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Comparative proteomics of Hirschsprung's disease

Yang Fan[a], Lili Wang[a], Yi Zhang[a], Tianchu Huang[a], Hui Li[a], Hui Gu[a], Weilin Wang[b], Zhengwei Yuan[a,*]

[a]Key Laboratory of Health Ministry for Congenital Malformation, Shengjing Hospital, China Medical University, Shenyang 110004, PR China
[b]Department of Pediatric Surgery, Shengjing Hospital, China Medical University, Shenyang 110004, PR China

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ABSTRACT

Hirschsprung's disease (HSCR) is a developmental disorder of the enteric nervous system characterized by aganglionosis in distal gut. The estimated population incidence of HSCR is 1/5000 live births, but higher in Asian populations. As the disease mainly manifested bowel motility disturbance, the underlying mechanism is unknown. Furthermore, in the long term up to 75% of patients showed unsatisfactory postoperative bowel function like incontinence or constipation, and 10% required a permanent colostomy. Improved therapy of HSCR is required, but the pathophysiological mechanism for postoperative bowel dysfunction is not clear. In this study, we perform a proteomics study in HSCR patients, expecting some findings in protein alterations to provide more information to reveal the pathophysiological mechanisms of disturbed bowel function before and after surgery therapy. As a result, we identified 16 proteins expressed differently in aganglionic segment of HSCR patients. These proteins function diversely, and included cytoskeleton proteins, regulatory proteins and some enzymes.

Biological significance
In the present study, we performed a 2-DE based proteomic research on HSCR patients, in order to find some clue for the pathomechanism of bowel motility of HSCR disease. As a character of this study, we also compared the expression of altered proteins in ganglionic segment of HSCR patients with that in normal children. Our results showed that some altered proteins found in aganglionic segment had also changed their expression in ganglionic segment comparing with normal children. This result suggested that the ganglionic segment of HSCR patients was not completely normal, and this is important because it provided more information to understand the pathophysiological mechanisms of bowel dysfunction and will help to the therapy of HSCR disease.

1. Introduction

Hirschsprung's disease (HSCR) is a developmental disorder of the enteric nervous system (ENS) characterized by the lack of ganglionic cells in distal gut (aganglionosis). The estimated incidence of HSCR is 1/5000 live births, which is higher in Asian populations (2.8/10,000) [1]. Multiple genes such as RET, NRG1, Hedgehog, and BMPs are reported to be involved in the etiology of HSCR [2–6]. However, as a disease characterized by motility disorder of aganglionic segment of the gut, less attention was paid to the pathophysiology underlying the motility dysfunction in HSCR which is still unclear. Furthermore, in the long term up to 75% of patients showed unsatisfactory postoperative bowel function like incontinence or constipation,

* *Corresponding author at:* Key Laboratory of Health Ministry for Congenital Malformation, Shengjing Hospital, China Medical University. No. 36, Sanhao Street, Heping District, Shenyang 110004, PR China. Tel./fax: +86 024 23929903.
E-mail address: yuanzw@hotmail.com (Z. Yuan).

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and 10% required a permanent colostomy [7–9]. But the exact mechanism causing the post-operative motility impairment is also unknown. Actually, the understanding of the pathophysiology for the gut motility dysfunction in children with HSCR might help formulating novel treatments in the future.

Gut motility is a complex process mediated by interaction between intestinal smooth muscle, interstitial cells of Cajal (ICC), and the ENS. ENS innervates the gastrointestinal tract intrinsically, and its formation requires the co-ordinated migration, proliferation, differentiation and survival of neural crest cells (NCC) within the developing gut. During the establishment of ENS, environmental signals like SOX9, PAX3, neurotrophin-3 and SEMA3A play a critical role [10–12]. Aganglionic segment was reported to be lack of nitrergic neurons in ENS causing inability to relax [13]. In HSCR, the distal colon is devoid of enteric neurons and glia which are derived from NCCs. The congenital defect of ENS development in HSCR may cause the fail in coordination with the gut smooth muscle, and participate in the pathophysiology for the gut motility dysfunction.

Except for the congenital defect of ENS development, derangement of ICCs also plays a role in the gut motility dysfunction of HSCR. There was a report that in the aganglionic colon, the network of ICCs was disrupted and ICCs were markedly reduced [14].

As a powerful tool to investigate the mechanism of a complex biological process, the proteomics approach can reveal the protein alterations corresponding to certain pathological condition in an integrated way. Considering the highly complex pathophysiology underlying the gut motility dysfunction in HSCR, we performed a proteomics study in HSCR patients, expecting to disclose the protein alterations related to bowel malfunction of HSCR.

2. Methods

2.1. Patients and specimens

Colon tissues were obtained from 32 patients (including 30 male and 2 female patients) diagnosed as HSCR in the Department of Pediatric Surgery, Shengjing Affiliated Hospital of China Medical University. The age of the patients ranged from 1 to 96 months old, with an average age of 12.8 months. HSCR was diagnosed by a barium enema x-ray and suction rectal biopsy. All HSCR patients showed aganglionosis restricted to the sigmoid colon. The aganglionic and ganglionic segments of the colon were immersed in liquid nitrogen immediately after surgery removal, and then stored in −80 °C. Colon tissues from 8 patients were used in 2-DE analysis, and those from the left 24 patients were used in verification by Western blot. In addition, there were six cases of newborn infants died from non-nervous or digestive system diseases as normal control. The study was approved by the local ethical committee and all the subjects involved in the study gave written informed consent.

2.2. Extraction of proteins from colon tissues

For 2-DE analysis, colon tissues were homogenized in fresh grinding solution containing 10% TCA and 2 mM TBP (tributylphosphine) in acetone. Proteins were extracted and precipitated by TCA/acetone. The homogenate was treated with precooled (−20 °C) solution of 10% TCA in acetone with 20 mM DTT. Proteins were allowed to precipitate overnight at −20 °C. After centrifugation at 20,000 ×*g* for 15 min at 4 °C, the pellet was washed with ice-cold acetone containing 20 mM DTT. The supernatant was discarded and the pellet dried in a SPD1010 SpeedVac system (Thermo Savant, Holbrook, NY, USA). The pellet was resolubilized in lysis buffer (7 M urea,2 M thiourea, 4% [V/W] CHAPS, 2% [V/V] IPG buffer [pH 3–10], 40 mM 1,4-dithioerythritol, 1 mM PMSF and 1% [V/V] cocktail protease inhibitor, Sigma). For immunoblot analysis, different specimens were homogenized with 5 volumes of lysis buffer containing 50 mM Tris (pH 7.4),150 mM NaCl,1% Triton X-100,1% sodium deoxycholate,0.1% SDS and 1% (V/V) cocktail protease inhibitor (Sigma) and sonicated as above. Then the solution was centrifuged at 20,000 ×*g* for 45 min at 4 °C. The supernatant was used as protein extract for immunoblot analysis. The protein concentration was determined by the Bradford method, and then stored in aliquots at −80 °C.

2.3. 2-DE

2-DE was performed as our previous report [15]. We loaded 900 μg protein extract onto an IPG strip (24 cm, pH 3–10; GE Healthcare, Uppsala, Sweden). For the first-dimension isoelectric focusing, the IPG strip was rehydrated with 450 μl of solubilized sample at 30 V for 12 h on an IPGphor (GE Healthcare). IEF followed a multi-step protocol: 100 V for 2 h, 300 V for3 h, 600 V for 2.5 h,1000 V for 2.5 h, gradient from 1000 V up to 8000 V in 2 h and finally 8000 V for held for 66,000 Vh at 20 °C. The IPG strips were equilibrated in 10 ml equilibration solutions (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate [SDS], 115 mM Tris–Cl [pH 8.8], 20 mM dithiothreitol [DTT]) for 15 min, then equilibrated in the same solution containing 100 mM iodoacetamide instead of DTT. SDS-PAGE involved use of 12.5% polyacrylamide gels in the Ettan DALT *twelve* system (GE Healthcare). Following SDS-PAGE, gels were stained with modified colloidal Coomassie Brilliant Blue (mcCBB) G-250 as described [16]. For 2-DE, we pooled the protein extracts of aganglionic and ganglionic colon tissues from 8 HSCR patients (Fig. 2). The experiment was repeated for three times.

2.4. Image acquisition and data analysis

CBB-stained gels were scanned by use of a PowerLook 2100XL image scanner (Umax, Taiwan). Spot detection, quantification, and matching involved use of 2-D gel analysis software (ImageMaster 2D platinum 6.0, GE Healthcare) with the CBB-stained gels. Percentage of volume of a spot representing a particular protein was determined in comparison with the total proteins present in the area of interest (relative volume, % vol). % vol of spots was obtained from 3 parallel experiments. Spots with at least 1.5-fold difference in % vol showing statistical significance ($P < 0.05$) were defined as differentially expressed proteins and were excised for further analysis.

2.5. In-gel digestion and MALDI-TOF MS

Selected spots were chosen manually. CBB-stained spots were destained in 50% acetonitrile (ACN) in 25 mM ammonium