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Original Article

Expression analysis of *BMP2*, *BMP5*, *BMP10* in human colon tissues from Hirschsprung disease patients

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Abstract: Objective: Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF β) superfamily. BMP2, BMP5 and BMP10 exert their biological functions by interacting with membrane bound receptors belonging to the serine/threonine kinase family. Hirschsprung disease (HSCR) is characterized by the absence of intramural ganglion cells in the nerve plexuses of the distal gut. However, putative Notch function in enteric nervous system (ENS) development and the etiology of HSCR is unknown. Methods: Aganglionic and ganglionic colon segment tissues of 50 HSCR patients were investigated for the expression pattern of BMP2, BMP5 and BMP10 using real-time RT-PCR, Western blot analysis and immunohistochemical staining. Results: The mRNA levels of BMP2, BMP5 and BMP10 in the stenotic colon segment from HSCR patients were significantly higher than those in the normal ones. Similar increased expressions of them in the stenotic colon segments were detected by Western blotting coupled with densitometry analysis. Lastly, immunohistologicl stain showed significant BMP2, 5 and 10 increases in mucous and muscular layers from stenotic colon segments compared to normal segments. Conclusions: BMP2, BMP5 and BMP10 are elevated in the stenotic colon segment of HSCR, and BMPs signaling plays a pivotal role in the development of HSCR.

Keywords: Bone morphogenetic proteins (BMPs), Hirschsprung disease (HSCR), enteric nervous system (ENS), stenotic colon, ganglion cell

Introduction

Hirschsprung disease (HSCR) is a common congenital intestinal defect in children, characterized by absence of focal intestinal ganglion cells. HSCR represents the main genetic cause of functional intestinal obstruction with an average incidence of 20/100,000 live births worldwide. Males are affected four times more often than females (a difference most prominent in short segment HSCR). There is a significant racial variation in the incidence of the disease and it is most often found among Asians (28 per 100,000 live births) [1]. Its main cause is that ganglion cells cannot cluster and locate in the intestinal wall. Genetic predisposition and intestinal microenvironment changes may contribute to development of HSCR. Nevertheless, the etiology and pathogenesis of HSCR are still incompletely understood. Symptoms in infants include difficult bowel movements, poor feeding, poor weight gain, and progressive abdominal distention. The early diagnosis of HSCR is often challenging, because no more than 10% of HSCR cases have a late presentation with classical chronic constipation and megacolon [2]. Therefore, effective tools or related genes for further understanding of HSCR are urgently needed.

Enteric nervous system (ENS) derived from migratory neural crest is the largest subdivision of the peripheral nervous system to regulate the gut behaviors, including the coordination of the smooth muscle contractions of the gut wall. The ENS neurons and glia cells organized into ganglia, which are interconnected to form two types of plexuses: the submucosal (Meissner's)

Table 1. Primer sequences for real-time PCR

Gene name	Sequence (5′-3′)	Annealing temperature (°C)	Amplicon (bp)
BMP-2	F GCC TGC TTC GCC ATC T	55	123
	R TGC CTC CTC CTT CTC CC		
BMP5	F CGC ATA CAG TTA TCT CG	50	160
	R CTT TGT AAT GCC TTC G		
BMP-10	F AGC TGG ACA ACT TGG G	51	116
	R TTT GGC GTT CCT TCT G		
GAPDH	F AGA GCT ACG AGC TGC CTG AC	57	184
	R AGC ACT GTG TTG GCG TAC AG		

and the myenteric (Auerbach's) plexus between the longitudinal and circular smooth muscle layers [3]. To date, at least 11 genes have been associated with sporadic or syndromic forms of HSCR. These 'HSCR genes' are generally related to the developmental program of neural crest cells, and include the RET Proto-oncogene, glial cell line-derived neurotrophic factor (*GDNF*), neurturin (*NTN*), endothelin3 (*EDN3*), endothelin receptor B (*EDNRB*), endothelin converting enzyme I (*ECE1*), transcriptional factors *SOX10* and *PHOX2B*, *ZFNX1B*, *KIAA1279*, and *TITF1* [4, 5].

Recent studies showed that bone morphogenetic protein (BMP) signaling has been proven to play a continuing role in the maintenance and function of neuronal cells in the ENS [6]. Several studies have demonstrated that BMP2 and BMP5 were highly expressed in colon tops, and BMP10 has been associated with inflammatory bowel disease (IBD) [7]. However, direct demonstration of BMPs expression in human tissues is few and is restricted to tumor samples. In this study, colon tissue samples from HSCR patients are examined for the expression of BMP2, BMP5 and BMP10, and significant increase of BMP2, BMP5 and BMP10 was detected in HSCR compared to normal colon tissues, suggesting that BMP signaling pathway plays a crucial role in the development of HSCR.

Materials and methods

Patients and specimens

Both stenotic and normal tissue samples were obtained from 50 HSCR patients. The patients ranged from 0.5 to 3.1 years old with an average of 1.5-years including 33 males and 17 females. Based on the results of barium enema and the intestinal morphology during and after the operation, the surgical specimens were divided into stenotic colon segments and normal colon segments, which were further confirmed by histological examination of the frozen section of each segment. The stenotic colon segments were defined by loss of the focal colonic ganglion cells by H&E stain. HSCR patients received definite pull-through operation at Shengjing Hospital of China Medical University, with adequate informed consent was signed by patients' supervisor. The study was approved by the local Institutional Research Board. ALL tissue samples were stored at -80°C immediately after operation.

Antibodies

Polyclonal anti-*BMP2* and anti-*BMP5* antibodies were purchased from Beijing biosynthesis biotechnology co., LTD (Rabbit anti-*BMP2* antibody-*BMP5* protein, Catalog Number: bs-1012R and bs-6614R). Polyclonal anti-*BMP10* antibody was purchased from ABgent (catalog ## AP1710a).

RNA extraction, reverse transcription and quantitative real-time RT-PCR

Approximately 100 mg tissues from stenotic and normal intestines were used for total RNA extraction using RNA extraction reagent TRIZOL (Invitrogen Life Technologies), according to manufacturer's instructions. The harvested RNA was diluted to a concentration of 1 μg/μl, aliquoted and stored at -80°C. For cDNA synthesis, 1 μg of total RNA was incubating with reverse transcriptase (Superscript™ IIRnase H-Reverse Transcriptase). Quantitative RT-PCR was performed in triplicate for each specimen in the presence of SYBR green PCR Master Mix; (TaKaRa Biotechnology Co.) in a LightCycler (Roche Molecular Biochemicals, Co.). The primers used were listed in **Table 1**. The amplification process was followed by a melting curve analysis, ranging from 50 to 90°C, with tem-

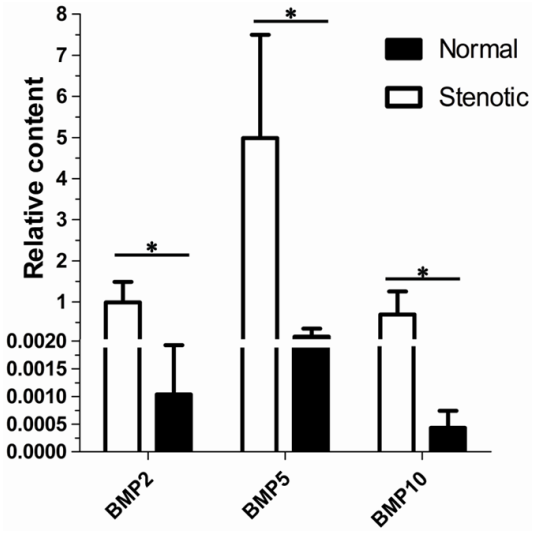

Figure 1. BMP2, BMP5 and BMP10 genes expression analysis by real time PCR of 2 groups. The results were expressed as the mean ± SD, where $P<0.05$ was considered significance. Data were normalized by the expression level of GAPDH. Significant differences in stenotic colon were observed from corresponding controls.

perature increasing steps of 0.2°C every 10 s. The threshold cycle (CT value) was recorded. To estimate the magnitude of the difference in expression for the individual samples, ΔCT is defined as $CT_{\text{gene of interest}}$ minus CT_{GAPDH} and the ΔΔCT ($=\Delta CT_{\text{HD samples}} - \Delta CT_{\text{control samples}}$) was transformed to $2^{-\Delta\Delta CT}$ as the trend of gene expression level in stenotic colon.

Hematoxylin and eosin staining and immunohistochemical staining

Immunohistochemical staining was performed as described. The stenotic and normal segment were immediately washed in cold phosphate buffered saline (PBS; pH 7.4), then fixed in 4% buffered paraformaldehyde at 4°C for 24 h. The samples were dehydrated, embedded in paraffin, and sectioned sagittally at a thickness of 4 μm. Slides were then incubated in boiling 0.01 mol/L citrate buffer (pH 6.0) for 10 minutes, cooled at room temperature, incubated with 3% H_2O_2 for 15 min, and 10% normal goat serum for 30 min. The sections were then incubated with primary anti-*BMP2, BMP5* and *BMPR10* (1:50) at 4°C for 14 to 16 hours; slides washed in PBS, incubated with anti-rabbit IgG-peroxidase antibody for 20 minutes at room temperature and stained with 3′3-diaminbezidine tetrahydrochloride (DAB). Dark brown granules in the cytoplasm and cytomembrane were considered as positive results. Negative controls were performed by incubating with equivalent concentrations of nonimmune rabbit antiserum. By integrated optical density (IOD) measurement [4], pictures were taken on color-positive films under the same illuminating setting for each sample. The optical density of the cells was measured in the microscopic fields of the seen malformed portion of the sample in cloaca or rectum tissue and the control tissues in those same fields, with a NISE Elements Basic Research (version 2.30) (NIS-Elements Basic Research, Kawasaki, Kanagawa, Japan) analysis system. Two researchers independently reviewed the immunohistochemical-stained slides.

Western blot analysis

Approximately 100 mg specimen was minced to small pieces using surgical blades and sonicated in protein lysis buffer. Protein concentrations were measured by the Bradford method, and specimens were adjusted to the same protein concentration, aliquoted and stored at -80°C. Equal amounts of total proteins from tissues were separated on SDS-polyacrylamide gels and then electro-transferred to PVDF membranes (Millipore, USA). The blots were incubated with following polyclonal antibodies: anti-BMP2, anti-BMP5 and anti-BMP10 (1:100, Beijing biosynthesis biotechnology co., LTD.) overnight at 4°C; washed, incubated with horseradish peroxidase-linked secondary antibodies (1:2000) for 1 h at room temperature and detected using an enhanced chemiluminescence (ECL) kit.

Statistical analysis

The Statistical Program for Social Sciences, version 16.0 (SPSS, Chicago, IL), was used for statistical analysis. Paired T test was used to compare the BMP2, BMP5 and BMP10 expression level between stenotic colon segments and normal colon segments.

Results

Real time quantitative PCR results

The concentration and purity of extracted total RNA from 50 pairs of HSCR patients were determined as the 260/280 ratio by NanoVue-Health care Bio-Sciences AB. Real time RT-PCR

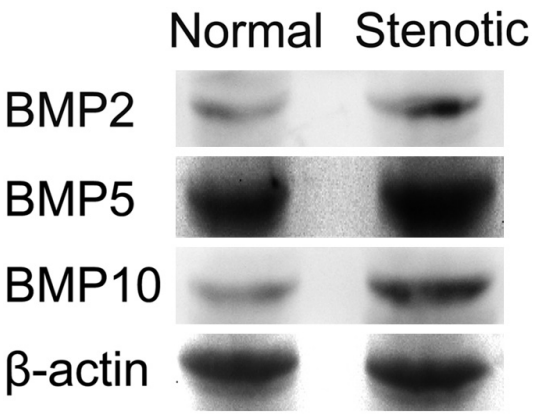

Figure 2. BMP2, BMP5 and BMP10 expression in HSCR by Western blot analyses. Data were normalized by the expression level of β-actin. Protein extracts from normal colon segment and stenotic colon segment in HSCR were probed by Western blot analysis with BMP2, BMP5 and BMP10 antibodies

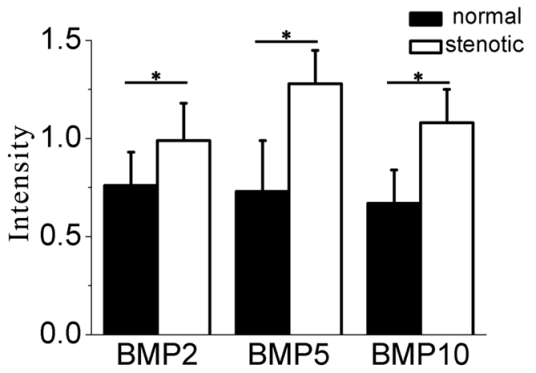

Figure 3. BMP2, BMP5 and BMP10 genes expression analysis by Western blot analyses. The results were expressed as the mean ± SD, where *P*<0.05 was considered significance. By statistical analysis with paired T test, the expressions of BMP2, 5 and 10 in stenotic colon segments were significantly higher than those in normal segments (P<0.05).

was performed and the expression level of BMP2, 5 and 10 was normalized to the mRNA level of *GAPDH* from the same specimen. Increased expression of BMP2, 5 and 10 were detected in stenotic colon segments from 50 HSCR patients respectively, compared to matched normal colons. By statistical analysis with SPSS 16.0 software, the expressions of BMP2, 5 and 10 in stenotic colon segments were significantly higher than those in normal colons (*P*<0.05). The mRNA level of BMP2, BMP5 and BMP10 in the stenotic colon segment from HSCR patients were significantly higher (953.0117, 31.83095 and 1596.147 times, separately) than those in the normal colon segments (p<0.05, **Figure 1**).

Western blot analysis

BMP2, BMP5 and BMP10 protein expression in the same group of 50 HSCR patients was evaluated by western blotting with specific antibodies. Consistent with the RT-PCR results, significant increase of BMP2, BMP5 and BMP10 was detected in stenotic colon segment compared to the matched normal colons (**Figures 2**-**4**).

Immunohistochemistry results

The increased expression of BMP2, BMP5 and BMP10 was further confirmed by immunohistochemistry analyses. The stenotic colon segments were defined by absence of the focal colonic ganglion cells by H&E stain (**Figure 5**). Of 50 cases of HSCR with BMP increase by RT-PCR and western blot, all of them had positive stains by immunohistochemical stain, which were mainly located in the mucous layer and muscular layer of aganglionic colon segment and appeared as light brown and brown particles (**Figure 6**).

Discussion

It is well-known that the onset of HSCR involves a series of complicated process including the distortion of ganglion cell development at different stages, and is caused by multiple factors [2, 5]. This developmental disorder is a neurocristopathy and is characterized by the absence of the intestinal ganglion cells of the nerve plexuses in variable lengths of the digestive tract. The cause of the arrest of the crest cells has not been clearly defined. Up to 50% familial and 15-35% sporadic cases could be explained by RET gene mutations excluding other genes [8].

In this study, the stenotic and normal tissue samples were obtained from 50 HSCR patients, which were ranged from 0.5 to 3.1 years old and included 33 males and 17 females (approximately 2:1 male to female ratio). The stenotic segments were identified as that in absence of the focal colonic ganglion cells by H&E staining; in contrast, the normal segments from the same patients were determined with the colonic ganglion cells. Due to that the stenotic segment and the normal segment were compared from the same patient, the paired T-tests were performed during the statistical analysis,

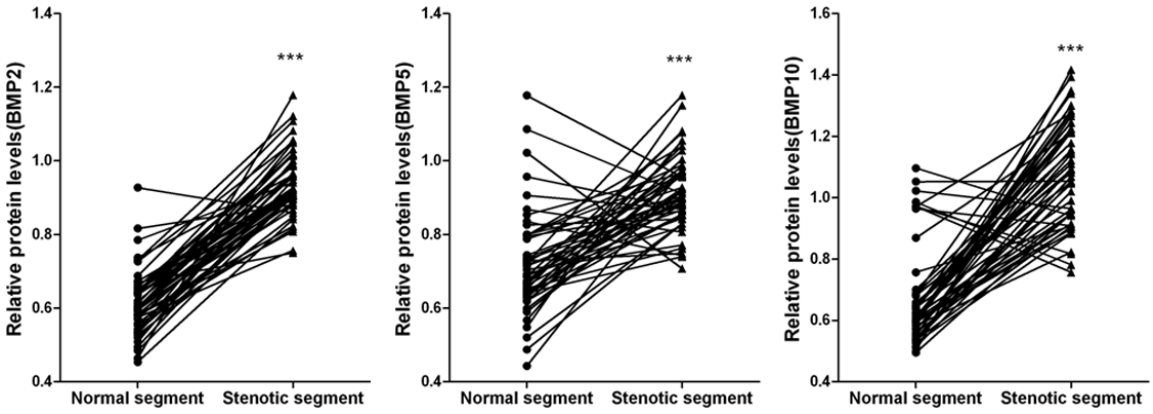

Figure 4. BMP2, BMP5 and BMP10 genes expression analysis by normal-stenotic diagram. Data from 50 patients was shown separately. BMP2, BMP5 and BMP10 genes expression level is significantly up-regulated in the stenotic colon.

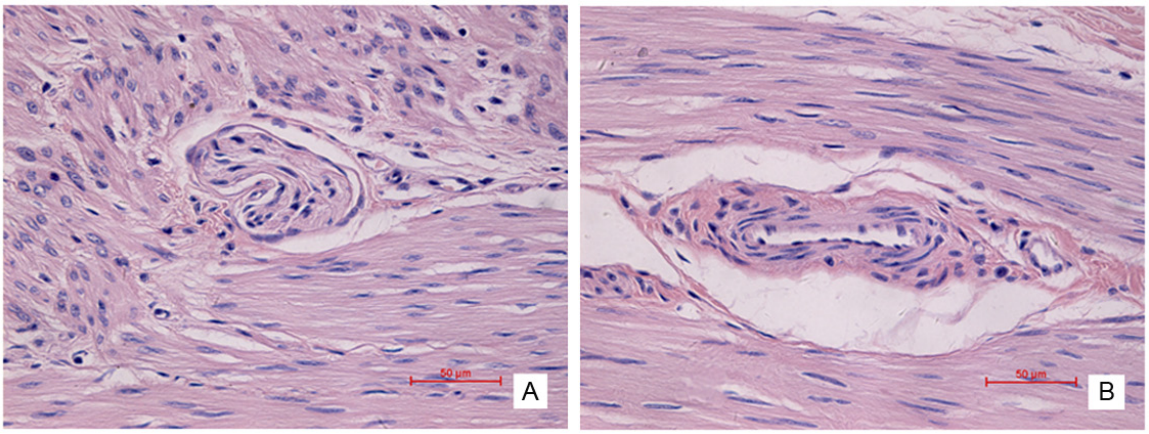

Figure 5. Photomicrographs of stenotic and normal intestine. A: Stenotic colon segment tissue (hematoxylin and eosin [H&E], ×400, aganglionic cell); B: Normal colon segment tissue (H&E, ×400, ganglionic cell).

although as heathy control, the children volunteers were hardly acquired.

BMPs belong to the transforming growth factor-β (TGF-β) superfamily and have been widely recognized as important molecules involved in tissue differentiation and functional modulation of various endocrine systems [9]. Currently, there are 21 known members in the human BMP family (BMP2-BMP7, BMP8A/B, BMP10, BMP15, GDF1-3, GDF5-7, myostatin, GDF911 and GDF-15 [10]. BMPs, some of which are called growth and differentiation factors (GDF), are dimeric molecules composed of two identical monomers linked with a disulfide bond [11-14]. BMP functions by binding to their receptors, namely, BMPRI (BMPRIA and BMPRIB) and BMPRII, which are located on chromosomes 10q23, 4q22-24, and 2q33-34. BMPRIA mediates growth stimulation signals, and BMPRIB transfers growth inhibition signals [15]. BMP bind to two separate transmembrane serine-threonine kinase receptors, type I and type II, forming a heterotetrameric complex. In the complex, type II receptor phosphorylates and activates the type I receptor that in turn phosphorylates and activates cytosolic Smad proteins. Smad1 complexes with Smad4, and this heterodimeric complex then translocates to the nucleus, where it affects transcription of genes specific for the BMP pathway.

BMPs are unique in their ability to initiate bone formation [16]. Besides inducing bone and cartilage formation, BMPs have been shown to play important roles in the regulation of various cellular processes, including cell proliferation, differentiation, cell motility, apoptosis, and self-

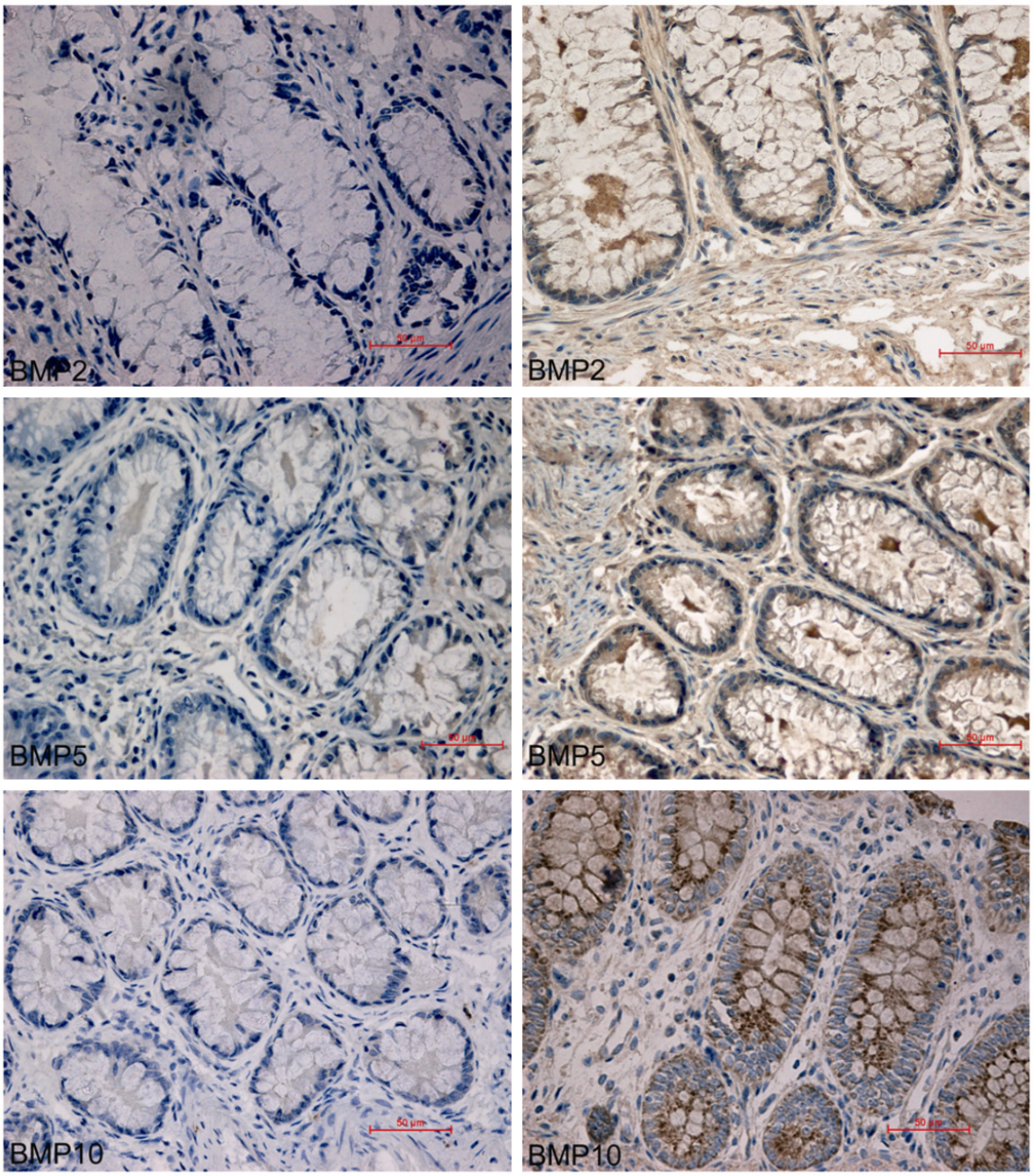

Figure 6. Expression of *BMP2*, *BMP5* and *BMP10* in stenotic and normal colon segment tissue detected by immunohistochemistry (original magnification×400).

renewal of embryonic stem cells [17, 18]. BMPs are also known to crosstalk with other major signaling pathways, such as Wnt, JAK/STAT, and Notch [19-22]. BMPs also play roles in the formation and development of multiple of key steps in vertebrate nervous system [23].

BMPs are a wide range of adjustment factors in life processes, and involved in cell proliferation, differentiation, apoptosis and morphogenesis. Some studies have confirmed that BMPs and their associated signaling molecules are closely related to the development in ENS [24]. Recent evidence showed that the low concentration of BMP2 elevated the neurons; on the other hand, at high concentration it limited the number of enteric neurons. Thus, the concentration-related abilities of BMPs to promote neuronal differentiation and reduce the size of the total population of crest-derived cells are

consistent with the idea that, by inducing neuronal precursors to differentiate into postmitotic neurons, BMPs limit precursor proliferation in the gut and thus regulate the ultimate size of the ENS [25]. Based on this role of BMPS in ENS development, some researchers found that BMP2 and receptor were strongly and specifically expressed in the gut from patients with HSCR, intestinal neuronal dysplasia, and immature ganglion cells [26].

Our studies here found, for the first time, that BMP2, BMP5 and BMP10 were significantly increased in the stenotic segments of HSCR compared to that in the normal segments, suggesting that these proteins could play important roles in the development of HSCR. The molecular study of endogenous BMP modulators is important for the understanding of mechanism in HSCR. Further studies on their mechanistic pathways may yield new therapeutic targets for HSCR.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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