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Original Article

Differential expressions of BMPR1α, ACTN4α and FABP7 in Hirschsprung disease

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Abstract: Hirschsprung disease (HSCR) is characterized by the absence of intramural ganglion cells in the nerve plexuses of the distal gut. Recent studies have shown that the bone morphogenetic protein receptor-type IA (BMPR1α), actinin-alpha 4 (ACTN4α) and fatty acid binding protein 7 (FABP7) play important roles in the differentiation and development of neurons. The aganglionic (stenotic) and the ganglionic (normal) colon segment tissues of 60 HSCR patients were collected to investigate the expression pattern of BMPR1α, ACTININ-4α and FABP7 using RT-PCR, quantitative real-time RT-PCR (qRT-PCR) and immunohistochemical staining. The mRNA and protein expressions of BMPR1α and ACTN4α were higher in the stenotic colon segment tissue than those in the normal colon segment tissue. However, the mRNA and protein expressions of FABP7 were lower in the stenotic colon segment tissue than those in the normal colon segment tissue. The study in HSCR patients, findings in mRNA and protein alterations to expecting provide more information to in order to find some clue for the pathomechanism of HSCR disease.

Keywords: Hirschsprung disease (HSCR), BMPR1α, ACTININ-4α and FABP7, aganglionic (stenotic) colon segment, ganglionic (normal) colon segment

Introduction

Hirschsprung disease (HSCR) is characterized by segmental aganglionosis of the terminal bowel. Neurons of the enteric nervous system (ENS) arise from neural crest, migrate and colonize intestinal muscle coat where they proliferate and differentiate. The first pathophysiologic hypothesis on HSCR suggests an absence of neural cell migration. The most recent hypothesis involves disorders of their homing and/or their differentiation due to an altered intestinal microenvironment. HSCR most occurs in neonates and early childhood, with symptoms ranging from chronic constipation to acute ileus. Its incidence is approximately one in 5000 live newborns, with a male predominance (3:1 to 5:1) [1]. The disease has a complex genetic etiology with susceptibility genes including the RET members [2, 3], endothelin-B receptor (EDNRB), endothelin 3 (EDN3), SOX-10 [4-6] and smad-interacting protein-1 (SIP1) [zinc finger homeobox (ZFHX1B)] [7].

Recent evidence suggests that bone morphogenetic protein receptor-type IA (BMPRs) can initiate colorectal tumorigenesis via the mixed juvenile-hyperplastic polyposis-carcinoma pathway by functioning in an analogous manner [8]. The functions of actinin-alpha 4 (ACTN4α) are likely to cross-link and bundle f-actin filaments and play a role in signal transduction [9]. Fatty acid binding proteins (FABPs) constitutes a multi-gene family encoding intracellular lipid-binding proteins [10].

In this study, we using RT-PCR, quantitative real-time RT-PCR (qRT-PCR) and immunohistochemical staining detected expression of mRNA and protein expressions of BMPR1α, ACTN4α and FABP7 between the stenotic colon segment tissue and the normal colon segment tissue in HSCR patients, expecting to disclose genes alterations related to bowel malfunction of HSCR.

Materials and methods

Patients and specimens

Tissue specimens from 60 HSCR patients. The patients ranged from 0.5 to 3.1 years old with

Table 1. RT-PCR and qRT-PCR primers

Gene name	GenBank accession no.	Sequence (5'-3')	Amplicon	Annealing temperature
BMPR1α	M_004329	F AAG TTC TGG TAG TGG GTC T	168 bp	50°C
		R CTG GCT TCT TCA GTG GTA		
ACTN4α	M_004924	F GAC GCC GAT AGG GA	109 bp	53°C
		R TGA CGG TGG TGT AGG G		
FABP7	M_001446	F AGG CTT TCT GTG CTA C	118 bp	51°C
		R ATT ACC GTT GGT TTG G		
GAPDH	M_002046	F GGG AAG GTG AAG GTC G	228 bp	51°C
		R GAA GAT GGT GAT GGG ATT		

Table 2. The relative coefficient of the BMPR1α, ACTN4α and FABP7 genes in the two segments (means ± SD)

Gene	Samples	Stenotic segment	Normal segment
BMPR1α mRNA	60	0.83 ± 0.19	0.31 ± 0.13
ACTN4α mRNA	60	0.79 ± 0.15	0.22 ± 0.15
FABP7 mRNA	60	0.19 ± 0.07	0.67 ± 0.18

an average of 1.5-years including 49 males and 11 females. Stenotic colon segment tissues and normal colon segment tissues were collected respectively and identified by pathological H&E staining and AChE staining. The aganglionic colon segment was taken as the stenotic segment, and the proximal end of the dilated segment was taken as the normal segment. Each tissue specimen was divided into two pieces, one piece was frozen at -80°C, the other piece was fixed in 10% neutral-formalin and embedded with paraffin. This study was approved by Ethics Committee of China Medical University (Ethical Number: 2012PS17K) and all the subjects involved in the study gave written informed consent.

RT-PCR

The total RNA of each sample was extracted respectively according to the description of the RT-PCR kit (QIAGEN Ltd., Crawley, West Sussex, UK). The optical density value (OD value) of RNA was determined and calculated by the equation, A260/A280. Then cDNA was synthesized by reverse transcription in 20 μl reaction buffer containing con-RNA 1 μg, M-MLV 200 U/μl, primer 10 μg/L. The reaction was continued for 1 hour at 37°C, and then stretched for 10 minutes at 95°C. The reactant was tacho-centrifuged. The amplification of GAPDH gene in separate tubes was used as internal semi-quantitative control. The primers used are listed in **Table 1**. Every primer pair was checked by sequencing the PCR product to ensure the specificity of amplification.

RT-PCR was done with a 50 μl reaction system composed of cDNA 0.1 μg/μl, each primer 10 μg/L, Taq-polymerase 1 U/μl, etc. The PCR condition included force-denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, renaturation shown (in **Table 1**) for 1 minute, elongation at 72°C for 1 minute, totally for 35 cycles. Then it stretched at 72°C for 7 minutes. The products were visualized in 2% agarose gel electrophoresis, the patterns were analyzed by the G-Box Synyene system software, and the quantity of genes was observed. The calculated relative coefficient was equal to the intensity of the goal gene as divided by the expressive intensity of GAPDH.

qRT-PCR

qRT-PCR was performed in triplicate for each cDNA (Superscript™ IIRnase H-Reverse Transcriptase, SYBR GreenI) with SYBR green PCR Master mix (TaKaRa Biotechnology Co.) and the LightCycler (Roche Molecular Biochemicals, Co.). Control reactions were performed omitting reverse transcriptase from the cDNA synthesis. The RNA content of samples was normalized based on GAPDH amplification. Conditions for PCR amplification of BMPR1α, ACTN4α and FABP7 genes fragments are shown in **Table 1**. SYBR Green I was used as the detection System. Reactions were done in 25 μl volumes containing 200 nM of each primer, 1 μl cDNA (corresponding 1~3 ng), and 12.5 μl 2 × SYBR Green Master Mix Reagents. At last, the qRT-PCR was done with a 25 μl reaction system composed of 2 × SYBR green mixed buffer 25 μl. The reaction condition was: force-degeneration, 50°C 10 seconds; degeneration, 95°C 10

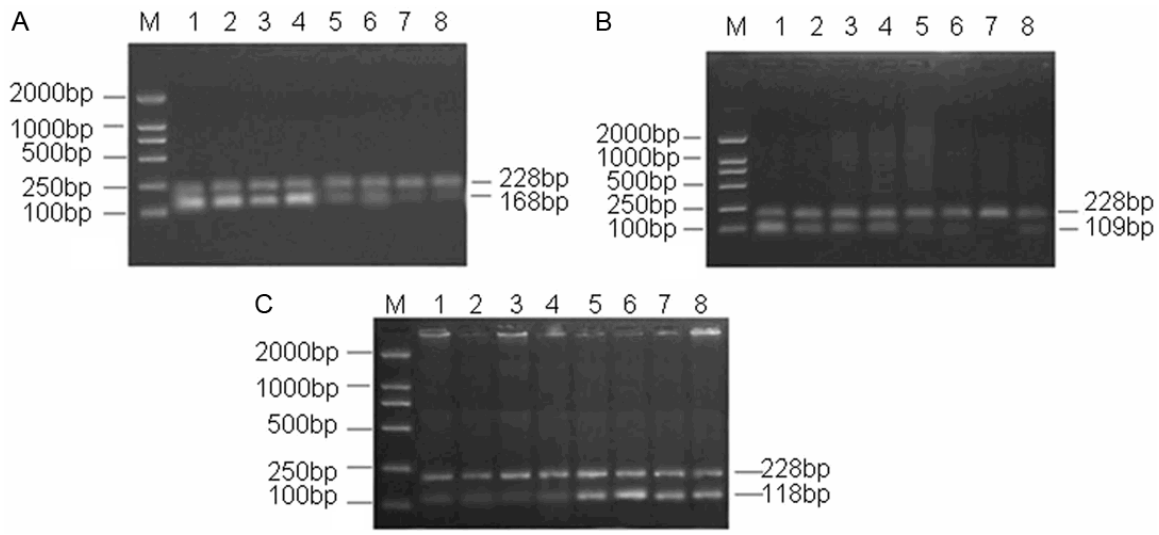

Figure 1. The expression of BMPR1α, ACTN4α and FABP7 genes mRNA by RT-PCR. A: BMPR1α gene, M was Marker DL 2000, lanes 1, 2, 3 and 4 were stenotic colon segment, lanes 5, 6, 7 and 8 were normal segment. B: ACTN4α gene, M was Marker DL 2000, lanes 1, 2, 3 and 4 were stenotic colon segment, lanes 5, 6, 7 and 8 were normal segment. C: FABP7 gene, M was Marker DL 2000, lanes 1, 2, 3 and 4 were stenotic colon segment, lanes 5, 6, 7 and 8 were normal segment.

minutes; denaturation, 95°C 15 seconds; elongation, shown (in **Table 1**) 1 minute; the course was circulated for 40 times. After the termination of PCR, the production was analyzed by the Germany GmbH D-68298 analysis system automatically. Each amplification curve of reaction and CT value was observed. The average CT value was the extreme CT value of the sample. The expression difference of the gene was calculated by the 2-ΔΔCT method [11]. ΔCT = CT value of the goal gene - CT value of the control gene. The normal segments were taken as the control group, ΔΔCT = ΔCT of the goal gene group - ΔCT of the control gene group. The expression of normal segment was taken as 1, 2-ΔΔCT CT would be the multiple genes of the goal segment compared with the control segment.

Immunohistochemistry

The colon segment tissue sections were obtained from stenotic and normal segment of HSCR patients with typical rectosigmoid. Consecutive paraffin embedded tissue sections (4-7 μm) were dewaxed and dehydrated. The slides were incubated at 50°C overnight, then soaked in xylene for 10 minutes for three times, and immersed in 100% ethanol for 5 minutes, 95% ethanol for 5 minutes, and 80% and 70% ethanol for 1 minute each. After rehydration for 5 minutes, the slides were boiled in citrate buffer (pH 6.1, Target Retrieval Solution; Sigma, CA, USA) for 25 minutes, rinsed with 1 × PBS for 5 minutes, and treated with 3% hydrogen peroxide for 10 minutes. After that, slides were placed flat on the bench top and sections were covered in a solution of rabbit anti-body to BMPR1α, ACTN4α and FABP7 (1:100 dilution; Chemicon, Temecula, California, USA) at room temperature for 30 minutes and then covered with a solution of goat anti-rabbit (1:500) (Dako) for 30 minutes, and finally in a solution of streptavidin–horseradish peroxidase (LSAB2 System; Dako) for 30 minutes. Color was with the Substrate - Chromogen Solution (LSAB2 System; Dako) for 5 minutes, and counter-stained with Mayer hematoxylin (Merck, Darmstadt, Germany) for 1 minute. At last, the slides were dehydrated in 50%, 75% and 100% ethanol for 5 minute each, and sealed with mounting medium (CytosealTM 60 Thermo Scientific, Waltham, MA, USA).

Statistical analysis

All the data were analyzed with SPSS 14.0. The area of positive staining of the two segments was compared by one-way ANOVA; the relative coefficient of the gene expression was also compared by one-way ANOVA. The average CT value of each segment of the 180 samples was

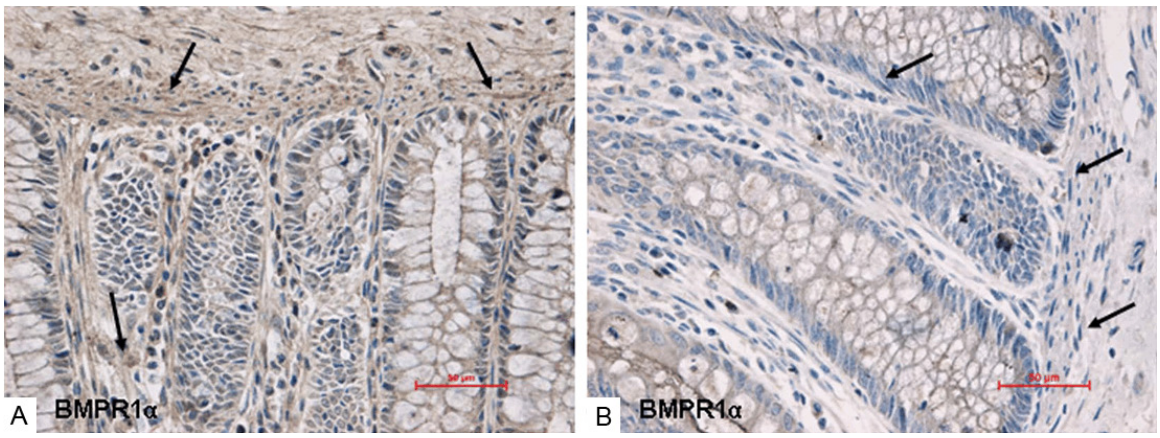

Figure 2. Immunohistochemical staining of BMPR1α. A: The mucous layer and the muscular layer of stenotic colon segment (The staining of BMPR1α were brown yellow); B: The mucous layer and the muscular layer of normal colon segment (The staining of BMPR1α were light yellow or colorless) (original magnification × 400).

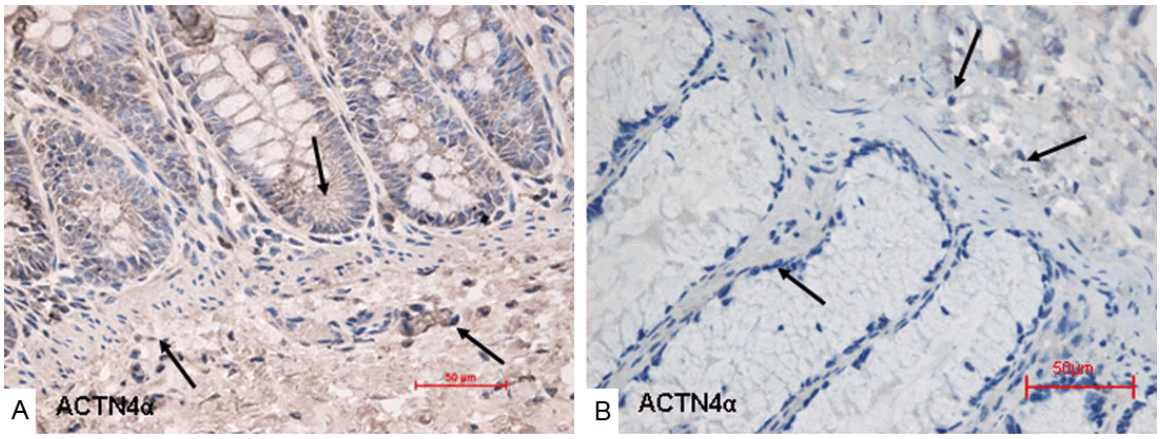

Figure 3. Immunohistochemical staining of ACTN4α. A: The mucous layer and the muscular layer of stenotic colon segment (The staining of ACTN4α were brown yellow); B: The mucous layer and the muscular layer of normal colon segment (The staining of ACTN4α were light yellow or colorless) (original magnification × 400).

calculated by SPSS for Windows 14.0 software, and was expressed by means ± SD. The correlation among the expressions of the BMPR1α, ACTN4α and FABP7 genes was calculated by Pearson's correlation. A *P* value less than 0.05 was considered statistically significant.

Results

mRNA expressions of BMPR1α, ACTN4α and FABP7 in HSCR colon tissues

The expressions of BMPR1α, ACTN4α and FABP7 in mRNA levels evaluated by RT-PCR were summarized in **Table 2**. The representative results were shown in **Figure 1**. The expression of BMPR1α and ACTN4α were higher in the stenotic colon segment tissues than those in the normal colon segment tissues (**Figure 1A** and **1B**). The expression of FABP7, however, was lower in the stenotic colon segment tissue than that in the normal colon segment tissue (**Figure 1C**).

To test if the was changed at the transcriptional level of BMPR1α, ACTN4α and FABP7 selected, we compared the mRNA level by performing qRT-PCR. The mRNA level of BMPR1α and ACTN4α were 4.6 fold and 3.7 fold higher in the stenotic colon segments than in the normal colon segments ($n = 60$, $P < 0.005$ and $P < 0.001$). The mRNA level of FABP7 was 3.3 fold higher in the normal colon segments than in the stenotic colon segments ($n = 60$, $P < 0.001$).

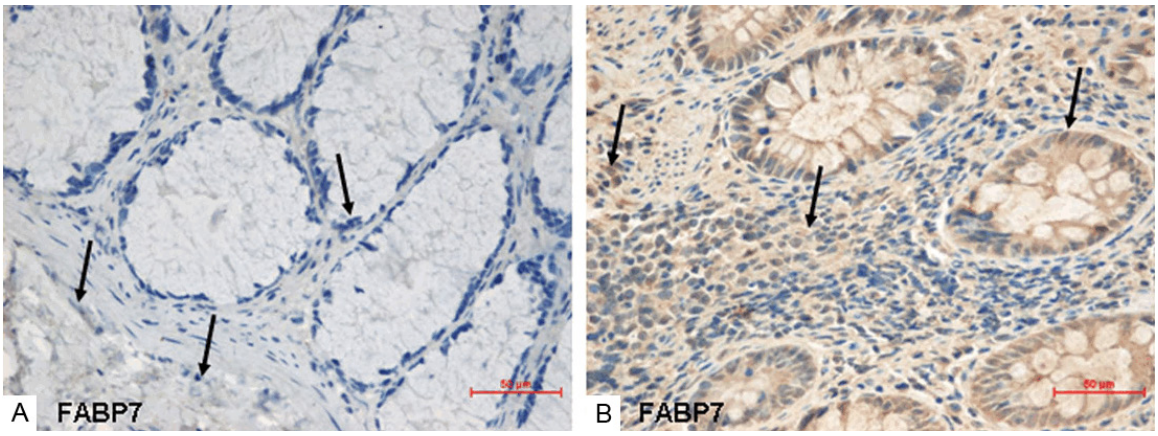

Figure 4. Immunohistochemical staining of FABP7. A: The mucous layer and the muscular layer of stenotic colon segment (The staining of FABP7 were brown yellow); B: The mucous layer and the muscular layer of normal colon segment (The staining of FABP7 were light yellow or colorless) (original magnification × 400).

Table 3. The density of BMPR1α, ACTN4α and FABP7 in two segments (percentage of staining area to whole area %, means ± SD)

Content	BMPR1α	ACTN4α	FABP7
stenotic segment	16.35 ± 3.15	13.85 ± 3.67	6.14 ± 4.72
normal segment	7.84 ± 5.43	7.47 ± 2.89	15.03 ± 6.12

$P < 0.05$.

Proteins expressions of BMPR1α, ACTN4α and FABP7 in HSCR colon tissues

We performed immunohistochemistry staining to compare the protein expressions of BMPR1α, ACTN4α and FABP7 in stenotic colon segment and normal colon segment of HSCR patients. As shown in **Figures 2**-**4**, positive reaction was mainly located in the mucous layer and muscular layer in the stenotic colon segment of HSCR. The protein levels of BMPR1α and ACTN4α were higher in the stenotic colon segment than those in the normal colon segment (**Figures 2A** and **3A**), while the protein level of FABP7 was lower in the stenotic colon segment than that in the normal colon segment (**Figure 4B**). The density analysis of the immunohistochemistry staining for BMPR1α, ACTN4α and FABP7 was summarized and shown in **Table 3**.

Discussion

Bone morphogenetic protein (BMP) signaling was recently shown to inhibit intestinal stem cell (ISC) renewal by suppressing the Wnt-β-catenin signalling pathway in a mouse model [12, 13]. In the study, the authors showed that inactivation of the BMP pathway via the loss of function of its receptor, Bmpr1α, could lead to an increase in ISC number and crypt fission and hence clonal expansion of aberrant crypts (hyperplasia) through increased nuclear localisation of β-catenin in the ISCs. In view of this recent evidence, it is conceivable that the loss of function mutation in the human homologue, BMPR1α, could initiate colorectal tumourigenesis via the mixed juvenile/hyperplastic/adenomatous polyposis-carcinoma pathway by functioning in an analogous manner. Based on their reported roles in polyposis and tumor suppression, the BMPR1α was selected for further analysis [14-16]. Here, we reported the expression of BMPR1α mRNA was higher in the stenotic colon segment tissue than that in the normal colon segment tissue (**Figure 1A**). In the mucous layer and muscular layer, the quantity of BMPR1α immunoreactivity showed a regional increase in the stenotic colon segment than that in the normal colon segment (**Figure 2**).

ACTN4α gene is located on chromosome 19q13 and encodes the 100-kDa protein. ACTN4α functions to crosslink and bundle F-actin filaments and plays a role in signal transduction [17-19]. Its biological functions are include maintaining normal structure and function of the podocyte and it participates in regulating cell mobility during tumor metastasis [20, 21]. In the present study, we found that the expression of ACTN4α mRNA was higher in the

stenotic colon segment tissue than that in the normal colon segment tissue in the HSCR patients (**Figure 1B**). In the mucous layer and muscular layer the quantity of ACTN4α immunoreactivity showed a regional increase in the stenotic colon segment than that in the normal colon segment (**Figure 3**). mRNA and protein of ACTN4α were significantly different in the stenotic and the normal colon segment tissue.

FABP7 is a human gene mapping to chromosome 6q22-23. It is a member of the FABP family, consisting of structurally related proteins that have specific cell, tissue, and development patterns of expression. FABP7 was first isolated from a foetal brain cDNA library, and the transcript was detected in adult human brain and skeletal muscle but not in other normal adult tissue [22]. Generally, FABP proteins are involved in the uptake and intracellular trafficking of fatty acids, bile acids, and retinoids, as well as in cell signalling, gene transcription, cell growth, and differentiation. The report showed that in glioblastoma cells FABP7 expression was associated with increased migration [23]. In the present study, we found that the expression of FABP7 was significantly lower in the stenotic colon segment than that in the normal colon segment by the qRT-PCR. In the mucous layer and muscular layer the quantity of FABP7 immunoreactivity showed a regional increase in the normal colon segment than that in the stenotic colon segment (**Figure 4**). mRNA and protein of FABP7 were significantly different in the stenotic and the normal colon segment tissue.

In summary, we detected differential changes of mRNA and protein expressions of BMPR1α, ACTN4α and FABP7 in stenotic colon segment tissues of HSCR patients, which help us to understand the pathogenesis of sporadic HSCR patients.

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Disclosure of conflict of interest

There is no interests of conflicts about this paper.

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