Gastroenterology 2015;148:158–169

MicroRNA 29 Targets Nuclear Factor-κB–Repressing Factor and Claudin 1 to Increase Intestinal Permeability

QiQi Zhou,[1,2,*] Stefan Costinean,[3,*] Carlo M. Croce,[3] Alan R. Brasier,[1] Shehzad Merwat,[1] Scott A. Larson,[1] Sarpreet Basra,[1] and G. Nicholas Verne[4]

[1]Department of Medicine, University of Texas Medical Branch, Galveston, Texas; [2]Department of Veteran Affairs, Cincinnati VA Medical Center, Cincinnati, Ohio; [3]Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio; and [4]Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana

BACKGROUND & AIMS: Some patients with irritable bowel syndrome with diarrhea (IBS-D) have intestinal hyperpermeability, which contributes to their diarrhea and abdominal pain. MicroRNA 29 (MIR29) regulates intestinal permeability in patients with IBS-D. We investigated and searched for targets of MIR29 and investigated the effects of disrupting *Mir29* in mice. **METHODS:** We investigated expression MIR29A and B in intestinal biopsies collected during endoscopy from patients with IBS (n = 183) and without IBS (controls) (n = 36). Levels were correlated with disease phenotype. We also generated and studied *Mir29*$^{-/-}$ mice, in which expression of Mir29a and b, but not c, is lost. Colitis was induced by administration of 2,4,6-trinitrobenzenesulfonic acid; intestinal tissues were collected and permeability was assessed. Microarray analysis was performed using tissues from *Mir29*$^{-/-}$ mice. Changes in levels of target genes were measured in human colonic epithelial cells and small intestinal epithelial cells after knockdown of MIR29 with anti-MIRs. **RESULTS:** Intestinal tissues from patients with IBS-D (but not IBS with constipation or controls) had increased levels of MIR29A and B, but reduced levels of Claudin-1 (CLDN1) and nuclear factor-κB–repressing factor (NKRF). Induction of colitis and water avoidance stress increased levels of Mir29a and Mir29b and intestinal permeability in wild-type mice; these increased intestinal permeability in colons of far fewer *Mir29*$^{-/-}$ mice. In microarray and knockdown experiments, MIR29A and B were found to reduce levels of *NKRF* and *CLDN1* messenger RNA, and alter levels of other messenger RNAs that regulate intestinal permeability. **CONCLUSIONS:** Based on experiments in knockout mice and analyses of intestinal tissue samples from patients with IBS-D, *MIR29* targets and reduces expression of *CLDN1* and *NKRF* to increase intestinal permeability. Strategies to block MIR29 might be developed to restore intestinal permeability in patients with IBS-D.

Keywords: Gene Regulation; mRNA Processing; Intestinal Barrier Function; Mouse Model.

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder in which patients suffer from chronic abdominal pain associated with diarrhea/constipation, urgency, and/or bloating.[1] Recent evidence has shown that some IBS-associated symptoms, such as abdominal pain and diarrhea, may result from increased intestinal permeability.[2–7] An intact intestinal barrier is key to preventing paracellular penetration of toxic macromolecules, bacteria, and cytokines from the gut lumen into the systemic circulation. Disruption of intestinal tight junctions may be a critical underlying pathophysiologic factor in patients with allergic (food) disorders, rheumatoid arthritis, chronic dermatologic conditions, and alcoholic cirrhosis.[8,9] Our current understanding of the in vivo molecular mechanisms of increased intestinal permeability in patients with gastrointestinal disorders is still limited.

MicroRNAs (miRNAs) are a class of 21–23 nucleotides, endogenously expressed RNAs that are small, noncoding RNA molecules that bind through partial sequence homology to the 3′-untranslated region (UTR) of target messenger RNAs (mRNAs) and block translation. miRNAs have emerged as regulators involved in gene expression of critical biologic processes, such as differentiation, apoptosis, and proliferation. miR-29a is an important miRNA that regulates intestinal barrier integrity in diarrhea-predominant IBS (IBS-D) patients through interaction with 2 seed sequence binding sites located at the 3′-end of the GLUL gene.[10] Indeed, miR-29 is critical for homeostasis of the intestinal barrier through gene regulation in terminally differentiated intestinal epithelial cells. Predicted targets of miR-29 are significantly enriched for key signaling pathway-related networks and genes involved in maintaining intestinal barrier integrity. Up-regulated genes highly represented in these networks include nuclear factor-κB–repressing factor (NKRF) and Claudin-1 (CLDN1), both critical signaling molecules involved in the regulation of intestinal permeability.

We investigated the functions of miR-29 in IBS patients and in an miRNA knockout mouse model (miR-29a/b$^{-/-}$) to determine the effects of the miR-29 family on intestinal permeability. A total of 219 subjects completed the study and underwent intestinal permeability testing and evaluation of gut miRNA to determine if enhanced expression of miR-29 regulates intestinal permeability. miR-29a/b$^{-/-}$ mice were used to study how silencing of the miR-29 family

*Authors share co-first authorship.

Abbreviations used in this paper: CLDN1, Claudin-1; IBS, irritable bowel syndrome; IBS-C, constipation-predominant irritable bowel syndrome; IBS-D, diarrhea-predominant irritable bowel syndrome; miRNA, microRNA; mRNA, messenger RNA; NKRF, nuclear factor-κB–repressing factor; TNBS, 2,4,6-trinitrobenzenesulfonic acid; UTR, untranslated region; WAS, water avoidance stress; WT, wild-type.

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2014.09.037

alters intestinal permeability. Subsequent microarray analysis was performed to detect the specific target gene changes after in vivo knockout of miR-29a/b. We report here that silencing miR-29 by generating knockout mice or by inhibiting cluster expression using anti-miRNAs prevents or reverses intestinal hyperpermeability. Thus, miR-29 may have important therapeutic implications for selected IBS patients with symptoms arising from increased intestinal permeability.

Materials and Methods

miR-29a/b$^{-/-}$ Mice

To determine the essential role of miR-29 in vivo, we generated miR-29a/b knockout mice. Homozygous floxed miR-29ab1 mice (C57BL/6 strain) were generated by 2 homologous recombination arms that were amplified by polymerase chain reaction on 129 SvJ/X1 genomic DNA, a 5′ sequence of 4171 bp, and a 3′ sequence of 3857 bp. The genomic fragment to be deleted, which had 600 bp, and which contained the miR-29a and miR-29b1 regions, was amplified and cloned in between 2 loxP sites, in a pFlox vector (Supplementary Figure 1*A*). Genotyping was performed by polymerase chain reaction in DNA extracted by tail clippings to identify the genotype of the first generation of recombinant mice with floxed miR-29a/b (Supplementary Figure 1*B*).

Animal Experiments

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committees at the Ohio State University and the University of Texas Medical Branch. Male miR-29a/b$^{-/-}$ and wild-type (WT) mice (10–13 weeks) were used. Intracolonic 2,4,6-trinitrobenzenesulfonic acid (TNBS) in 50% ethanol was used to produce colitis as described previously.[11] The water avoidance stress (WAS) model was also used to induce visceral hyperalgesia.[12] Testing for visceral hypersensitivity to nociceptive colonic distension was performed as described previously.[13]

Human

All human studies were approved by the Institutional Review Boards at the Ohio State University and the University of Texas Medical Branch. All IBS patients met the Rome III criteria and had IBS symptoms for >5 years. All participants underwent a hydrogen breath test for bacterial overgrowth and had an antiendomysial antibody titer drawn. All participants underwent a 24-hour urine collection for intestinal permeability testing after ingestion of a solution of lactulose and mannitol.[7,10] Increased intestinal permeability was defined as an elevated urinary lactulose/mannitol ratio (≥0.07). Within 1 week of permeability testing, all subjects underwent endoscopy with small intestinal/colonic biopsies that were stained with H&E and processed for measurement of miR-29a/b, CLDN1, and NKRF.

Subjects were excluded from participation if they had history of inflammatory bowel disease or microscopic colitis, lactose intolerance, bacterial overgrowth, or celiac sprue; positive hydrogen breath test or antiendomysial antibody titer; used nonsteroidal anti-inflammatory drugs and/or alcohol for a period of 21 days before the study; history of diabetes, pancreatitis, cirrhosis, food allergies, rheumatoid arthritis, collagen vascular disease, kidney disease, or chronic dermatologic condition.

Cell Culture

Human colonic epithelial cells (FHC) and small intestinal epithelial cells (ATCC CCL-241; FHs74Int) (CRL-1831, ATCC, Manassas, VA) were cultured as described previously.[10]

Microarray Analysis

Microarray analysis for gene and microRNA profiling was performed in SeqWright and LC Sciences (Houston, TX) as described previously.[10]

miRNA Target Prediction

Target mRNAs of the miR-29 family were determined using the miRGen web tool for the algorithms TargetScanS (release 3.1) and PicTar (4-species conservation). Targets predicted by 2-way intersection of these algorithms were further analyzed. Ensembl gene identification numbers of putative targets retrieved from miRGen were referenced to Ensembl builder. The convergence of these miRNA targets with mRNAs that were differentially expressed in a microarray study of miR-29a/b$^{-/-}$ vs WT mouse colon tissue was determined. mRNA probe sets were considered differentially expressed if the fold change was $\geq +1.5$ or ≤ -1.5 at cyberT $P \leq .05$.

Additional Methods

Detailed methodology is described in the Supplementary Methods.

Statistical Analysis

All statistical analyses were done using GeneSpring GX software version 7.3 (Agilent Technologies, Santa Clara, CA), Prism version 6 (GraphPad Inc, San Diego, CA), and ASA software Version 9.1.3. One-way analysis of variance was done, followed by Tukey's comparison or by the Benjamini and Hochberg correction for false-positive reduction. *T* tests were also used. Values are expressed as mean ± SD. Human tissue samples were paired for comparisons based on matching for age and sex. Pearson correlation coefficients were calculated to explore the association between intestinal permeability and miR-29a and miR-29b expression.

Results

Intestinal Hyperpermeability and miR-29 Expression in Humans

We enrolled 233 subjects; 219 (94%) of which completed the study; 14 dropped out, including 12 IBS patients and 2 controls. Of the 219, there were 109 IBS-D patients (28.6 ± 2.9 years old, 34 male and 75 female); 74 constipation-predominant IBS (IBS-C) patients (30.4 ± 4.3 years, 20 male and 54 female); and 36 healthy controls (mean age 31.5 ± 3.6 years; 10 male and 26 female). There was no significant difference in age or sex between the groups (controls, IBS).