Close association between abnormal expressed enzymes of energy metabolism and diarrhea-predominant irritable bowel syndrome

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Abstract

Background: Irritable bowel syndrome (IBS) is one of the most common functional intestinal diseases, but its pathogenesis is still unknown. The present study aimed to screen the differentially expressed proteins in the mucosa of colon between IBS with diarrhea (IBS-D) patients and the healthy controls.

Methods: Forty-two IBS-D patients meeting the Rome III diagnostic criteria and 40 control subjects from July 2007 to June 2009 in Chinese PLA General Hospital were enrolled in the present study. We examined the protein expression profiles in mucosa of colon corresponding to IBS-D patients ($n=5$) and controls ($n=5$) using 2-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Secondly, Western blot and immunohistochemical analysis were carried out to validate the screened proteins in 27 IBS-D patients and 27 controls. Thirdly, high-performance liquid chromatography (HPLC) was further carried out to determine ATP concentration in the mucosa of colon between 10 IBS-D patients and 8 controls. Comparisons between 2 groups were performed by Student's *t*-test or Mann-Whitney *U*-test.

Results: Twelve differentially expressed proteins were screened out. The α-enolase (ENOA) in the sigmoid colon (0.917 ± 0.007 *vs.* 1.310 ± 0.100, $t=2.643$, $P=0.017$) and caecum (0.765 ± 0.060 *vs.* 1.212 ± 0.122, $t=2.225$, $P=0.023$), Isobutyryl-CoA dehydrogenase (ACAD8) in the sigmoid colon (1.127 ± 0.201 *vs.* 1.497 ± 0.392, $t=7.093$, $P=0.008$) of the IBS-D group were significantly lower while acetyl-CoA acetyltransferase (CT) in the caecum (2.453 ± 0.422 *vs.* 0.931 ± 0.652, $t=8.363$, $P=0.015$) and ATP synthase subunit d (ATP5H) in the sigmoid (0.843 ± 0.042 *vs.* 0.631 ± 0.042, $t=8.613$, $P=0.007$) of the IBS-D group was significantly higher, compared with the controls. The ATP concentration in the mucosa of the sigmoid colon in IBS-D group was significantly lower than that of control group (0.470 [0.180, 1.360] *vs.* 5.350 [2.230, 7.900], $U=55$, $P<0.001$).

Conclusions: Many proteins related to energy metabolism presented differential expression patterns in the mucosa of colon of the IBS-D patients. The abnormalities in energy metabolism may be involved in the pathogenesis of IBS which deserves more studies to elucidate.

Keywords: ATP; Irritable bowel syndrome; Metabolism; Proteome

Introduction

Irritable bowel syndrome (IBS) is a functional bowel disorder in which abdominal pain or discomfort is associated with defecation or a change in bowel habit, and with features of disordered defecation.[1] The world prevalence of IBS among adults and adolescents is approximately 5% to 20%.[2-4] Although IBS does not end up with the development of serious disease and associated mortality, it does have a significant negative impact on patients' quality of life and social functioning and can increase healthcare costs.[5,6]

Unfortunately, the pathogenesis of IBS is still unclear and IBS remains incurable. Previous studies have shown that changes in intestinal reactivity (including abnormalities in motor and secretory functions), intestinal infection and dysbacteriosis, organ hypersensitivity, functional disturbance in brain-gut axis, stress and other factors may be related to IBS or involved in the pathogenesis of IBS. With the intensification in the studies on IBS, it was found that mast cells (MC) and enterochromaffin cells in the mucosa of colon in IBS patients were more than those in controls,[7] and colonic permeability also increased,[8] serotonin (5-HT), 5-HT receptor and serotonin transporter (SERT) concentrations were also abnormal.[9] Furthermore, the

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pathogenesis of IBS is also related to abnormalities in nuclear genes,[10] mitochondrial DNA,[11] protease signaling system,[12] immunological system and immune-related factors (interleukin-1β, interleukin-6, tumor necrosis factor-α (TNF-α).[13] Most of previous studies focus on a certain cell or a certain molecule, but the regulations on pathogenesis always simultaneously involve the changes or functions of several proteins, and it is still unclear which proteins in the mucosal membrane of colon play crucial roles in the pathogenesis of IBS. Therefore, it is of great importance to screen and identify IBS-related proteins in the mucosa of colon for comprehensive understanding of pathogenesis, diagnosis and treatments of this disease. Ding *et al*[14] established the model for IBS in rats and found that some proteins were differentially expressed in the mucosal membrane of IBS rats by using proteomic methods, but further confirmation for clinical data was not provided.

In the present study, comparative investigations were carried out using proteomic techniques in the mucosa of colon between IBS with diarrhea (IBS-D) patients and controls. Furthermore, we validated the expression patterns of these identified abnormal proteins in different positions of the colon from more IBS-D patients and controls by immunohistochemical (IHC) method and western blot. This represents the first look at abnormal expression proteins in the mucosa of the colon in IBS-D patients and the findings may provide new ideas for elucidating the pathogenesis of IBS.

Methods

Ethical approval

The protocol for this study was approved by the Ethics Committee of the Chinese PLA General Hospital (No. 2009070112). Informed patient consent for use of tissues was obtained in all cases. The protocol was registered at www.clinicaltrials.gov (No. NCT01028898).

Study subjects

Forty-two IBS-D patients meeting the Rome III diagnostic criteria and 40 control subjects from July 2007 to June 2009 in Chinese PLA General Hospital were enrolled in the present study.

Inclusion criteria for IBS: non-postinfectious IBS-D patients meeting the Rome III diagnostic criteria.[15]

Exclusion criteria for IBS: (1) patients having organic diseases in intestinal tract and medical records for operations; (2) patients with organic diseases in psychological, nervous or other systems; (3) women in pregnancy and lactation; (4) IBS patients with medical records for alimentary infection, in other words, post-infectious IBS (PI-IBS); (5) patients with diabetes, obesity and other metabolism-related diseases.

Controls included patients who were diagnosed as piles after re-examination, half a year after colonic polypectomy or colonoscopy due to small amount of blood in stools. They should meet the criteria as below: (1) no abnormality was detected in total colonoscopy and conventional pathological examinations in mucosa of colon; (2) no symptom and physical sign in digestive tract; (3) no organic and functional enteropathy; (4) no medical records for immunological diseases and infectious diseases; (5) no recent medical record for drug medication.

Using daily questionnaires over a 10-day period, the following parameters were recorded in all participants: (1) intensity of abdominal pain or discomfort by a 100-point visual analogue scale; (2) stool frequency (average bowel movements per day in the 10-day period); (3) frequency of abdominal pain or discomfort (number of days with pain or discomfort in the 10-day period); and (4) stool consistency assessed by the Bristol Stool Form Score (if more than one bowel movement per day, the Bristol Stool Form Score was averaged, and the grand mean over the 10-day period was calculated).[16]

Twenty-seven patients in the IBS-D group and 27 subjects in the control group were subjected to immunohistochemical (IHC) examination and western blot analysis. Ten patients in the IBS-D group and 8 subjects in the control group were subjected to ATP concentration determination in the mucosal membrane in colon. The distributions of gender and age between the 2 groups were matched.

Tissue specimens

Mucosal membrane tissues were collected during the biopsy at caecum and sigmoid colon during the coloscopy for the IBS-D patients and the controls respectively, 5 pieces were collected at each position and 4 pieces were rinsed in ice saline and immediately preserved in liquid nitrogen; the other piece was fixed in 10% formalin solution and subjected to paraffin embedding and conventional pathological examinations.

Two-dimensional gel electrophoresis and image analysis

Protein samples were obtained by lysis of caecum and sigmoid colon tissue from each IBS-D patients and controls in buffer containing 40 mmol/L Tris-Hcl, 7 mol/L urea, 2 mol/L thiorea, 4% CHAPS, 1% DTT, 1 mmol/L EDTA and protease inhibitor cocktail (Roche, Mannheim, Germany). The lysate was sonicated on ice followed by adding 5 μL (10 μg/μL) RNAase and DNAase respectively and centrifugation (14,000 × *g*; 4 °C; 20 min). Protein concentration was determined using the method of Bradford.

A total of 130 μg protein sample (silver staining) or 1.3 mg (coomassie brilliant blue staining) was dissolved in a sample buffer (8 mol/L Urea, 0.02% CHAPS, 0.02 mol/L DTT and 0.05% IPG buffer) and then added into IPG strip holder. After rehydration, the immobilized pH gradient (IPG) strip (18 cm, pH range 3–10, Amersham Biosciences, Uppsala, Sweden) was subjected to isoelectric focusing on an IPGphor System (Amersham Biosciences) for a total of 8000 V (10 h) at 20 °C. The focused IPG strip was equilibrated in SDS buffer (30% glycerol, 2.0% SDS and 6 mol/L urea and trace bromophenol blue in 50 mmol/L

Tris-HCl, pH 8.8) for 15 min twice. The equilibrated IPG strip was placed onto the top of a 13% gradient polyacrylamide gradient gel (ExcelGel SDS, Amersham Biosciences) (20 cm × 20 cm × 1 mm). The second dimensional SDS-PAGE was performed at 40 mA for 40 min, 60 mA for 5 h using Amersham Biosciences Ettan-Dalt II System. After separation in SDS-PAGE, the proteins on the gel were fixed and visualized by silver staining using a Silver Stain Plus Kit (Bio-Rad Laboratories Inc., Hercules) as described previously.[17] Scanning was carried out with an ImageScanner (Amersham Pharmacia Biotech) and image analysis was performed using ImageMaster 2D Platinum software, version 5.0. Proteins separated by 2-DE gels were quantitated in terms of their relative volume (% vol). All the results of 2-DE and the analysis by software were repeated at least 3 times. After normalizing the quantity of each spot by total valid spot intensity, we selected differentially expressed protein spots for determination of significant expression levels that deviated more than or equal to 2-fold ($P < 0.05$) compared to the control group. The total 10 gels from IBS-D patients and control were performed 2-D PAGE at same time, including isoelectric focusing (IEF), SDS-PAGE, silver staining and quantitive analysis to reduce the error among sample groups.

In-gel digestion, Q-TOF analysis and database searching

To obtain gel spots of the differentially expressed protein, a preparative gel was run and stained by Coomassie Brilliant Blue (CBB) R-250 in 50% methanol and 10% acetic acid. Gel spots corresponding to proteins differentially expressed were picked out manually. The gel specimens were then destained with different concentration CH3CN/NH4HCO3 and digested by trypsin (Roche, Mannheim, Germany).

After gradient desalinization by using capillary liquid chromatography, 1.4 μL was directly loaded and subjected to quadrupole time-of-flight (Q-TOF) analysis (after [Glu1]-Fibrinopeptide B calibration). The spectra obtained from secondary mass spectrometry can be used for the identification on the amino acid sequence of the peptide after processing with MaxEnt4 software. The searching was carried out in the SwissProt database by using Mascot method in www.matrixscience.com.

Immunohistochemical analysis

The specimens were fixed with 10% neutral formalin and embedded in paraffin or directly embedded in OCT. Four-μm-thick sections were deparaffinized in xylene, rehydrated with graded alcohols. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in absolute methanol for 15 min, and non-specific binding was blocked with 1% BSA for 15 min. Mouse α-enolase (ENOA) antibody (Abnova Inc., Taipei, Taiwan, China) and goat anti-acetyl-CoA acetyltransferase (CT) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were incubated with the paraffin and frozen sections respectively at 4°C for overnight. Goat anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used as a secondary antibody respectively. After washing 3 times in PBS, the sections were incubated with streptavidin-biotin conjugated with horse-radishperoxidase, and visualized by demonstration of conjugated peroxidase with diaminobenzidine as the substrate. The slides were counterstained with hematoxylin. For the negative control, primary antibodies were omitted.

Western blot analysis

Aliquots of 20 μg proteins were separated by 12% SDS-polyacrylamide gel, and transferred to nitrocellulose filters. The filters were blocked with TBST buffer (10 mmol/L Tris-HCl, PH 8.0, 0.15 mol/L NaCl, 0.05% Tween 20) containing 5% skimmed milk, incubated with mouse anti-ENOA (Abnova Inc., Taipei, Taiwan, China); mouse anti-Isobutyryl-CoA dehydrogenase (ACAD8; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); goat anti-CT (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); mouse anti-ATP synthase subunit d (ATP5H; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); mouse anti-β-actin (C4; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight, and followed by the addition of horseradish peroxidase-linked anti-mouse or anti-goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and Enhanced Chemiluminescence (ECL) visualization (Sigma Inc., USA) of the bands. The expression of β-actin was used as an internal control to normalize the expressions of other proteins. Bands were scanned using a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) and results were quantified using Quantity One software (Bio-Rad).

Determination of ATP concentration by high-performance liquid chromatography (HPLC)

Fresh biopsy tissue of caecum and sigmoid colon from IBS-D patients and controls were weighed, rapidly homogenized on ice by adding 200 μL perchloric acid and then centrifugation (10,000 × *g*; 4°C; 5 min). The supernatant (pH 7.0) was stored at 4°C for 1 h and then centrifuged for 5 min at 10,000 × *g* again. The supernatant was directly analyzed by Aligent 1100 HPLC system (Aligent Inc., Palto Alto, CA, USA). Chromatographic separation was achieved by using a Zoabox SB-C18 column (5 μm, 150.0 mm × 4.6 mm; AligentInc.USA) serially connected to a UV absorbance diode array detector. For measurements of samples, we used the mobile phase, which consisted of 50 mmol/L KH2PO4, 0.24 mmol/L methanol, and HPLC grade water, pH 5.2 . The flow rate was maintained at 1.8 ml/min. A 20 μL volume of sample was injected from an autosampler. The detection wavelength was 254 nm. Daily calibration curves were prepared by a 3 point standard (3.0, 1.0, 0.3 μmol/L) of adenosine triphosphate (ATP), adenosine triphosphate (ADP), adenosine monophosphate (AMP), respectively.

Retention times of the standard preparation peak and the sample peak as well as the comparison in ultraviolet spectra between them were used as the criteria for quantitative judgment. The sample concentrations were determined according to the peak areas of samples, the peak area of standard preparation and the concentration of the standard preparation, and the ATP concentrations in

each gram of tissue were calculated according to the weight of samples.

Statistical analysis

Statistical calculations were performed by SPSS 13.0 statistical package (SPSS Inc., Chicago, USA). Continuous data with normal distribution were presented as a mean ± standard deviation (SD) and the data were compared using an independent *t*-test. Continuous data with nonnormal distribution were shown as median (P_{25}, P_{75}) and analyzed using the Mann-Whitney *U*-test. All tests were two-tailed with a 0.05 significance level.

Results

Demographic and clinical data of IBS-D and controls

Demographic characteristics of the controls and IBS-D patients indicated comparable samples for analysis ($P > 0.05$) [Table 1]. Among the 40 control patients, 20 subjects were male and 20 subjects were female, they aged 21.0 to 56.0 years and the average age was 43.5 years; among the 42 IBS-D patients, 20 patients were male and 22 patients were female, they aged 21.0 to 58.0 years and the average age was 42.9 years. Demographic details of the subjects are summarized in Table 1.

Clinical characteristics including bowel movement, stool consistency, intensity and frequency of abdominal symptoms in IBS-D are also illustrated in Table 1. The controls presented no abdominal symptom and abnormal bowel movement.

2-DE analysis of IBS colonic mucosa

Considering the differences among individuals, 5 patients in the IBS-D group and 5 subjects in the control group were included in the proteomics analysis. More than 1000 spots were displayed on the 2 dimensional gel, the pI ranged between 3 and 10 and the relative molecular weights ranged between 10 and 200 k. After the comparisons between the 2 groups, it was found that 41 spots showed differences for more than 2 folds; among them 11 spots were up-regulated in the IBS-D group and 30 spots were down-regulated [Figure 1A and 1B]. We cut 12 spots out from the gels for Q-TOF identification. The selection criteria for these spots were shown as below: (1) good reproducibility in silver stained gels and CBB stained gels, and the spots were clearly displayed on the CBB-stained gels; (2) these differentially expressed proteins should show consistent changing tendency in three samples at the least (an incidence rate higher than 60%); (3) they can be successfully identified after Q-TOF analysis. We found that 5 spots were up-regulated and 7 spots were down-regulated in the specimens from colonic mucosal membrane in the IBS group. After the searching against SWISS PROT database, we successfully obtained the information for these proteins [Table 2]. Most of the proteins belong to structure related proteins or metabolic enzymes, and some proteins were transcription factors and molecular chaperones.

Table 1: Demographic and clinical characteristics of IBS-D patients and controls

Characteristic	2-DE				ENOA			
	Control	IBS-D	*t*	*P*	Control	IBS-D	*t*	*P*
Cases, *n*	5	5			9	9		
Age (years), mean ± SD	44.0 ± 3.4	46.4 ± 2.4	−1.3	0.210	49.6 ± 6.3	43.5 ± 6.4	2.0	0.070
Gender (F/M), *n*	2/3	2/3	–	–	4/5	4/5	–	–
BMI (Kg/m^2), mean ± SD	24.2 ± 2.9	22.8 ± 1.4	0.97	0.380	23.4 ± 1.0	23.2 ± 1.0	0.4	0.630
Bowel movements (times/day), mean ± SD	1.2 ± 0.4	4.0 ± 1.6	−3.8	0.010	1.9 ± 0.5	5.2 ± 2.8	−3.5	0.010
Stool consistency (Bristol score), mean ± SD	3.6 ± 0.5	6.0 ± 0	−10.7	<0.001	3.6 ± 0.5	5.6 ± 0.9	−5.8	<0.001
Frequency of abdominal pain or discomfort (days in a 10-day period), mean ± SD	0	5.6 ± 1.3	–	–	0	5.7 ± 2.2	–	–
Intensity of abdominal pain or discomfort (score), mean ± SD	0	40.0 ± 12.0	–	–	0	50.3 ± 25.0	–	–
Duration of IBS-D (years), mean ± SD	–	3.8 ± 3.5	–	–	–	3.2 ± 2.1	–	–

Characteristic	CT and ACAD8				ATP5H				ATP			
	Control	IBS-D	*t*	*P*	Control	IBS-D	*t*	*P*	Control	IBS-D	*t*	*P*
Cases, *n*	9	9			9	9			8	10		
Age (years), mean ± SD	42.8 ± 5.3	40.5 ± 3.2	1.1	0.230	42.3 ± 2.6	41.5 ± 3.4	0.6	0.500	40.7 ± 7.1	43.0 ± 5.2	−0.8	0.250
Gender (F/M), *n*	5/4	5:4	–	–	4/5	4/5	–	–	5/3	5/5	–	–
BMI (Kg/m^2), mean ± SD	23.5 ± 1.2	24.2 ± 1.5	0.4	0.630	23.5 ± 1.2	22.2 ± 1.5	2.0	0.070	22.5 ± 1.9	23.7 ± 1.2	−1.6	0.310
Bowel movements (times/day), mean ± SD	2.0 ± 0.7	5.4 ± 2.6	−3.8	0.010	2.0 ± 0.7	5.2 ± 2.6	−3.6	0.010	1.8 ± 2.4	4.9 ± 0.3	−3.6	0.030
Stool consistency (Bristol score), mean ± SD	3.2 ± 0.6	5.3 ± 0.7	−5.8	<0.001	3.2 ± 0.6	5.2 ± 0.5	−7.7	<0.001	3.0 ± 0.6	6.1 ± 0.3	−13.3	<0.001
Frequency of abdominal pain or discomfort (days in a 10-day period), mean ± SD	0	5.8 ± 2.3	–	–	0	5.5 ± 2.2	–	–	0	4.5 ± 2.3	–	–
Intensity of abdominal pain or discomfort (score), mean ± SD	0	44.3 ± 17.0	–	–	0	46.3 ± 17.0	–	–	0	48.3 ± 25.0	–	–
Duration of IBS-D (years), mean ± SD	–	3.7 ± 2.5	–	–	0	3.3 ± 2.5	–	–	–	3.4 ± 2.3	–	–

2-DE: 2-dimensional gel electrophoresis; ACAD8: Isobutyryl-CoA dehydrogenase; ATP5H: ATP synthase subunit d; BMI: Body mass index; CT: Acetyl-CoA acetyltransferase; ENOA: α-enolase; F: Female; IBS-D: IBS with diarrhea; M: male; SD: Standard deviation.

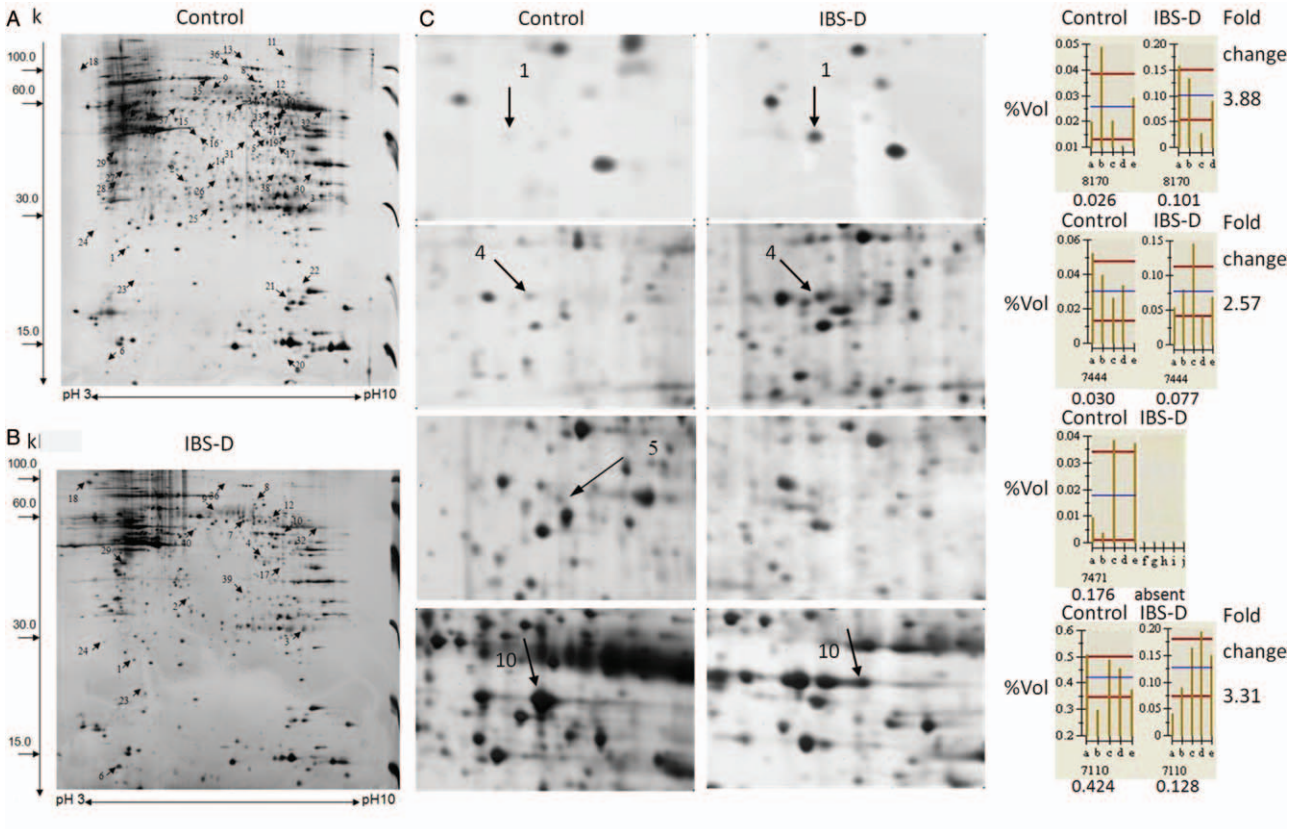

Figure 1: Silver-stained 2-DE analysis of IBS-D colonic mucosa ($n=5$) and control colonic mucosa ($n=5$). (A) Representative silver-stained 2-DE image of control colonic mucosa; (B) Representative silver-stained 2-DE image of IBS-D colonic mucosa; (C) Silver-stained 2-DE gel images and corresponding histogram showing levels of expression in Vol% of ATP5H, CT, ACAD8 and ENOA in colonic mucosa. Arrows indicate the protein spot corresponding to ATP5H (dot 1), CT (dot 4), ACAD8 (dot 5) and ENOA (dot 10). 2-DE: 2-dimensional gel electrophoresis; IBS-D: Irritable bowel syndrome with diarrhea; ATP5H: ATP synthase subunit d; CT: Acetyl-CoA acetyltransferase; ACAD8: Isobutyryl-CoA dehydrogenase; ENOA: α-enolase.

Table 2: Identification of 12 differential expressed proteins by mass spectrometry and matching with proteins in SwissProt database

Spot No.	Vol Ratio	Protein	THEO PI	THER MW	Subcelluar location	Mascot score	Sequence coverage	Swiss-Prot accession no.	Queries matched	*P*
1	3.88	ATP synthase subunit d	4.41	23650	Mitochondrion	134	19%	O75947	12	0.005
2	3.99	Sulfotransferase 1A3/1A4	5.62	33912	Cytoplasm	399	33%	P50224	13	0.001
3	−2.28	Ig kappa chain C region	8.10	28768	Extracellular region	1264	88%	P01834	54	0.001
4	2.57	Acetyl-CoA acetyltransferase	7.26	45620	Cytoplasm	196	20%	Q9BWD1	6	0.034
5	IBS-D absent	Isobutyryl-CoA dehydrogenase	7.35	44752	Mitochondrion	239	15%	Q9UKU7	8	0.045
6	7.97	Thioredoxin	3.99	14484	Cytoplasm	72	22%	P10599	3	<0.001
7	3.49	Aldehyde dehydrogenase 2	6.85	57694	MitochondrionMatrix	241	16%	P05091	8	0.011
8	−2.12	WD repeat-containing protein 1	7.10	75169	Cytoplasm;Cytoskeleton	141	33%	075083	18	0.029
9	−3.07	T-complex protein 1 subunit alpha	6.23	62344	Cytoplasm	599	35%	P17987	26	0.030
10	−3.31	Alpha-enolase	7.65	54065	Cytoplasm;Cell membrane;myofibril; sarcomere;M-band	2788	73%	P06733	110	<0.001
11	IBS-D absent	C-1-tetrahydrofolate synthase	7.76	114873	Cytoplasm	33	6%	P11586	7	0.002
12	−7.81	Dihydrolipoyl dehydrogenase	7.48	62700	Mitochondrion matrix	207	17%	P09622	15	0.042

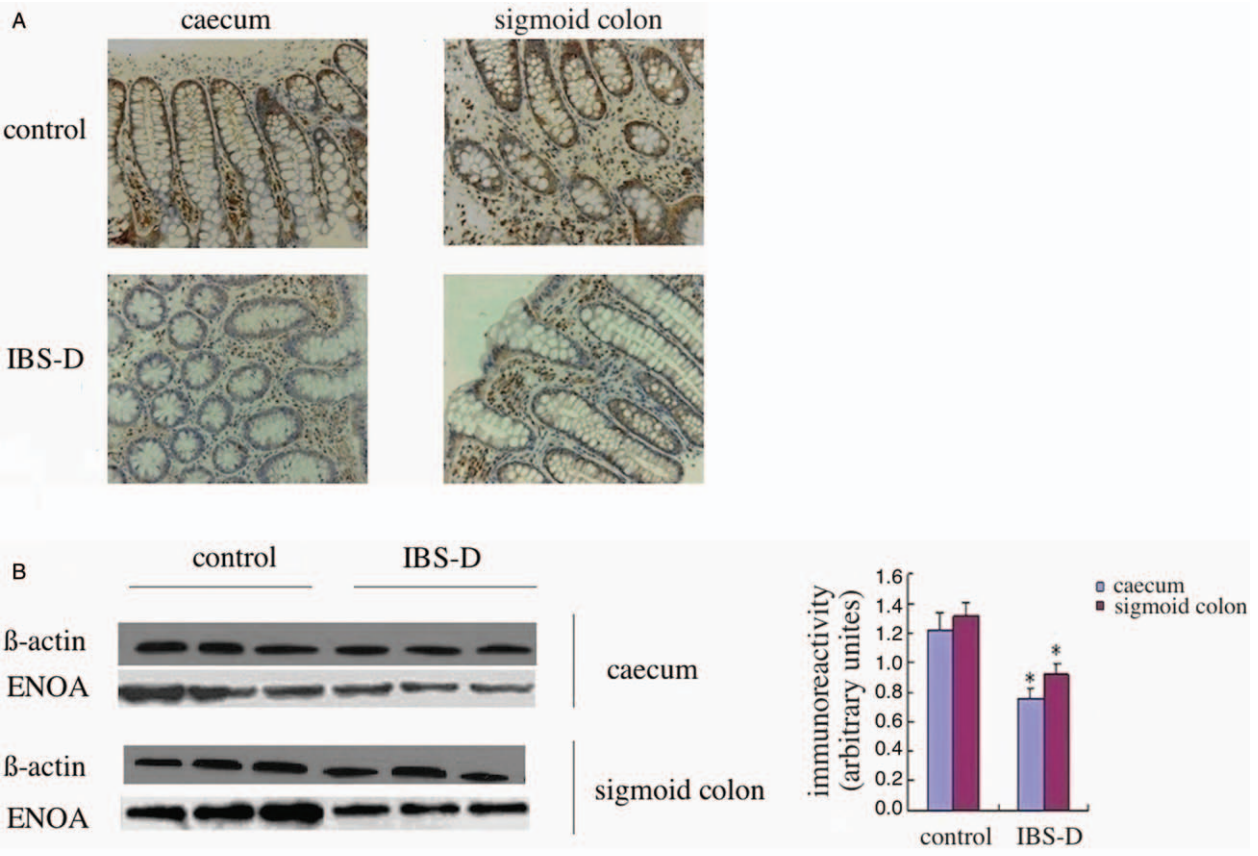

Figure 2: ENOA expression in sigmoid colon and caecum in the IBS-D group ($n=9$) compared with those in the control group ($n=9$). (A) Immunohistochemical analysis (×200) for ENOA expression in the colonic mucosal membrane. (B) Western blot detection for ENOA expression in colonic mucosal membrane, with ß-actin as a loading control. Data were expressed as mean ±SD. $^{*}P<0.05$, versus control group. ENOA: α-enolase; IBS-D: Irritable bowel syndrome with diarrhea; SD: Standard deviation.

Expression of ENOA in the colonic mucosal membrane of IBS patients

ENOA showed highly positive expression in the cytosol of glandular epithelium, basement membrane of epithelium and the cytosol of lymphocytes in proper coat in the colonic mucosal membrane of the control group, and they were shown as dark brown particles [Figure 2A]; yellow filamentous expression of ENOA can be detected in the cytosol of epithelium and basal membrane in the IBS-D group, and the coloration intensity was lower than that in the control group [Figure 2A]. Western blot detection showed that the expression levels of ENOA in sigmoid colon (0.917 ± 0.007 *vs.* 1.310 ± 0.100, $t=2.643$, $P=0.017$) and caecum (0.765 ± 0.060 *vs.* 1.212 ± 0.122, $t=2.225$, $P=0.023$) in the IBS-D group were significantly lower than those in corresponding positions in the control group, and the differences were statistically significant [Figure 2B]. No significant difference was detected in the expression levels of ENOA in sigmoid colon and caecum in the control group (1.212 ± 0.122 *vs.* 1.310 ± 0.100, $t=2.524$, $P=0.230$).

Expression of ACAD8 in colonic mucosal membrane of IBS patients

ACAD8 expression in colonic mucosal membrane among the 2 groups was detected by Western blot detection. The expression level of ACAD8 in sigmoid colon of the IBS-D group was significantly lower than that in the control group (1.127 ± 0.201 *vs.* 1.497 ± 0.392, $t=7.093$, $P=0.008$). No significant difference was detected in the expression of ACAD8 in caecum between the 2 groups (1.040 ± 0.072 *vs.* 1.185 ± 0.459, $t=2.31$, $P=0.170$). The difference in the expression levels of ACAD8 between sigmoid colon and caecum in the control group were not statistically significant [Figure 3].

Expression of CT in colonic mucosal membrane between the 2 groups

CT expression was detected in the cytosol of endothelial cells in colonic mucosal membrane in the control group and IBS-D group and they were shown as buffy particles [Figure 4A]. Western blot detection indicated that the expression level of CT in caecum in the IBS-D group was higher than that in the control group, and the difference was statistically significant (2.453 ± 0.422 *vs.* 0.931 ± 0.652, $t=8.363$, $P=0.015$); the difference in the expression level of CT in sigmoid colon from the IBS-D group was not statistically significant in comparison to that in the control group (1.431 ± 0.114 *vs.* 1.431 ± 0.213, $t=2.543$, $P=0.067$) [Figure 4B].

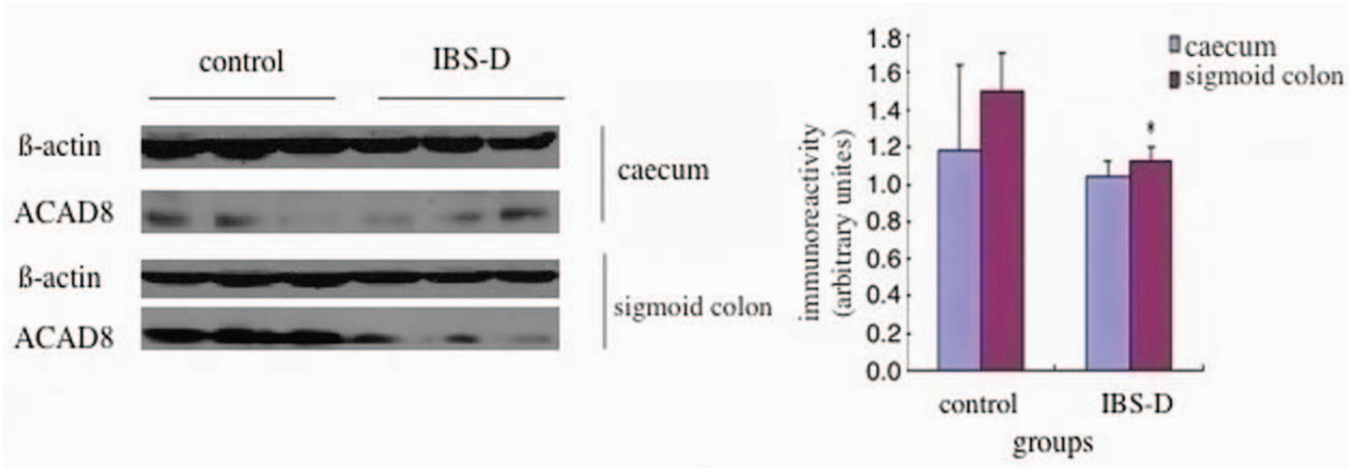

Figure 3: Western blot analysis of ACAD8 in sigmoid colon and caecum of the IBS-D group ($n=9$) compared with those of control group ($n=9$), with ß-actin as a loading control. Data were expressed as mean ± SD. $^{*}P<0.01$, versus control group. ACAD8: Isobutyryl-CoA dehydrogenase; IBS-D: Irritable bowel syndrome with diarrhea; SD: Standard deviation.

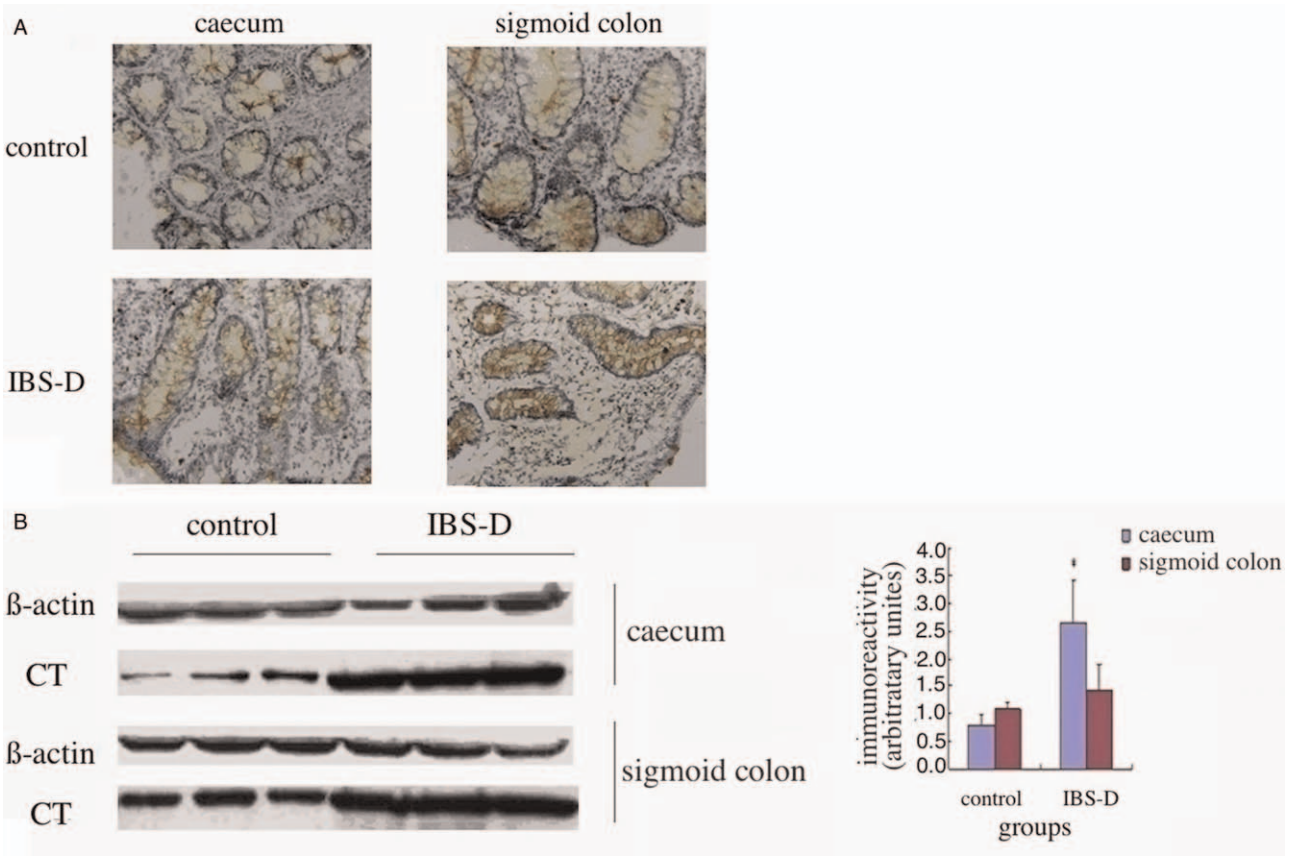

Figure 4: The expression of CT in sigmoid colon and caecum in the IBS-D group ($n=9$) compared with those in the control group ($n=9$). (A) Immunohistochemical analysis (×200) for CT expression in the colonic mucosal membrane; (B) Western blot detection for CT expression in colonic mucosal membrane between the 2 groups, with ß-actin as a loading control. Data were expressed as mean ± SD. $^{*}P<0.05$, versus control group. CT: Acetyl-CoAacetyltransferase; IBS-D: Irritable bowel syndrome with diarrhea; SD: Standard deviation.

Expression of ATP5H in colonic mucosal membrane between the 2 groups

Western blot detection indicated that the expression level of ATP5H in sigmoid colon in the IBS-D group was higher than that in the control group, and the difference was statistically significant (0.843 ± 0.042 *vs.* 0.631 ± 0.042, $t=8.613$, $P=0.007$); the difference in the expression level of ATP5H in caecum from the IBS-D group was not statistically significant in comparison to that in the control group (0.623 ± 0.042 *vs.* 0.601 ± 0.043, $t=7.649$, $P=0.150$) [Figure 5].

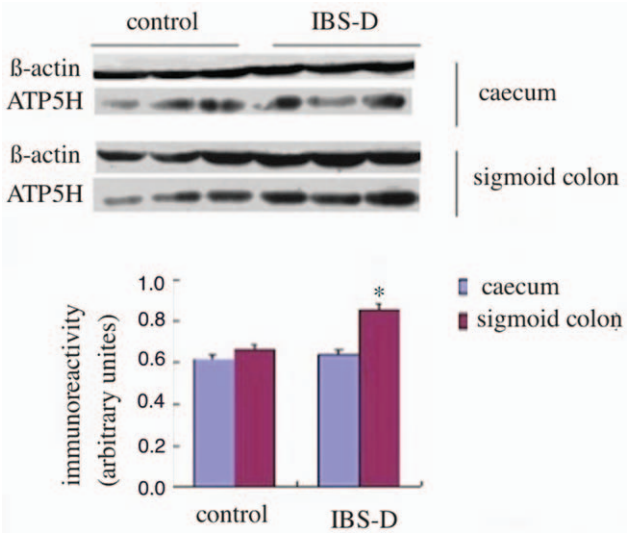

Figure 5: Western blot analysis of ATP5H in sigmoid colon and caecum of the IBS-D group ($n=9$) compared with those of control group ($n=9$), with ß-actin as a loading control. Data were expressed as mean ± SD. $^{*}P<0.01$, versus control group. ATP5H: ATP synthase subunit d; IBS-D: Irritable bowel syndrome with diarrhea; SD: Standard deviation.

ATP concentration in colonic mucosal membrane

In comparison to the ATP concentration in colonic mucosal membrane in controls, the ATP concentration in sigmoid colon in the IBS-D group was statistically significantly lower than that in corresponding position in the control group (0.470[0.180,1.360] *vs.* 5.350[2.230, 7.900], $U=55$, $P<0.001$), though the ATP concentration in caecum of the IBS-D group was lower than that in the control group, the difference was not statistically significant (3.530 ± 0.212 *vs.* 5.700 ± 2.900, $t=2.142$, $P=0.360$).

Discussion

Our laboratory carried out studies on the protein expression profile in colonic mucosal membrane in the IBS patients by using differential proteomic technique. More than 1000 protein spots were displayed and 41 spots showed differences more than 2 folds; among them 11 spots were up-regulated in the IBS-D group and 30 spots were down-regulated. Twelve spots were selected for further identification, among them 5 spots were up-regulated, including ATP5H, sulfotransferase 1A3/1A4, CT and Aldehyde dehydrogenase 2 (ALDH2), while the down-regulated proteins were Igkappa chain C region, ACAD8, WD repeat-containing protein (WDR1), T-complex protein 1 subunit alpha (TCP-1α) and ENOA. Among them ENOA, ACAD8, CT and ATP5H were all important enzymes involved in energy metabolism. Therefore, we validated the expression of ENOA, CT, ATP5H and ACAD8 in IBS-D patients and the controls.

A highly conserved cytosolic enzyme in the glycolytic pathway, ENOA has also been reported to be closely related to many diseases as a kind of multifunctional protein, and the interaction between enolase and diseases is mainly derived from its immunogenic activities, DNA binding capability and fibrinolysin receptor.[18] Up to now, most studies on ENOA involving intestinal diseases focus on the pathogenesis of intestinal tumors and inflammatory bowel disease, while only few studies were related to FGIDs.[14,19,20]

The nutritional substances that can be used by healthy colonocytes are successively butyrates>glucose>ketonebodies>glutamine.[21] Short-chain fatty acids (SCFAs), produced by fermentation of enteric bacteria, provide the colonocyte with about 70% of its energy, while the remaining 30% energy for physiological activities of colon is mainly provided by blood circulation, among them glucose is one of suppliers for energy.[22] Because IBS has been associated with disruptions to the intestinal microbiota,[23] the major energy sources from the fermentation of enteric bacteria may be disturbed. If the expression of key enzymes for glycolytic pathway such as ENOA in the colonic mucosa of IBS patients were reduced, glycolytic pathway is interfered and the changing degree of glycolysis may affect the tricarboxylic acid (TCA) cycle and levels of subsequent substrates for oxidative phosphorylation, which can further affect ATP level and ATP-related reactions. Disorders in ATP production may decrease energy supply for colonocyte and have an effect on its physiological functions, which finally lead to functional disturbance in intestinal tract, such as diarrhea or constipation. Ding *et al*[14] have found that the expression of ENOA was down-regulated in the rat model for IBS. The present study reported that the expression of ENOA in the mucosal membrane of sigmoid colon and caecum of the IBS-D patients significantly decreased compared with those of the controls, indicating that disorder in energy supply or utilization may exist in the colonic mucosal membrane of IBS-D patients.

The expression levels of enolase may be different in different physiological, pathological, metabolic, developmental conditions of cells,[24] mitogenic stimuli in lymphocytes can increase the expression of ENOA,[25] similarly, hypoxia, production of inflammatory cytokines, bacterial lipopolysaccharide and other factors can also increase its expression.[26] The reason why ENOA was reduced in the colonic mucosa of IBS-D is unclear. Additional work is needed to further confirm the abnormal ENOA in IBS and clarify the causal relationship of ENOA and the pathogenesis of IBS-D.

The physiological function of CT is to catalyze the synthesis of acetyl-acetyl coenzyme A, and this is the first step for the synthesis of cholesterol and ketone bodies; [27] simultaneously, CT catalyzes the degradation of ketobodies in order to obtain acetyl coenzyme A required for cholesterol synthesis,[28] therefore, increase or decrease in CT expression has an effect on the status of cholesterol synthesis.

We found that the expression level of CT was elevated in caecum of the IBS-D group, indicating that cholesterol anabolic metabolism in the mucosal membrane of caecum in the IBS-D group may be abnormal. Ahmad found that in the dextran sulphate sodium (DSS)-induced colonitis, DSS can disturb butyrate oxidation in colonocytes and colonic mucosal membrane can utilize other substrates to restore

energy disorder, finally glucose oxidation is compensatively enhanced.[27] Glucose can stimulate lipid synthesis in the colonocytes.[29] The significantly increased level of CT in colon, particularly sigmoid colon, in IBS patients may be attributed to some factors related to IBS pathogenesis triggering the increase of enzyme production in the colonocytes. The findings also indicate lipid metabolism disorder may be disturbed in the colon of IBS patients. It is still unclear whether this change is related to the decrease in ENOA expression in the colonic mucosa of IBS or the altered CT may be the compensative enhancement in cytoplasmic lipid metabolism due to abnormality in glucose metabolisms.

ACAD8 is one of the members in the acyl-CoA dehydrogenase family in mammals.[30] The dehydrogenation of acyl-CoA intermediates in the catabolism of fatty acids and branched-chain amino acids is catalysed by the mitochondrial acyl-CoA dehydrogenase enzymes.[31]

Our laboratory found the expression of ACAD8 in sigmoid colon in the IBS-D group was statistically significantly lower than that in the control, but the differences in the caecum of 2 groups were not statistically significant. The down-regulation in ACAD8 expression could have an effect on SCFAs, Leucine, isoleucine, antidiuretic hormone and other branched-chain amino acids metabolism.[31,32] The substances are particularly important for growth and development of colonocytes, and abnormalities in the metabolism in colon tissues of IBS may inevitably affect normal functions of colonocytes. It still needs further studies to evaluate whether decrease in ACAD8 expression is related to gene mutation and whether there are abnormalities in amino acid and SCFAs metabolism, which may further lead to symptoms of IBS patients.

In the present study, it was found that the expression level of ATP5H in sigmoid colon of the IBS-D group was increased. ATP synthase enables protons to flow back to the matrix and uses the released energy to synthesize ATP.[33] Mitochondrial ATPase is composed of two linked multi-subunit complexes: the soluble catalytic core, F_1, and the membrane- spanning component, F_0, which comprises the proton channel. The F_0 has 9 subunits and the ATP5H gene encodes the d subunit of the F_0 complex. [34] The function of ATP5H is not clear. Because F_0F_1-ATPase is a complex of multiple subunits, it is not possible to increase ATPase activity by increasing expression level of individual subunits. Since F_0F_1-ATPase is involved in cellular energy metabolism, the cells may have a defensive role by increasing energy supply and respiration rates with increased expression of mitochondrial ATPase in colonocytes of IBS-D.

In order to confirm whether energy production is abnormal in colonic mucosal membrane of IBS patients, we further examine the ATP concentrations in colon tissues and found that the ATP concentration in sigmoid colon of IBS-D patients was statistically significantly lower than that in corresponding position in the control group, further indicating that abnormality of energy metabolism in sigmoid colon led to decrease or deficiency in energy in colon tissues at this position. It may be attributed to the increase in energy requirement and capability of utilizing local nutrition by colon mucosal epithelium due to disturbance in colon functions in IBS patients. In caecum and right hemicolon, the pH is 5 to 6 and bacteria rapidly increase, the fermentation is extremely intense and the yield of short-chain amino acids is high.[35,36,37] However, in left hemicolon or colon at distal end, the pH is almost neutral, rancidification is predominant and the bacterial population is in static status, only few substances can be used.[38] Caecum is the major place for energy absorption in colon, therefore, local energy in caecum in IBS patients may not change significantly. However, sigmoid colon is located at the distal end of colon and the local energy absorbed is statistically significantly less than that from caecum, thus energy insufficiency or shortage may be more obvious, but new evidences for these hypotheses are still required.

In conclusion, our laboratory utilized proteomic techniques and carried out comparative investigations on the colonic mucosal membrane between IBS-D patients and controls by using 2-DE and MS technique. It was found that 12 proteins significantly and reproducibly changed between IBS-D patients and controls. Among them ENOA, ACAD8, CT and ATP5H were all important enzymes involved in energy metabolism. Abnormalities in the expression of the enzymes in the colon of IBS patients and the decrease in ATP concentration in colon indicated that some abnormalities in energy metabolism existed in the colon of IBS-D patients. It was found for the first time that the ATP concentration in the colon particularly sigmoid colon in IBS patients decreased. We will further confirm the expression of other proteins in subsequent studies. The twelve differentially expressed proteins in IBS-D patient may be new diagnostic markers or therapeutic targets which will play an important role in the clinical practice. In general, the findings in this study indicated that the pathogenesis of IBS may involve the abnormalities in colon metabolism. Different molecular mechanisms affecting pathogenesis of IBS may exist for different positions in colon. The present study may provide new ideas for new mechanisms for the pathogenesis of IBS.

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Conflicts of interest

None.

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