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The Colonic Mucosal MicroRNAs, MicroRNA-219a-5p, and MicroRNA-338-3p Are Downregulated in Irritable Bowel Syndrome and Are Associated With Barrier Function and Mitogen-Activated Protein Kinase (MAPK) Signaling

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Abstract

Background and Aims: Alterations in microRNA (miRNA) and in the intestinal barrier are putative risk factors for irritable bowel syndrome (IBS). We aimed to identify differentially expressed colonic mucosal miRNAs, their targets in IBS compared to healthy controls (HCs), and putative downstream pathways.

Methods: Twenty-nine IBS patients (15 IBS with constipation [IBS-C], 14 IBS with diarrhea [IBS-D]), and 15 age-matched HCs underwent sigmoidoscopy with biopsies. A nCounter array was used to assess biopsy associated miRNA levels. A false discovery rate (FDR) <10% was considered significant. Real-time PCR (RT-PCR) was used to validate differentially expressed genes. To assess barrier function, trans-epithelial electrical resistance (TEER) and dextran flux

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Conflict of interest statement

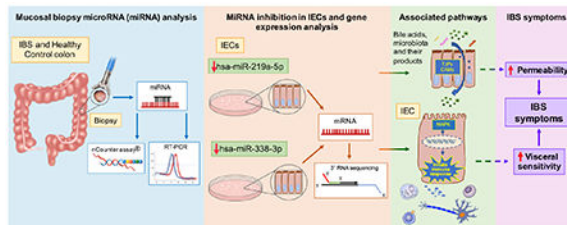
No conflict of interest to declare.

assays were performed on Caco-2 intestinal epithelial cells that were transfected with miRNA-inhibitors or control inhibitors. Protein expression of barrier function associated genes was confirmed using western blots.

Results: Four out of 247 miRNAs tested were differentially expressed in IBS compared to HCs (FDR<10%). RT-PCR validation suggested decreased levels of miR-219a-5p and miR-338-3p in IBS (0.026 and $p=0.004$), and IBS-C ($p=0.020$ and 0.06) vs. HCs as the strongest associations. Inhibition of miR-219a-5p resulted in altered expression of proteasome/barrier function genes. Functionally, miR-219a-5p inhibition enhanced the permeability of intestinal epithelial cells as TEER was reduced (25-50%, $p<0.05$) and dextran flux was increased ($p<0.01$). Additionally, inhibition of miR-338-3p in cells caused alterations in the mitogen-activated protein kinase (MAPK) signaling pathway genes.

Conclusion: Two microRNAs that potentially affect permeability and visceral nociception were identified to be altered in IBS patients. MiR-219a-5p and miR-338-3p potentially alter barrier function and visceral hypersensitivity via neuronal and MAPK signaling and could be therapeutic targets in IBS.

Graphical Abstract



IBS, irritable bowel syndrome; miRNA, microRNA; RT-PCR, real time PCR; IECs, Intestinal epithelial cells; TJPs, tight junction proteins; CAMs, cell adhesion molecules; MAPK, mitogen activated protein kinase; dotted lines represent proposed mechanisms

Keywords

miRNA; miR-338-3p; miR-219a-5p; MAPK signaling; barrier function

Introduction

Irritable bowel syndrome (IBS) is a prevalent chronic gastrointestinal (GI) condition characterized by abdominal pain and altered bowel habits (BH) including diarrhea (IBS-D), constipation (IBS-C), or a mixture of both diarrhea and constipation (IBS-M). IBS is a disorder of altered gut-brain interactions¹ and is associated with significant morbidity². Reported findings in IBS include alterations in central sensory processing, neurohormonal regulation, motility and secretion, bile acid metabolism, gut microbiome, immune activation, and epithelial barrier function, and some of these alterations may contribute to IBS symptoms. Intestinal barrier dysfunction associated with altered BH and abdominal pain has been reported in some patients with IBS^{3,4}. Some studies have reported the presence of immune activation via mast cells and T-lymphocytes⁵, which may mediate intestinal barrier

dysfunction, motor abnormalities, and visceral pain in IBS^{6,7}. Although the etiology of IBS is incompletely understood, there is evidence that genetic, environmental, and epigenetic⁸ factors play a role.

Expression of protein-coding genes (mRNAs) has been previously investigated in IBS^{9,10}, however, a majority of transcripts are non-coding¹¹. MicroRNAs (miRNAs) are small (21-23 bp) non-coding RNAs that regulate gene expression either by base-pairing to target mRNAs or via endonucleolytic mRNA cleavage¹². MiRNAs have been implicated in several GI physiologic and pathophysiologic mechanisms and studied widely in intestinal immune and inflammatory diseases, however, studies in IBS are highly heterogeneous¹³⁻²⁰. Most IBS-related miRNA studies were limited to IBS-D women. Some of the miRNAs studied were suggested to play a role in visceral hypersensitivity and barrier dysfunction, which are important pathophysiological mechanisms in IBS²¹. For example, miR-29a targets the glutamine synthetase gene (GLUL) and increases intestinal permeability²⁰, and miR-199a/b targets transient receptor potential cation channel subfamily V member 1 (TRPV1), and a decreased expression of this miRNA correlates with visceral hypersensitivity¹⁵. However, there is a lack of a global overview of validated miRNA changes, differences in target gene expression, and associated pathways in IBS, specifically IBS-C. We hypothesize that 1) IBS and BH subtypes are associated with changes in expression of mucosal miRNA and their target genes 2) IBS-associated miRNAs regulate functions/pathways associated with IBS pathophysiology.

We addressed these hypotheses by aiming to identify: 1) differentially expressed miRNAs between IBS and BH subtypes vs. healthy controls (HCs), 2) targets of differentially regulated miRNA and associated pathways by silencing or overexpressing them in intestinal epithelial cell lines, 3) differentially regulated miRNA target genes in the colonic mucosa of IBS patients, and 4) testing potential functional roles for the miRNAs identified.

Methods

Study Population

IBS patients and HCs ages 18-55 were recruited primarily by community advertisement. The diagnosis of IBS and BH subtypes was based on Rome III criteria²² and confirmed by a clinician with expertise in IBS. HCs had no personal or family history of IBS or other chronic pain conditions. Additional exclusion criteria for all subjects included: infectious or inflammatory disorders, active psychiatric illness over the past six months assessed by structured clinical interview for the DSM-IV (MINI)²³, use of corticosteroids or narcotics, or current tobacco or alcohol abuse. Participants were compensated. The study was approved by the UCLA Institutional Review Board, and subjects signed a written informed consent prior to the study. Overall IBS symptoms, abdominal pain, and bloating severity over the prior week were assessed with numeric rating scales (0-20)²⁴. Current anxiety and depression symptoms were measured with the Hospital Anxiety and Depression (HAD) scale²⁵. Scores were classified as non-case (0-7), doubtful case (8-10), or definite case (>11).

Colonic mucosal tissue collection

Sigmoid biopsies (30 cm from the anal verge) were obtained by flexible sigmoidoscopy following tap water enemas. Specimens were flash-frozen in liquid nitrogen. Multiple biopsies were obtained from each patient and one biopsy each was used for miRNA and mRNA sequencing. All participants were instructed to hold aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) for 72 hours before the procedure.

NanoString nCounter assay for miRNA expression

Human v2 miRNA CodeSet was used to measure miRNA expression. RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and quantified using NanoDrop (Thermo Scientific, Waltham, MA). The nCounter assay was performed using Nanostring® Analysis Technologies Systems (Nanostring Technologies, Seattle, USA) as described in the Supplementary Methods. All the miRNAs referred to as miRs are human miRNAs (Hsa-miRs).

Real-time PCR (RT-PCR)

Semi-quantitative RT-PCR was used to determine the expression levels of selected miRNAs in biopsies and NCM460 cell line. A miRCURY LNA Universal cDNA synthesis Kit was used to reverse transcribe the RNA (Exiqon). ExiLent SYBR® Green master mix (203421; Exiqon) and a CFX384 RT-PCR detection system (Bio-Rad, Hercules, CA) were used to perform RT-PCR. LNA miRNA PCR assays were used to measure miRNA expression as listed in Supplementary Table 1.

In vitro target identification

The human immortalized colonic epithelial cell line NCM460 (INCELL, San Antonio, TX; referred to as intestinal epithelial cell-lines, IECs herein) were cultured as described in the Supplementary Methods. At 48 hours post-transfection, the cells were harvested and TRIzol was used to extract RNA. The expression of selected miRNAs in the cell lines was confirmed by RT-PCR.

Assessment of epithelial barrier function

For barrier function experiments, Caco-2 intestinal epithelial cells were used (American Type Culture Collection, Manassas, VA). Trans-epithelial electrical resistance (TEER) was measured every 24 hours over three days and dextran flux was measured every two hours over a 6 hour period as described in the Supplementary Methods.

3' RNA sequencing of miRNA targets

Total RNA was extracted from biopsies and from IECs transfected with miRNA inhibitors or mimics using RNeasy Plus Mini Kit (Qiagen, 74134, Hilden, Germany). RNA was quantified using QuantiT™ RiboGreen™ RNA Assay Kit (Fisher Scientific, Waltham, MA) and samples were normalized to a concentration of 20 ng/μL. RNA quality (RNA integrity number, RIN) was assessed by Agilent TapeStation capillary electrophoresis (most samples had a RIN>7). Gene expression was measured using QuantSeq 3' mRNA sequencing²⁶. QuantSeq generates highly strand-specific next-generation sequencing libraries close to the

3' end of polyadenylated RNA. RNA sequencing (RNA-seq) was performed in the UCLA Neuroscience Genomics Core Laboratory using Lexogen QuantSeq 3' FWD cDNA library synthesis²⁶ and multiplex DNA sequencing on an Illumina HiSeq 4000 instrument with single-strand 65 nucleotide sequence reads following standard manufacturer protocols.

Statistical and bioinformatic analysis:

nCounter—Data preprocessing and normalization: nCounter expression data was analyzed using the NanoStringNorm²⁷ and limma²⁸ packages in R. Sample content normalization was performed using the geometric mean of the top 75 expressed genes. The background was calculated using the geometric mean of negative controls and the data were log-transformed. Hierarchical clustering was used to identify clusters of similar miRNA expression patterns and to identify outliers. One outlier was detected and was excluded from the analysis. **Differential expression analysis:** Differential expression was determined using a linear model for arrays implemented in 'limma' package in R. The adjusted p-values were generated using the function 'p.adjust' in R. A false discovery rate (FDR) < 0.1 was considered significant, which is acceptable when additional validation experiments are planned²⁹. MiRNA clusters and individual miRNAs were correlated with the clinical symptoms using logistic regression.

RT-PCR—Bioinformatic methods used for the selection of miRNAs for RT-PCR validation are described in Supplementary Methods. Experiments were performed in triplicates for each condition. MiRNA expression levels were normalized to the average level of 5S rRNA and U6 snRNA. Normalized expression levels were quantified to the plate control. Comparative Ct [DeltaDeltaC(T)] method³⁰ was used to analyze the data. A p-value < 0.05 was considered significant.

QuantSeq RNA sequencing—Samples yielded ~3 million reads. De-multiplexed raw reads (FASTQs) were then subjected to the QuantSeq 3' mRNA-Seq Integrated Data Analysis Pipeline on the Bluebee® Genomics platform (<https://www.bluebee.com/lexogen/>), which uses standard tools but with parameter settings optimized for processing QuantSeq data. Additional analysis steps are described in the Supplementary Methods. A FDR < 5% was considered significant. For gene ontology and pathway analyses, a FDR < 0.05 and a fold change > 1.2 was used.

Western Blot Analysis—Antibodies for tight junction protein-1 (TJP1/ZO1 cat# 61-7300) and E-cadherin (CDH1, clone 4A2C7) antibodies were from Invitrogen. The GAPDH antibody was from Cell Signaling Technologies (Danvers, MA; clone 14C10). The experimental methods are outlined in Supplementary Methods.

GO pathways and network analysis of miRNA targets

TarBase v.8 and Diana miRpath V3 were used to make *in-silico* predictions of miRNA targets and associated pathways³¹. The network of Gene Ontology (GO) terms associated with differentially expressed genes for selected GO terms in IEC models was visualized using ClueGO 2.5.4 in Cytoscape 3.5 (Cytoscape Consortium, <http://www.cytoscapeconsortium.org/>). The CluePedia plugin was used to enrich the genes with

publicly available information from databases, including STRING (<https://string-db.org/>), IntAct (<https://www.ebi.ac.uk/intact/>), MiMI (<http://www.ncbi.org/mimi.html>), miRbase (<http://www.mirbase.org/>), and miRecords (<http://c1.accurascience.com/miRecords/>)³².

Identification of miRNA targets for drug development

For the identification of genes that are targets of known prescribed or experimental drugs, we used The International Union of Basic and Clinical Pharmacology (IUPHAR) as described in the Supplementary Methods.

Results

To identify miRNAs altered in IBS patients, we used a total of 44 subjects including 29 IBS patients (52% IBS-C and 48% IBS-D) and 15 HCs. Baseline characteristics are displayed in Table 1. IBS patients and HCs were similar in sex, age, BMI, ethnicity, and race ($p>0.05$). Overall symptom severity of IBS patients was moderate. Mean anxiety and depression scores were low (non-case) in both groups. Twenty-eight participants (IBS=22, HC=6) reported medication use; most used laxatives or antidiarrheals and NSAIDs only on an as-needed basis. One IBS patient used a benzodiazepine. None of the subjects were taking probiotics or antibiotics.

MiRNAs associated with IBS and BH subtypes

nCounter platform was used to assess 800 miRNAs simultaneously. Of these, 247 miRNAs were expressed above the background and were tested for differential expression between IBS and BH subtypes, and HCs. Four out of 247 miRNAs were differentially expressed between IBS and HCs, and two were deregulated between IBS-C and HCs ($FDR<0.1$). MiR-363-3p and miR-338-3p were downregulated, whereas miR-106b-5p and miR-532-5p were upregulated in IBS vs. HCs ($FDR=0.06$, all miRNAs). When comparing IBS-C vs. HCs, the levels of miR-338-3p and miR-100-5p were decreased ($FC=-1.82$ and -1.72 , $FDR=0.04$) and the levels of miR-106b-5p were increased ($FC=1.31$, $FDR=0.04$, Supplementary Table 2). In IBS-D vs. HCs, a marginal association of eight miRNAs was observed ($p<0.05$), with miR-219-5p being 3-fold decreased in IBS-D compared to HCs ($p<0.05$).

To validate the high-throughput miRNA data, we performed RT-PCR on 12 differentially expressed miRNAs shown in Figure 1, that were selected from significantly ($FDR<0.1$) and differentially expressed miRNAs at $p<0.05$, in IBS and BH subtypes vs. HCs. We prioritized the miRNAs to be included in the validation set using the 'random forest' classification algorithm (Supplementary Figure 1). The miRNAs were sorted based on their ability to discriminate between IBS and HCs as detailed in the Supplementary Results. Additionally, we included bowel habit subtype associated miRNAs for validation. Hierarchical clustering of the 12 miRNAs identified subtypes within IBS, however, they were not associated with IBS symptom severity. Of the 12 miRNAs validated, the strongest associations were decreased levels of miR-338-3p and miR-219-5p in IBS vs. HCs ($p = 0.004$ and 0.026 respectively, Supplementary Figure 2), and in IBS-C vs. HCs ($p = 0.03$ and 0.06 ,

respectively). When comparing nCounter and RT-PCR results of the genes that were altered between IBS and HCs, 83% were in congruence (Table 2).

Identification of change in mRNA expression associated with miR-219a-5p and miR-338-3p

For both nCounter miRNA and RT-PCR data, decreased levels of both miR-219a-5p and miR-338-3p were observed in IBS (and IBS bowel habit subtypes) compared to HCs. Additionally, computationally predicted targets of miR-219a-5p were associated with barrier function, which is important in IBS pathogenesis. To identify the targets of miR-219a-5p and miR-338-3p, we inhibited miRNAs in IECs and measured their expression (Supplementary Figure 3).

Inhibition of miR-219a-5p in NCM460 cells alters the expression of permeability associated genes—To study the role of miR-219a-5p downregulation in the pathophysiology of IBS, we inhibited miR-219a-5p in normal human IECs and performed 3' mRNA sequencing. 1066 genes were upregulated, and 1187 genes were downregulated between miR-219a-5p-inhibited cells and control cells (FDR<0.05, absolute fold change > 1.2 fold). GO terms associated with the genes upregulated in miR-219a-inhibited cells vs. controls included, “cell-cell adherence junction” (count=55 genes, Benjamini_p= 2.1E-11) and “Neuropathy” (count=14 genes, Benjamini_p= 7.4E-3) among others.

GO network analysis of the differentially expressed genes resulted in a network of terms such as “cell-cell adherence junction”, “Wnt signaling”, “Parkinson’s disease” and “proteasome”, with miR-219-5p (Figure 2). Go term ‘cell-cell adherence junction’ was associated with genes including, Tight junction (TJ) protein 1 (*TJPI* or *ZO-1*, FDR=0.03), E-Cadherin (*CDH1*), Carcinoembryonic antigen-related cell adhesion molecule 5 (*CEACAM5*) and, Catenin delta 1 (*CTNND1*, FDR=0.040), and ‘Wnt signaling’ was associated with Kruppel-like factor 5 (*KLF5*, FDR=0.02) and Zinc finger 148 (*ZNF148*, p=0.046), which were upregulated in miR-219-inhibited cells, and are suggested to play a role in barrier function³³. Increased proteasome-mediated degradation of tight TJ has been associated with altered barrier function, increased permeability, and low-grade inflammation in IBS³⁴.

Expression of barrier function-related miR-219a-5p associated genes in NCM460 cells.—The mRNA sequencing data showed an upregulation of barrier function associated genes including *ZO1* and *CDH1*, which play an important role in barrier function. To test the differential expression at the protein level, we measured the protein expression of ZO1 and CDH1 in miR-219a-inhibited NCM460 cells compared to control cells by Western blot (Figure 3). ZO1 protein was significantly upregulated in miR-219a-inhibited cells compared to control cells. However, E-cadherin levels were decreased in miR-219a-inhibited cells compared to controls, which is in agreement with the previous studies³⁵. A decreased E-cadherin expression has been associated with inflamed epithelium in Crohn’s disease and ulcerative colitis patients³⁶.

Inhibition of miR-219a-5p disrupts intestinal barrier function—Given the observed association of miR-219a-5p inhibition and expression of cell-cell adherence junctions genes, we hypothesized that a decreased miR-219a-5p expression would result in perturbed barrier function and consequently increased paracellular permeability. Since NCM460 cells formed only weak barriers, we inhibited miR-219a-5p expression in a barrier-forming, intestinal epithelial cell line, Caco-2³⁷, and measured permeability. After one day, a reduced TEER was observed in miR-219a-5p inhibitor-treated monolayers compared to control-treated monolayers and this difference increased over time, with some experiments exhibiting a significant 50% reduction in TEER on the final day ($p < 0.05$, Figure 4A). As another measure of barrier function, dextran flux was then measured at the end of each experiment. It was observed that 2, 4, and 6 hours after the addition of dextran, almost no dextran crossed the control-treated monolayers, however, both 4 and 10 kDa dextran were able to significantly cross miR-219a-5p treated monolayers ($p < 0.01$, Figure 4B).

MiR-219a-5p is associated with neuronal genes—In addition to changes in barrier function associated genes, miR-219a-5p inhibition resulted in deregulation of mitochondrial genes along with the proteasome complex genes. The ubiquitin-proteasome system plays an important role in mitochondrial biogenesis and mitochondrial function^{38,39}, which modulates neuronal function and participates in neuronal signaling⁴⁰.

Additionally, Growth differentiation factor-15 (*GDF15*), which is a hormone conveying somatic distress to the brain⁴¹ was among the top upregulated expressed genes. Also, we observed an upregulation of Neuferricin (*CYB5D2*) which promotes neurogenesis, in addition to several physiologic functions including cholesterol/steroid biosynthesis, drug metabolism, and response, among others⁴².

Targets of miR-219a-5p are deregulated in the colonic mucosa of IBS patients—To identify the overlapping genes differentially regulated in the cellular model and colonic mucosa of IBS vs. HCs, we performed 3' mRNA sequencing on the colonic mucosa of subjects associated with this study. We identified 134 genes, which included genes associated with the mitochondrial function such as oxidation-reduction process, including cytochrome b561 (*CYB561*), cell-cell adhesion function, including (integrin subunit beta 1 binding protein 1 (*ITGB1BPI*), channel proteins including, TRPM8 channel-associated factor 1 (*TCAFI*), ABC transporter genes, *ABCC1* and *ABCA5*, and calcium/calmodulin dependent protein kinase ID (*CAMK1D*), that were deregulated in both the colonic mucosa of IBS patients compared to HCs as well as in the miR-219a-5p-inhibited cells ($p < 0.05$ for colon and $FDR < 0.1$ for cells, congruent fold changes, Supplementary Table 3).

MiR-338-3p inhibition is associated with MAPK signaling and immune pathway alterations—To uncover the role of miR-338-3p in the pathophysiology of IBS, we inhibited miR-338-3p in NCM460 cells and performed 3' mRNA sequencing. Inhibition of miR-338-3p was associated with deregulation of 1368 genes ($FDR < 0.05$, 737 up, and 631 downregulated). The deregulated genes were significantly associated with GO terms including “kinase activity” (Enrichment p -value = $8.5E-3$, # of genes = 58). A network of kinase and MAPK (mitogen-activated protein kinase or protein serine/threonine kinase) pathway genes, some of which are the predicted targets of has-miR-338-3p included kinase-

related and immune response genes (Figure 5). MAPK pathways are implicated in inflammation pain and hypersensitivity in animal models^{43,44}. While the MAPK genes including *MAPK1*, *MAPK8IP3*, and *MAPK9* were upregulated, genes associated with the ‘kinase inhibitor’ term, including tribbles related protein 3 (*TRIB3*), were downregulated in miR-338-3p-inhibited cells, suggesting activation of the MAPK pathway. *TRIB3* inhibits key inflammatory signaling pathways, including the MAPK and phosphatidylinositol 3 kinase (PI3K) networks⁴³. Additionally, innate immune response related and Wnt signaling related genes were upregulated in miR-338-inhibited cells compared to control cells. Additional MAPK associated genes included Stratifin (*SFN*, Keratinocyte-releasable 14-3-3-sigma) and Fatty acid amide hydrolase (*FAAH*) which were upregulated in miR-338 inhibited cells compared to controls. *SFN* is a proinflammatory cytokine that binds to CD13 (also known as aminopeptidase N, APN) which plays a role in pain sensation via MAPK pathway⁴⁵. Fatty acid amide hydrolase (*FAAH*), which catalyzes the degradation of endocannabinoids (anandamide, 2-arachidonoylglycerol) and mediates promigratory effects via MAPK pathway⁴⁶, was increased in miR-338-inhibited cells. *FAAH* inhibitors ameliorate signs of acute, inflammatory, visceral, and neuropathic pain in animal models⁴⁷.

MiR-338-3p associated genes are deregulated in the colonic mucosa of IBS patients—When comparing protein-coding genes that were differentially expressed in the colonic mucosa of IBS patients to HCs as well as miR-338-3p-inhibited IECs, 47 were downregulated and 45 were upregulated in both ($p < 0.05$ for colon and $FDR < 0.1$ for cells), Supplementary Table 4). These included upregulation of kinase domain-containing genes such as Serine/Threonine kinase (*AKT2*), and LIM domain kinase 1 (*LIMK1*) and downregulation of ATM serine/threonine kinase (*ATM*) and inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (*IKBKB*). This suggests an involvement of the MAPK pathway, which has been associated with pronociception in a rat visceral pain model⁴⁸. Additionally, solute carrier (SLC) family 2 member A3 (*SLC2A3*, *GLUT3*, *facilitated glucose transporter*) was downregulated in both IBS patients and the IEC miR-338-3p inhibition model. Mice deficient in *GLUT3* show increased startle response, which is a functional pain paradigm in IBS women⁴⁹.

Genes associated with miR-219a-5p and miR-338-3p deregulation are targets of known drugs

Next, we investigated whether the differentially expressed genes in colonic mucosa (IBS vs. HCs) and IEC miRNA inhibition models were targets of known drugs. Using the pharmacology database as described in the Methods, we identified genes including *CAMK1D* and TRPM8 Channel Associated Factor 1 (*TCAFI*), which were upregulated, and interleukin-7 receptor subunit alpha (IL-7R), which was downregulated in IBS vs. HCs and miR-219a-5p inhibited cells (Supplementary Table 5).

Additionally, we identified inhibitors for LIM Domain Kinase 1 (LIMK1) and Serine/Threonine Kinase 2 (AKT2) protein kinases, which were upregulated miR-338-3p inhibited cells as well as between IBS and HCs (Supplementary Table 6).

Discussion

The key findings of this study are 1) identification of two miRNAs (miR-219a-5p, miR-338-3p) in the colonic mucosa which are downregulated in IBS 2) miR-219a-5p dysregulation results in impaired barrier function in IECs, and 3) identification of pathways and networks through combined bioinformatic analysis of gene expression profiling in colonic mucosal biopsies from IBS and IEC models. Additionally, we identified a miRNA that was altered in IBS, which plays a potential role in visceral hypersensitivity via the MAPK pathway.

Our study identified novel colonic mucosal miRNAs associated with IBS. There is ample evidence that alterations in miRNAs may be important contributing factors in IBS pathogenesis. For example, downregulation of miR-16 and miR-125b have been associated with a dysregulation of barrier function in IBS-D patients via modulation of TJ signaling⁵⁰. Changes in colonic mucosal expression of miR-29a²⁰ are associated with altered permeability via regulation of glutamine synthase gene in IBS⁵¹. Similarly, miR-199 has been associated with visceral hypersensitivity via regulation of *TRPV1* in IBS-D, further supporting the importance of miRNAs in IBS pathophysiology. These miRNAs were not associated with IBS or within either BH subset of IBS patients in this study, which may be due to differences in the IBS patient populations (race/ethnicity), tissue sampling (e.g., sigmoid colon vs. jejunum), sample size differences, and/or variable analysis platforms. The potential sources of differences between this and the previous miRNA studies on IBS and animal models of IBS are listed in Supplementary Table 7 and 8, respectively. While many studies focused on IBS-D women, a subset of patients with increased permeability, or miRNA in the small intestine, our study included colonic mucosal biopsies from men and women with IBS-C or IBS-D. Similar to our previous findings, we found most differences in IBS-C vs. HCs⁵².

While increased permeability is most often associated with IBS-D, it has also been observed in IBS-C⁵³. We observed downregulation of miR-219a-5p in both IBS-C and IBS-D compared to HCs. Our findings and previous studies on miR-219a-5p suggest that its downregulation may impact many pathways with potential relevance to IBS. For example, miR-219a-5p inhibition was associated with *TJPI/ZO1* upregulation and impaired barrier function. ZO1 is a TJ protein that interacts with claudins and occludins on epithelial cell membranes⁵⁴. Although some studies have reported an association of decreased ZO1 expression in the colonic mucosa with increased permeability in IBS^{53,55}, our results are consistent with studies demonstrating decreased permeability in cells depleted for ZO1⁵⁶. Another explanation for the discordance of phenotype and gene expression changes is that the mechanism may not involve direct alterations in TJ protein expression or that they may be compensatory. Increased Wnt-signaling has been associated with altered barrier function in epithelial cells³³. Additionally, we found E-cadherin to be downregulated at the protein level. Mice models that lack E-cadherin highlighted its importance in maintaining intestinal epithelial integrity and in the defense against enteropathogenic bacteria³⁶. Furthermore, an increase in expression of barrier function genes may be related to increased ion transport and increased fluid or electrolyte secretion as suggested in the previous studies⁵⁷.

Functional analysis of miR-219-inhibited cells showed a significant difference in the TEER and dextran flux between controls and miR-219a-5p inhibited cells suggesting a role for miR-219a-5p in regulating barrier function. The differences in the baseline levels of rate of dextran flux between controls and the 219a-5p-inhibited cells may be due to the role of this miRNA in regulating cortical actomyosin contraction.

Additionally, we identified an association of miR-219a-5p with the proteasome and mitochondria-associated genes, linked to neurological disorders including Parkinson's disease, which is associated with altered GI motility⁵⁸. The proteasome system modulates neuronal function and plays an important role in neuronal signaling⁴⁰. A study in a mouse model of spinal cord injury found downregulation of miR-219a-5p associated with an increase in inflammatory cytokines and reactive oxygen species (ROS), which were normalized by transfection with miR-219a mimics⁵⁹. Similarly, reduced expression of miR-219 in spinal neurons in mouse models of chronic inflammation was significantly associated with inflammatory pain, while overexpression was associated with a reversal of thermal hyperalgesia, mechanical allodynia and, spinal neuronal sensitization^{60,61}. These data suggest a role for miR-219a in reducing mitochondrial oxidative stress, which has been previously associated with IBS^{62,63}. Colonic mucosal gene expression analysis corroborated the gene expression findings in IECs suggesting a deregulation of neuro-motor and neuronal cell adhesion functions associated with downregulation of miR-219a-5p in IBS. This is supported by our previous finding that colonic mucosal expression in IBS-C is involved in pathways mediating neuronal signaling¹⁰. Further studies are needed to determine if inhibition of miR-219a-5p is associated with visceral hypersensitivity or mucosal immune activation in IBS.

Similarly, changes in permeability with altered Wnt signaling may also result in alterations in homeostatic mechanisms associated with a proliferative vs. differentiated fate, which may include metabolism and apoptosis in addition to alteration in cellular junctions⁶⁴. Both increased apoptosis and oxidative stress can increase permeability⁶⁵. Upregulation of *KLF5* and *CTNND1* in IECs with miR-219-5p depletion is also supportive of a role of miR-219-5p in Wnt signaling^{66,67}. Additionally, there is bioinformatic evidence for miR-219-5p regulating *ZNF148*, which was upregulated in our miR-219-5p depletion model and is a positive regulator of Wnt signaling⁶⁸. Another cadherin-binding protein, cortactin (*CTTN*), was downregulated, a change that was associated with increased permeability in mice⁶⁹. Oxidative stress-related barrier dysfunction could also be due to other signaling mechanisms as discussed above.

Our study identified differentially expressed genes common to both IBS colon and miR-219-inhibited cells that can be potential drug targets. *TCAFI*, which was increased in the colon and miR-219-inhibited cells codes for an ion binding protein that regulates TRPM8 trafficking and activity and plays a role in temperature sensing⁷⁰. TRPM8 antagonists have been investigated to treat chronic pain and migraine and can be a potential therapeutic agent in IBS⁷¹. Additionally, *CAMKID* has been associated with epigenetic changes associated with the transition from acute to chronic pain in mouse prefrontal cortex following nerve injury⁷² and was identified as a potential drug target (Supplementary Table 5).

Another interesting finding from this study was that miR-338-3p targets the MAPK pathway and its downregulation, as observed in IBS vs. HCs, results in downregulation of MAPK inhibitors including *TRIB3*. *TRIB3* is regulated by cannabidiol (CBD), a non-psychotropic phytocannabinoid that modulates allodynia⁷³ via TRPV4 signaling⁷⁴. Additionally, miR-338-3p depletion resulted in deregulation of several MAPK pathway genes including *MAPK1* and *MAPK9*, activated in response to stressful stimuli⁷⁵. Animal studies showed that activation of MAPKs and PI3K pathways in dorsal horn neurons involved in the production of proinflammatory cytokines mediate inflammatory pain and visceral hypersensitivity^{43,44}. Additionally, inhibitors of MAPKs have been shown to effectively alleviate inflammatory and neuropathic pain in animal models⁷⁶. Colonic gene expression analysis corroborated the involvement of genes associated with MAPK and cell adhesion pathways in IBS. The role of the MAPK pathway in IBS, which is not a primarily inflammatory disorder, is unclear. However, there is evidence of immune activation and microscopic inflammation in some patients, particularly post-infection IBS (PI-IBS). In patients with PI-IBS, alterations in the innate and adaptive immune systems were associated with increased intestinal permeability which persisted after the enteric infection^{77,78}.

Genes congruently deregulated in the colon of IBS and miR-338-inhibited cells included targets of known drugs. For example, we identified channel blockers for *KCNJ14* and ligands for *SLC2A3* that can be investigated as potential therapeutic agents.

This study has limitations. Although our study's sample size was comparable to most other miRNA studies and included relatively well-characterized IBS and HC populations, some of the findings warrant replication in larger cohorts. We tried to overcome the sample size limitation in part by validating the findings independently using RT-PCR. Interestingly, some of the miR-219a-5p targets were neuronal even though we studied epithelial cell lines. Since the NCM460 cells have a multilineage capability for in vitro differentiation, they express neuroendocrine markers including chromogranin⁷⁹, which may explain the expression of neuronal genes. Additionally, there are limitations associated with drawing conclusions regarding neuronal physiology based on findings from mucosal biopsies. While the mucosa is innervated by sensory nerve fibers, and biopsies frequently include intestinal submucosal elements, an alternative explanation is that the expression changes may reflect alterations in glial cells or enteroendocrine cells, both of which have neuronal properties. Both miR-219a-5p and miR-338-3p have well-defined roles in the maturation of oligodendrocytes which have some functional overlap with enteric glial cells⁸⁰. Additionally, the drug targets predicted here are based on the assumption that increased mRNA translates into increased protein expression, which is not always true. Nevertheless, our study provides evidence for multiple new drug targets that can be potentially explored for IBS.

In conclusion, using integrative analysis on high throughput miRNA and 3' quantseq data, followed by validation of individual targets on a well-characterized, age and sex balanced IBS and HC groups, our study showed several altered miRNAs and miRNA-associated pathways that may play a role in intestinal permeability and visceral hypersensitivity, which are characteristics of IBS. Based on our observations, future studies investigating some of

the proposed drug targets and focusing on pathways that lead to neuro-immune dysfunction in IBS may be warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

AKT2	serine/threonine kinase
ATM	ATM serine/threonine kinase
BH	bowel habits
CAMK1D	calcium/calmodulin dependent protein kinase ID
FAAH	fatty acid amide hydrolase
FDR	false discovery rate
GI	gastrointestinal
GO	Gene Ontology
GPCR	G protein-coupled receptors HC, healthy control
IBS	irritable bowel syndrome
IBS-C	irritable bowel syndrome with constipation
IBS-D	irritable bowel syndrome with diarrhea
IEC	intestinal epithelial cell
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
LIMK1	LIM domain kinase 1
MAPK	mitogen-activated protein kinase
miRNA	microRNA
mRNA	messenger RNA
PCR	polymerase chain reaction
TCAF1	TRPM8 channel-associated factor 1
TEER	transepithelial electrical resistance

TJ	tight junction
TJP1	tight junction protein 1
TRIB3	tribbles related protein 3
TRPV1	transient receptor potential cation channel subfamily V member 1
ZO1	zonula occludens-1

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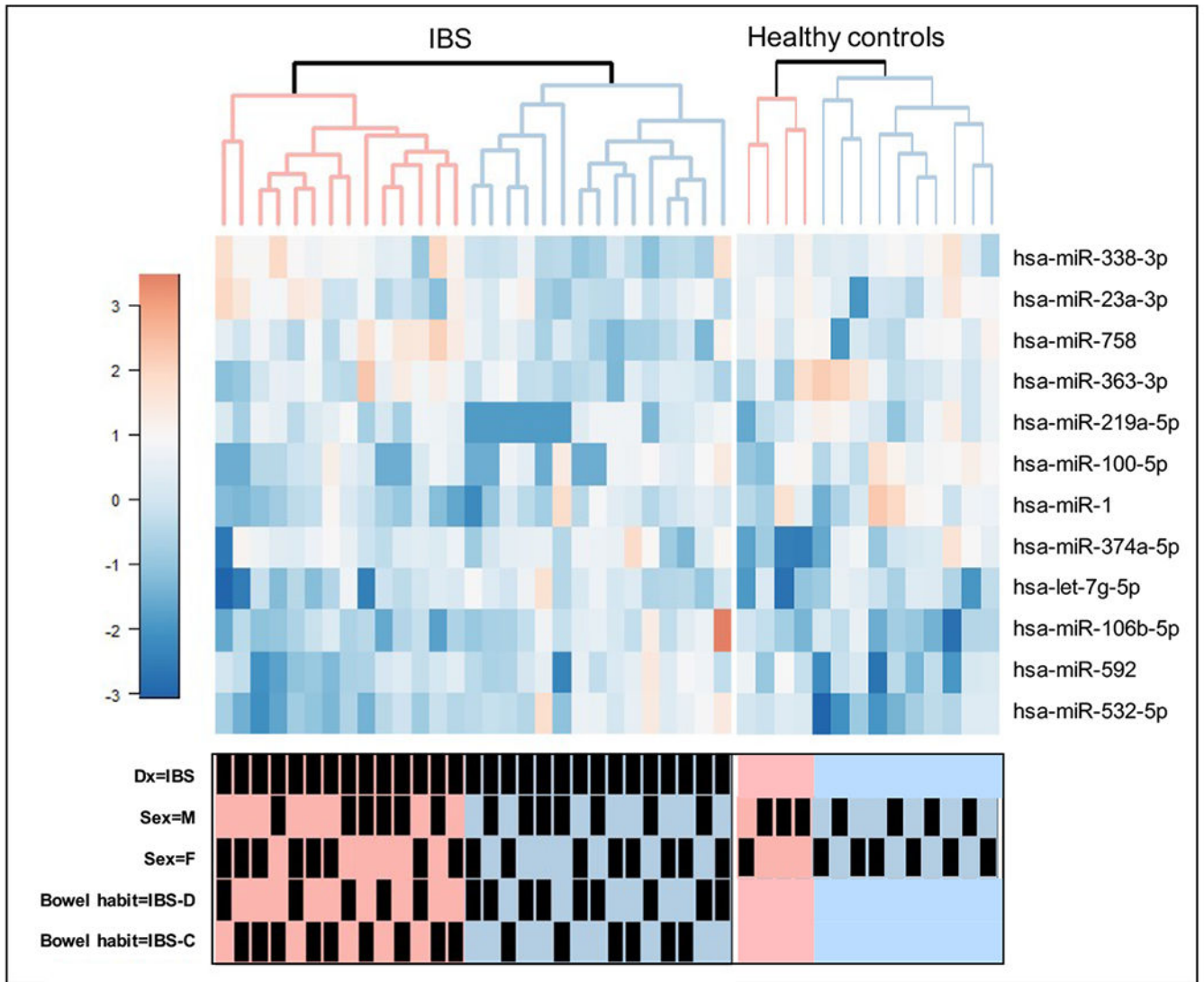


Figure 1. shows the heatmap of the normalized expression of 12 differentially expressed miRNAs. Heatmap colors range from dark blue, white, and dark red, representing low, average, and high expression, respectively. Dx, diagnosis; M, Male; F, Female; IBS-C constipation-predominant IBS; IBS-D, diarrhea-predominant IBS. The bottom-panel shows variables associated with the samples.

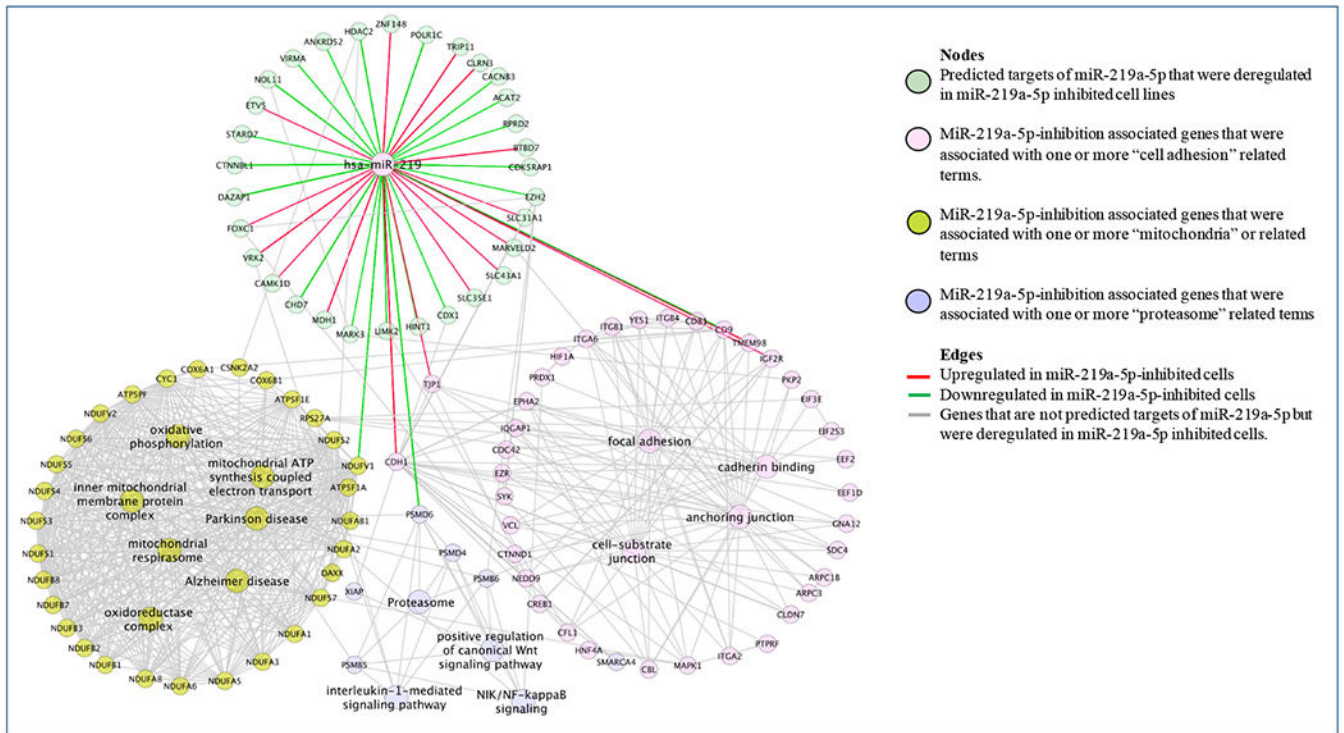


Figure 2. shows a network of deregulated genes and select gene ontology (GO) terms associated with the inhibition of miR-219a-5p. Nodes and edges are described in the top right panel of the figure.

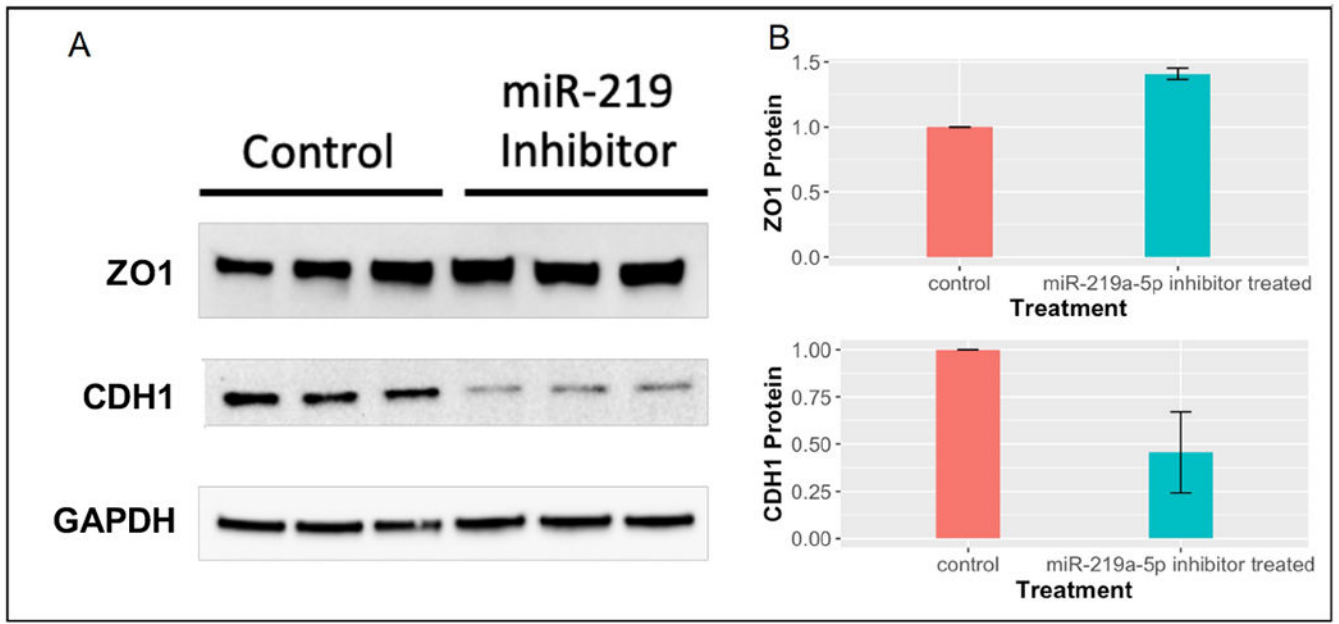


Figure 3. shows A) western blots of tight junction protein 1 (TJP1 or ZO1) and E-cadherin (CDH1) proteins performed in NCM460 cells, B) Protein levels of ZO1 and CDH1 by densitometry. The error bars represent mean \pm standard deviation, and the data represent fold changes relative to control. The experiments were performed in duplicate.

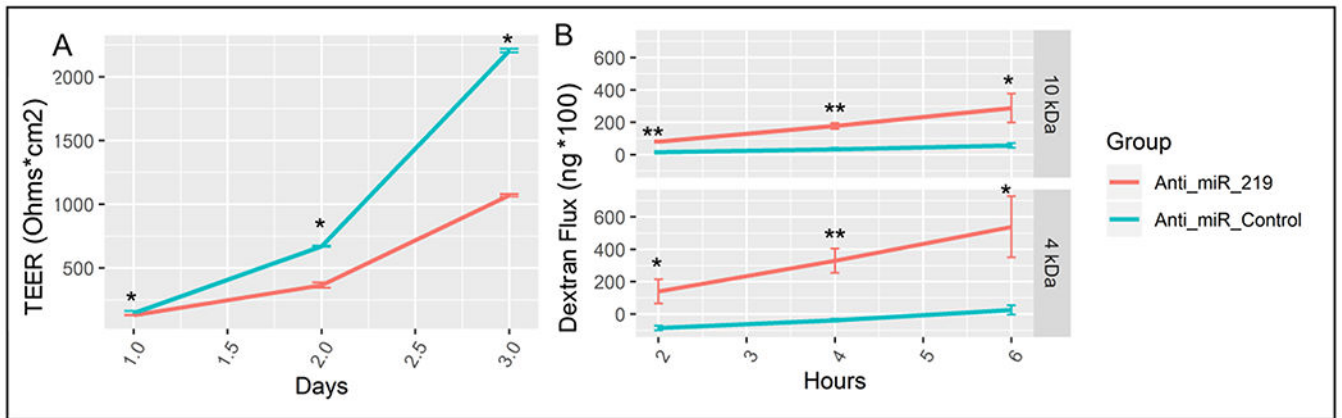


Figure 4. shows, A) a progressive decrease in trans-epithelial electrical resistance (TEER) over 3 days in Caco-2 cells treated with miR-219a-5p inhibitor (Anti_miR_219, red line) compared to cells treated with control miRNA (Anti_miR_Control, blue line), and B) an increased dextran flux over 6 hours (measured every 2 hours) in cells treated with miR-219a-5p inhibitor (Anti_miR_219, red line) compared to cells treated with control miRNA (Anti_miR_Control, blue line). *, $p < 0.05$; **, $p < 0.01$.

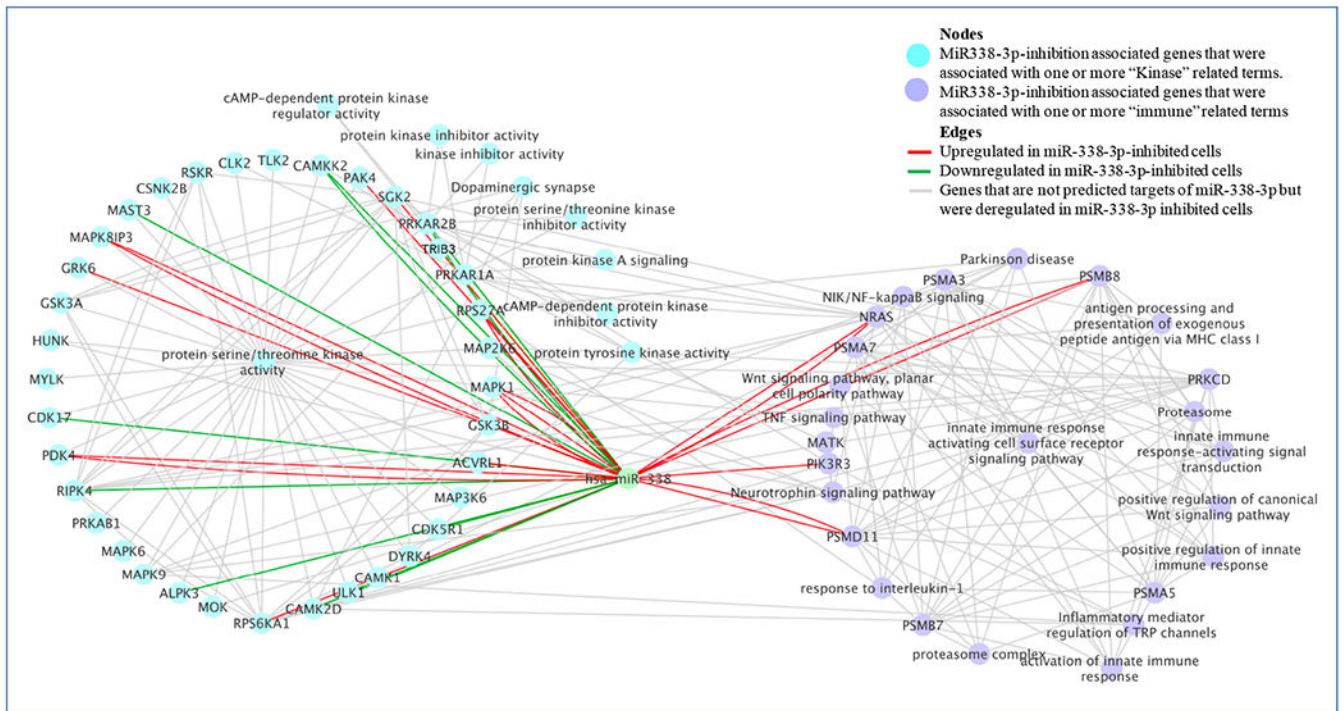


Figure 5. shows a network of deregulated genes and select gene ontology (GO) terms associated with the inhibition of hsa-miR-338-3p. Nodes and edges are described in the top right panel of the Figure.

Table 1:

Demographic characteristics of IBS patients and healthy controls

Group	IBS	IBS-C	IBS-D	HCs
N	29	15	14	15
Women (%)	55	67	43	47
Age [mean(sd)]	33.9 (12.2)	32 (13.7)	35.93 (10.5)	34.2 (9.2)
BMI [mean(sd)]	24.69 (5.0)	23.98 (3.47)	25.45 (6.29)	25.75 (3.1)
Ethnicity				
Hispanic N (%)	4 (14)	3 (20)	1 (7)	2 (13)
Race				
Asian (%)	8 (28)	3 (20)	5 (36)	3 (20)
Black (%)	4 (13)	4 (27)	0 (0)	1 (7)
White (%)	12 (41)	7 (47)	5 (36)	8 (53)
Other/mixed (%)	5 (18)	1 (7)	4 (28)	3 (20)
Bowel habit subtype				
IBS-C (%)	15 (52)	NA	NA	NA
IBS-D (%)	14 (48)	NA	NA	NA
HAD anxiety score (0-21) [mean(sd)]	6.38 (5.9)	4.27 (4.53)	8.64 (6.57)	4.0 (3.1)
HAD depression score (0-21) [mean(sd)]	3.03 (2.9)	2.07 (2.43)	4.07 (3.08)	1.73 (1.94)
Overall IBS severity (0-20) [mean(sd)]	10.4 (4.3)	9.43 (4.47)	11.29 (4.05)	NA
Abdominal pain (0-20) [mean(sd)]	10.4 (4.2)	9.14 (4.4)	11.57 (3.88)	NA
Bloating [mean(sd)]	12.5 (4.1)	12.07 (4.4)	13.07 (3.97)	NA

Table 1 shows the demographic characteristics of IBS subjects and healthy controls (HCs). N, number of subjects; sd, standard deviation; HAD, Hospital anxiety depression scale; IBS-C constipation-predominant IBS; IBS-D, diarrhea-predominant IBS, NA, not applicable.

Table 2:

Differentially expressed microRNAs in IBS and IBS bowel habit subtypes compared to healthy controls

	nCounter P Values	FDR	nCounter Fold Change	RT-PCR p values	RT-PCR Fold Change
IBS vs. HCs	N=29,15			N=24,14	
hsa-miR-363-3p	0.0004	0.063	-1.750	0.056	-1.138 *
hsa-miR-338-3p	0.001	0.063	-1.602	0.004	-1.342 *
hsa-miR-106b-5p	0.001	0.063	1.249	0.056	-2.177
hsa-miR-532-5p	0.001	0.063	1.352	0.396	1.043 *
hsa-miR-100-5p	0.006	0.301	-1.448	0.223	-1.186 *
hsa-miR-23a-3p	0.020	0.598	-1.198	0.348	-1.193 *
hsa-miR-758	0.022	0.598	-2.355	0.936	-1.038 *
hsa-miR-592	0.032	0.626	1.343	0.525	-1.582
hsa-miR-1	0.038	0.626	-1.367	0.120	-2.318 *
hsa-miR-219-5p	0.09	0.911	-2.280	0.026	-1.441 *
IBS-C vs. HCs	N=15, 15			N=13, 14	
hsa-miR-338-3p	0.0002	0.039	-1.820	0.020	-1.325 *
hsa-miR-100-5p	0.0004	0.039	-1.720	0.230	-1.221 *
hsa-miR-106b-5p	0.0005	0.039	1.310	0.025	-1.782
hsa-miR-532-5p	0.002	0.128	1.380	0.699	-1.008
hsa-miR-363-3p	0.003	0.166	-1.690	0.100	-1.172 *
hsa-miR-23a-3p	0.010	0.342	-1.260	0.379	-1.173 *
hsa-miR-1	0.016	0.430	-1.520	0.008	-1.814 *
hsa-miR-592	0.037	0.537	1.390	0.131	-1.404
hsa-miR-374a-5p	0.048	0.537	1.120	0.049	-1.600
hsa-miR-219-5p	0.5	0.235	-1.610	0.060	-1.246 *
IBS-D vs. HCs	N=14, 15			N=11, 14	
hsa-miR-338-3p	0.034	0.999	-1.390	0.119	-1.364 *
hsa-miR-106b-5p	0.024	0.869	1.190	0.048	-2.823
hsa-miR-532-5p	0.009	0.869	1.320	0.720	1.113 *
hsa-miR-363-3p	0.001	0.262	-1.820	0.078	-1.089 *
hsa-miR-758	0.021	0.869	-2.750	0.199	-1.116 *
hsa-miR-219-5p	0.016	0.172	-3.100	0.129	-1.315 *

Table 2 shows differentially expressed microRNAs in IBS and IBS bowel habit subtypes compared to healthy controls (HCs).

*direction of differential expression in nCounter was in agreement with real-time PCR (RT-PCR);

FDR, false discovery rate; IBS-C constipation-predominant IBS; IBS-D, diarrhea-predominant IBS; N= number of subjects.