Effects of SCN9A gene modification on Na^+ channel and the expression of nerve growth factor in a rat model of diarrhea-predominant irritable bowel syndrome

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Abstract. The aim of the present study was to identify whether the sodium voltage-gated channel alpha subunit 9 (SCN9A) gene modification is a potential treatment for diarrhea-predominant irritable bowel syndrome (D-IBS), via regulating the Na^+ channel and the expression of nerve growth factor (NGF). The recombinant adenovirus vector of the SCN9A gene was established, and rat colon cells were isolated for SCN9A gene modification. All subjects were divided into four groups: i) The SCN9A-modified (D-IBS rat model implanted with SCN9A-modified colon cells), ii) negative control (NC; D-IBS rat model implanted with colon cells without SCN9A gene modification), iii) blank (D-IBS rat model without any treatment) and iv) normal (normal rats without any treatment). Western blotting and reverse transcription-quantitative polymerase chain reaction were used to detect the protein and mRNA expression levels of SCN9A, NGF and voltage gated sodium channels (Na_v)1.8 and Na_v1.9 in rat colon tissues. Compared with the normal group, the rats in the SCN9A, NC and blank groups had significantly elevated mRNA and protein expression levels of NGF, SCN9A, Na_v1.8 and Na_v1.9. The rats in the SCN9A group demonstrated significantly increased mRNA and protein expression levels of NGF, SCN9A, Na_v1.8 and Na_v1.9 compared with the NC group and the blank group (all $P<0.05$). SCN9A gene modification can promote the expression of Nav1.8 and Nav1.9 channels, in addition to NGF which may provide a novel therapeutic basis for treating of D-IBS.

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Introduction

As a functional gastrointestinal disorder, irritable bowel syndrome (IBS) may be divided into three primary types: Constipation predominant (C-IBS), diarrhea predominant (D-IBS) and mixed/alternating IBS, which have significant influences on life quality for nearly 10-20% of the population (1). The diagnosis of IBS is on the basis of the exclusion of the Rome I, II and III criteria in addition to other organic or functional disorders (2). Current data indicates that IBS is increasing in the Asia-Pacific region, particularly in developing countries, and a study conducted in China has reported that the prevalence of IBS (according to the Rome III criteria) is 15.9% in outpatient clinics (3). IBS is characterized by discomfort, recurrent abdominal pain, and altered bowel habits in the absence of any organic disorder, and D-IBS patients demonstrate visceral hypersensitivity and damaged colonic motility with elevated frequency and enhanced amplitude of giant migrating contractions (GMCs) which leads to mass movements, stool propulsion and the initiation of defecation (1,4). The concrete pathogenesis of IBS is multifaceted and not completely understood, however several risk factors for IBS have been identified including genetic, epigenetic, environmental and behavioral factors (2). The development of D-IBS is correlated with psychosocial stress, altered gut flora, intestinal barrier dysfunction, disturbed gastrointestinal motility, mucosal immune activation, visceral hypersensitivity, euro-endocrine abnormality and genetic susceptibility (5). There is still no agreement over optimal pharmacological treatment for D-IBS and as ion channels are important in gastrointestinal function, disrupted ion channels may result in disease, therefore the present study aimed to investigate whether a gene associated with ion channels may act as a novel target to treat the disease (4,6).

Sodium voltage-gated channel alpha subunit 9 (SCN9A) encodes the subunit of the voltage-gated sodium channel (VGSC Na_V1.7), is expressed at a rather high density in sensory, sympathetic and nociceptive neurons and may be important in nociception and vasomotor regulation (7,8). SCN5A-encoded Na_v1.5 has previously been demonstrated to exist in human intestinal interstitial cells of Cajal, and ion channels may be associated with a subset of patients with IBS, a heterogeneous

and poorly understood disorder. Due to the fact that SCN5A and SCN9A encode sodium channels which share common evolutionary origins, it is plausible to conjecture that SCN9A may be important in IBS (9-11). Nerve growth factor (NGF) is critical to the functional regulation and development of sensory neuron (nociceptor) signaling events that result in pain and has a primary role in the pathophysiology of inflammatory pain (12). Chronic abdominal pain and low-grade mucosal inflammation are the predominant manifestations of D-IBS and NGF is associated with chronic inflammatory pain, therefore there is a possibility that NGF may be a potential therapeutic target in treating D-IBS, and a study conducted by Willot *et al* (13) verifies this conjecture. The present study, aimed to investigate the effects of SCN9A gene modification on sodium ion channels (Na^+ channel) and the expression of NGF in a rat model of D-IBS.

Materials and methods

Ethical statement. All experiments in the present study were conducted in accordance with public institution conventions and strictly complied with relevant standards for the care and use of laboratory animals according to the National Research Council or National laws. The study was approved by the Ethics Committee of Cangzhou Central Hospital (Cangzhou, China).

Study subjects. A total of 56 healthy, mature, specific pathogen-free Sprague-Dawley rats, male (n=28) and female (n=28), aged 3 months, weighted 172±15 g, were provided by the Laboratory Animal Center of Xiangya Medical College in Central South University [Changsha, China; animal certificate no. SCXK (XIANG) 2016-0007]. All rats were randomly kept in cages with 8 rats in each and they had free access to food and water. Rats were fed under controlled temperature (25±3˚C) and humidity (55-68%). All cages were cleaned once a day; windows were opened twice a day for ventilation for 0.5 h each time. A complete disinfection of all cages, equipment and the room was conducted once a week.

Model establishment and grouping. A total of 56 rats were divided into two groups: 32 were assigned for the model group and 24 for the normal control group. From the 32 rats assigned to the model group, 8 were randomly selected and were used to conduct the model evaluation. The remaining 24 rats were divided into 3 sub-groups, each containing 8 rats as follows: SCN9A gene modification group (model rats with insertion of cells with SCN9A gene modification), negative control (NC) group (model rats with insertion of cells with non-transfected SCN9A gene modification), the blank group (model rats without any treatment) and the normal group (normal rats without any treatment).

Rats in the model group had free access to water however a 12 h fasting period was required prior to experiments. On the first day of experimentation, all rats were put in the specifically prepared plastic containers with tail position higher than head. The rat tail was raised for 30 sec to expose the anus for inserting the infusion catheter (with 8 cm outside anus) with the other end connected to a syringe. Following this, 1 ml glacial acetic acid (40 ml/l, analytically pure, provided by Tianjin Bodi Chemical Co. Ltd. (Tianjin, China) was infused into the colon, then the infusion catheter was removed by pressing the anus with a cotton swab soaked in normal saline, and 1 ml PBS (0.01 mol/l, provided by Beijing Zhongshan Jiqiao Biotechnology Co. Ltd., Beijing, China; batch no. ZLI.9062) was used to clean the feces or other waste in the colon. From the fourth day of experiment, restraint stress treatment was applied for 5 days consecutively for 2 h each day (front shoulders, front limbs and chest were restrained by a wide paper tape to keep rat from scratching head and face, other body parts were free to move). Model rats had increased rectal sensitivity, defecation and visceral sensitivity which is consistent with the characteristics of IBS patients.

Model evaluation. For the model evaluation, rats were sacrificed under anesthesia with 10% pentobarbital sodium (300 μl/100 g) following blooding. Rat abdomens were cut open for the observation of organs with the naked eye. Colon tissue (2 cm; 1 cm distance from cecum) was cut and opened longitudinally, and then was conventionally fixed with 10% neutral formalin solution for 4-6 h, dehydrated by 85% ethanol, cleared by xylene twice (each for 15 min), paraffin-embedded at 54-56˚C, sliced into 4 μm thickness and stored at -80˚C.

The following indicators were observed: i) The color and texture of rat fur and rat mental status and activity; ii) calculation of body mass growth rate: Following finish of feeding, rat body mass was weighed and recorded as the initial body mass; rat body mass was then observed and weighed every day during the experimental period. Growth rate of body mass = (body mass of the day - initial body mass)/initial body mass x100%; iii) Comparison of the loose stool rates: Starting time of diarrhea on each day was recorded according to the filter paper marks; total frequencies of stools and the frequencies of mucks were recorded in 24 h a day. Loose stools rate = frequencies of mucks/total frequencies of stools x100%; iv) calculation of viscera indexes of thymus and spleen: Following finish of the experiment, rat body mass was weighed following a 24 h fasting period and recorded as final body mass.

Rats were sacrificed via exsanguination; thymus and spleen were removed from body and then rats were weighed following clearing damp with filter paper. Viscera indexes = viscera weight/final body mass x100%.

Cell separation and culture. Colon tissues of 8 normal rats were used for cell separation and culture. Following removal of connective tissues, colons were put in the culture dish with prepared D-hanks (Hyclone; GE Healthcare, Logan, UT, USA; batch no. NSG0054). Colons were cut open longitudinally and D-hanks solution with antibiotics was used to clean feces and other waste. Colons were then cut into tissue blocks of 1 mm^3 and transferred to the 50-ml centrifuge tube following washing with D-hanks solution with antibiotics 2-3 times. D-hanks solution containing 10 mM dithiothreitol (cat no. 10197777001; Beijing Solarbio Science & Technology Co., Ltd.; solarbio.bioon.com.cn) and 1 mM EDTA (batch no. B1227012; Beijing Meike Mei Biotechnology Development Co. Ltd.) was added to the centrifuge tube with colon tissues to 20 ml in total. Following a still-standing period at room temperature for 15 min, colon tissue blocks were centrifuged for 5 min (4˚C, 300 x g) using a centrifugal machine (L420;

Hunan Xiangyi Instrument Development Co., Ltd., Hunan, China). Sediments were transferred to a 100-ml conical beaker, shaken and digested following addition of 50 ml hyaluronidase (cat. no. A0701203; Shanghai Gaochuang Chemical Technology Co. Ltd., Shanghai, China; www.gaochem.cn) resulting in a turbid solution. The turbid solution was then centrifuged for 5 min (4˚C, 100 x g) and then sediments were transferred to the conical beaker with 20 ml type I collagenases (batch no. 080203; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). It was then shaken for 30 min at 37˚C (80 r/min), and centrifuged for 5 min (4˚C, 200 x g). Sediments were taken, triturated for 5 min with 10 ml icy precooling Hanks solution (batch no. 971272; Sino American Shanghai Squibb Pharmaceuticals Ltd., Shanghai, China), filtered using a 200-mesh sieve, and then washed with icy precooling Hanks. The washing fluid was collected and centrifuged for 10 min (150 x g). Cells sediments were suspended and put into a T25 cell culture bottle (batch no. TCF-25 Shanghai Baiyan Biotechnology Co., Ltd., Shanghai, China) for 90 min. And cell supernatants were then transferred to a 5 ml Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for another culture. After trituration with a pipette, cells were counted by an inverted microscope (MI12, Mshot Technology Ltd., Guangzhou, China). Then cells were inoculated into a 25 cm^2 culture bottle containing $1x10^6$ cells at 37˚C in 5% CO_2. Three days later, cells were observed under an inverted microscope and medium was changed.

Construction of SCN9A gene adenovirus vector. DNA of SCN9A gene was extracted using Easy Pure Genomic DNA Kit (EE101-01; Trans Gen Biotech Co., Ltd., Beijing, China) in accordance with the manufacturer's protocol and gene modifications were done according to the manufacturer's protocol, using hydrosulfite (American Epigentek Company, Farmingdale, NY, USA). Polymerase chain reaction (PCR) (14) amplifications were applied to the modified DNAs with methylation specific primers and non-methylation specific primers. Double enzyme digestions of adenovirus vector pDC316-EGFP (Microbix Biosystems Inc., Mississauga, ON, USA) and SCN9A gene segment/SCN9A gene modified segment were performed with *Bgl*II and *Hind*III endonucleases (New England Biolabs, Hitchin, Herts, UK), followed by collection with agarose gel electrophoresis. The collected vector segments and target gene cDNA were connected with T4Ligase (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 16˚C overnight. TG1 competent bacteria (Guangzhou Medical University, Guangzhou, China) was directly transformed from 5 μl connected products and 3 clones were selected following shaking overnight, through a lysogeny broth culture dish (Gibco; Thermo Fisher Scientific, Inc.) with ampicillin. The plasmids were extracted and recombinant adenovirus vectors were correctly verified by double-enzyme digestion and termed pDC316-EGFP-SCN9A/hSCN9A. The obtained recombinant adenoviruses were amplified and purified, and the infectious titers (TCID50) were tested. Virus titer was calculated according to the Karber method: Titer $(T)=10^{1+d(s-0.5)}$.

Adenovirus transfection of target cells and grouping. Cells deriving from the gene modification SCN9A (transfected with plasmid modified by SCN9A), NC (transfected with plasmid not modified by SCN9A gene) and the blank group were used for this experiment. Briefly, cells were put under a fluorescence microscope for the observation of green fluorescent protein expression. When cell density increased to 30-50%, cell transfection was conducted using Lipofectamin 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells of each group (100 pmol) were incubated with 250 μl serum-free Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nM at room temperature for 5 min. Lipofectamin 2000 (5 μl) was incubated with 250 μl serum-free Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nM at room temperature for 5 min. Then the aforementioned two compositions were mixed and incubated at room temperature for two min and then transplanted into a cell culture. After incubation at 37˚C in 5% CO_2 for 6~8 h, medium was replaced by complete medium, followed by a 24 h transfection incubation. Rates of green fluorescence cells in 400 cells were calculated under different magnetic optic imaging (MOI) values (MOI=0.0%; MOI=5, 15.9±1.6%; MOI=20, 42.5±2.1%; MOI=40, 95.2±1.9%; MOI=80, 99.1±1.7%; MOI=100, 99.4±2.5%), and results demonstrated that the rates of cells expressing green fluorescence were increased with the increasing of MOI values. When MOI was 40, green fluorescence appeared in various cells (Fig. 1); when MOI was 80 and 100, almost 100% of cells revealed green fluorescence; however, following a culture period of 48 h, cells with MOI of 80 and 100 decreased significantly and floating dead cells in the culture dish were observed. It was hypothesized that the virulence may have been too strong to inhibit cell proliferation and finally led to cell apoptosis. Therefore, considering green fluorescence expression and cell growth, MOI=40 was selected for the optimum transfection time.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNAs were extracted with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription reactions were conducted according to the manufacturer's protocol of the reverse transcription kit (18091200; Thermo Fisher Scientific, Inc., Shanghai, China). PCR reactions (20 μl) were performed in a mixture containing 10 μl SYBR-Green Mix (Thermo Fisher Scientific, Shanghai, China), 1 μl of forward primer, 1 μl of reverse primer, 1 μl of cDNA and 7 μl of ddH_2O (batch no. 4385618; Shanghai solarbio Bioscience & Technology Co., Ltd., Shanghai, China). PCR reactions were performed in ABI7 100 real time PCR equipment (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: pre-denaturation at 95˚C for 5 min, 95˚C for 40 sec, 57˚C for 40 sec, 72˚C for 40 sec; extension at 72˚C for 10 min and at 4˚C for 5 min. GAPDH was used as internal reference. PCR primer are listed in Table I.

Western blotting. Colon tissues of all groups were cut into small fragments (measuring about 1 mm^3). Following washing with PBS (0.01 mol/l) and precooling with ice, colon tissue fragments were incubated at 4˚C for 2 h, prior to addition of an adequate amount of protein extracting solution (including 50 mmol/l Tris-HCl, 1% SDS, 50 mmol/l NaCl and 0.5% proteinase inhibitor). Centrifugation of the prepared samples

Table I. RT-qPCR primer sequences.

Name	Sequence (5'-3')
NGF	
Forward	CCGAGCCCCGAATCCTGTA
Reverse	GGGAAGGGGGCTGCAGGCAAG
SCN9A	
Forward	TCTCCCTTCAGTCCTCTAA
Reverse	AACAAAGTCCAGCCAGTT
Nav1.8	
Forward	GGACTCCCTGAAGACCAATATGGAG
Reverse	GCATTGAGCTAGATGGGTTAATGTTG
Nav1.9	
Forward	CCCTGCTGCGCTCGGTGAAGAA
Reverse	GACAAAGTAGATCCCAGAGG
GAPDH	
Forward	CAAGGTCATCCATGACAATTTG
Reverse	GTCCACCACCCTGTTGCTGTAG

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NGF, nerve growth factor; Nav, voltage gated sodium channel; SCN9A, sodium voltage-gated channel alpha subunit 9.

was performed at 94,553 x g for 10 min. Then, buffer solution (20% glycerinum, 1 mmol/l Tris-HCl, 10% SDS, 10% β-mercaptoethanol) was added for degeneration at 100˚C for 4 min. The supernatant was collected with sediments removed and kept for use, or preserved at -80˚C. Polypropylene gel (10%) was prepared, and electrophoresis was done with addition of sample (10 μl/well) and 3 μl Marker (Tiangen Biotech Co., Ltd.). Quantitative analysis of proteins was performed by Kjeldahl method. Protein was transferred to a polyvinylidene fluoride membrane (PVDF) (Jiangsu Jie LV Mo Technology Co., Ltd., Jiangsu, China). Subsequently, PVDF were soaked in 5% non-fat milk and sealed at 37˚C for 2 h. Subsequently, the PVDF nitrocellulose membrane was incubated with rabbit polyclonal antibody Anti-SCN9A (1:1,000, cat. no. ab65167; Abcam, Cambridge, UK) diluted with Tris-buffered saline with Tween-20 (TBST), on a shaking table at 4˚C overnight. Following the overnight incubation, the membrane was washed three times with TBS with Tween-20 (10 min each). Following this, the membrane was soaked in the prepared secondary antibody horseradish peroxidase labeled sheep anti-rabbit IgG (HRP-IgG) diluent (1:1,000; cat. no. DF109489; Shanghai Yaoyun Biological Technology Co., Ltd., Shanghai, China), stirred at 4˚C (WD-9405B; Beijing Liuyi Instrument Factory, Beijing, China) and incubated for 1 h. The nitrocellulose membrane was fully cleaned, anti-incubated, and cleaned with TBST 5 times (5 min each time). Adequate amounts of developing solution A and B (Tiangen Biotech Co., Ltd.) were mixed evenly away from light. Following addition of the mixed solution, the PVDF was put into UV Transilluminator (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for imaging, and then was preserved for analysis. Detection procedure of $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ protein levels were the same as the aforementioned one.

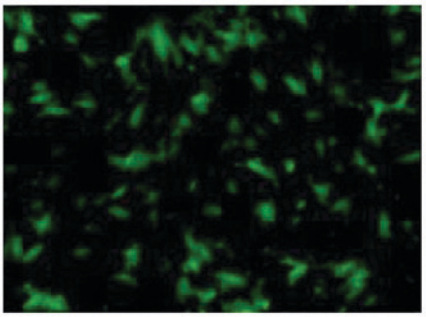

Figure 1. Green fluorescent protein expression in colon tissue cells transfected with Ad-SCN9A (MOI=40). Magnification, x200. MOI, magnetic optic imaging; Ad-SCN9A, adenovirus vector-sodium voltage-gated channel alpha subunit 9.

Statistical analysis. SPSS software, version 20.0 (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. All measurement data were presented as the mean ± standard deviation. A paired Student's t-test was used for comparison between two groups. As for the comparisons among multiple groups, one-way analysis of variance was applied. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparisons of model evaluation based on rat fur, mental status, activity, growth rate of body mass, loose stool rate and viscera indexes between the normal control and model groups. Fur color and texture, mental status and activity of rats in all groups were observed. Results demonstrated that rats in the normal control group had good mental status, well-shaped body and shining dorsal fur; whereas rats in the model group were slightly dispirited and exhausted, the glossiness of dorsal fur was decreased markedly, and bodies were comparatively thinner. No mortalities occurred during experiments.

Comparison of growth rate of body mass revealed a persistent increasing of body mass for rats in the normal control group from day 1 to day 5; whereas body mass of rats in the model group was significantly decreased compared with the normal control group ($P<0.05$) and presented a continuously decreasing trend (Table II).

Comparison of loose stool rate revealed that stools of rats in the normal control group were dry and in particle forms, and there was no stain on filter papers; whereas loose stool rate of rats in the model group increased significantly compared with that in the normal control group ($P<0.05$; Table III).

Results demonstrated that the spleen index of rats in the normal control group was 0.251±0.023 and the thymus index was 0.354±0.041; whereas spleen index of rats in the model group was 0.153±0.017 and the thymus index was 0.285±0.036. Compared with the normal control group, viscera indexes of the model group decreased significantly ($P<0.05$; Table IV), which demonstrated that (D-IBS models were successfully established.

Titer determination of recombinant adenovirus. Cells with recombinant adenovirus were collected, frozen and dissolved repeatedly 3 times at 37˚C and -80˚C. Following centrifugation at 560 x g and precipitation, part of the virus supernatant was used to infect cells for the amplification of recombinant virus, as

Table II. Comparisons of mean growth rate of body mass of rats between the normal control and model groups.

Groups	n	1 day	2 days	3 days	4 days	5 days
Normal control	8	0.935±0.098	2.573±0.265	3.507±0.382	5.796±0.623	6.878±0.731
Model	8	-5.856±0.623[a]	-7.395±0.754[a]	-8.171±0.925[a]	-8.580±0.792[a]	-8.835±0.956[a]

[a]P<0.05 vs. normal control group at the same time.

Table III. Comparisons of mean loose stool rates of rats between the normal control and model groups.

Groups	n	1 day	2 days	3 days	4 days	5 days
Normal control	8	0	0	0	0	0
Model	8	22.597±3.630[a]	28.432±3.344[a]	28.784±3.455[a]	29.457±3.068[a]	31.771±2.797[a]

[a]P< 0.05 vs. normal control group at the same time.

Table IV. Comparisons of viscera indexes of rats between the normal control and model groups.

Groups	n	Spleen index	Thymus index
Normal control	8	0.251±0.023	0.354±0.041
Model	8	0.153±0.017[a]	0.285±0.036[a]

[a]P<0.05 vs. normal control group at the same time.

previously described (15). The aforementioned procedures were repeated various times to acquire recombinant adenoviruses with high titers. Virus titer tested by TCID50 was 2.8x108 PFU/ml.

SCN9A expression levels increase in SCN9A transfected group of rats. RT-qPCR demonstrated that the SCN9A mRNA expression in the SCN9A group, the NC group, the blank group and the normal group were 0.985±0.116, 0.614±0.081, 0.558±0.073 and 0.292±0.035. Compared with the NC group and the blank group, SCN9A mRNA expression in the SCN9A group was comparatively higher (P<0.05; Fig. 2). Western blotting revealed that the expression of SCN9A in the SCN9A, the NC, the blank and the normal groups were 1.115±0.146, 0.637±0.083, 0.591±0.075 and 0.344±0.029. Compared with the normal group, SCN9A protein expression levels in the SCN9A, the NC and the blank groups were comparatively increased (P<0.05), and the protein expression in the SCN9A group was further increased compared with the NC and the blank groups (P<0.05; Fig. 3A and B). These results demonstrated that transfection had been successful.

NGF expression levels increase in SCN9A transfected group of rats. RT-qPCR demonstrated that the NGF expression levels in the SCN9A, the NC, the blank and the normal groups were 2.215±0.324, 1.162±0.152, 1.089±0.127 and 0.576±0.078. Compared with the normal group, the NGF mRNA expression in the SCN9A group, the NC and the blank group were increased

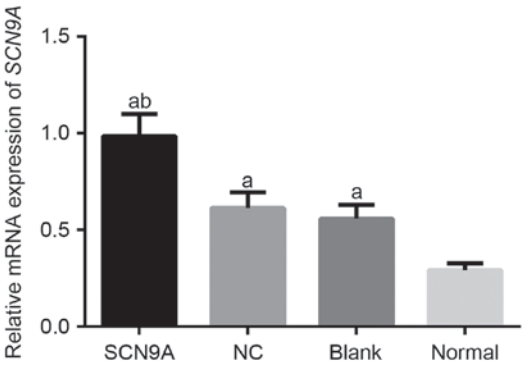

Figure 2. SCN9A mRNA expression in colon tissues. mRNA expression levels of SCN9A in the 4 groups were detected via reverse transcription-quantitative polymerase chain reaction. [a]P<0.05 vs. normal group; [b]P<0.05 vs. NC and blank group. SCN9A, sodium voltage-gated channel alpha subunit 9; NC, negative control.

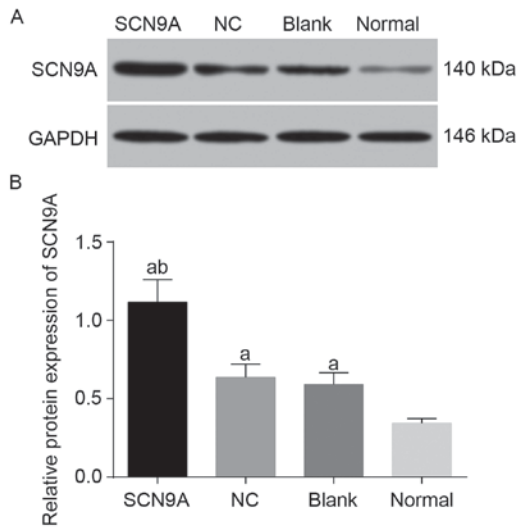

Figure 3. SCN9A protein expression in colon tissues. (A) Representative image and (B) quantitative analysis of protein expression levels of SCN9A in the 4 groups, detected by Western blotting. [a]P<0.05 vs. normal group; [b]P<0.05 vs. NC blank group. SCN9A, sodium voltage-gated channel alpha subunit 9; NC, negative control.

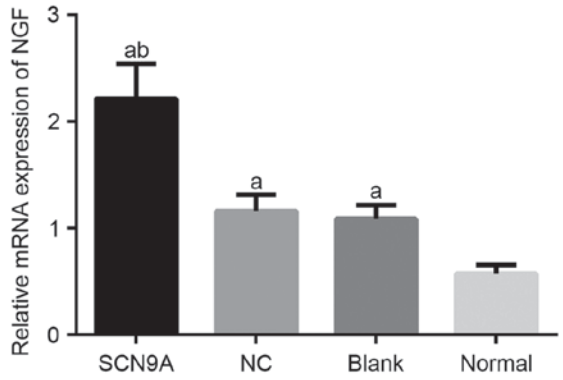

Figure 4. NGF mRNA expression in colon tissues. mRNA expression levels of NGF in the 4 groups were detected via reverse transcription-quantitative polymerase chain reaction. [a]P<0.05 vs. normal group; [b]P<0.05 vs. NC and blank group. NGF, nerve growth factor; NC, negative control; SCN9A, sodium voltage-gated channel alpha subunit 9.

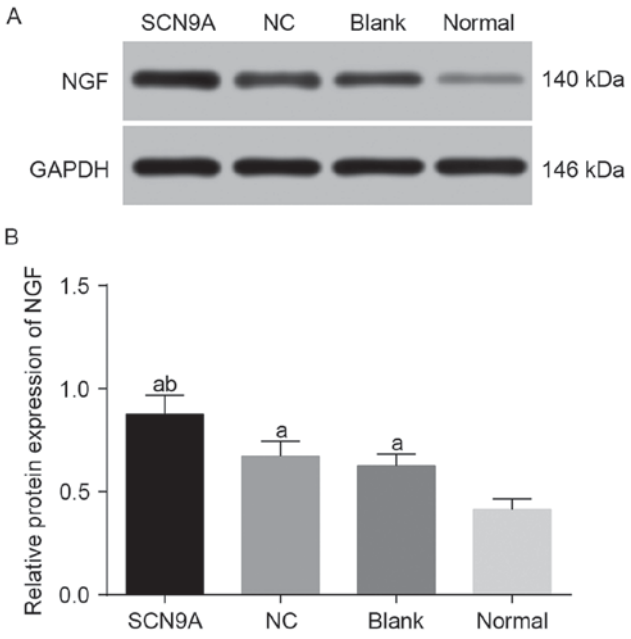

Figure 5. NGF protein expression in colon tissues. (A) Representative image and (B) quantitative analysis of protein expression levels of NGF in the 4 groups, detected by western blotting. [a]P<0.05 vs. normal group; [b]P<0.05 vs. negative control and blank group. NGF, nerve growth factor; NC, negative control; SCN9A, sodium voltage-gated channel alpha subunit 9.

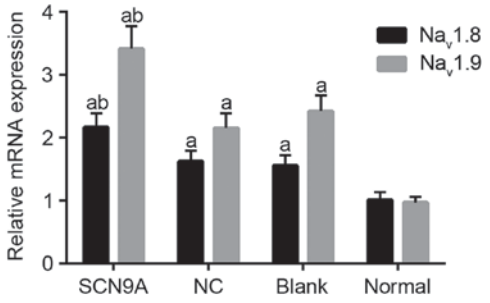

Figure 6. $Na_v1.8$ and $Na_v1.9$ mRNA expression in colon tissues. mRNA expression levels of $Na_v1.8$ and $Na_v1.9$ in the 4 groups were detected via reverse transcription-quantitative polymerase chain reaction. [a]P<0.05 vs. relevant normal group; [b]P<0.05 vs. negative control and blank group. NC, negative control; SCN9A, sodium voltage-gated channel alpha subunit 9; Na_v, voltage gated sodium channel.

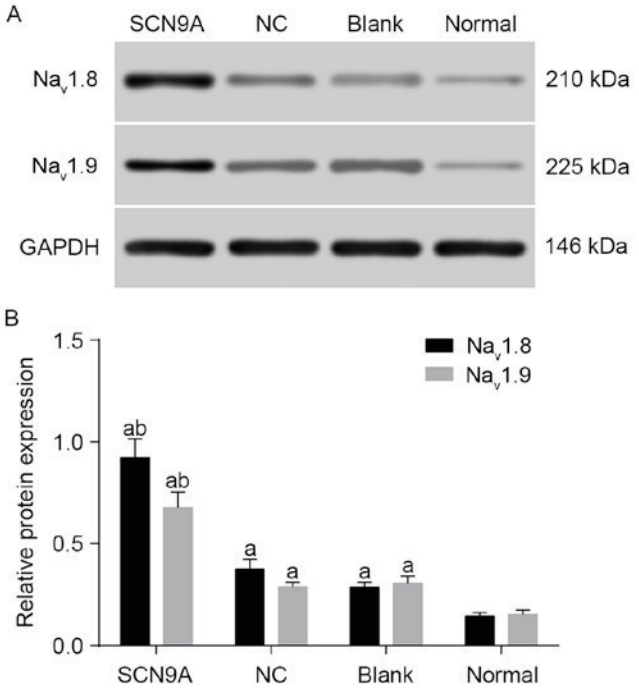

Figure 7. $Na_v1.8$ and $Na_v1.9$ protein expression in colon tissues. (A) Representative image and (B) quantitative analysis of protein expression levels of $Na_v1.8$ and $Na_v1.9$ in the 4 groups, detected by western blotting. [a]P<0.05 vs. relevant normal group; [b]P<0.05 vs. negative control and the blank group. NC, negative control; SCN9A, sodium voltage-gated channel alpha subunit 9; Na_v, voltage gated sodium channel.

(P<0.05), and NGF mRNA expression in SCN9A group was further enhanced compared with in the NC group and the blank group (P<0.05; Fig. 4). Western blotting revealed that the expression levels of NGF in the SCN9A, the NC, the blank and the normal group were 0.876±0.092, 0.671±0.074, 0.625±0.058 and 0.413±0.053. Compared with the normal group, the NGF protein expression in the blank, the SCN9A and the NC groups were increased (P<0.05), and protein expression in the SCN9A group was significantly increased compared with the NC group and the blank group (P<0.05; Fig. 5A and B). Results demonstrated that SCN9A gene modification of colon tissues of rats with D-IBS stimulated the expression of NGF.

SCN9A gene modification promotes expression of $Na_v1.8$ and $Na_v1.9$ in rats. RT-qPCR demonstrated that the $Na_v1.8$ expression levels in the SCN9A, the NC, the blank and the normal groups were 2.167±0.224, 1.624±0.173, 1.559±0.167 and 1.016±0.123. Compared with the normal group, $Na_v1.8$ and $Na_v1.9$ mRNA expression levels in the SCN9A, the NC and the blank group were relatively increased (P<0.05), and $Na_v1.8$ and $Na_v1.9$ mRNA expressions in the SCN9A group was significantly increased compared with the NC group and the blank group (P<0.05; Fig. 6). Western blotting demonstrated that $Na_v1.8$ protein levels in the SCN9A, the NC, the blank and the normal groups were 0.923±0.091, 0.375±0.048, 0.286±0.025 and 0.145±0.017. $Na_v1.8$ and $Na_v1.9$ protein expression levels in the SCN9A, the NC and the blank group were significantly increased compared with the normal group (P<0.05), and compared with the NC and the blank group, protein expression

in the SCN9A group was increased ($P<0.05$; Fig. 7A and B). Results demonstrated that SCN9A gene modification promoted the expression of $Na_v1.8$ and $Na_v1.9$ in rats with D-IBS.

Discussion

The present study aimed to investigate the role of the SCN9A gene in Na^+ channels and the expression of NGF in D-IBS rats, and the data suggested that SCN9A gene modification promoted the expression of sodium channels-$Na_v1.8$, $Na_v1.9$, and increased the expression level of NGF in rats with D-IBS.

VGSCs, which are additionally termed Na_v, are responsible for the conversion of chemical and/or mechanical stimuli into electrical signals in excited cells, and currently 9 different sodium channels which are encoded by 9 different genes named SCN (1-9A) have been identified (16). SCN9A (sodium channel $Na_V1.7$) with physiological traits including slow closed-state inactivation has previously been demonstrated to be expressed in trigeminal ganglia, dorsal root ganglia (DRG) and sympathetic ganglion neurons, and its mutations have been suggested to be associated with pain due to its ability to amplify stimuli (17,18). Sodium channel $Na_v1.8$ is abundantly expressed in DRG neurons and peripheral nerve axons (19). $Na_V1.9$ has an important role in regulating afferent sensitivity in visceral pain (a rather common symptom for patients with gastrointestinal diseases including IBS-D) to inflammatory and mechanical stimuli (20). It has been acknowledged that highly conserved Na_V channels sharing common evolutionary origins and a large variety of voltage-sensitive ion channels are expressed in contractile organs including gastrointestinal organs, which may lead to the consideration of mechanical modulation of ion channel function (11,21). Ion channels existing in gastrointestinal smooth muscle (including rat and human colon) are excitatory for slow waves, therefore ion channels may act as a therapeutic and pathophysiological target, as they directly participate in gastrointestinal motility and visceral pain, thus ion channelopathies including $Na_v1.8$ and $Na_V1.9$ have the potential to result in IBS (22). In addition, disturbed gastrointestinal motility is a risk factor for D-IBS, and ion channels including $Na_v1.8$ and $Na_V1.9$ may influence the contractile ability of these organs, suggesting that the development of D-IBS may require the active participation of ion channels. Since expression of $Na_v1.8$ and $Na_v1.9$ channels, as well as NGF were enhanced by SCN9A gene modification, this may lead to a novel direction for D-IBS treatment (5). Designer genetic recombination tools and mammalian genetic modification technology are currently novel, reliable and efficient methods to target genomic sequences, which may result in further advances in understanding of gene expression and its associated influence (23).

Furthermore, it was demonstrated that NGF mRNA was expressed most frequently in the SCN9A group compared with the other groups, following the establishment of D-IBS in rats, implicating that the expression of NGF may be stimulated in SCN9A gene-modified colon tissue cells. As a protein with a high degree of conservation and homology, NGF is produced by a single gene located at chromosome 1, which codes for 2 transcripts that generate 27 kDa and 35 kDa precursors (24). NGF may exert influence on paracellular permeability via altering the expression of claudins in tight junctions or on the transcellular uptake route via increasing macro-pinocytosis during stress and inflammation mast cell mediators (25). Therefore, NGF may interrupt the barrier to antigens and bacteria, and as D-IBS has risk factors including psychosocial stress, intestinal barrier dysfunction and altered gut flora, it may be hypothesized that NGF is closely associated with D-IBS (5). A previous study reveals that NGF as a pro-inflammatory mediator is critical in the sensitization of peripheral visceral pain hypersensitivity, and as colonic hypersensitivity is generally regarded as a biological marker for IBS, NGF thus may be considered as a potential novel therapeutic target for IBS treatment (26). Furthermore, NGF is expressed in colonic mucosa and its influences on the induction and maintenance of visceral sensitivity in animals have been acknowledged, and it has been identified to be upregulated in the colonic wall and rectal mucosa in conditions of chronic stress present in IBS patients (13,27).

In conclusion, the present study investigated the influence of SCN9A gene modification on Na^+ channels and NGF in rats with D-IBS. Results demonstrated that $Na_v1.8$, $Na_v1.9$ and NGF were expressed more frequently in the SCN9A group, providing a promising novel therapeutic target for the clinical treatment of D-IBS. However, the present study did not investigate how the SCN9A gene regulates and influences associated signaling pathways and factors, therefore further studies are required in the future.

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