Original Article

Down-regulation of fibronectin and the correlated expression of neuroligin in hirschsprung disease

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Abstract

Aim: The goal of this study was to investigate the expression of fibronectin (FN) and the correlated abundance of neuroligins (NLs) in the enteric nervous system (ENS) and to find a novel diagnostic marker in the serum of Hirschsprung disease (HSCR) patients.

Methods: The expression levels of FN, neuroligin-1 and neuroligin-2 were detected in 114 children with or without HSCR. The expression and localization of the NLs and FN were assessed morphologically by immunohistochemical staining. Western blot analysis and real-time fluorescence quantitative PCR (qPCR) were performed to examine the correlated expression of the NLs and FN in aganglionic, transitional, and normal ganglionic colon tissues. An enzyme-linked immunosorbent assay (ELISA) was performed to evaluate and compare serum FN levels between HSCR and non-HSCR and between long-type HSCR and short-type HSCR.

Results: These studies showed that both neuroligin-1 and neuroligin-2 were expressed at low levels in aganglionic segments and at intermediate levels in transitional segments compared to their high level of expression in normal tissue. In contrast, FN expression was negatively correlated, with expression in these three samples transitioning from highest to lowest. The serum FN level was higher in HSCR than in non-HSCR, but no significant difference between short-type HSCR and long-type HSCR was observed.

Conclusion: FN affects the expression of both neuroligin-1 and neuroligin-2 in HSCR, which may lead to the hypoplasia of ganglion cells in the ENS. This correlation may play a key role in the pathogenesis, diagnosis, or classification of HSCR.

Key words
enteric nervous system, fibronectin, Hirschsprung disease, neuroligin

1 | Introduction

Hirschsprung disease (HSCR), which is a congenital disease that mostly occurs in infants, with an incidence of one in 5000 human births, is characterized by the absence of ganglion cells. According to the length of the affected segment, 80% of patients are classified as short-segment HSCR, 15% of cases are classified as long-segment HSCR, and the remaining 5% of cases unfortunately show involvement of the entire colon and small intestine. Ultimately, the disease manifests as distal intestinal obstruction neonatally, chronic constipation in older children, or enterocolitis due to the disordered development of enteric neurons in the distal gut. Although surgical options such as "pull-through"
surgery or colostomy have been a consensus therapy and performed in clinical practice for years, the pathogenesis of HSCR remains unclear.4

To date, there are two major theories that explain the morbidity of HSCR in children. One is that mutations in a variety of genes are responsible for the pathogenesis of HSCR, including ret proto-oncogene (RET), sex determining region Y-box 10 (SOX10), glial cell-derived neurotrophic factor (GDNF), and endothelin receptor B (EDNRB); such mutations mostly occur in familial cases.5,6 Neuroligins (NLs) are a family of post-synaptic transmembrane cell-adhesion proteins that feature a long extracellular domain and a short intracellular domain and have been shown to be involved in synaptogenesis with neurexins.7 The aberrant expression of both NLs and neurexins has been widely reported in central nervous system (CNS) diseases that affect cognition, such as Alzheimer’s disease and autism.8,9 Although much less research has been conducted in the enteric nervous system (ENS), we know that the CNS affects and communicates with the ENS through neuroendocrine functions.10 The other theory to explain the morbidity of HSCR is that an abnormal extracellular matrix or dysregulation of a proportion of extracellular matrix components might affect the microenvironment surrounding the enteric neural crest cells (eNCCs). These changes may be responsible for the innervation deficiency due to incomplete colonization of eNCCs in the gut in HSCR.11-13 Here, we chose to further investigate the pathogenic mechanism for the expression of both NLs and fibronectin (FN) in HSCR. We proposed the hypothesis that the synapse-related genes and the extracellular matrix microenvironment collectively affect intrinsic innervation of the distal bowel. Currently, for patients in which HSCR is highly suspected based on clinical features and radiological investigations, a definitive diagnosis can be made only on histological evaluation of a rectal biopsy.4 Therefore, we also wanted to develop a more convenient and less painful diagnostic method to prevent the suffering of pediatric patients and the anxiety of their parents that is caused by biopsy.

2 | MATERIALS AND METHODS

2.1 | Patients and sample preparation

These studies were reviewed and approved (NO. 12025) by the Institution Review Board of Qilu Hospital, Shandong University. All samples used in this study were collected from surgical excision waste tissues and routine preoperative blood tests, none of which caused any harm to the pediatric patients.

From January 2013 to December 2015, 57 children with HSCR (1 month to 5 years old, 37 boys and 20 girls) and 57 children with indirect inguinal hernia (IIH), both treated at the Department of Pediatric Surgery of Qilu Hospital of Shandong University, were involved in this study. All these patients were matched by age and nutritional status (Table 1), and no other diseases were found. The HSCR patients (33 short-segment HSCR and 24 long-segment HSCR) showed obvious clinical symptoms (abdominal distension and constipation), with diagnosis confirmed by pathology tests. The IIH patients used as a control group were diagnosed by ultrasonography. Tissue samples were obtained from surgically excised waste colon of HSCR patients. From each patient, tissue samples were collected from three regions of the colon: aganglionic, transitional, and ganglionic. From each region, a 100 mg tissue sample was collected in a sterile tube and stored at −80°C for Western blot analysis and quantitative PCR assay; an additional 3 cm of colon was collected in PBS and fixed in 4% paraformaldehyde for immunohistochemical staining. Blood samples were obtained from each patient as part of the first blood test during the hospital stay. After the serum samples were clotted for two hours at room temperature, they were centrifuged for 20 minutes at 1000xg and stored at −80°C for enzyme-linked immunosorbent assay (ELISA).

2.2 | Functional study of the down-regulatory effects of fibronectin on neuroligins

The down-regulatory effects of fibronectin on neuroligin-1 and neuroligin-2 were studied in PC12 cell cultures, and the fibronectin gene was knocked down with the RNA interference technique. The rat PC12 cell line (a rat pheochromocytoma derived from neural crest cells that is widely used in studies of neuronal disease and in vitro neurobiological studies14) was purchased from the cell bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). The PC12 cells were cultured in 24-cell culture dishes (ø=55 mm) in RPMI-1640 medium (Gibco) with 10% horse serum (Gibco, MA, USA) and 5% fetal bovine serum (Gibco, MA, USA) at 37°C, 5% CO₂. The PC12 cell culture dishes were randomly divided into

| TABLE 1 | Comparison of nutritional state information between Hirschsprung’s disease patients and non-Hirschsprung’s disease patients |
|----------------|----------------|----------------|----------------|
| Item                        | HSCR (n=57) | non-HSCR (n=57) | P-value |
| Age (month)                 | 9.1±3.07    | 8.9±2.45       | NS       |
| Serum total protein (g/L)   | 65.1±4.27   | 68.2±4.19      | NS       |
| Serum albumin (g/L)         | 46.6±6.93   | 49.1±6.12      | NS       |
| Hemoglobin (g/L)            | 123.1±11.27 | 129.4±8.99     | NS       |
| Body height (cm)            | 71.2±5.04   | 68.7±5.11      | NS       |
| Bodyweight (kg)             | 8.3±2.21    | 8.9±2.36       | NS       |

NS, Not significant; HSCR, Hirschsprung’s disease.
two groups: the fibronectin gene knock-down group and the negative control group, with 12 dishes in each group. When they were cultured with nerve growth factor (NGF) (Peprotech, Rocky Hill, NJ, 50 ng/mL) for 24 hours, the PC12 cells gradually responded reversibly to NGF by induction of the neuronal phenotype. In the fibronectin gene knock-down group, silencing of fibronectin gene expression was performed by transfecting small interfering RNA (siRNA) into the PC12 cells using Lipofectamine 6000 (Beyotime, Shanghai, China) according to the manufacturer’s instructions. The sequence of the fibronectin (rat Fibronectin; NM_019143) siRNA was 5′-AGAGATGATTCCAGAGAGTAA-3′. The PC12 cell cultures were incubated in opti-MEM containing siRNA for 12 hours, and then the medium was replaced with normal medium and the cultures were incubated for another 60 hours. In the negative control group, the PC12 cell cultures were incubated under the same conditions but did not receive the siRNA treatment. The cell cultures were photographed at 24, 48, and 72 hours and the morphology of the cells was examined using the photographs. Finally, the culture dishes in each group were randomly divided into two sub-groups, with six dishes in each sub-group. The cells in one sub-group were harvested for total RNA isolation, while those in the other sub-group were used for total protein isolation. The RNA was used for quantitative real-time PCR to compare fibronectin gene expression levels between the knock-down group and the negative control group. The total protein was used for Western blotting to compare fibronectin, neuroligin-1, and neuroligin-2 protein levels between the knock-down group and the negative control group. In all samples, β-actin was studied as a protein loading control.

2.3 | Immunohistochemical single staining

Tissue samples of aganglionic, transitional, and normal segments of the colon of HSCR patients were used in the study. The immunohistochemical procedure used was similar to that used previously. Briefly, fixed tissue samples were embedded in paraffin, and 4 μm sections were cut and collected on glass slides. The sections were dewaxed with xylene and rehydrated in graded alcohols. After the sections were processed for antigen retrieval in 0.01 M sodium citrate buffer at 95°C for 20 minutes, they were incubated in 3% H2O2 for 10 minutes to inactivate endogenous peroxidases. The sections were then blocked in 10% normal goat serum in PBS for 30 minutes to prevent non-specific staining. Primary antibodies were used to detect the abundance of neuroligins (goat antihuman polyclonal antibodies for neuroligin-1 and neuroligin-2, Santa Cruz, CA, USA, 1:150 dilution) and fibronectin (rat antihuman polyclonal for fibronectin, Abcam, MA, USA, 1:400 dilution) via incubation at 4°C overnight. On the negative control slides, PBS was used to replace the primary antibodies during the incubation. After the sections were washed in PBS three times (10 minutes each), they were incubated with an appropriate secondary antibody (mouse antirat and mouse antigoat 1:500, Beyotime, Shanghai, China) at 37°C for 1 hour. The Polink-2 plus Polymer HRP Detection System (ZSGB-BIO, Beijing, China) and DAB Kit (ZSGB-BIO, Beijing, China) were used to visualize the immunoreactivity. The sections were then counterstained with hematoxylin, differentiated with hydrochloric acid ethanol, blued with tap water, and dehydrated with graded alcohols. Finally, the sections were permanently mounted. The sections were viewed with an Olympus microscope (Olympus, Tokyo, Japan), and photographs were taken with Image-Pro Plus 2D image analysis software (Media Cybernetics, Bethesda, MD, USA).

2.4 | Immunohistochemical double staining

The paraffin sections of aganglionic, transitional, and ganglionic segments of the colon from HSCR patients were also processed for immunohistochemical double staining. After the sections were dewaxed and rehydrated, they were blocked with 10% normal goat serum in PBS for 30 minutes at room temperature. The sections were incubated in primary antibodies (the same as used in immunohistochemical single staining) in a dark room at 4°C overnight to demonstrate the localization of the neuroligins and fibronectin protein. After the sections were washed with PBS containing 1% Tween-20 three times (10 minutes each), they were incubated with secondary antibodies (Texas red-conjugated donkey antibody, FITC-conjugated donkey antirat, ZSGB-BIO, Beijing, China, 1:400 dilution) in a dark box at 37°C for 1 hour. After the sections were washed with PBS three times (10 minutes each), they were mounted with neutral gum, and the immunoreactivity was observed using a laser scanning fluorescence microscope (Olympus, Tokyo, Japan). Fluorescence photographs were digitally captured.

2.5 | Western blot analysis

From each segment of each patient, 100 mg samples were thawed in the sterile tubes at room temperature and divided equally into two parts (50 mg each). One part of the sample was homogenized with 500 μL lysis buffer (RIPA: PMSF=1:100, Beyotime, Shanghai, China), and the other part was used for quantitative PCR assay. The homogenate was centrifuged at 12 000 r/min for 30 minutes, and then, the supernatant containing 30 μg protein was loaded onto a 10% SDS-PAGE gel for protein separation. The separated proteins were then electroblotted onto polyvinylidene fluoride (PVDF) membranes and immersed in blocking buffer containing 5% non-fat dry milk in Tris base-sodium chloride buffer (TBS) for 1 hour at room temperature. The blot was washed with TBS containing 0.05% Tween 20 (TBST) and finally incubated with primary antibodies against the neuroligins, fibronectin (the same antibodies as used in immunohistochemical single staining, working concentration 1:1000), and β-actin (1:2,000 Abcam, MA, USA) overnight at 4°C. After the blot was washed three times with TBST, it was then incubated with HRP-conjugated IgG (1:5000) for 1 hour at room temperature. An enhanced chemiluminescence (ECL) kit (Millipore, MA, USA) was used for detection according to the manufacturer’s instructions. The expression of the proteins was calculated using the relative gray values (measurable protein IOD/β-actin IOD), which were analyzed with Gel-Pro analyzer 4.0 software (Media Cybernetics, Bethesda, MD, USA).

2.6 | Quantitative PCR (qPCR) assay

Total RNA was isolated with a method described previously. Briefly, total RNA from 50 mg tissue samples of three different segments of colon from the HSCR patients was extracted using the Total RNA...
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Isolation Kit (RNAiso Plus, Takara, Shiga, Japan) according to the manufacturer’s instructions. The quantity of RNA was assessed spectrophotometrically. The OD value at 260/280 for RNA ranged between 1.8 and 2.0. An amount of 1 μg of each RNA sample was used in a 20 μL cDNA synthesis reaction conducted using a SYBR® Premix Ex Taq™ II kit (Takara, Shiga, Japan). Quantitative PCR was performed with a SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) quantitative fluorescence kit (Takara, Shiga, Japan) according to the manufacturer’s instructions. The reaction solution consisted of 10 μL PCR reaction buffer (2x), 1 μL forward primer (10 μmol/L), 1 μL reverse primer (10 μmol/L), and 2 μL cDNA. The PCR was carried out in a Roche Applied Science LightCycler 480 Real-time PCR system. After the reaction, the Ct of each sample was measured, and the 2^{-△△Ct} value was calculated. The results were expressed relative to the number of β-actin transcripts used as an internal control. All reactions were repeated three times. The primers and annealing temperatures used for PCR are shown in Table 2.

2.7 | ELISA

In all, 114 serum samples (57 from HSCR patients and 57 from IIH patients) were stored at −80°C. All samples were thawed on ice for preparation, and a serum ELISA kit (R&D Systems, MN, USA) was used to detect the level of fibronectin. Samples were then added to 96-well detection plates (10 L per well), and each sample was analyzed in triplicate. After the reaction, the OD values were measured, and the actual serum fibronectin concentration was calculated according to the standard curve.

2.8 | Statistical analysis

The data were analyzed with GraphPad Prism® 5 software (La Jolla, CA, USA) and expressed as the mean±SD. Unpaired t tests were used for comparisons between two groups, and one-way analysis of variance (ANOVA) was used for comparisons among three groups. All P values were calculated by two-sided analysis, and values less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Immunohistochemical single staining

Immunohistochemical single staining was performed to detect the localization and intensity of neuroligin-1 (Figure 1, B1-3) and fibronectin (Figure 1, C1-3) immunoreactivity in paraffin-embedded sections of aganglionic, transitional, and ganglionic colonic segments of HSCR patients. The intensity of fibronectin immunoreactivity was strongest in aganglionic segments (C1) and gradually decreased from transitional segments (C2) to ganglionic segments (C3). However, neuroligin-1 and neuroligin-2 immunoreactivity was strongest in ganglionic segments (A3 and B3), mild in transitional segments (A2 and B2) and virtually absent in aganglionic segments (A1 and B1).

3.2 | Immunohistochemical double staining

Immunohistochemical double staining (Figure 2) was used to examine the co-expression of neuroligin-1 (A1-C1, red) and neuroligin-2 (D1-F1, red) with fibronectin (A2-F2, green) in the myenteric plexus (A3-F3). However, neuroligin-1 and neuroligin-2 immunoreactivity was strongest in ganglionic segments (A2 and D2) and decreased gradually from transitional segments (B2 and E2) to ganglionic segments (C2 and F2). In contrast, neuroligin-1 and neuroligin-2 immunoreactivity was strongest in ganglionic segments (C1 and F1), mild in transitional segments (B1 and E1), and almost absent in aganglionic segments (A1 and D1). From merged photographs, we could see that the fibronectin immunoreactive product was primarily located around the neuroligin immunoreactive product in the myenteric plexus of the colon.

3.3 | Expression of fibronectin and neuroligins in HSCR

Western blot analysis was used to detect the expression of neuroligin-1 (NL1), neuroligin-2 (NL2) and fibronectin (FN) immunoreactivity (Figure 3A). Neuroligin-1, neuroligin-2, and fibronectin immunoreactivity was present in aganglionic, transitional, and ganglionic colonic segments of HSCR patients. Neuroligin-1 and neuroligin-2 were highly expressed in ganglionic (normal) segments (1.60±0.33 and 0.82±0.21, respectively), moderately expressed in transitional segments (the relative gray values were 0.77±0.11 and 0.50±0.13, respectively), and

| Primers          | Primer sequences (5’-3’) | Annealing temperature (°C) | Product size (bp) |
|------------------|--------------------------|-----------------------------|-------------------|
| Neuroligin-1     | F: GCAAGACCCAGACAGACT    | 59                          | 314               |
|                  | R: CACCACCAAAGAATCAAATTT |                             |                   |
| Neuroligin-2     | F: CCATCTCCGGCTCTACCTTTACA | 59                          | 316               |
|                  | R: CTGGTTTCTCTTGCTTTGAAAT |                             |                   |
| Fibronectin      | F: TGGAACCTTTCACAGTCGCCAC | 59                          | 451               |
|                  | R: TGACATCCCCATCATCGTAAACG |                             |                   |
| β-actin          | F: AGGAGCATCCCCCAAAGTT    | 60                          | 285               |
|                  | R: GGGCAGGAAGGCTCATCATT   |                             |                   |

TABLE 2 | Detail information of primers
weakly expressed in aganglionic segments (0.51±0.25 and 0.32±0.13, respectively). However, fibronectin was highly expressed in aganglionic segments (1.70±0.45), moderately expressed in transitional segments (0.98±0.32) and significantly down-regulated in ganglionic (normal) segments (0.70±0.33). qPCR assay was used to detect the gene expression of neuroligin-1, neuroligin-2, and fibronectin (Figure 3B). Transcripts encoding neuroligin-1 (NL-1), neuroligin-2 (NL-2) and fibronectin (FN) were expressed in aganglionic, transitional, and ganglionic colonic segments of HSCR patients. The abundance of these three transcripts relative to the abundance of those encoding β-actin was determined by qPCR. The abundance of transcripts encoding neuroligin-1 and neuroligin-2 was significantly lower in aganglionic segments (the relative expression level was 0.99±0.30, 0.90±0.27, respectively) than in transitional segments (1.25±0.41, 1.06±0.33, respectively) and ganglionic segments (1.49±0.40, 1.23±0.30, respectively). The abundance of transcripts encoding fibronectin was significantly higher in aganglionic segments (1.39±0.31) than in transitional segments (1.19±0.34) and ganglionic segments (0.95±0.32). The mRNA expression levels of neuroligin-1, neuroligin-2, and fibronectin detected by qPCR were consistent with the intensity of the immunoreactivity of these three proteins demonstrated by Western blot and immunohistochemical staining.

### Functional study of the down-regulatory effects of fibronectin on neuroligins

The down-regulatory effects of fibronectin on neuroligin-1 and neuroligin-2 were studied in PC12 cell cultures in which the fibronectin gene was knocked down with siRNA. The cell cultures of these two groups were photographed at 24, 48, and 72 hours (Figure 4A). In the cultures, when the PC12 cells were incubated with nerve growth factor, the cell proliferation rates were lower and neurite outgrowth was seen, indicating neuron-like differentiation. There were no significant differences of the PC12 cell sizes and cell densities between KD group and NC group at those three periods, suggesting that the siRNA treatment did not affect the growth of the PC12 cells in the cultures. Quantitative PCR analysis revealed that the fibronectin gene expression level in the fibronectin KD group was significantly lower than that in the NC group (P<.001, n=6) (data not shown), which indicated that the fibronectin gene was successfully silenced with the siRNA technique. Western blot analysis was used to compare the protein levels of fibronectin (FN), neuroligin-1 (NL1), neuroligin-2 (NL2), and β-actin between the fibronectin knock-down (KD) group and the negative control (NC) group (Figure 4B). The protein levels of fibronectin (FN), neuroligin-1 (NL1), and neuroligin-2 (NL2) were normalized to that of the β-actin loading control. The protein levels of fibronectin in the fibronectin KD group were significantly lower than those in the NC group (P<.001, n=6). The protein levels of neuroligin-1 and neuroligin-2 in the fibronectin KD group (1.08±0.09, 0.70±0.07, respectively) were significantly higher than those in the NC group (0.69±0.06, 0.51±0.06, respectively) (P<.05, n=6).

### Enzyme-linked immunosorbent assay

Serum fibronectin (FN) was measured by ELISA, as shown in Figure 5. The concentration of fibronectin (FN) in serum was compared between HSCR and non-HSCR patients and between long-HSCR and short-HSCR patients. The fibronectin serum concentrations in HSCR patients (0.57±0.10) were significantly higher than those in non-HSCR patients (0.47±0.11) (P<.01). However, there was no significant difference between the serum fibronectin concentrations of long-segment HSCR patients (0.56±0.10) and those of short-segment HSCR patients (0.58±0.09) (P=.36).
**DISCUSSION**

Currently, it is widely accepted that the development and coordinated function of synapses in the CNS plays a key role in human learning and memory. Particularly, the formation and connection of synapses affects embryonic brain development. When the excitatory/inhibitory synaptic balance or the pathways of neuronal networks are altered, mental diseases are more likely to occur in the CNS. As similarity and bidirectional communication exist between the CNS and ENS, the pathogenesis of CNS diseases may be similar to that of ENS diseases. NLs are postsynaptic transmembrane cell-adhesion proteins that mediate the formation and regulation of synapses between neurons. Deletions or mutations in the neuroligin-3 and neuroligin-4 genes have been implicated in autism spectrum disorder (ASD) and intellectual disability because of synapse dysfunction. Furthermore, ASD patients commonly suffer from gastrointestinal (GI) problems.
related to abnormal changes in GI structure and function. We observed, in our previous study, that the expression of neuroligin-2 in the ENS was significantly decreased in aganglionic colonic segments from HSCR patients; this effect may correlate with ENS dysplasia and ultimately result in excessive intestinal contraction in HSCR. In the present study, we examined NL (both neuroligin-1 and neuroligin-2) expression in the aganglionic, transitional, and ganglionic colonic segments of 57 HSCR patients. Our data demonstrated that the expression of both NLs was decreased in the aganglionic and transitional colonic segments compared with the ganglionic segments. It has been reported that acetylcholine receptors (AchRs), which are critical due to their functional organization at neuronal synapses and at the neuromuscular junction, are co-expressed with neurexin in hippocampal neurons and that neurexin is a synaptic binding partner of neuroligin. In our current studies, the immunoreactive products of both FN and NLs mainly located in the myenteric plexus of the colon, suggesting that the expression of FN may affect the development of these neurons. Therefore, it is possible that the excessive contraction of the abnormal colon in HSCR is due to the absence of neuroligins. Morphologically, the myenteric plexus exists in all segments, although the hypogenetic ganglion cells are much smaller in transitional segments than in ganglionic segments. Whether the high recurrence of chronic constipation after patients accept surgery results from these smaller ganglion cells, which can be detected by fast icing of cross-sectional tissue sampled from the resection margin during the operation, is still unclear, and more research is needed. Thus, perhaps both the abnormal number and morphology of ganglion cells result in the gastrointestinal dysfunction observed in HSCR patients.

Among all of the HSCR patients, only 20% of cases were familial, but these cases had a high rate of recurrence of 7.6%. Of these cases, 62% occurred in siblings, 22% occurred between parents and offspring and 16% occurred in other relatives. Thus, the majority of cases were sporadic, and the genes associated with the ENS were normal. The remaining 80% of sporadic cases highlights the need to understand the etiology and pathogenic mechanism of this disease. As mentioned in the literature review, the components and proportion of the extracellular matrix surrounding neurons may affect the developing and postnatal ENS. In a transgenic mouse model that expresses high levels of collagen VI protein, increased expression during ENS development resulted in slower migration of eNCCs. FN, as an important component of the extracellular matrix, plays a major role in cell adhesion, growth, migration, and differentiation as well as in the processes of embryonic development. We hypothesized that FN may affect the development and migration of ganglion cells due to alterations of the surrounding microenvironment. The data in this study showed differential expression of FN in the three regions of colonic tissues; expression gradually decreased from the aganglionic segment to the transitional segment and ganglionic segment, while the opposite increasing pattern was observed for neuroligin-1 and neuroligin-2 in HSCR samples. This phenomenon was also examined in PC12 cells in vitro. Reduced fibronectin expression in PC12 cells would aggravate neuroligin-1 and neuroligin-2 production (Figure 4B). After 72 hours of incubation with NGF, the undifferentiated PC12 cells switched to a well-differentiated form that exhibited structures such as synaptic connections (Figure 4A). The fibronectin gene was knocked down with RNA interference, and Western blotting was used to compare the protein levels of fibronectin, neuroligin-1 and neuroligin-2 between the fibronectin knock-down (KD) group and the negative control (NC)
The protein levels of fibronectin in the fibronectin knock-down (KD) group were significantly lower than those in the negative control (NC) group, and the protein levels of neuroligin-1 and neuroligin-2 in the KD group were significantly higher than those in the NC group. These data suggested that fibronectin might have a down-regulatory effect on the expression of neuroligin-1 and neuroligin-2.

Indeed, our statistical analysis identified a strong negative association between the expression of the NLs and FN. Morphological examination of double-labeled immunofluorescence staining revealed the location of the NLs and FN and showed that the NLs were primarily expressed in the ganglion cells in the myenteric nerve plexus, while FN surrounded these cells. In aganglionic segments, FN was expressed in the circular muscle and longitudinal muscle instead of the original location of the myenteric nerve plexus. It has been reported that FN accumulated on the surface of a gelatin sponge (GS) scaffold promotes neurite elongation among neuronally differentiating mesenchymal stem cells (MSCs) and that this accumulation progressively decreased during neural induction. Our results suggest that abnormal autocrine FN may have a dynamic influence on the disorderly migration of eNCCs. Once the colonization of the eNCCs is influenced by a change in the microenvironment, such as an increase in FN in the extracellular matrix, various ENS disorders may occur, leading to distal colorectal aganglionosis, which is the primary problem in HSCR. From the results observed in the ganglionic segment of HSCR patients, although FN was expressed in proximity to neuroligin-1 and neuroligin-2 in myenteric nerve plexus, ganglion cells in transitional segments continued to show similar dysplasia and were much smaller and sparser than those in ganglionic segments. This neuronal dysplasia may be caused by the decrease in NLs accompanied by the increase in FN compared with that in ganglionic segments.
the basis for further studies of the potential role of the extracellular matrix in the development of the ENS. In particular, greater numbers of samples and additional data are required to further investigate this molecular mechanism. Due to the high level of non-specific expression of FN in various tissues, we suggest using serum neurotransmitter detection together with serum FN in the diagnosis of HSCR. Therefore, the currently available diagnostic applications have a long way to go, but we have confidence that by combining research and clinic work, new treatment modalities may be developed.

CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest to this work.

AUTHOR CONTRIBUTIONS

Aiwu Li and Yi Zheng designed the research study; Yi Zheng, Xiaona Lv, Dongming Wang, and Ni Gao performed the research works; Ni Gao contributed new reagents/analytic tools; Qiangye Zhang analyzed the data; and Aiwu Li and Yi Zheng wrote the manuscript.

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