Colonic mucosal gene expression and genotype in irritable bowel syndrome patients with normal or elevated fecal bile acid excretion

Michael Camilleri, Paula Carlson, Andres Acosta, and Irene Busciglio

Clinical Enteric Neuroscience Translational and Epidemiological Research (C.E.N.T.E.R.), Mayo Clinic, Rochester, Minnesota

Submitted 11 March 2015; accepted in final form 28 April 2015

Camilleri M, Carlson P, Acosta A, Busciglio I. Colonic mucosal gene expression and genotype in irritable bowel syndrome patients with normal or elevated fecal bile acid excretion. Am J Physiol Gastrointest Liver Physiol 309: G10–G20, 2015. First published April 30, 2015; doi:10.1152/ajpgi.00080.2015.—The mucosal gene expression in rectosigmoid mucosa (RSM) in irritable bowel syndrome with diarrhea (IBS-D) is unknown. Our objectives were, first, to study mucosal gene expression [by RT² PCR of 19 genes pertaining to tight junctions, immune activation, intestinal ion transport and bile acid (BA) homeostasis] in RSM in IBS-D patients (n = 47) and healthy controls (n = 17) and study expression of a selected protein (PDZD3) in 10 IBS-D patients and 4 healthy controls; second, to assess RSM mRNA expression according to genotype and fecal BA excretion (high ≥2.337 μmol/48 h); and third, to determine whether genotype or mucosal mRNA expression is associated with colonic transit or BA parameters. Fold changes were corrected for false detection rate for 19 genes studied (P < 0.00263). In RSM in IBS-D patients compared with controls, mRNA expression of GUC2AB, PDZD3, and PR2Y4 was increased, whereas CLDN1 and FN1 were decreased. One immune-related gene was upregulated (C4BP4) and one downregulated (CCL20). There was increased expression of a selected ion transport protein (PDZD3) on immunohistochemistry and Western blot in IBS-D compared with controls (P = 0.02). There were no significant differences in mucosal mRNA in 20 IBS-D patients with high compared with 27 IBS-D patients with normal BA excretion. GPR11 (P < 0.05) was associated with colonic transit. We concluded that mucosal ion transport mRNA (for several genes and PDZD3 protein) is upregulated and barrier protein mRNA downregulated in IBS-D compared with healthy controls, independent of genotype. There are no differences in gene expression in IBS-D with high compared with normal fecal BA excretion.

neurotransmitters; ion channels; cytokines; barrier; immune; secretion; PDZD3; GUC2AB

THE CAUSE OF LOOSE BOWEL MOVEMENTS in patients with irritable bowel syndrome with diarrhea (IBS-D) is partly attributed to acceleration of colonic transit, documented in ~45% of patients with IBS-D (15), and to intestinal secretory mechanisms (reviewed in Refs. 7 and 8). Documented secretory mechanisms include increased duodenal and rectosigmoid expression of secretory transmitters (e.g., 5-HT), reduced expression of the serotonin reuptake protein, and fecal excretion of secretogranins or chromogranins (25, 26, 46). There is also evidence of reduced expression of proabsorption mechanisms (e.g., mucosal PYY, somatostatin, NPY). IBS has also been associated with changes in RSM expression of immune factors, barrier function, and mucus secretion (1, 4, 5, 9, 22, 33, 51, 56, 60, 63, 65, 68). In a few instances, altered mucosal gene expression in tissues was associated with the inherited genotype, such as TNFSF15 gene, which is associated with IBS and has been linked with functional alterations of mucosal immune and protective functions (51, 68).

Differences in jejunal mucosal expression (at gene and protein levels) and distribution of apical junction complex proteins between IBS patients and controls support the observed alterations in barrier function in colonic mucosa in patients with IBS-D (36, 37). We also demonstrated a borderline difference in the zonula occludens 1 (ZO-1) intensity score in the small bowel mucosa (P = 0.06) of patients with IBS-D compared with healthy controls, with lower intensity in HLA-DQ2/8-positive relative to HLA-DQ2/8-negative patients with IBS-D (56).

In a prior study, based on next-generation RNA sequencing and confirmation by reverse transcriptase polymerase chain reaction (RT-PCR), we examined RSM from nine patients with IBS-D and nine healthy controls and identified differential expression of secretory and barrier genes, suggesting that the transcriptome is different in IBS-D compared with controls (11). The upregulated mechanisms associated with changes in ion transport included PDZD3. PDZ adapter proteins are involved in multiple ion transport functions in the intestine, including sodium absorption through sodium-hydrogen exchange (NHE3), as well as guanylyl cyclase C receptor (GC-C, or GUCY2C)-induced chloride and water secretion through cGMP signaling that leads to cystic fibrosis transmembrane conductance regulator phosphorylation and chloride-bicarbonate exchange through the SLC26 anion exchanger (35). These ion-exchange mechanisms also result in alterations in intestinal fluid transport.

Another upregulated ion transport mechanism identified in our prior study was increased GUC2AB mRNA. GUC2AB encodes uroguanylin, an endogenous ligand for GC-C receptor, increasing cyclic GMP, chloride, and water secretion.

The present study hypotheses were as follows: first, there is upregulation of genes and proteins associated with intestinal secretion in the colonic mucosa of patients with IBS-D, particularly in patients with high fecal bile acid (BA) excretion; second, variations in inherited genes are associated with the mRNA expression of the same genes in the colonic mucosa; and third, genetic variation and/or RSM expression of the genes are associated with alterations in intermediate phenotype in IBS-D, specifically colonic transit and parameters of BA homeostasis.

The aims of this study were, first, to replicate the prior pilot study by targeted mRNA analysis using quantitative RT-PCR of RSM biopsies from 47 IBS-D patients and 17 healthy controls and to analyze the protein expression of one of the...
proteins associated with ion transport, PDZD3, in 10 IBS-D patients and 4 healthy controls; second, to determine whether there are differences in mucosal mRNA expression in subgroups of IBS-D with high compared with normal fecal BA excretion; third, to determine whether genotype was associated with level of expression of mRNA in RSM, colonic transit, or BA parameters; and fourth, to assess whether the level of expression of mRNA in RSM was associated with colonic transit.

METHODS

Ethical Approval

The study was approved by Mayo Clinic Institutional Review Board on October 28, 2011. Written, informed consent was received from participants prior to inclusion in the study.

Study Design

We appraised bowel functions, total fecal BA excretion over 48 h, fasting serum C4 (7α-hydroxy-4-cholesten-3-one) and FGF19, colonic transit, genotype, and RSM mRNA expression in 47 patients with IBS-D (by Rome III criteria). Fecal total BA excretion was used to differentiate patients with high or normal BA excretion, suggestive of BA diarrhea.

Patient Selection

Patients were recruited by public advertisement or by invitation to participate from a database of ~1,200 patients with IBS living in communities within ~120 miles of Mayo Clinic in Rochester, MN. Inclusion criteria were based on symptoms by use of a validated diary questionnaire that characterized IBS symptoms and, particularly, bowel functions (53). Participants also completed the Hospital Anxiety and Depression Inventory (67). These patients had been evaluated at Mayo Clinic, and alternative diagnoses such as inflammatory bowel disease, cancer, and celiac disease were excluded. The main exclusion criteria were intake of medications that could interfere with the study, including 19 genes to assess the effect of IBS-D on the expression of tight junction proteins, chemokines, markers of innate immunity, ion channels, and transmitters that have been demonstrated to be differentially expressed in RSM of patients with IBS (11). The custom profile included two housekeeping genes for normalization and three control genes that check for sample quality and reaction quality (Table 1).

Stored Biospecimens

We used stored samples from patients who had consented to the use of biospecimens for future research in prior studies (1, 10, 57) conducted at Mayo Clinic in Rochester, MN. These samples were obtained in 47 patients with IBS-D and 17 healthy controls. To appraise specificity of the observations in IBS-D, we analyzed RSM obtained in 47 patients with IBS-D and 17 healthy controls. To determine whether genotype was associated with level of expression of mRNA in RSM, colonic transit, or BA parameters; and fourth, to assess whether the level of expression of mRNA in RSM was associated with colonic transit.

Identification of Subgroups of IBS-D Patients Based on Fecal BA Excretion

Total and main fecal BA excretions (per 48 h on 100 g fat diet) were measured by HPLC/tandem mass spectrometry (48, 57, 64). This assay was adapted from a method used with serum samples (52).

IBS-D subgroups were identified by fecal BA excretion >2,337 μmol/48 h based on 90th percentile of 45 healthy volunteers studied in our laboratory (64). The 90th percentile was used to define the upper limit of normal range, consistent with the observation that a normal distribution (estimated from 5th and 95th percentiles) requires sampling of 500 normal people (2).

Other BA Parameters

Fasting serum C4 (a measure of hepatic synthesis rate of BAs sampled in the morning) was measured by HPLC/tandem mass spectrometry (16); serum C4 is a validated method for detecting BA malabsorption (6). Similarly, fasting levels of fibroblast growth factor-19 (FGF19) were measured by a commercial enzyme-linked immunosorbent assay (FGF19 Quantikine Enzyme-Linked Immunosorbent assay Kit; R&D Systems, Minneapolis, MN) as in prior studies (43, 64).

Gene Expression Method by RT2 PCR Array

Selection of genes of interest. We developed a custom profile including 19 genes to assess the effect of IBS-D on the expression of tight junction proteins, chemokines, markers of innate immunity, ion channels, and transmitters that have been demonstrated to be differentially expressed in RSM of patients with IBS (11). The custom profile included two housekeeping genes for normalization and three control genes that check for sample quality and reaction quality (Table 1).

Assay method. For mRNA expression, RNA was purified from human RSM biopsies by using the Qiagen RNeasy Kit (Qiagen, Valencia, CA), including on-column DNase treatment to remove genomic DNA. RNA quality was assessed on the Agilent Bioanalyzer. The resulting RNA (RIN > 7) was reverse transcribed by use of the RT2 First Strand Kit (Qiagen), and samples were analyzed for expression by a Custom Profiler RT2 PCR Array (Qiagen).

Genotyping Method

DNA was extracted from venous blood, and candidate genotype analysis was conducted by established PCR-based methods, as previously detailed in prior publications: rs4795541 [SLC6A4 (34)]; rs4263839 [TNFSF15 (68)]; rs11554825 [FGFR4 (18)]; rs434434 [FGFR4 (13)]; rs188096 [SLC10A2 (18)]; rs17618244 [KLBl (18)]; rs1966265 [FGFR4 (18)]; and rs351855 [FGFR4 (18)]. Farnesoid X receptor (FXR) single-nucleotide polymorphisms (SNPs) rs17030285 and rs4764980 were also assessed by TaqMan assay (catalog no. C_34126156_10; catalog C_3127933_20). Gene names are given according to Hugo Gene Nomenclature.

Briefly, genomic DNA was isolated from whole blood by use of the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA) and stored at −80°C until genotyping. The serotonin transporter protein promoter polymorphism (SLC6A4 rs4795541), also referred to as 5-HTTLPR, was determined by PCR-based fragment length. The remaining eight SNPs were analyzed by TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) per the manufacturer’s instructions. Following polymerase chain reaction amplification, end reactions were analyzed by using ABI 7300 Real-Time PCR System by Sequence Detection Software (Applied Biosystems).

Confocal Immunofluorescence Microscopy of PDZD3

We chose to study expression of PDZD3 because of its involvement in both sodium absorptive and chloride secretory mechanisms.
### Table 1. Genes of interest included in the RT-PCR analysis of rectosigmoid mucosa

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Refseq No.</th>
<th>Official Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4BPA</td>
<td>NM_000715</td>
<td>Complement component 4 binding protein, alpha</td>
</tr>
<tr>
<td>CCL20</td>
<td>NM_004591</td>
<td>Chemokine (C-C motif) ligand 20</td>
</tr>
<tr>
<td>CLDN1</td>
<td>NM_021101</td>
<td>Claudin 1</td>
</tr>
<tr>
<td>FGRFR1</td>
<td>NM_002011</td>
<td>Fibroblast growth factor receptor 4</td>
</tr>
<tr>
<td>FN1</td>
<td>NM_002026</td>
<td>Fibronectin 1</td>
</tr>
<tr>
<td>GPBAR1</td>
<td>NM_170699</td>
<td>G protein-coupled bile acid receptor 1 (syn. TGR5)</td>
</tr>
<tr>
<td>GUCA2B</td>
<td>NM_007102</td>
<td>Guanylate cyclase activator 2B (uroguanylin)</td>
</tr>
<tr>
<td>IFT3</td>
<td>NM_001549</td>
<td>Interferon-induced protein with tetrapeptide repeats 3</td>
</tr>
<tr>
<td>NR1H4</td>
<td>NM_005123</td>
<td>Nuclear receptor subfamily 1, group H, member 4 (syn. Farnesoid X receptor)</td>
</tr>
<tr>
<td>OCLN</td>
<td>NM_002538</td>
<td>Occludin</td>
</tr>
<tr>
<td>P2RY4</td>
<td>NM_002565</td>
<td>Purinergic receptor P2Y, G-protein coupled, 4</td>
</tr>
<tr>
<td>PDZD3</td>
<td>NM_024791</td>
<td>PDZ domain containing 3</td>
</tr>
<tr>
<td>RBP2</td>
<td>NM_004164</td>
<td>Retinol binding protein 2, cellular</td>
</tr>
<tr>
<td>SLC10A2</td>
<td>NM_000452</td>
<td>Solute carrier family 10 (sodium/bile acid cotransporter family), member 2 (syn. Apical Sodium-coupled Bile acid transporter)</td>
</tr>
<tr>
<td>SLC6A4</td>
<td>NM_001045</td>
<td>Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (syn. Serotonin transporter)</td>
</tr>
<tr>
<td>TFF1</td>
<td>NM_003225</td>
<td>Trefoil factor 1</td>
</tr>
<tr>
<td>TJP1</td>
<td>NM_175610</td>
<td>Tight junction protein 1 (syn. zonula occludens 1)</td>
</tr>
<tr>
<td>TNFSF15</td>
<td>NM_005118</td>
<td>Tumor necrosis factor (ligand) superfamily, member 15</td>
</tr>
<tr>
<td>VIP</td>
<td>NM_003381</td>
<td>Vasooactive intestinal peptide</td>
</tr>
<tr>
<td>ACTB</td>
<td>NM_001101</td>
<td>Actin, beta (housekeeping gene)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene)</td>
</tr>
<tr>
<td>HGDC</td>
<td>SA_00105</td>
<td>Human Genomic DNA Contamination</td>
</tr>
<tr>
<td>RTC</td>
<td>SA_00104</td>
<td>Reverse Transcription Control</td>
</tr>
<tr>
<td>PPC</td>
<td>SA_00103</td>
<td>Positive PCR Control</td>
</tr>
</tbody>
</table>

Syn., synonym.

Confocal immunofluorescence microscopy was performed as previously described (44). Briefly, unstained RSM sections were deparaffinized and rehydrated, boiled in antigen unmasking solution (Vector Laboratories, Burlingame, CA), quenched with Image-iT FX signal enhancer (Life Technologies, Grand Island, NY), and blocked with 1% BSA/10% FBS/0.1% Triton X-100 in PBS. Slides were then incubated overnight at 4°C with primary antibodies PDZD3 (1:1,000, LifeSpan BioSciences, Seattle, WA) and cytokeratin 8/18 (1:50, Santa Cruz, Dallas, TX). Fluorescent-labeled goat anti-rabbit IgG H+L (Alexa Fluor 568, Abcam, Cambridge, MA) and donkey anti-mouse IgG H+L (Alexa Fluor 488, Life Technologies) were applied to the slides; the slides were then rinsed and mounted with ProLong Gold with DAPI (Molecular Probes, Life Technologies) and were visualized on a Zeiss LSM 510 confocal microscope with a ×63 magnification oil objective.

**PDZD3 Protein Measurements by Western Blots**

Whole cell lysates were isolated from human colon biopsy sections with the RIPA Lysis Buffer System (Santa Cruz), and concentrations were determined by BCA quantification (Pierce, Rockford, IL). Proteins were separated by use of 4–15% Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA) and blotted onto nitrocellulose membranes. The membranes were blocked with 5% milk in PBS/0.2% Tween, after which PDZD3 primary antibody (1:4,000, LSBio Systems, Seattle, WA) was applied overnight at 40°C.
excretion. Thus, on average, there was 8% of mean BA excretion of 5.167 μmol per 48 h or −0.41 mmol as CDCA in stool of IBS-D with high fecal BA excretion, compared with average 5% of mean BA excretion of 1.025 μmol per 48 h or −0.05 mmol as CDCA in stool of IBS-D with normal fecal BA excretion.

mRNA Fold Change in RSM from IBS Relative to Healthy Controls

The mean (and 95% confidence interval) fold changes (based on 2^−ΔΔCT) in IBS-D and subgroups of IBS-D patients relative to healthy controls are illustrated in Table 3 and Fig. 1.

By using FDR for 19 gene comparisons, these observations suggest, first, no significant fold changes in expression of genes associated with absorption [SLC10A2 (ASBT)], effects [GBP1R1(TGR5)] or feedback regulation of synthesis (FGFR4) of BAs; however, there were borderline changes in expression of FXR (P = 0.0081); second, increased expression of GUCAB2, PDZD3, and P2RY4, reflecting ion transport mechanisms; there was borderline reduction in expression of vasoactive intestinal peptide (VIP) (P = 0.0030); third, increased expression of C4BPA and reduced expression of CCL20, reflecting immune functions; and fourth, decreased expression of several barrier proteins, claudin-1 and fibronectin.
tin-1, and borderline increased expression of retinol binding protein (RBP-2, $P = 0.0031$).

There were no differences in the mucosal expression of any of the genes of interest among IBS-D patients with high compared with normal fecal BA excretion; however, there were numerical (univariate, FDR-uncorrected values, $P = 0.10$) reductions in expression of the mRNA of tight junction proteins [ZO-1 (TJP1), and FN-1] and decreased expression of TNFSF15 (immune marker).

The mean (and 95% confidence interval) fold changes (based on $2^{-\Delta\Delta CT}$) in IBS-D, and subgroups of IBS-D and IBS-C patients relative to healthy controls are illustrated in Table 3. The only significant change, corrected for FDR, was increased expression of PR2Y4, VIP, and occludin (OCLN).

**PDZD3 Protein Expression by Immunohistochemistry and Western Blot**

We used immunofluorescence to assess protein expression of the regulatory protein, PDZD3. Figure 2 shows the increased expression of this ion transport protein in RSM of patients with IBS-D compared with normal healthy volunteers by Western blot and immunohistochemistry. Western blots quantitating PDZD3 protein (with vinculin as control) showed increased expression of PDZD3 ($P = 0.02$ by Student’s $t$-test) in IBS-D patients ($n = 10$) compared with healthy controls ($n = 4$).

**Relationship of Genotype and mRNA Expression (corrected for $\beta$-Actin) in IBS**

Using general genetic model. mRNA expressions (corrected for the expression of $\beta$-actin) showed no significant associations with genotypes except for a trend in the expression of GPBAR1 (Table 4); however, the observation that homozygous genotypes were both associated with higher expression than the heterozygotes questions any biological significance of this finding.

Using dominant genetic model. There was a significant association of GPBAR1 genotype and mRNA expression ($P = 0.043$) in RSM, based on analysis using the dominant genetic model (Table 5).

**Spearman Correlation of mRNA Expression with Ascending Colon Emptying $T_{1/2}$**

We sought the association of mRNA expression (corrected for $\beta$-actin) in RSM with ascending colon emptying $T_{1/2}$. There were no significant relationships between mRNA expression of FGFR4 ($r = -0.06, P = 0.71$), GPBAR1 ($r = -0.08, P = 0.63$), GUCA2B ($r = 0.22, P = 0.13$), P2RY4 ($r = 0.16, P = 0.27$), NR1H4 ($r = -0.11, P = 0.48$), TNFSF15 ($r = -0.63, P = 0.673$), SLC10A2 ($r = -0.08, P = 0.62$), and SLC6A4 ($r = 0.147, P = 0.39$) and ascending colon emptying $T_{1/2}$.

**Relationship of Genotype with Colonic Transit in IBS-D** (using Dominant Genetic Model)

GPBAR1 was significantly associated with colonic transit at 24 and 48 h, as well as ascending colon emptying $T_{1/2}$ (Table 6); a borderline association of 5-HTTLPR with colonic transit at 24 and 48 h was also observed ($P \leq 0.081$).

**Association of BA Homeostasis Genes (SLC10A2, KLB and FGFR4 Genotypes) and BA Measurements in Patients with IBS-D (using Dominant Genetic Model)**

In these 47 patients with IBS-D, the variants in the genes associated with BA homeostasis were not significantly associated with serum FGF19, serum C4, and 48-h fecal BA excretion (Table 7).
DISCUSSION

Our study has provided several novel insights about mucosal pathobiology of IBS by means of studies of RSM biopsies from 47 IBS-D patients or controls. The clinical and biochemical characteristics of the participants in this study are consistent with those described recently in a somewhat larger cohort (n = 64) that included the same 47 patients. However, the present study could only include the 47 patients who consented to undergo rectosigmoid biopsies. One difference in the present group of patients is that fasting serum FGF19 was not significantly different between IBS-D and healthy controls, in contrast to the original report by Walters et al. (62) and our prior studies (10, 64) with larger sample sizes. We perceive that the lack of significant difference in fasting serum FGF19 between IBS-D and healthy controls represents a type 2 error: among the 17 healthy controls who had undergone rectosigmoid biopsies, only 4 had fasting serum FGF19 measurement.

The following observations were made in the present study:

Fig. 2. Top: Western blots quantitating PDZD3 protein (with vinculin as control). Note the increased expression (P = 0.02) of PDZD3 in IBS-D patients (n = 10) compared with healthy controls (n = 4). Bottom: immunofluorescence for PDZD3 in rectosigmoid colon mucosa of a patient with IBS-D compared with a normal, healthy control (×63 magnification). Stains show nuclear staining (DAPI), cytokeratin (C8/18, marker of epithelial cells), PDZD3, and merge. Note the localization of increased PDZD3 protein in epithelial cells in mucosa from the patient with IBS-D compared with the healthy control.
Table 4. Relationship of genotype and mRNA expression (median, IQR; corrected for β-actin) by general genetic model (comparing expression in 3 genotypes: AA, Ab, bb) in IBS (analysis used ANOVA on ranks to compare 3 genotypes)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MAF</th>
<th>N</th>
<th>mRNA Expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC6A4</td>
<td>0.447</td>
<td>7</td>
<td>16.59 (15.47, 17.44)</td>
<td>0.463</td>
</tr>
<tr>
<td>SLC6A4</td>
<td>0.287 (A)</td>
<td>20</td>
<td>10.83 (10.62, 11.17)</td>
<td>0.48</td>
</tr>
<tr>
<td>SLC6A4</td>
<td>0.287 (A)</td>
<td>20</td>
<td>10.83 (10.62, 11.17)</td>
<td>0.48</td>
</tr>
<tr>
<td>TNF5F15</td>
<td>0.38 (A)</td>
<td>6</td>
<td>12.94 (12.11, 13.85)</td>
<td>0.27</td>
</tr>
<tr>
<td>TNF5F15</td>
<td>0.38 (A)</td>
<td>6</td>
<td>12.94 (12.11, 13.85)</td>
<td>0.27</td>
</tr>
<tr>
<td>TNF5F15</td>
<td>0.38 (A)</td>
<td>6</td>
<td>12.94 (12.11, 13.85)</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Table 5. Relationship of genotype and mRNA expression (median, IQR; corrected for β-actin) by dominant genetic model (comparing expression in homozygous major allele genotype vs. combining heterozygotes and minor allele homozygous) in IBS (comparisons with Mann-Whitney test)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MAF</th>
<th>N</th>
<th>mRNA Expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC6A4</td>
<td>0.447 (L)</td>
<td>7</td>
<td>16.59 (15.47, 17.44)</td>
<td>0.70</td>
</tr>
<tr>
<td>SLC6A4</td>
<td>0.447 (L)</td>
<td>7</td>
<td>16.59 (15.47, 17.44)</td>
<td>0.70</td>
</tr>
<tr>
<td>TNF5F15</td>
<td>0.38 (A)</td>
<td>17</td>
<td>12.28 (12.07, 12.96)</td>
<td>0.12</td>
</tr>
<tr>
<td>TNF5F15</td>
<td>0.38 (A)</td>
<td>17</td>
<td>12.28 (12.07, 12.96)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

MAF, minor allele frequency.

First, targeted mRNA analysis by quantitative RT-PCR replicates our prior results showing generally increased expression of intestinal ion transport mechanisms and generally reduced intestinal barrier and up- or downregulation of mucosal immune mechanisms. The significance of the observed associations is limited by the relatively small number of biopsies assessed (total 64); however, we present both uncorrected P values and, more importantly, we present the significance of observations corrected for the comparison of 19 selected genes. One of the well-recognized ion transport mechanisms (PDZD3) showing increased mRNA expression was also associated with increased expression of PDZD3 protein on immunohistochemistry and Western blot analysis.

Second, there were no differences in mucosal expression in IBS-D associated with degree of fecal BA excretion.

Our third general finding was that genotype was not significantly associated with the level of expression of mRNA in RSM, colonic transit, or BA parameters, with the exception of the GPBAR1 gene, which is significantly associated with mucosal expression of GPBAR1 and with colonic transit.

Fourth, the level of expression of mRNA in RSM was not associated with ascending colon emptying time.

Mucosal mRNA Expression in IBS-D

Among the intestinal secretory mechanisms, increased mRNA expression of GUCA2B and P2RY4 could all support fluid and electrolyte secretion through actions on enterocytes or submucosal neurons. On the other hand, the significantly increased expression of PDZD3 is associated with either increased sodium ion and fluid absorption (through effects on NHE3) or increased chloride ion and fluid secretion through CFTR, and borderline decreased expression of VIP (P = 0.003) may conceivably be associated with reduced intestinal secretion.

The largest fold increases in mRNA expression in our IBS-D patients were observed for the purinergic receptor, P2RY4. Purinergic receptors are divided into adenosine P1 [A(1), A(2A), A(2B), A(3)], ionotropic ATP-gated P2X receptors [P2X(1–7) that form ion channels or pores], or metabotropic P2Y(1, 2, 4, 6, 11–14) receptors. Metabotropic receptors are indirectly linked with ion channels on the plasma membrane through transduction mechanisms, often G proteins. The purinergic hypothesis is based on ATP (or a related nucleotide, e.g., ADP or AMP) release at the neurotransmitter synapses or on neuromuscular transmission, and these may involve βNAD+ and ADP ribose in neurotransmission in rodents, primates, and humans (24, 29, 30). Mechanically evoked reflex electrogenic chloride secretion in rat distal colon is triggered by endogenous nucleotides acting at P2Y1, P2Y2, and P2Y4 receptors (21). P2Y2, 4, and 6 receptors regulate Cl−, Na+, and K+ secretion in the intestinal tract, and absorption mechanisms, in particular, P2Y4 receptors, are involved in chloride secretion and potassium secretion (20, 28, 38). Thus our observation of marked increase in expression of P2RY4 is consistent with the increased expression of secretory mechanisms (in addition to GUCA2B) in IBS-D patients. In addition to the novel mechanisms potentially related to ion transport in the manifestations of IBS-D, the present findings provide the basis for further hypothesis testing and, possibly, testing novel pharmacological approaches to reverse electrolyte secretion (41) in IBS-D. These findings complement the observation of...
increased small intestine secretion in response to BA infusion in IBS-D (42).

The specificity of the observed fold changes in mRNA expression is enhanced by the differences in the observations for IBS-D and the additional control group of IBS-C. The biological significance of the significant associations with IBS-D is discussed in the next section.

Increased mRNA expression of GUCA2B is associated with the endogenous GC-C ligand, uroguanylin, which is secreted by intestinal goblet cells (50). In the same way that guanylate cyclase C agonists are effective in the treatment of IBS-C, it is conceivable that antagonists at the guanylate cyclase receptors or GC-C ligands (guanylin and uroguanylin) with gastric motor dysfunction and intestinal dilatation (49). The activation observed in IBS-D (45).

We observed borderline increased mRNA expression of FXR in colonic mucosa in the whole IBS-D group (P = 0.0081, relative to significance value with FDR, P = 0.00263); this was also observed in both IBS-D subgroups. FXR activation prevents chemically induced intestinal inflammation, with improvement of colitis symptoms, inhibition of epithelial permeability, and reduced goblet cell loss in a mouse model of inflammatory bowel disease (27). FXR expression is decreased in colonic mucosa of patients with primary sclerosing cholangitis [PSC (54)], and PSC is associated with higher circulating levels of the conjugated primary BAs (3). These data are consistent with a potential role of colonic mucosal FXR in protecting the colonic mucosal integrity in patients with IBS-D. Increased expression of C4BPA and reduced expression of CCL2 reflect changes in immune functions that may ultimately lead to the immune activation observed in IBS-D (45).

Decreased expression of several barrier proteins, especially claudin-1 and fibronectin-1, may reflect the observed increase in intestinal mucosal permeability in IBS-D (14). The observed borderline increased expression of RBP-2 may be a compensatory mechanism to maintain intestinal barrier integrity.

Table 6. Relationship of genotype with CT and ascending colon emptying T1/2 in IBS-D

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Colonic GC24</th>
<th>P</th>
<th>Colonic GC48</th>
<th>P</th>
<th>AC Emptying T1/2 h</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HTTLPR rs4795541 LL</td>
<td>7</td>
<td>3.9 (3.01, 4.42)</td>
<td>0.079</td>
<td>4.00 (4.17, 5.00)</td>
<td>0.081</td>
<td>15.14 (5.41, 17.41)</td>
<td>0.362</td>
</tr>
<tr>
<td>5-HTTLPR rs4795541 LS/SS</td>
<td>40</td>
<td>2.43 (1.78, 3.89)</td>
<td>4.57 (3.33, 4.99)</td>
<td>0.40</td>
<td>16.39 (10.46, 19.47)</td>
<td>0.825</td>
<td></td>
</tr>
<tr>
<td>TNFSF15 rs4263839 GG</td>
<td>17</td>
<td>2.97 (1.70,4.56)</td>
<td>0.79</td>
<td>4.67 (3.99, 5.00)</td>
<td>0.40</td>
<td>15.57 (10.67, 18.57)</td>
<td>0.825</td>
</tr>
<tr>
<td>TNFSF15 rs4263839 AA/AG</td>
<td>30</td>
<td>2.72 (1.90,3.90)</td>
<td>4.58 (3.16, 5.00)</td>
<td>0.99</td>
<td>16.60 (5.30, 20.15)</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

Data show median and interquartile range; analysis is by Mann-Whitney rank-sum test. Note the significant association with GPBAR1 genotype and the borderline association with SLC6A4 (5-HTTLPR polymorphism). S is minor allele for 5-HTTLPR; T is minor allele for GPBAR1. *Number of AA KLB genotype identified. AC, ascending colon.

Table 7. Associations of variants in genes (associated with BA absorption and feedback regulation of synthesis) with serum FGF-19, serum C4, and 48-h fecal BA excretion in IBS-D

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Fasting Serum FGF-19, pg/ml</th>
<th>P</th>
<th>Fasting Serum C4, ng/ml</th>
<th>P</th>
<th>Total Fecal BA, μmol/48 h</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC10A2 rs188096 AC</td>
<td>12</td>
<td>2.26 (1.87, 3.39)</td>
<td>0.80</td>
<td>4.52 (3.69, 4.93)</td>
<td>0.88</td>
<td>15.62 (7.69, 19.9)</td>
<td>0.96</td>
</tr>
<tr>
<td>SLC10A2 rs188096 CC</td>
<td>35</td>
<td>2.80 (1.70, 4.31)</td>
<td>4.66 (3.20, 5.00)</td>
<td>0.99</td>
<td>3.18 (7.63, 19.3)</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

Data show median and interquartile range.
satory change to correct the decreased expression of the other tight junction proteins.

On the other hand, there were no significant differences (see Table 3) in the mucosal expression of any of the genes of interest among IBS-D patients with high compared with normal fecal BA excretion. The significance of the increased mucosal expression in P2RY4 and VIP in IBS-C is unclear, although their increased expression may be related to muscle function (e.g., relaxation) rather than ion secretion. The increased OCLN in mucosa from IBS-C patients may reflect greater mucosal barrier functions in these patients; prior work (5) had documented the preservation of occludin and claudin expression in IBS-C in contrast to IBS-D.

There are only a few other papers in the published literature that describe alterations in mucosal mRNA expression of several mechanisms by next-generation sequencing methods in patients with IBS-D. Our previous study used next-generation RNA sequencing and RT-PCR of RSM in nine patients with IBS-D and nine healthy controls; we identified differential expression of secretory and barrier genes (11). In the present study in IBS-D patients, we confirmed the mRNA fold changes for 8 of the 10 genes evaluated in the prior study: FN1, IFIT3, PDZD3, TFF1, GUC2A2B, RBP2, C4BPA, and P2RY4. The Barcelona group used combinations of microarray and PCR, focused their studies on mucosal tight junction expression and immune activation in jejunal mucosal biopsies, and identified reduced expression of ZO-1 in IBS-D at both gene and protein levels (37), as well as higher mucosal immune activity in IBS-D, with upregulation of germline transcripts and immunoglobulin genes (58). A recent combined study from Helsinki and Nottingham explored the association of host rectal mucosal expression and the fecal microbiome in different subgroups of IBS, including IBS-D and postinfectious IBS (31), rather than the differences in mucosal expression between IBS subgroups and healthy controls. However, one of the strongest associations between microbial populations and rectal mucosal mRNA expression in IBS patients pertains to NR1H4 or FXR, which was a change of borderline significance (P = 0.0081) in our present study.

Relation of mRNA expression and genotype and phenotype in IBS-D. Among the genotypes studied, the only one that is significantly associated with colonic transit is GPBAR1 genotype. We had previously observed this relationship in studies in several hundred patients (17), but we present it now in only 47 patients with IBS-D and present this unique statistically significant finding in the context of a broad spectrum of genes associated with absorption, feedback regulation, and action of BAs, and selected immune function and serotonin transporter genes. None of the other genes of interest was associated with alteration in colonic transit. Importantly, we did not observe association with variation in ASBT, which has been reported rarely in familial or sporadic diarrhea (39, 40).

We found no relation between genotype and expression for all the candidate genes of interest, and, similarly, we did not find significant associations of BA-related genotypes with serum C4, serum FGF19, and fecal BA excretion. This suggests that posttranslational modification may be a more relevant mechanism controlling these phenotypic parameters of BA control and expression than genotype.

Finally, there was no relation between mRNA expression and ascending colon emptying or transit; we focused on the latter parameter of transit, since it appears to be more closely related to the state of secretion within the colon. Thus ascending colon emptying is accelerated in carcinoid diarrhea [a classical secretory diathesis (61)], and proximal colon emptying is positively correlated with stool weight (55).

Strengths and Limitations

We have studied more than five times as many IBS-D patients in the present study compared with the prior pilot study, and we evaluated mRNA expression in RSM for 19 genes; the statistical analysis was corrected for multiple comparisons. We also replicated the numerically increased or decreased mRNA fold changes (identified as FDR uncorrected changes in the prior study) in 8 of the first 10 genes of interest. Overall, these findings support the potential role of altered mucosal functions in IBS-D.

The limitations include the following: First, we restricted our study to 19 candidates, for which there was a strong biological rationale for each biological process (e.g., ion transport, immune mechanisms, barrier proteins) in IBS. However, several different potential mechanisms were explored and, therefore, we did not bias the study, other than using the prior pilot study conducted with RNA sequencing and RT-PCR confirmation of the main findings (11) as a starting point. In designing such studies, one has to balance the need to explore as many potentially relevant mechanisms with the sample size available and the need to correct for FDR.

Second, although genetic variation in GPBAR1 was associated with colonic transit (with P < 0.05 for colonic transit at 48 h), the association of genotype with colonic transit appraised nine variants in seven genes [SLC6A4, GPBAR1, TNFSF15, KLB, FGFR4, FXR, SLC10A2 (ASBT)] and three measurements of colonic transit. Therefore, the observation should be regarded as hypothesis generating and not definitely proven in this study of 64 people. Nevertheless, the present sample of patients is much smaller than the ~650 IBS patients and healthy controls that were used to demonstrate in prior studies the association of GPBAR1 (17, 18) and other genes [e.g., KLB, FAAH (summarized in Ref. 12)] with small bowel or colonic transit.

Third, we do not know that the parallel expression (mRNA and protein) of PDZD3 is necessarily a good “representation” for the other mRNA changes.

Conclusion

In conclusion, the present data demonstrate that mucosal ion transport mechanisms (mRNA for several factors, and PDZD3 protein) are generally upregulated and barrier genes downregulated in IBS-D compared with healthy controls, independent of genotype. There are no differences in gene expression in IBS-D with high compared with normal fecal BA excretion. These pathobiological mechanisms deserve further study to further advance the understanding of pathophysiological mechanisms in patients with IBS and diarrhea.

Acknowledgments

The authors thank Cindy Stanislav for excellent secretarial assistance.

Grants

This research was funded by RO1-DK92179 grant from National Institutes of Health to M. Camilleri.
REFERENCES


46. Ohman L, Stridsberg M, Isaksson S, Jerlstad P, Simrén M.
47. Sogawa C, Abe A, Tsuji T, Koizumi M, Saga T, Kunieda T.
50. Ohman L, Simrén M.