regional DNA bank con-

Table 1 General study outline

Study design[1]	Cohort and disease	Number of families	Number of individuals	Women
Case control study	Healthy unrelated controls		333	162
	Swedish IBD families	191		
	IBD		347	157
	CD		150	69
	UC		166	71
	IBD-U		31	17
Family based approach	Non-Swedish families	463		
	IBD		715	398
	CD		528	297
	UC		151	83
	IBD-U		36	18

[1]Including Claudin (*CLDN*)*1, CLDN2, CLDN4*. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Unclassified colitis.

sisting of randomly selected individuals (n = 800) living in the southeastern part of Sweden were used as controls in the case-control studies. The study was conducted under approval by the ethics committees of Linköping University (DNR 97271) and Karolinska Institutet (DNR 97-327).

SNP selection for genetic association studies

Linkage blocks were defined using SNP data from the HapMap CEPH collection and the SNPbrowser software version 3.5 (Applied Biosystems, Foster City, CA, United States) and a default value of 0.3 linkage disequilibrium units (LDU)[29]. SNP markers were selected for *CLDN1*, *CLDN2* and *CLDN4* (Table 2). A distance less than 1 LDU has been considered useful for allelic association[30]. SNP markers were also chosen from adjacent linkage blocks.

Genotyping

Allele discrimination was carried out using TaqMan SNP genotyping assays (Table 2) and TaqMan Genotyping Master Mix or TaqMan Fast Universal polymerase chain reaction (PCR) Master Mix without AmpErase UNG (Applied Biosystems), using either a 7300 Real-Time PCR System or a 7500 Fast Real-Time PCR System (Applied Biosystems). All genotype data were analyzed using the 7500 Fast System SDS Software version 1.3.1.21 (Applied Biosystems).

In addition, a nonsynonymous coding SNP in *MORC4* (rs6622126; Applied Biosystems assay ID C_22273025_10) and two novel *CLDN2* SNP markers (this study; rs62605981 and rs72466477) were genotyped. The two novel *CLDN2* sequence variants were ordered as custom assays from Applied Biosystems. Primer and probe sequences are available from the authors upon request.

Resequencing of CLDN2

The promoter region, exon-intron boundaries and exons harbouring the 5' untranslated region and protein coding region of *CLDN2* were amplified by PCR and resequenced (Table 3). PCR amplifications were in accordance with the manufacturer's recommendations, using HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 2.0 mmol/L $MgCl_2$, and 0.2 μmol/L per PCR primer (Operon Biotechnologies GmbH, Cologne, Germany, and Scandinavian Gene Synthesis AB, Köping, Sweden). The following PCR cycle was repeated 45 times: 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 60 s. A total of 93 individuals (21 CD, 21 UC, 5 IBD-U, and 46 healthy; 60 females) were resequenced.

All PCR products were purified in accordance with the QIAquick PCR purification kit protocol (Qiagen), and analyzed using the DNA 1000 assay on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). Cycle sequencing was carried out using the CEQ8800 system and accompanying sequencing reagents from Beckman Coulter (Fullerton, CA, United States) and conducted using half concentrated CEQ DTCS quick start kit, purification with an ethanol-precipitation protocol. Sequence data were analyzed with the heterozygote detection option activated in the software (version 8.0.52) and relative a reference sequence. New sequence variants were deposited in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and provided with rs-numbers.

Prediction of transcription factor binding sites

Transcription factor binding sites were predicted using the Alibaba 2.1 software[31] available through Biobase (www.gene-regulation.com), in conjunction with the Transfac database public release version 7.0[32] at Biobase.

Statistical analysis

Allelic OR with accompanying 95%CI, and P values based on χ^2 statistics were calculated using a likelihood-based analysis for genetic association with the Unphased software version 3.0.13[33], both in the case-control approach and in the family-based study. In order to avoid bias due to genetic relatedness in the case-control study, one case per family was randomly selected from 191 Swedish IBD families. These random samplings of cases were repeated 15 times and the median OR was used as a representative measure of association. The case-control studies with respect to IBD, CD and UC were based on 191, 103 and 102 cases of IBD, CD, and UC, respectively, and 333 controls. No deviations from Hardy-Weinberg equilibrium were observed.

Since *CLDN2-MORC4* are located in a non-pseudo-autosomal region of the X-chromosome, males contribute one allele and females two alleles. Because the analysis did not identify sex as a confounder, males and females were analyzed jointly. Association to clinical features was tested for using a chi-square test for qualitative variables and one way ANOVA for quantitative variables, and carried out using the GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, United States). For all statistical

Table 2 Single nucleotide polymorphism markers in the genetic regions of *Claudin-1*, *Claudin-2* and *Claudin-4*

Candidate gene	SNP rs number[1]	MAF[2]	Assay ID[3]	Position in kbp relative candidate gene and location[4]	Coverage[5]
CLDN1	rs1491991	0.25	C_7550365_10	-66.3 (5′-flanking region)	*CLDN16*
	rs3732923	0.41	C_27509271_10	5.5 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs3732924	0.29	C_8528578_10	5.6 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs9848283	0.49	C_2057729_10	6.4 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs12629166	0.47	C_2057718_10	13.8 (intron 3)	*CLDN1* (from second intron until 3′-flanking region)
	rs7620166	0.41	C_8528273_10	45.9 (3′-flanking region)	*CLDN1* (from second intron until 3′-flanking region)
	rs567408	0.42	C_1587588_10	94.5 (3′-flanking region)	intergenic block
	rs536435	0.42	C_1587674_10	155.3 (3′-flanking region)	*LOC391603*
CLDN2	rs4409525	0.34	C_382795_10	-23.3 (5′-flanking region)	*RIPPLY1*, *TBC1D8B*
	rs5917027	0.48	C_11771710_10	-1.0 (5′-flanking region)	*CLDN2*, *MORC4*
	rs12014762	0.21	C_2013132_20	20.0 (3′-flanking region)	*CLDN2*, *MORC4*
CLDN4	rs4131376	0.43	C_26657639_10	-56.9 (5′-flanking region)	*ABHD11*, *CLDN3*, *CLDN4*, *WBSCR27*, *WBSCR28*
	rs8629	0.18	C_7493975_10	0.3 (exon 1)	*ABHD11*, *CLDN3*, *CLDN4*, *WBSCR27*, *WBSCR28*

[1]Initially all of the selected single nucleotide polymorphism (SNP) markers were evaluated, using the Hapolview software 4.0[52], for linkage disequilibrium (LD) and association to inflammatory bowel disease (IBD) in a case-control study using a subset of Swedish IBD patients (73, 39 and 42 individuals with IBD, Crohn's disease (CD) or ulcerative colitis, respectively). Because of significant associations to CD, SNP markers for claudin (*CLDN)1* (rs7620166) and *CLDN2* (rs12014762) were chosen for further evaluation on the complete case-control. Even though non of the *CLDN4* SNP markers showed evidence of association to any of the disease categories rs8629 was included for further evaluation; [2]Minor allele frequency (MAF) according to SNP data from the HapMap CEPH collection; [3]TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, United States); [4]With respect to each candidate gene, SNP positions are defined relative a genomic reference sequence. The first nucleotide of a reference cDNA sequence has been designated +1, with the preceeding genomic position being -1. The following reference sequences have been used for *CLDN1* NT_005612.15 (genomic) and NM_021101.3 (mRNA), *CLDN2* NT_011651.16 (genomic) and NM_020384.2 (mRNA) and *CLDN4* NT_007758.11 (genomic) and NM_001305.3 (mRNA); [5]Linkage blocks have been defined using SNP data from the HapMap CEPH collection and the SNP browser software with 0.3 LDU (linkage disequilibrium units) as threshold for linkage block computation.

Table 3 Primers for polymerase chain reaction amplification and sequencing of *Claudin-2*

Candidate gene	Exon	Forward primer[1]	Reverse primer[2]	Size of PCR-product
CLDN2	1	GTAGGACCTGCTCTTTGAACTC[2,3]	TGAATTTAAAAGGCAGCAACTA[2,3]	708 bp
	1	TCAATCTTTCCCAGCCTCCA[3]	TTTCTGTCAAAAACTCCACTCC[3]	
	2	TTGTAGAGAATGGGGAGGTGTGC[2,3]	TCAATTGCAGACTGAGGCCAAA[2]	599 bp
	2		TGCAGACTGAGGCCAAACTG[3]	
	2	CTAGGCCCCTGGAGATTCAAGA[2,3]	TGTGCCCAAAAGCCCCAGAA[2,3]	682 bp
	2	TTCCTTTCTCATGTGTTATTTCTAA[3]	GAGAAAGGAAAAAAAAAACAAC[3]	
	2	TGAGGGATTAGAGGTGTTCAA[2,3]	GCAGCACCTTCTGACACGA[2,3]	892 bp
	2	ATCCTACGGGACTTCTACTCA[3]	ACTCCACCTGCTACCGCCACT[3]	

[1]q, Q-solution for polymerase chain reaction (PCR)-amplification; [2]Primer used for PCR; [3]Primer used for sequencing. CLDN: Claudin.

tests, two-sided *P* values < 0.05 were considered significant. Correction for multiple testing was not performed.

RESULTS

Genetic association – case-control approach

Based on a subset of Swedish IBD patients (73, 39 and 42 individuals with IBD, CD or UC, respectively) one marker per gene was selected for analysis in the full study material (Table 2). Allelic association between any of the three markers for the genetic regions of *CLDN1*, *CLDN2* or *CLDN4* and patients with IBD, CD or UC were investigated using a case-control study approach (Table 4). Significant associations were observed between the *CLDN2* marker (rs12014762; associated C allele frequency of 0.776 among controls) and CD (*P* = 0.007), and the *CLDN1* marker (rs7620166; associated T allele frequency of 0.470 among controls) and IBD (*P* = 0.025). No associations were observed for the *CLDN4* marker (rs8629; C allele frequency of 0.772 among controls), neither to IBD, CD nor UC.

Genetic association - family-based approach

The same three SNP markers were further investigated using a family-based approach in non-Swedish families (Table 4). The significant associations identified in the case-control study were not confirmed (*P* = 0.126 and *P* = 0.177 for the *CLDN2* and *CLDN1* marker, respectively). The *CLDN2* marker rs12014762 was not related to any demographic or disease characteristics in CD-patients (Table 5).

Resequencing of CLDN2

Based on the most significant association (CD and the *CLDN2* marker), *CLDN2* was chosen for resequencing in an approach to identify novel sequence variants of putative importance for the risk of developing CD. Two novel non-coding sequence variants were identified for

Table 4 Inflammatory bowel disease-phenotypes were investigated by performing family-based and case-control approach in genetic association studies

		rs7620166 (*CLDN1*)		rs12014762 (*CLDN2*)		rs8629 (*CLDN4*)	
		allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value
IBD	Swedish case-control	1.33 (1.04–1.72)	0.025	1.39 (0.95–2.01)	0.083	1.21 (0.89–1.65)	0.225
	Non-Swedish families	0.87 (0.72-1.06)	0.177	1.25 (0.89–1.77)	0.195	1.09 (0.88–1.33)	0.432
CD	Swedish case-control	1.17 (0.86–1.60)	0.319	1.98 (1.17-3.35)	0.007	1.25 (0.84–1.85)	0.258
	Non-Swedish families	0.80 (0.64-1.00)	0.052	1.37 (0.91–2.07)	0.126	1.14 (0.89–1.46)	0.287
UC	Swedish case-control	1.35 (0.98–1.84)	0.064	1.27 (0.80–2.02)	0.304	1.18 (0.80-1.73)	0.409
	Non-Swedish families	1.19 (0.77-1.84)	0.436	0.91 (0.39-2.14)	0.827	1.15 (0.75-1.77)	0.512

The family-based association studies included a total of 463 families. For the single nucleotide polymorphism -markers rs7620166 [claudin (*CLDN*)*1*], rs12014762 (*CLDN2*) and rs8629 (*CLDN4*) genotyping failed for 5 [including 2 Crohn's disease (CD)], 9 (including 3 CD) and 7 (including 2 CD) samples, respectively. Results were based on 191, 103 and 102 cases of inflammatory bowel disease (IBD), CD and ulcerative colitis (UC), respectively and 333 controls. OR (and its associated 95%CI) and *P* values (based on log likelihood ratio χ^2 statistics) were calculated for the T allele of rs7620166, the C allele of rs12014762, and the C allele of rs8629.

Table 5 Genotype-phenotype correlation between *Claudin-2* marker (rs12014762) polymorphism and clinical characteristics of 677 patients with Crohn's disease (all families)

rs12014762 Crohn's disease	At least one C	T	*P* value
Sex			
Male	265	47	
Female	354	11	
Age at diagnosis (yr)			
Mean (SD)	24.48 (12.11)	24.36 (11.46)	0.90
Median (range)	22 (3-70)	22 (8-63)	
Location at onset:	*n* = 583	*n* = 54	
Pure colonic disease	78	6	0.64
Pure ileal disease	117	14	0.31
Ileocolonic disease	274	309	0.87
Any colonic disease	414	37	0.70
Any ileal disease	424	42	0.42
Upper digestive tract	118	12	0.73
Perineal disease	147	11	0.53
Granuloma	237/478	26/43	0.12
Penetrating disease	218/478	22/43	0.48
Strictures	221/478	15/43	0.15
Extra-digestive symptoms	190/619	15/58	0.44
Smoking habits	*n* = 505	*n* = 51	
Non-smoker	272	21	0.08
Smoker	157	21	0.14
Ex-smoker	76	9	0.62

CLDN2 (Table 6).

Prediction of transcription factor binding sites in the CLDN2 promoter and analysis of genetic association to the newly identified sequence variants

The two novel *CLDN2* polymorphisms were located in a region corresponding to the *CLDN2* promoter, as described by Sakaguchi *et al*[34] Analyzing the promoter region of *CLDN2* for the presence of possible transcription factor binding sites, revealed that these new variants were located in a putative specificity protein 1 (Sp1) binding site/GC-box (rs62605981) and a putative upstream stimulatory factor (USF) binding site/E-box (rs72466477). The genotyping results of these two novel SNPs were based on 166, 89 and 89 cases of IBD, CD and UC, respectively, from the Swedish families and their 333 non-related controls (Table 1). Neither rs62605981 (C allele frequency of 0.860 among controls) nor rs72466477 (AT allele frequency of 0.842 among controls) showed any significant associations to IBD overall or to any sub-entities (Table 7).

MORC4 and genetic association

In addition to *CLDN2*, *MORC4* is located on the same linkage block as the CD associated marker (rs12014762). A nonsynonymous coding SNP (rs6622126) was identified in the *MORC4* gene using the NCBI SNP database, for which a high minor allele frequency (A allele = 0.440) was present in the non-related Swedish control samples. This SNP was investigated for genetic association to IBD overall and to sub-entities, using the same individuals as used for the two novel *CLDN2* variants. A significant association was observed between the G allele (frequency of 0.560 among controls) and CD ($P = 0.018$), but not to UC or IBD (Table 7).

DISCUSSION

TJ proteins are considered key components in the regulation of paracellular permeability of both epithelial and endothelial cell linings[20,21], and due to their barrier-forming properties we considered claudin genes as candidate genes affecting a leaky gut phenotype of IBD.

Using the case-control approach, an association was identified between the *CLDN2-MORC4* region (rs12014762) and the occurrence of CD. This SNP marker was associated with an overall increased risk of having CD, but not to any distinctive phenotypic pattern. This association was not confirmed in the family-based approach, where non-Swedish families were included. Such a regional heterogeneity may in part be explained by different genetic backgrounds. Substantial genetic heterogeneity due to geographic stratification has been demonstrated in genome-wide association studies[6]. A geographically homogenous population (this study) should be advantageous for unveiling regionally restricted genetic risk factors. The case of underrepresented *NOD2* mutations in CD patients from the Nordic countries[35-37] together with our data on the presence of *CLDN2* vari-

Table 6 Sequence variants identified by resequencing of selected gene regions

Gene[1]	Position of SNP	rs-number	Part of gene/predicted consequence	VAF/control[2]	VAF/patients[2]
CLDN2	c.-178-678C>G (g.29466911)	rs62605981	Intron/5′ gene flanking region[3]	0.175	0.139
	c.-178-104_-103delAT (g.29467485_29467486delAT)	rs72466477	Intron/5′ gene flanking region[3]	0.175	0.083

[1]The following reference sequences have been used for claudin (*CLDN*)2 NT_011651.16 (genomic) and NM_020384.2 (mRNA); [2]Variant allele frequencies (VAF) are defined with respect to the corresponding reference sequence; [3]The intronic region of *CLDN2*, according to the *CLDN2* reference mRNA (NM_020384), correspond to the promoter region described by Sakaguchi *et al*[34]. SNP: Single nucleotide polymorphism.

Table 7 Genetic association between inflammatory bowel disease-phenotype and either newly discovered *Claudin-2* promoter polymorphisms or a nonsynonymous coding polymorphisms in *MORC4* was analyzed by performing case-control studies

	rs62605981 (*CLDN2*)		rs72466477 (*CLDN2*)		rs6622126 (*MORC4*)	
	allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value	allelic OR(95%CI)	*P* value
IBD	1.11 (0.71–1.73)	0.659	1.23 (0.79–1.91)	0.350	1.24 (0.91–1.70)	0.179
CD	0.83 (0.49–1.41)	0.501	1.16 (0.68–2.00)	0.584	1.61 (1.08–2.41)	0.018
UC	1.24 (0.69–2.26)	0.463	1.19 (0.68–2.06)	0.543	0.903 (0.61–1.33)	0.606

Results were based on 166, 89 and 89 cases of inflammatory bowel disease (IBD), Crohn's disease (CD) and Ulcerative colitis (UC), respectively, available from the Swedish families and 333 controls (Table 1). OR (and its associated 95%CI) and *P* values (based on log likelihood ratio chi-square statistics) were calculated for the C allele of rs62605981, the AT allele of rs72466477, and the G allele of rs6622126. CLDN: Claudin.

ants in Swedish patients add support for such an assumption taking into account that IBD, and especially CD, are polygenic disorders[2,3]. When we investigated a possible interaction between *NOD2* and the *CLDN2-MORC4* region (rs12014762) with individuals stratified on the basis of carrying none or at least one *NOD2* mutant allele, no significant association was identified between these two genetic regions (data not shown).

Both CD and UC have been associated with an increase in intestinal permeability[9-11]. Experimental data reveal an altered expression of claudin-2, and other claudin proteins, in the intestinal epithelium of IBD patients, and such alterations affect the permeability characteristics[38]. Claudin-2 expression was increased in a cell culture of human colonic epithelial cells (HT-29/B6) in response to tumor necrosis factor-α (TNF-α)[38], a finding consistent with an increased expression of claudin-2 in the inflamed epithelium of CD patients[38,39]. Using another human colonic epithelial cell line (T84), Prasad *et al*[39] demonstrated an increase both in paracellular permeability and claudin-2 expression in response to interleukin-13, but not in response to interferon-γ/TNF-α treatment.

Within the resequenced parts of *CLDN2* we identified two novel polymorphisms in the promoter region. These two sequence variants were located in different putative transcription factor binding sites, a Sp1 binding site/GC-box (rs62605981) and an USF binding site/E-box (rs72466477). It has been shown that the *CLDN2* transcription is affected by several transcription factors[34], and both E-box binding[40-42] and Sp1 binding site[43,44] transcription factors have been identified as important determinants of claudin gene expression. However, neither of the two novel promoter variants were associated to CD.

MORC4 is present on the same linkage block as *CLDN2* and the CD associated marker (rs12014762). It is therefore possible that the C allele of rs12014762 is a marker for a functional variant of *MORC4* that results in an increased overall risk of developing CD. A significant association was observed between CD and the G allele of the nonsynonymous coding SNP in the *MORC4* gene (rs6622126), resulting in a substitution of the more hydrophobic amino acid isoleucine at position 473, located immediately outside the region predicted to be a zinc finger (420-472), with threonine.

MORC4 encodes a member of the MORC family of CW-type zinc finger proteins, which contain a number of predicted domains and motifs suggestive of being transcription factors[45]. *MORC4* exhibits a low-level mRNA expression in a variety of normal tissues, including the intestine. Using the SKI-like protein as bait in a two-hybrid screen, MORC4 has been identified as a putative interacting protein, linking MORC4 to the transforming growth factor-β (TGF-β) signaling pathway and the SMAD family of signal transduction proteins[46]. Increased intestinal expression of TGF-β has been observed in patients with CD[47] and, in monolayers of intestinal epithelial cells, TGF-β has been shown to preserve or enhance the paracellular barrier[48,49]. These two findings seem to contradict the increased intestinal permeability that has been associated to both CD and UC[9-11]. Possibly a genetic variant in the *CLDN2-MORC4* region could disturb a TGF-β mediated signal that preserves or enhances the paracellular barrier, or exert an effect on *CLDN2* expression that dominates a TGF-β mediated effect on paracellular permeability. In addition, a SMAD4-dependent, but TGF-β-independent, repression of *CLDN1* transcription[50] and a ZEB2-mediated repression of *CLDN4*[42] support a role for the SMAD signal transduction pathway in the regulation of claudin genes.

An increased intestinal permeability has been associated with the CD susceptibility allele CARD15 3020insC[18], and TJ associated genes have been suggested as suscepti-

bility genes for UC (*e.g.*, *GNA12*[2]) and for both UC and CD (*MAGI2*[51]).

In conclusion, our findings add further support for a genetically impaired intestinal epithelial barrier as one predisposing factor in the etiology of CD, either directly through *CLDN2* or indirect *via* a tentative link between *MORC4*, TGF-β/SMAD signalling and an effect on paracellular permeability. This putative genetic link between the *CLDN2-MORC4* region, intestinal epithelial integrity and the risk of developing CD needs to be further explored.

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COMMENTS

Background

For chronic inflammatory bowel disease (IBD) - Crohn's disease (CD), ulcerative colitis - a number of studies have demonstrated a substantial genetic predisposition. These inflammatory conditions have been associated with an increased intestinal permeability. Consistent with the phenotype of these diseases, several genetic association studies have implicated mainly components of the immune response, but also factors implicated in intestinal permeability. In order to further investigate intestinal permeability as a predisposing genetic risk factor for IBD, the authors have conducted genetic association studies on claudin genes (*CLDN1*, *CLDN2* and *CLDN4*), key components in the regulation of permeability.

Research frontiers

Although large-scale genome-wide association studies have uncovered a large number of genetic susceptibility loci in relation to IBD these factors still explain only a minority of the total genetic risk for IBD. The genetic background regulating intestinal barrier functions largely remains unknown.

Innovations and breakthroughs

The barrier of epithelial cells is critical for the permeability properties of the intestine. The tight junction structure is a multicomponent protein complex that serves to seal and regulate permeability across the space between adjacent epithelial cells, with significant contribution from members of the claudin family. This is the first study to report genetic link between claudin gene (*CLDN2*) and the risk of developing CD.

Applications

In this study the authors have identified a genetic link between the *CLDN2-MORC4* region and the risk of developing CD, and thereby highlighted claudins as therapeutic targets.

Terminology

The tight junction structure is critical for the permeability properties of the intestine. The structure is a multicomponent protein complex that serves to seal and regulate permeability across the paracellular space between adjacent epithelial cells.

Peer review

This is a small study on the importance of claudins (*CLDN1*, *CLDN2* and *CLDN4*) in Swedish and non-Swedish case-control and family-based approach. A weak suggestive association was reported in the case-control setting, while this was not replicated in the non-Swedish sample. The paper is interesting and well written: its main limitation lies in the study design, comparing Swedish and non-Swedish individuals using two different approaches.

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