Supplementary Materials

1. Establishment of Diarrhea-predominant Irritable Bowel Syndrome Rats Model

Forty (20 male and 20 female) Sprague-Dawley (4 weeks, weight 180 ± 10 g) rats were purchased from Guangzhou Experimental Animals Center (Guangzhou, China) and housed individually in a specific-pathogen-free facility (22 ± 2°C), under a 12/12 hours light/dark cycle (lights on from 6 AM). Standard rat food and tap water were available without restriction unless otherwise noted. After a 1-week acclimation, rats were randomly exposed to one of the following conditions: unpredictable chronic stress for 3 weeks followed by 7 days of rest and 1 hour acute restraint stress (rat model of CAS). Food (but not water) was removed 10 hours prior to the acute restraint stress exposure. The unpredictable chronic stress included the following seven stressors at random order: overnight illumination for 12 hours, 45°C environment for 5 minutes, water deprivation for 24 hours, 4°C environment for 3 minutes, tail clamp for 1 minute, level vibration (120/min) for 40 minutes, and food deprivation for 24 hours. After completion of the nociceptive visceral hypersensitivity test, myeloperoxidase (MPO) activity assay, electromyographic (EMG) activity, and restraint stress-induced defecation, the rats were sacrificed to obtain the distal colon.

2. Nociceptive Visceral Hypersensitivity

A Flat Bottom Holder (Kent Scientific Corp, Torrington, CT, USA) plastic restrainer was used to hold the unsedated animals during colonic distension. It was large enough to allow the rats to move inside of it. Because the holder is clear plastic, we were able to monitor all abdominal movement and contractions. The abdominal contractions in response to balloon distension were clearly distinct and readily recognizable compared to normal abdominal movements.

A 5-cm long and 2-cm wide balloon made of polyethylene was secured to tubing attached to an automated distension device (G & J Electronic Inc., Toronto, Canada) that was used to perform colonic distension. The balloon was lubricated and placed into the rat's distal colon so that the tip of the balloon was 0.5-1 cm from the anus. Rats were restrained in a plastic containment device and allowed to acclimate for 15-20 minutes before testing. Then, rats received phasic distension of the colon (to pressures of 0-60 mmHg in 5 mmHg ascending increments) until the first contraction of the testicles, tail, or abdominal musculature occurred, which was defined as the visceral nociceptive pain threshold and which was indicative of the first nociceptive response. Colonic distensions were repeated two times within 5-10 minutes. Inter-stimulus intervals and the mean pressures at the nociceptive threshold were recorded for each animal. The technicians involved in the study were blinded to the specific group the rats belonged to.

3. Electromyographic Activity

To obtain EMG measurements of visceromotor responses, under anaesthesia with pentobarbital sodium 50 mg/kg intraperitoneally, 2 electrodes were implanted in the external oblique muscle and externalized behind the head. Colorectal distention was performed as described previously (Supplement 1_2) with 20 seconds of distention followed by 2-minute rest between distentions of 20, 40, 60, and 80 mmHg. EMG was recorded continuously during the experiment on a Biopac Systems EMG 100 C, MP100A-CE (Biopac Systems, Inc, Santa Barbara, CA). The EMG signal was amplified, filtered at 300 Hz, and digitized using Acknowledge (Biopac Systems, Inc). The area under the curve for the EMG signal (during each 20 seconds of distention plus 10-second post-distention period for a total of 30 seconds) was calculated using an in-house written computer program. The net value for each distention was calculated by subtracting the baseline value derived from the average area under the curve (30-second interval) for the 2-minute pre-distention period.

4. Myeloperoxidase Activity Assay

MPO is a peroxidase enzyme abundantly expressed in neutrophil granulocytes. In this study, distal colon tissue was sheared into pieces and homogenized in hexadecyltrimethylammonium bromide (HETAB) buffer. The homogenates were centrifuged at 10 000 × g for 15 minutes. Next, 50 μL of supernatant was incubated with 150 mL of 0.0005% o-dianisidine dihydrochloride so-

lution containing 0.001% hydrogen peroxide in potassium phosphate buffer (pH 6.0). The alteration of absorbance at 470 nm was then detected for 1 minute using a microplate reader. A unit of MPO activity was defined as that converting 1 μmol of hydrogen peroxide into water. The value of MPO activity was normalized with the weight of colon tissue.

5. MicroRNA Microarray Analysis

The distal colon of the two groups was assessed with a miRNA microarray assay, which was performed at Guangzhou RiboBio Co., Ltd. The assay consisted of four steps: pre-hybridization, hybridization, hybridization washing and imaging. The CustomArray microarray were assembled using hybridization cap and clips. Pre-hybridization was first performed: The hybridization chambers were filled with nuclease-free water, incubated at 65°C for 10 minutes, and gradually reduced to room temperature. After the water was drawn out of hybrid chamber, the pre-hybridization solution was filled into the chamber, incubated at 37°C for 60 minutes, and gently rotated in the hybridization oven. Next, the hybridization was performed: The hybridization solution was prepared according to the following procedure. The total RNA from distal colon in the two groups were extracted using TRIzol and labelled with cy3 as fluorescence labelling using ULS notation. The solution was denatured at 95°C for 3 minutes and cooled on ice for 20 seconds to prepare the hybridization steps. The hybridization chambers were filled with hybridization solution, followed by removal of the pre-hybridization solution, gently mixed and incubated at 37°C for 16 hours. Finally, the microarray was washed to reduce the specific hybridization background, covered with the imaging solution and loaded into a GenePix 4000B Microarray Scanner to scan.

6. Isolation and Culture of Colonic Epithelial Cells

The distal colons obtained from the two groups were cut into small pieces, washed and digested with 0.1% collagenase I and hyaluronidase for 25 minutes at 37°C to separate colonic epithelial cell clusters. After digestion, the supernatant was transferred to a new tube, and Dulbecco's modified eagle medium (DMEM) was added. After centrifuging 3 times, cells were cultured in a DMEM solution containing 100 mL/L foetal bovine serum in a CO_2 incubator at 37°C with saturated humidity. Fibroblasts were removed using phase difference digestion and adherence. When 80-90% of the cells were adherent to culture plates, the cells were passaged using trypsin digestion.