ADVANCES IN THE DIAGNOSIS AND TREATMENT OF HIRSCHSPRUNG DISEASE AND ITS COMPLICATIONS

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ADVANCES IN THE DIAGNOSIS AND TREATMENT OF HIRSCHSPRUNG DISEASE AND ITS COMPLICATIONS

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Laparoscopic vs. Transabdominal Treatment for Overflow Fecal Incontinence Due to Residual Aganglionosis or Transition Zone Pathology in Hirschsprung's Disease Reoperation

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Objective: The aim of this study was to describe the details of laparoscopic-assisted reoperative surgery for Hirschsprung's disease (HSCR) with overflow fecal incontinence, and to retrospectively compare laparoscopic-assisted surgery with transabdominal pull-through surgery.

Methods: We retrospectively analyzed patients with HSCR with overflow fecal incontinence after the initial surgery in our center between January 2002 and December 2018. Pre-operative, peri-operative, and post-operative data were recorded for statistical analysis.

Results: Thirty patients with overflow fecal incontinence after initial megacolon surgery [17 who underwent transanal pull-through (TA-PT) and 13 who underwent laparoscopic-assisted pull-through (LA-PT)] required a secondary surgery [reoperation with LA-PT (LAR-PT) ($n = 16$) or reoperation with transabdominal pull-through (TR-PT) ($n = 14$)]. Indications for reoperation were residual aganglionosis (RA) (7/30, 23.3%) or transition zone pathology (TZP) (23/17, 76.7%). Blood loss was significantly decreased in the LAR-PT group (75 ± 29.2 ml) compared to the TR-PT group (190 ± 51.4 ml) ($P = 0.001$). The length of hospital stay was significantly shorter in the LAR-PT group (10 ± 1.5 days) than that in the TR-PT group (13 ± 2.4 days). No significant differences were found between two groups in surgical methods, defecation function score, or post-operative complications except for wound infection (LAR-PT vs. TR-PT 0 vs. 28.6%, $P < 0.05$).

Conclusions: It is necessary to make a comprehensive analysis of the causes of fecal incontinence after HSCR surgery and make an accurate judgment using appropriate methods. If a reoperation was inevitable for patients with overflow fecal incontinence due

to RA or TZP, a comprehensive evaluation prior to the operation is required to maximize the benefit from reoperation. Although laparoscopic reoperation with heart-shaped anastomosis was safe and feasible for patients with failed initial Soave technique, unnecessary reoperation should be avoided as much as possible.

Keywords: Hirschsprung disease, laparoscopy, reoperative surgery, incontinence, outcomes

INTRODUCTION

Since Swenson (1) first successfully performed surgical treatment for Hirschsprung's disease (HSCR) in 1948, many modifications and advanced techniques have been used to improve this intervention. With the development of the less invasive surgical techniques, quality of life for patients with HSCR have been improved vastly. However, many post-operative complications may occur, such as constipation, abdominal distention, soiling, and incontinence (defined as a patient with involuntary bowel movements) (2). Fecal continence refers to the ability to voluntary defecate without soiling (defined as a patient with voluntary bowel movements, wherein the patient had more than one involuntary bowel movement between two voluntary bowel movements with few or only liquid feces) and using an enema. The physiologic elements needed to maintain continence include intact anal sensation, voluntary sphincter control, and appropriate colonic motility (3). Once these constituent become damaged, partial or total fecal incontinence will occur. This type of fecal incontinence is defined as true incontinence.

In theory, children with HSCR have an anatomically intact continence mechanism after birth, and all HSCR radical surgical techniques are designed to preserve intact anal sensation and voluntary sphincter control mechanism. Therefore, children with HSCR after surgery should not have true fecal incontinence. However, post-operative fecal incontinence may still occur for the following two reasons. Firstly, the intact anal sensation and the sphincter accidentally injured during surgery may lead to the occurrence of true incontinence. Secondly, the obstruction of the distal colon is not completely relieved during the operation, which led to the persistence of post-operative distal colon obstruction. The causes of the post-operative obstruction could be anatomical or histopathological: including residual aganglionosis (RA), transitional zone pathology (TZP), stricture, a retained dilated segment, or a Soave muscular cuff (4, 5). In mechanical bowel obstruction, a blockage in the distal colon causes the pressure in the proximal colon to increase, and over time, the rectum and anal sphincters expand and relax. When the colon pressure gradually exceeds the anal pressure, feces overflow from the anus, which is called overflow fecal incontinence.

Currently, most patients and surgeons have accepted the concept of minimally invasive laparoscopic surgery, which has become the most popular surgical procedure for HSCR (6–8). The aim of this study was to share our experiences in the treatment of overflow fecal incontinence due to TZP or RA, with comparing the endpoints of open surgery and laparoscopic surgery for HSCR.

METHODS

Materials

We retrospectively analyzed all patients with HSCR in our center between January 2002 and December 2018. This study was approved by the Institutional Review Board of Tongji Medical College. Patients were included if they continually complained of soiling or fecal incontinence for more than 12 months after the initial surgery. Patients were excluded if they were younger than 4 years (before the age of toilet training), had total colonic aganglionosis, had hypothyroidism, had neurological diseases, or had a colostomy at the time of evaluation. At last, 71 patients were subjected to our protocol. Signed consent form, medical history, physical examination (checking the integrity of the anal canal), and barium enema (BE) were obtained from each subject. If patients had an intact anal canal, they had the potential for bowel control, and may become continent over time. It was vital to carefully determine whether the anus was intact. We classified the type of fecal incontinence in children with incontinence after HSCR surgery according to an algorithm (**Figure 1**). (1) The anal canal was intact: if a BE showed that the colon was dilated, and the patient had a history of constipation, then the disorder was considered overflow fecal incontinence caused by intestinal hypomotility; if a BE showed an undiluted colon, it was considered as intestinal hypermotility, and loperamide, pectin, and dietary modifications were offered. (2) The anal canal was damaged in a previous surgery: this condition was considered as true incontinence, and daily colonic irrigation was chosen to improve the patient's quality of life by reducing the frequency of soiling.

According to the above scheme and process, 47 patients with overflow fecal incontinence were evaluated. After the primary operation in other hospital, all children experienced constipation, soiling or fecal incontinence gradually, which eventually attracted the attention of their parents. Although intermittent anal dilation, glycerine or colonic irrigation were given during the course of the disease, the symptoms persisted. We chose conservative treatment, including diet control, laxative treatment, anal dilation, enema, and colonic irrigation for at least 3 months. A secondary surgery was considered after such

Abbreviations: HSCR, Hirschsprung's disease; RA, residual aganglionosis; TZP, transitional zone pathology; BE, barium enema; PT, pull through; TA-PT, transanal pull-through; LA-PT, laparoscopic-assisted pull-through; LAR-PT, reoperation by the laparoscopic-assissted pull through; TR- PT, reoperation with the transabdominal pull through; AChE, histochemical acetylcholinesterase; HAEC, Hirschsprung-associated enterocolitis; RAIR, rectoanal inhibitory reflex; 24hDAR, 24-h delayed abdominal radiograph.

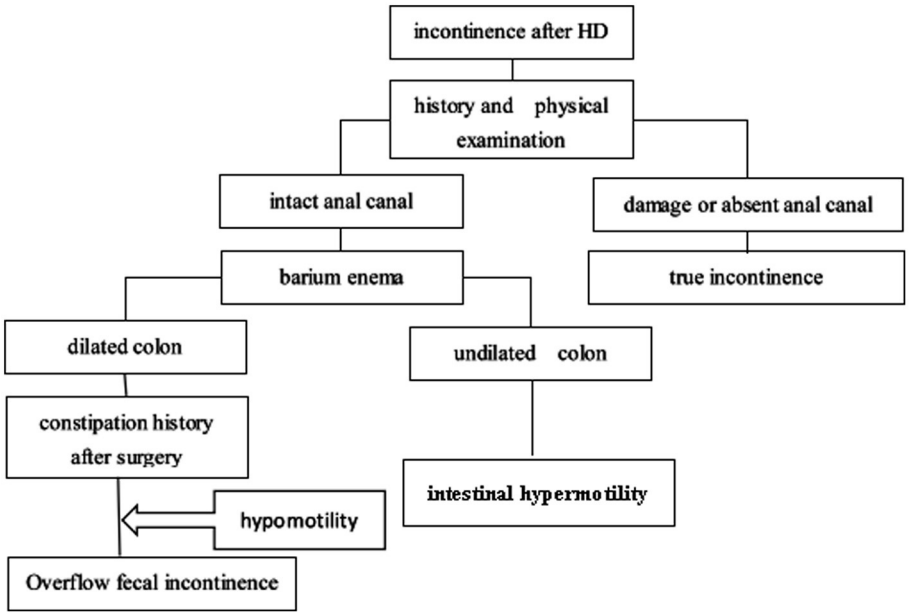

FIGURE 1 | Incontinence diagnosis procedure.

conservative treatments failed. Among them, 12 patients were successfully treated with conservative treatment, and 35 patients underwent reoperation. Among these 35 patients, five cases were excluded to minimize bias because these patients initially underwent laparotomy. We ultimately selected 30 patients with HSCR with overflow fecal incontinence as subjects. All children underwent the primary operation and treatment in other hospitals. Furthermore, the type of primary operation was either transanal or laparoscopic-assisted pull-through (TA-PT or LA-PT, respectively) and all patient's anastomosis was performed in Soave technique.

Before reoperation, surgeons reviewed the patients' medical history and performed physical examinations, BE, anorectal manometry, and histochemical acetylcholinesterase (AChE) staining of the rectal biopsy. All patients underwent rectal biopsy of mucosal/submucosal or full thickness, which was performed 3 and 6 cm above the dentate line. The interpretation of AChE staining was similar to that described by El-Badawi (9). All procedures were performed by a very experienced and single pediatric surgeon at the department of Pediatric Surgery of Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China). Before deciding to proceed with the operation, the children's guardians were properly informed of all risks and potential benefits, and surgical procedure decision was made according to individual circumstances and preferences of each family. The diagnosis was confirmed by intraoperative frozen section, and furtherly confirmed by the post-operative pathology. The speculative extent of the diseased intestine was determined by the surgeon prior to the operation, based on the BE and a 24-h delayed abdominal radiograph (24hDAR, barium residue). During the operation, the length of the resected bowel was determined by intraoperative frozen section and pre-operative findings. Furthermore, an experienced pathologist was assigned to examine the submucosal ganglia and nerve trunk in the intestine. Aganglionosis was defined as the absence of ganglion cells in the submucosa and myenteric plexus with hypertrophied and hyperplastic nerves. A transition zone was defined as the presence of ganglion cells in the submucosa or myenteric plexus with hypertrophied and hyperplastic nerves.

The pre-operative, intraoperative, and post-operative data of the children were recorded in detail in both groups, including demographic information, operation time, type of primary PT, type of repeat PT, length of the aganglionic segment, early complications of surgery and short-term outcome [anastomotic leak, twisted PT, wound infection, perforation, Hirschsprung-associated enterocolitis (HAEC), soiling, and uroschesis], and late complications and long-term outcome (constipation, stricture, fistula, soiling, and HAEC).

Surgical Procedures

The Laparoscopic-Assisted Pull-Through Reoperative Technique With Heart-Shaped Anastomosis

A 1-cm skin incision was first made below the umbilical margin, and a 5-mm trocar was placed into the abdomen after incising the peritoneum. Two 5-mm trocars were then placed on both sides of the umbilicus. Next, we explored the abdomen by inserting a 5-mm, 30° optic fiber, found the suspected intestinal lesion, and cut 2–3 pieces for colonic seromuscular level biopsies for rapid frozen pathological examination to determine whether normal ganglion cells were present in the submucosal nerve plexus. Then, according to the pre-operative examination, intraoperative exploration, and biopsy results, we determined the resection range. Next, the adhesions were gently and completely

separated, and the affected portions of the descending, transverse and ascending colon were then mobilized distally beyond the peritoneal reflection level of the rectum and 5 cm proximal to the most distal biopsy site showing normal ganglion cells by cutting down the mesentery using an ultrasonic scalpel. Finally, a heart-shaped anastomosis was performed: the sponge forceps were inserted into the upper part of rectum through anus. With the assistance of laparoscopy, a segment of colon which had been mobilized was clamped. Then the colon was pulled out and everted. After the distal colon was incised, the affected intestine was pulled out until the normal colon was determined by intraoperative biopsy. Subsequently, the affected intestine was removed, and a longitudinal incision was made at the back wall of anorectal tube, about 0.5–1.0 cm above the dentate line to avoid damage to the sphincter and anal sinus. Finally, the remaining rectum and the normal colon were sutured in layers. The anastomosis was high at the front and low at the back, and the front was about 3–4 cm higher than the back, similar to the heart shape [The new method was reported by our center (10, 11)].

The Transabdominal Pull-Through Reoperative Procedure With Heart-Shaped Anastomosis

An incision was made in the vertical paramedian and left lower abdomen. The following abdominal and anal procedures were similar to the laparoscopic surgery described above.

After surgery, all patients had an anal tube placed for 7–14 days to facilitate defecation and to prevent anastomotic rupture. The first rectal digital examination was performed after 14 days, and routine anal dilatation was required in all patients once or twice weekly for at least 3 months.

Statistical Analysis

Statistical analyses for all variables were performed using SPSS Version 20.0. The normality was checked for each parameter using Shapiro-Wilk test. The results are expressed as ranges and means ± standard deviations (SD). Fisher's exact test was used to analyze dichotomous variables, and Student's *t*-test was used to analyze continuous parameters. The Kruskal-Wallis test was used to analyze the defecation function scores. A P-value < 0.05 was considered statistically significant.

RESULTS

Distribution of Patient Data

All 30 children underwent the first surgical treatment for HSCR in other hospitals, including 17 patients who underwent TA-PT and 13 who underwent LA-PT. Review of the surgical records and post-operative routine pathology showed that the rectum or rectosigmoid had been resected, and the normal intestine with normal ganglions was anastomosed at the distal end. The lengths of removed segments ranged from 12 to 25 cm. Details of the patient characteristics are listed in **Supplementary Table 1**, including symptoms, primary operation, pre-operative examination, reoperation, and histopathological findings. Among all patients, BE showed HSCR, and 24hDAR after BE revealed that barium remaining in the transverse colon, sigmoid colon or the proximal to descending colon. The secondary operation consisted of LAR-PT ($n = 16$) or TR-PT ($n = 14$). For all patients, the histopathology findings showed RA (7 of 30, 23.3%) or TZP (23 of 30, 76.7%) after reoperation.

TABLE 1 | Information and surgical features of patients.

	LAR-PT ($n = 16$) Mean ± SD	TR-PT ($n = 14$) Mean ± SD	*P*-value
Male:Female[a]	8:1	7:1	0.735
Age Redo (month)[a]	60.44 ± 13.24	58.93 ± 14.08	0.765
Follow-up time (month)[a]	33 ± 11.5	30 ± 15.6	0.656
Blood loss (ml)[b]	75 ± 29.2	190 ± 51.4	0.001
Length of resection (cm)[a]	51 ± 16	49 ± 8.2	0.722
Length of hospital stay (day)[b]	10 ± 1.5	13 ± 2.4	0.008

LAR-PT, reoperation by the laparoscopic-assissted pull through; TR- PT, reoperation with the transabdominal pull through.
[a] $P > 0.05$.
[b] $P < 0.05$.

Information and Surgical Features of Patients

Table 1 shows that there were no differences in gender, age, follow-up time, or length of bowel resection between the groups. However, significant differences were observed in the volume of blood loss and days spent in the hospital. Blood loss in the LAR-PT group (75 ± 29.2 ml) was significantly less than that in the TR-PT group (190 ± 51.4 ml). The length of hospital stay in the LAR-PT group (10 ± 1.5 days) was significantly shorter than TR-PT group (13 ± 2.4 days).

The distribution of surgical procedures was not significantly different between children underwent primary and repeated surgery, as shown in **Supplementary Table 2** ($P > 0.05$). **Supplementary Table 3** shows the Heikkinen (12) clinical continence scoring criteria, which divided the defecation continence into four levels: excellent, good, fair, and poor. All patients were followed up by telephone or face-to-face meeting after surgery for more than 1 year to obtain the corresponding defecation function scores for the LAR-PT and TR-PT groups, as shown in **Supplementary Table 4**. However, no significant difference was observed in the defecation continence score between two groups. The results shown in **Supplementary Tables 2**, **4** suggest that no significant correlation exists between the choice of surgical approach and post-operative defecation continence function.

Post-operative Complications and Outcome

No significant differences in post-operative early and late complications were found between the two groups during the follow-up period, except for wound infection. We defined early post-operative complications and short-term outcome (listed in **Table 2**) as those that occurred within 1 year, and late post-operative complications and long-term outcome (listed in **Table 3**) as those that remained 1 year post reoperation. Among

TABLE 2 | Early post-operative complications and short-term outcome.

	LAR-PT ($n = 16$)	TR-PT ($n = 14$)	*P*-value
Anastomotic leak, *n* (%)	0 (0)	0 (0)	NS
Twisted PT, *n* (%)	0 (0)	0 (0)	NS
Wound infection, *n* (%)[b]	0 (0)	4 (28.6%)	0.037
Perforation, *n* (%)	0 (0)	0 (0)	NS
Enterocolitis, *n* (%)[a]	7 (43.8%)	7 (50%)	0.509
Constipation, *n* (%)	0 (0)	0 (0)	NS
Soiling, *n* (%)[a]	7 (43.8%)	7 (50%)	0.509
Incontinence, *n* (%)	0 (0)	0 (0)	NS
Uroschesis, *n* (%)[a]	0 (0)	2 (14.3%)	0.209

LAR-PT, reoperation by the laparoscopic-assissted pull through; TR- PT, reoperation with the transabdominal pull through; NS, not significant.
[a]P > 0.05.
[b]P < 0.05.

TABLE 3 | Late post-operative complications and long-term outcome.

	LAR-PT ($n = 16$)	TR-PT ($n = 14$)	*P*-value
Constipation, *n* (%)	0 (0)	0 (0)	NS
Stricture, *n* (%)	0 (0)	0 (0)	NS
Fistula, *n* (%)	0 (0)	0 (0)	NS
Enterocolitis, *n* (%)[a]	4 (25%)	3 (21.4%)	0.581
Soiling, *n* (%)[a]	2 (12.5%)	2 (14.3%)	0.648
Incontinence, *n* (%)	0(0)	0 (0)	NS

LAR-PT, reoperation by the laparoscopic-assissted pull through; TR- PT, reoperation with the transabdominal pull through; NS, not significant.
[a]P > 0.05.

the early complications, there were four cases (28.6%) of incision infection and two case (14.3%) of urinary retention in the TR-PT group; none of the patients in the LAR-PT group experienced these conditions. The children with incision infections were treated by cleansing and dressing the incision, and the incision then healed after debridement. Children with urinary retention were treated by placing an indwelling catheter for 1 week to restore urinary function. HAEC and soiling occurred in the two groups, during both the early and late complication stages, but no other complications such as constipation or incontinence occurred. The symptoms of HAEC improved with anti-inflammatory and colonic treatments, colon lavage, and regulation of the intestinal microflora. Among the early and late complications, the incidence rates of HAEC decreased from 43.8 to 25% in the LAR-PT group and from 50 to 21.4% in the TR-PT group, respectively. Similarly, the symptoms of soiling were improved with the training of defecation function and the relief of anal sphincter spasms. The incidence of soiling decreased from 43.8 to 12.5% in the LAR-PT group and from 50 to 14.3% in the TR-PT group. However, no significant difference was observed in the incidence of HAEC or soiling between the two groups when comparing both early and late complications (all $P > 0.05$).

Long-term follow-up was obtained in both groups after operation, and there was no difference in follow-up time (Listed in **Table 1**). During the first month of follow-up after surgery, most patients had more than 15 bowel movements per day frequently; however, the frequency of bowel movements decreased significantly over time. At the last follow-up, 26 patients had complete fecal control (the frequency of stools was 2–3 times a day), and only 4 patients had soiling occasionally without social issues.

DISCUSSION

Children with megacolon can benefit from surgical treatment. However, even after a successful surgery, some patients still experience a wide array of stooling disorders. These disorders can range from intermittent enterocolitis to far more significant issues, such as severe stool retention and intestinal obstruction, as well as incontinence (13, 14). In 2010, Levitt et al. (15) reported a need for a high index of suspicion for those patients who were not recovering well after a PT procedure to detect those with anatomically or histopathologically correctable problems. Although there are many reasons for distal colon obstruction after HSCR surgery, most current reports focus on RA or TZP (16). In our study, 30 patients were eventually confirmed to have RA or TZP despite undergoing TA-PT or LA-PT surgery. Children with obstruction of the distal colon might gradually develop constipation, soiling, or fecal incontinence.

Based on epidemiological research, Rajindrajith et al. (17) reported that the incidence of fecal incontinence in 2,686 children was 2%. They also discovered that fecal incontinence contributed to developing an unsociable personality, social dysfunction, and mental disorders in children. In turn, mental and psychological disorders could aggravate incontinence. The appearance of constipation after HSCR surgery is often mild and lagging. The patient's parents may not notice the symptoms or may manage the symptoms inappropriately, which results in prolonged problems and eventually leads to "fecal incontinence." Through our diagnostic process, we can identify the type of fecal incontinence. Patients with overflow fecal incontinence have bloating stools in their underwear, and a massive fecal mass can be reached by digital rectal examination. Anorectal manometry can help to understand the peristaltic function of the intestine by detecting the rectoanal inhibitory reflex (RAIR), the anal canal resting pressure, the rectal resting pressure, and the anal canal peristalsis frequency, rhythm, amplitude, and compliance. In the current study, increased anorectal pressure was detected in all of the patients, and a RAIR was induced in most children. The BE could show the shape of the intestine, which appeared as a narrow or dilated segment. Previous studies (18) in our center showed the density of interstitial cells of Cajal was significantly decreased in the bowel segments that displayed barium retention. The 24hDAR reflected the peristalsis and transport of the intestine and might be a valuable tool for predicting the length of bowel resection in patients with HSCR. As a result, a preliminary determination could be made on the range of the lesion in the colon and the length of the pre-operatively resected bowel (19). Of course, the frozen section pathology results during surgery could be referenced to determine the length of the intestine to be removed. In all 30 cases, pre-operative 24hDAR showed barium residue in the distal end of the ascending colon or in

the transverse colon, and intraoperative frozen section suggested that normal ganglion cells did not exist before the transverse colon to the distal end of the ascending colon. Therefore, the pre-operative 24hDAR might be used as a good reference for the extent of suspicious intestinal biopsy in surgery, and ultimately for the achievement of the goal that removing all diseased bowel. Finally, all patients underwent left hemicolectomy or subtotal colectomy based on the results of intraoperative frozen pathology and pre-operative findings. In addition, the results of the routine pathological examination of the resected bowel segment were consistent with our pre-operative judgment and intraoperative frozen pathology. No children had recurrence of constipation and fecal incontinence through long-term follow-up.

RA or TZP will cause the post-operative symptoms of persistent constipation, eventually leading to the occurrence of soiling or fecal incontinence, thus, a strong indication exists to remove the diseased intestine. However, the risk of a repeated surgery is greater than the primary surgery. When the primary operation was laparoscopic-assisted or transanal surgery, the intraoperative abdominal cavity was less disturbed. As a result, the adhesions seen during the reoperation were relatively mild, as noted by many authors (20, 21), allowing greater feasibility of laparoscopy for the secondary surgery. In our study, none of the children undergoing laparoscopic surgery were converted to open surgery. Recent studies have shown that LA-PT could significantly reduce the surgical trauma for children, speed up the recovery time of bowel movement, promote early recovery, reduce hospitalization time, and result in more visually pleasing incision scars (22, 23). Our results were similar. Compared with laparoscopic surgery, open surgery had more trauma, more bleeding and greater impact on intestinal function, leading to a longer recovery time, slower recovery of intestinal peristalsis, and a longer duration of hospitalization. However, regarding the secondary surgery in our study, no significant differences were found in the incidence of post-operative constipation and HAEC complications between the two groups. Langer (22) and Weber et al. (24) also reported no correlation in the outcomes based on the type of PT, either at primary PT or repeated PT. Of course, when deciding the surgical technique, the preferences of the patient's guardians and the technical level of the surgeons should be considered. Open surgery might be a better choice in some circumstances, such as when a child presented with severe abdominal adhesions or a frozen pelvic cavity pre-operatively. With the improvement in laparoscopic techniques and the accumulation of surgical experience in this field, laparoscopic surgery for HSCR reoperation may become more popular over time.

Until now, there is no recommendation for the method of anastomosis during HSCR reoperation. Schweizer et al. (25) believed that the Duhamel procedure was the best method in complicated cases because this procedure considers the anatomy of the pelvis and has advantages in various surgical techniques, including surgical procedure caused alterations in the pelvis. Sheng (26) and Teitelbaum (27) recommended the Soave procedure as the first choice for secondary anastomosis. However, in our cases, the initial anastomosis was performed in Soave technique, so we chose the heart-shaped anastomosis for patients in whom the mucosectomy was difficult to perform. In addition, the "heart-shaped anastomosis" with high front and low back was performed to expand the anastomotic caliber so as to effectively avoid post-operative stenosis. Since 2000's, the modified operation for HSCR had been used in many medical centers in China. As for the long-term outcomes (11), the incidence of anal sphincter dysfunction and intraoperative nerve injury, as well as the incidence of post-operative constipation and soiling have been reduced.

Our research showed no significant difference in the stooling, regardless of the type of PT used for primary PT or repeated PT. This result was consistent with those reported by other scholars (25, 28, 29), indicating that the management of recurrent HSCR requires a detailed and cogent evaluation of the patient's condition by an experienced team of pediatric surgeons and pathologists. Surgical and other treatment options should be elaborated and personalized, rather than focusing solely on the choice of surgical technique.

The limitations of our study are its retrospective nature and small sample size. Larger prospective randomized studies and detailed long-term follow-ups are required to explore additional differences between these two procedures.

CONCLUSION

It is necessary to make a comprehensive analysis of the causes of fecal incontinence after HSCR surgery and make an accurate judgment with various appropriate methods. Children with overflow fecal incontinence after PT for HSCR may have RA or TZP of the bowel and may benefit from re-operative treatment. When a reoperation was inevitable in a patient with overflow fecal incontinence after initial HSCR surgery, it was very important to select the appropriate operation. Laparoscopically assisted reoperation with heart-shaped anastomosis was feasible and more advantageous than open surgery, with less operative bleeding, faster recovery, and shorter hospital stay. Of course, larger prospective randomized studies and detailed long-term follow-ups are needed. Even if precise reoperation and other comprehensive treatments may cure RA or TZP, we should reduce the necessity of reoperation as least as possible.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Tongji Medical College. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FC and JF: study conception and design. FC and LX: data acquisition. FC, XW, LX, and XC: analysis and data

interpretation. FC: drafting of the manuscript. XW and JF: critical revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Colonoscopic Diagnosis of Postoperative Gastrointestinal Bleeding in Patients With Hirschsprung's Disease

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Aim: Postoperative lower gastrointestinal bleeding in children with Hirschsprung's Disease (HSCR) is a non-specific symptom, which may be caused by various etiologies. Our current study aims to utilize colonoscopy to diagnose the causes of postoperative hematochezia and to analyze its feasibility, accuracy, and safety.

Methods: Twenty-four patients with HSCR with postoperative lower gastrointestinal bleeding or occult blood in the stool were enrolled in this study. The postoperative onset duration, age at examination, accompanied anomalies were recorded. After bowel preparation, all patients underwent colonoscopy. According to visual findings, mucosal biopsy was performed, followed by pathological diagnosis. Further treatment was determined according to the visual findings and pathological diagnosis. All patients were followed up for 6 months including therapeutic outcomes and recurrence of symptoms.

Results: The mean onset duration was (221.3 ± 216.8) days postoperatively (ranging from 25 to 768 days). The mean age at examination was (41.0 ± 29.4) months. There was no significant difference in the onset days among each group (all, $p > 0.05$). Based on visual and pathological findings, there were 11 cases of HSCR associated enterocolitis (HAEC), 4 cases of anastomotic leakage, 7 cases of anastomotic inflammation, 1 case of juvenile polyp, and 1 case of inflammatory pseudopolyp. Intraluminal saline irrigation, thrombin treatment or colorectal polyp electrocision was performed according to intraoperative diagnosis. Patients with HEAC and anastomotic inflammation underwent antibiotics therapy and colorectal irrigation. Patients with leakage underwent reoperation. The highest incidence of accompanied symptoms of diarrhea existed in HEAC group ($p = 0.02$) and fever in leakage group ($p = 0.02$), respectively. No perforation or aggravated bleeding occurs in any patients. All patients gained uneventful recovery during follow-up period.

Conclusions: Colonoscopy is a safe, accurate and timely examination for HSCR patients with postoperative lower gastrointestinal bleeding. The visual findings and biopsy may provide accurate diagnosis and guide treatment for this subset of patients.

Keywords: Hirschsprung disease, gastrointestinal bleeding, enterocolitis, colonoscopy, hematochezia

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INTRODUCTION

Although the symptom of rectal bleeding was usually considered to be non-specific (1), when patients with Hirschsprung's disease (HSCR) experienced hematochezia or occult stool postoperatively, great attention should be paid to the postoperative HSCR associated enterocolitis (HAEC) (1), anastomotic lesions (2), leakage (3) or other complications. Timely diagnosis and treatment can avoid further progress of these complications. For most of patients with hematochezia or positive occult blood, colonoscopy was reported to be a safe, crucial, and accurate examination (4). It help doctors make macroscopical diagnosis through direct vision and histological diagnosis by mucosal biopsy, therefore, it helps pediatric surgeons carry out corresponding treatments (5). Although some sporadic reports showed the application of colonoscopy in diagnosing HAEC or other minor lower gastrointestinal bleeding, till now, there is no report on colonoscopic diagnosis for the group of HSCR patients experiencing postoperative gastrointestinal hematochezia presents or occult blood, nor there is any consensus of the technique and appropriate case target selection.

This retrospective study reported a group of children with HSCR experiencing hematochezia or fecal occult blood after pull-through procedures. All patients underwent colonoscopy and received endoscopic mucosal biopsy. According to specific diagnosis, corresponding treatments or reoperation were chosen by doctors. The importance and safety of colonoscopy in this subset of patients were evaluated, analyzed and discussed.

PATIENTS AND METHODS

From October 1st 2016 to June 30th 2020, a total of 301 cases of children with HSCR in our department received pull-through operations. The length of resected bowels was evaluated preoperatively based on results of barium enema and determined intraoperatively by rapid frozen section followed by histological examinations.

Among them, 24 cases had recurrent hematochezia or occult blood after stool examination. All patients had more than or equal to 2 times of onsets. The mean postoperative onset duration, presence of clotting abnormalities and associated anomalies including diarrhea, fever, and abdominal distension were retrieved from the clinical database.

Before colonoscopy, electrocardiogram, blood routine examination, and coagulation test were performed. Colonoscopy was performed in the digestive endoscopy room under intravenous anesthesia. The digestive tract was prepared 1 day before operation, and saline irrigation was performed once using a 16Fr silicone anal tube. Patients need to be fasting for 2 h before colonoscopy. Intravenous Cephalosporins (100 mg/Kg) twice a day and metronidazole (10 mg/Kg) once a day were given. Nasogastric tube was intubated in patients with abdominal distension. According to the macroscopic findings, mucosal biopsy, colorectal polyp electrocision, or intraluminal saline or thrombin treatment were performed. The corresponding further treatment was determined according to the visual findings of colonoscopy and pathological diagnosis. Patients with HAEC were treated with abovementioned antibiotics and colorectal saline irrigation until relief of all symptoms, and disappearance of red blood cell or white blood cell in stool. Patients with anastomotic leakage underwent enterostomy after colonoscopy. Patients with unabsorbed thread head and anastomotic inflammation were given Cephalosporins orally for a week, and we did not perform saline irrigation unless occult blood was persistent. Under the colonoscope, local, or extensive submucosal congestion, mucosal edema, mucosal ulcer accompanied with or without intraluminal bleeding were considered to be the macroscopic signs of HAEC. According to Elhalaby et al.'s (6) histological criteria, pathological diagnosis of HAEC was made.

During examination, patients took the right lateral position, and the surgeons stood by the left hand side of patients. When operating the colonoscope, as long as the view was clear, we did not frequently inflate or flush saline to ensure low pressure in the bowel cavity.

All patients were followed up for 6 months, including outcomes and recurrence of hematochezia. The model of digestive endoscopy system was vp-4450hd (Fujinon, Japan), and the model of colonoscopy was ec-530wm (Fujinon, Japan). Continuous data were analyzed by t-test after analysis of variance, and category data were compared by Fisher's exact test. $P < 0.05$ showed statistical difference.

RESULTS

In the 24 patients, including 13 males and 11 females, the average onset of gastrointestinal bleeding was (221.3 $\pm$ 216.8) days postoperatively (ranging from 25 to 768 days). The average age at examination was (41.0 $\pm$ 29.4) months. There was no significant difference in the onset days among the groups ($P > 0.05$). There were 12 cases of hematochezia and 12 cases of occult blood. Ten cases were accompanied with diarrhea, 11 with fever, and 4 with abdominal distension which were relieved after antibiotics treatment and colorectal saline irrigation.

Sixteen cases of short segment HSCR were treated with left hemicolectomy, five cases of long segment HSCR, and three cases of HSCR complicated with intestinal neuronal dysplasia were treated with subtotal colectomy. Eighteen cases were treated with heart-shaped anastomosis (7, 8), four cases with Soave anastomosis, one case with Rehbein anastomosis, and one case with Duhamel anastomosis. Overall, only the average prothrombin time at admission (15.4 $\pm$ 1.0 s) slightly increased compared to the reference value (11.5–14.0 s). The average values of other parameters including prothrombin activity, international normalized ratio, fibrinogen, activated partial thromboplastin time, and thrombin time were all within the normal range. Based on visual and pathological findings, there were 11 cases of HAEC, four cases of anastomotic leakage, seven cases of anastomotic inflammation, one case of juvenile polyp, and one case of inflammatory pseudopolyp. No lesion was observed in ileocecum region. None of the patients had perforation, aggravated bleeding or other endoscopy

TABLE 1 | Overview of pathology, symptoms, and signs.

Pathological changes	Cases	Mean postoperative onset duration (days)	Symptoms and signs			
			Hematochezia /fecal occult blood	Diarrhea	Fever	Abdominal distention
Hirschsprung's disease associated enterocolitis	11	216.8 ± 261.7	4/7	9*	2**	3
Anastomotic leakage	4	173.5 ± 61.5	1/3	1	4	1
Anastomotic inflammation	7	241.3 ± 235.3	5/2	1	4	0
Polyp	2	271.5 ± 188.8	2/0	0	1	0

*$P = 0.02$, compared to anastomotic inflammation group.
**$P = 0.02$, compared to anastomotic leakage group.

related complications after examination. There was no significant difference in the ratio of hematochezia to occult blood between patients with HAEC and other patients (all, $P > 0.05$). The incidence of diarrhea in HAEC group was significantly higher than that in anastomotic inflammation group (9/11 vs. 1/7, $^{*}P = 0.02$), but there was no significant difference compared with anastomotic leakage group or polyp group (both, $P > 0.05$). The incidence of fever in HAEC group was significantly lower than that in anastomotic leakage group (2/11 vs. 4/0, $^{**}P = 0.02$), but there was no significant difference compared with other groups (both, $P > 0.05$). There was no significant difference in the incidence of abdominal distension among the groups ($P > 0.05$; **Table 1**).

According to the severity of clinical episodes of HAEC (9), nine patients were scored as Grade 1 and the other 2 were scored as Grade 2. In 11 children with HAEC, extensive congestion and mucosal edema of the colon and neorectum were observed under the colonoscopy (**Figure 1A**). Some patients had mucosal ulceration and bleeding with mucosal annular protrusion and mucosal ulcer formation (**Figure 1B**). Some children had remote hemorrhage in the colorectal cavity (**Supplementary Video 1**). Mucosal biopsy showed that eight cases had extensive chronic inflammation in colonic and rectal mucosal tissues, where some of the mucosa disappeared with mucoid and inflammatory effusion (**Figure 1C**). Two cases were accompanied by infiltration of a small amount of foam cells, lymphoid cells, plasma cells and formation of lymphoid follicles. One case was accompanied by lymphoid hyperplasia in the lamina propria, lymphoid follicles, and scattered eosinophilic infiltration. After completion of the examination, 11 children were treated with intravenous antibiotics and colorectal saline irrigation, and all recovered and discharged. Two cases had recurrent HAEC during a followed-up period of 6 months after discharge.

There were four cases of anastomotic leakage, including one case of short segment HSCR with intestinal neuronal dysplasia (heart-shaped anastomosis), two cases of long segment HSCR (one case of Rehbein anastomosis, one case of heart-shaped anastomosis), and one case of short segment HSCR (Soave anastomosis). Colonoscopy showed fistula formation near the anastomotic leakage (**Figure 1D**), mucosal edema and congestion around the leakage (**Figure 1E**). In one case, a sacrococcygeal external fistula was formed. Diluted methylene blue injection injected from the external fistula was observed flowing out of the internal fistula (**Supplementary Video 2**). Mucosal biopsy near the internal fistula showed chronic inflammatory cell infiltration and focal erosion in the intestinal mucosa (**Figure 1F**). All of the four patients underwent enterostomy after colonoscopy. Three months later, the fistulectomy, re-pull-through, and Soave anastomosis were performed again. Two months later, the stoma was closed. All the patients recovered well during follow-up period.

In six children with anastomotic inflammation, mucosal erosion, edema, and bleeding near the anastomotic site were observed under colonoscope (**Figure 1G**). Part of the mucosa was locally uplifted and brittle, and bleeding was obvious after scratching (**Figure 1H**). After scratching the uplifted and edematous mucosa, unabsorbed thread head was observed in some cases, which was removed under colonoscope. Anastomotic stenosis could be seen in 1 patient. Duhamel's procedure was performed in one of them, and the formation of blind rectal pouch was observed (**Supplementary Video 3**). Macroscopically, the mucosa on the septum after Duhamel's procedure was swollen and congestive, with sporadic punctate hemorrhage. Mucosal biopsy showed chronic infiltration of inflammatory cells in the mucosa near the anastomotic site, accompanied by focal hemorrhage and extensive erosion (**Figure 1I**). After 6 months of follow-up, symptoms disappeared in 4 cases after antibiotics treatment and saline irrigation. In 1 case accompanied with anastomotic stenosis, after antibiotics therapy and saline irrigation for 1 week, no recurrence of hematochezia was observed. Three weeks later, anal dilation was performed for 2 months.

Two children were diagnosed as polyps by colonoscopy. One patient had a polyp with a diameter of 8 mm at ∼30 cm from the anastomotic site, which was diagnosed as juvenile polyp by pathological examination (**Figure 1J**). In the other case, a polyp with a diameter of about 1.5 cm was found at the anastomotic site, with the width of about 4 mm at the bottom (**Figure 1K**). These 2 cases were diagnosed as juvenile polyp (**Figure 1L**) and inflammatory pseudopolyp, respectively. After 6 months of follow-up, there was no recurrent hematochezia or occult blood in stool in the two cases.

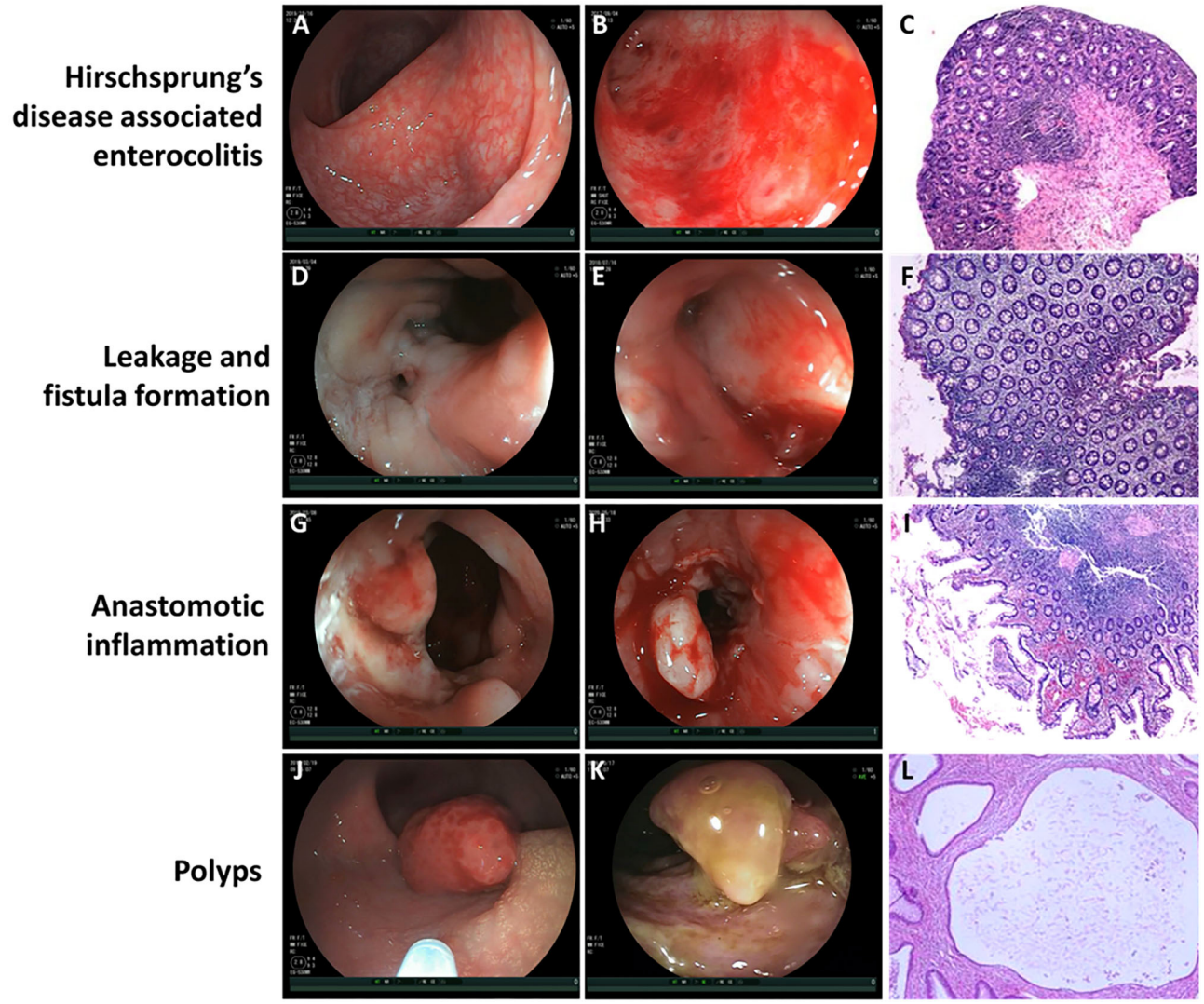

FIGURE 1 | The visual finding of colonoscopy and pathological changes under microscope. **(A)** Extensive congestion and mucosal edema of the colon and neorectum in patients with Hirschsprung's disease associated enterocolitis (HEAC). **(B)** Mucosal ulceration and bleeding with mucosal annular protrusion and mucosal ulcer formation in patients with HAEC. **(C)** Extensive chronic inflammation in colonic and rectal mucosal tissues with mucoid and inflammatory effusion under microscope. Magnification ×100. **(D)** Fistula formation near the anastomotic leakage. **(E)** Mucosal edema and congestion around the leakage. **(F)** Chronic inflammatory cell infiltration and focal erosion in the intestinal mucosa under microscope. Magnification ×100. **(G)** Mucosal erosion, edema, and bleeding near the anastomotic site in patients with anastomotic inflammation. **(H)** Mucosa was locally uplifted and brittle, and bleeding was obvious after scratching in patients with anastomotic inflammation. **(I)** Chronic infiltration of inflammatory cells in the mucosa near the anastomotic site, accompanied by focal hemorrhage and extensive erosion under microscope. Magnification ×100. **(J)** Juvenile polyp. **(K)** Inflammatory pseudopolyp. **(L)** Focal areas of mucosal hyperplasia with cystic dilatation of the crypts and accumulation of mucus within the cysts under microscope (Juvenile polyp). Magnification ×100.

DISCUSSION

In 1998, Balkan et al. (10) reported sigmoidoscopy in minor lower gastrointestinal bleeding in 2 patients with HAEC. Although Balkan's report is the first one focusing on application of endoscopy for HAEC, our report focused on lower digestive endoscopy for hemorrhagic issues following surgery for HSCR, with different targeted subset. The postoperative hematochezia needs to be identified by pediatric surgeons in time, because the most common cause may usually be HAEC, anastomotic inflammation, anastomotic leakage, and polyps, as shown in our current study. In our study, hematochezia or occult blood occurs at 25 days to more than 2 years after primary pull-through operation. Early diagnosis could avoid poor prognoses such as septic shock, perforation, acute/chronic anemia and hemolysis shock in this subset of patients (11). Pediatric surgeons were usually used to diagnosing HAEC from clinical manifestations and laboratory examinations (1), however hematochezia of a part of patients were caused by local anastomotic site. The two groups of patients were sometimes difficult to differentiate,

meanwhile their treatments are quite different (12). When anastomotic leakage occurs, it usually needs staged reoperation (13), while inflammatory diseases usually need conservative therapy (14). Therefore, timely and accurate diagnosis is of great significance for determining strategies of treatment for postoperative hematochezia with various etiologies.

Colonoscopy has been widely used in the diagnosis and treatment of lower gastrointestinal bleeding in children (5). Total colonoscopy is now very common for patients over 1 year old (15). However, in our study, this subset of patients has distinctive features, and the operating technique of colonoscopy is different from the reports when diagnosing lower gastrointestinal bleeding. Firstly, the children have already undergone pull-through endorectal anastomosis. According to the length of the removed bowel segment, the length and shape of the residual bowel after operation are different from unoperated children. For children with short segment HSCR, the left hemicolectomy is usually performed. The anatomical position has changed after the splenic flexure is dissociated, so that the angle of bowel is sharper when entering the transverse colon. Especially when postoperative HAEC occurred, the mucosal becomes swollen and fragile, and the intestinal wall becomes weak due to long-term explosive diarrhea and overproduction of aerogenic bacteria (6). Therefore, great attention should be paid to the total colonoscopy to avoid perforation or mucosal injury. For children with long segment HSCR and IND, subtotal colectomy is often performed, and ileocecum can be observed easily by colonoscopy. Secondly, through our observation, there are very few lesions only located in ileocecum in children with postoperative gastrointestinal bleeding. Based on the abovementioned points of view, we think that it is unnecessary for all children to undergo total colonoscopy, except for those who had no obvious lesions near the anastomotic site and needed to be examined until the ileocecum. For example, in one patient of our study, a juvenile polyp was found at the transverse colon at 30 cm proximally to the anastomotic site. Thirdly, we consider that it is unnecessary to fully inflate the colon to expose the space during colonoscopy, because the operation of biopsy does not need to fully dilate the colon, and excessive inflation may increase the risk of perforation in children with HAEC. During colonoscopy, we used low pressure inflation, therefore, none of the children in this study had perforation. Our practice confirmed the safety of colonoscopy for HSCR patients postoperatively. Meanwhile, we consider that patients with extreme abdominal distension or peritonitis should not undergo colonoscopy unless the symptoms were relieved after conservative treatment, otherwise the risk of perforation may be very high (1).

Besides safety issues, through our practice, we also consider that colonoscopy is an efficient and accurate examination for the diagnosis of postoperative hematochezia or fecal occult blood. One of the most important reasons is that obtaining tissue samples at the suspicious lesions for pathological diagnosis is the most direct evidence and most accurate scientific fundament, which provides evidence for determining clinical treatment (16). We found that the pathological characteristics of HAEC were acute or chronic inflammatory changes with lymphoid tissue hyperplasia and lymphoid follicle formation in lamina propria, and most of the bleeding may be caused by mechanical stress stimulation when feces pass through and destruction of mucosal barrier after toxin absorption (17). In those with anastomotic inflammation, biopsy of inflammatory and ulcerative surface near the anastomotic site indicated chronic inflammatory cell infiltration, which was accompanied by focal bleeding and extensive erosion. It is often due to mechanical stimulation of stool caused by poor anastomosis techniques, delayed suture reaction, suture shedding, and anastomotic stenosis, resulting in long-term inflammatory stimulation of the anastomotic site and adjacent mucosal tissue, which lead to ulceration and bleeding (18). If the anastomotic inflammation does not heal in time, part of the anastomotic site may form inflammatory pseudopolyps secondary to repeated bouts of intense inflammation (19), leading to periodic bleeding which is similar to the symptoms of juvenile polyps. For this part of patients with inflammatory hemorrhagic changes, saline, thrombin, and other drugs for local irrigation treatment were applied directly under colonoscopy. What is more, colonoscopy provides us a timely and objective basis for the reoperation of children with leakage or blind rectal pouch formation after Duhamel's procedure, so as to avoid delaying the opportunity of surgical treatment (20). Finally, through retrospective analysis, we found that the incidence of diarrhea in HAEC group was significantly higher than that in anastomotic leakage group, and the incidence of fever was the highest in patients with leakage, which were both consistent with a previous literature (21).

We have to recognize that there were some limitations in this study. Since our department started to set up independent children's digestive endoscopy center in 2016, the number of cases collected was relatively small, accounting for 7.97% of all children with HSCR undergoing operations in the same period. By using our technique and preoperative preparation, although there was no complication in colonoscopy and its accuracy was 100%, it still needs to further expand the sample size to fully evaluate the safety and accuracy of colonoscopy in children with HSCR after operation, and set up excluding criteria to avoid site injuries. Additionally, the colonoscopy does not apply to examining bleeding in short term after surgery, because we worried that it may affect the healing of the anastomosis. Thus, there is selection bias in this study. Lastly, the retrospective assessment limits the interpretation of our results, and a prospective study is expected in future.

CONCLUSION

In summary, for HSCR patients with postoperative gastrointestinal bleeding, colonoscopy is safe and effective if appropriate cases were selected carefully. Colonoscopy can accurately provide location of the lesions, clarify the characteristics and pathological changes, and therefore provide timely and reliable basis for corresponding treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JY designed and executed the study, performed statistical analyses, drafted the figures and tables, and wrote the consecutive versions of the manuscript. TZ, XW, MW, and GW performed statistical analyses, critically reviewed and commented all versions, interpreted findings, and contributed to the discussion. JF conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors agree to be accountable for the content of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.672767/full#supplementary-material

Supplementary Video 1 | Remote hemorrhage in the colorectal cavity.

Supplementary Video 2 | Diluted methylene blue injection injected from the external fistula was observed flowing out of the internal fistula.

Supplementary Video 3 | Blind rectal pouch.

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REVIEW
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The Emerging Genetic Landscape of Hirschsprung Disease and Its Potential Clinical Applications

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Hirschsprung disease (HSCR) is the leading cause of neonatal functional intestinal obstruction. It is a rare congenital disease with an incidence of one in 3,500–5,000 live births. HSCR is characterized by the absence of enteric ganglia in the distal colon, plausibly due to genetic defects perturbing the normal migration, proliferation, differentiation, and/or survival of the enteric neural crest cells as well as impaired interaction with the enteric progenitor cell niche. Early linkage analyses in Mendelian and syndromic forms of HSCR uncovered variants with large effects in major HSCR genes including *RET*, *EDNRB*, and their interacting partners in the same biological pathways. With the advances in genome-wide genotyping and next-generation sequencing technologies, there has been a remarkable progress in understanding of the genetic basis of HSCR in the past few years, with common and rare variants with small to moderate effects being uncovered. The discovery of new HSCR genes such as neuregulin and *BACE2* as well as the deeper understanding of the roles and mechanisms of known HSCR genes provided solid evidence that many HSCR cases are in the form of complex polygenic/oligogenic disorder where rare variants act in the sensitized background of HSCR-associated common variants. This review summarizes the roadmap of genetic discoveries of HSCR from the earlier family-based linkage analyses to the recent population-based genome-wide analyses coupled with functional genomics, and how these discoveries facilitated our understanding of the genetic architecture of this complex disease and provide the foundation of clinical translation for precision and stratified medicine.

Keywords: Hirschsprung disease, aganglionosis, genetics, genetic architecture, rare variants, GWAS, next-generating sequencing, common variants

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INTRODUCTION

Hirschsprung disease (HSCR), also known as colonic aganglionosis, is the leading cause of neonatal functional intestinal obstruction. It is a rare congenital developmental defect of the enteric nervous system (ENS) with a global incidence of 1 in 3,500–5,000 live births. The incidence of the disease varies widely among ethnic groups and is the highest among Asians (2.8/10,000 live births) (1, 2). The disease is characterized by the absence of enteric ganglia in the distal colon due to the failure of enteric neural crest cells (ENCCs) to fully colonize the hindgut during embryonic development. The incomplete innervation may result from any or a combination of genetic defects

and environmental factors affecting migration, proliferation, differentiation, and survival of the ENCCs as well as impaired extracellular milieu or impaired interaction between the migrating neural crest-derived precursor cells and the mesenchymal microenvironment through which they migrate (3, 4).

HSCR is traditionally classified by the extent of aganglionosis. In short-segment HSCR (S-HSCR; 80% of cases), aganglionosis does not extend beyond the sigmoid region whereas in long-segment HSCR (L-HSCR; 20% of cases), it extends proximal to the sigmoid colon. Among the L-HSCR, if the aganglionosis extends to at least ileocecal valve, it is termed total colonic aganglionosis (TCA), which represents approximately 5% of all HSCR cases. The majority of HSCR cases (∼70%; isolated HSCR) occur as isolated anomalies. Other cases are syndromic (syndromic HSCR) and often encountered with chromosomal aberrations and/or a range of other congenital malformations (1, 2, 5–8). The inheritance of HSCR is considered to be complex. It most commonly presents as sporadic forms (80–90%), which are more often S-HSCR and follow a multifactorial inheritance pattern. The remaining 10–20% of cases are familial and tend to be of L-HSCR/TCA with autosomal dominant inheritance (2, 9). HSCR also exhibits significant sex bias with a marked male preponderance. Especially for S-HSCR, the disease prevalence is four times higher in males than in females. Up till now, the only definitive treatment available for HSCR is surgery.

Over the past decades, through human genetic studies—beginning with the classical positional cloning, linkage analyses, and candidate gene screening—through genome-wide association studies (GWAS)—to the recent next-generation sequencing (NGS) studies, coupled with validation by functional genomic studies, many genetic variants and genes have been linked to HSCR. Majority of these genes belong to several key biological pathways that often crosstalk and have been shown to orchestrate the dynamic process of ENS development and HSCR pathogenesis, though some initially unsuspected candidate genes have also been uncovered by the bias-free approaches. These studies have provided solid evidence that most HSCR cases have complex (oligogenic/polygenic) genetic basis wherein multiple HSCR-associated common variants contribute to the genetic predisposition and modify the penetrance of rare damaging variants in disease-relevant genes and hence disease manifestation.

In this review, we explore the paradigm shift in genetic discoveries of HSCR from family-based, candidate gene studies to population-based, genome-wide analyses with the advent of genotyping and sequencing technologies and how these discoveries furthered our understanding of the genetic architecture of this complex disorder. This review primarily focuses on the emerging genetic landscape of rare coding variants, common regulatory variants, and the interplay between them as well as their differential contribution to HSCR and the disease subtypes, and how the new knowledge may pave the way for genome data to be incorporated as part of "routine" in the precision-medicine era.

PRE-GWAS ERA: IDENTIFICATION OF CORE HSCR PATHWAYS THROUGH LINKAGE MAPPING AND CANDIDATE GENE STUDIES ON SYNDROMIC AND FAMILIAL HSCR CASES

Genetic analyses to uncover the genes underlying HSCR began in late 1980s/early 1990s. **Table 1** lists the main genes known to be involved in either syndromic or isolated HSCR. In the early days, analyses were primarily focused on the more Mendelian forms of syndromic and familial HSCR. These studies built upon the fundamental idea that disease causal variant and nearby genetic markers tend to be transmitted together due to linkage disequilibrium (LD). Such approach of positional cloning and linkage analysis have been applied to multiplex families where highly informative genetic markers are used to map the disease-associated loci of large effect. Once a locus is linked, a search for rare damaging mutations (i.e., variants with minor allele frequency <1% in general population) in candidate genes within the locus is ensued. This strategy remained very popular especially before the GWAS era. In fact, the two major HSCR genes—*RET* (in 10q11.2) and *EDNRB* (in 13q22.3)—representing the two core HSCR pathways as well as transcription factors underlying syndromic form of HSCR were initially discovered with this approach in the early 1990s (**Table 1**).

RET Signaling

RET (Rearranged during Transfection) proto-oncogene encodes a transmembrane tyrosine-protein kinase receptor. It is activated by glial cell line-derived neurotrophic factor (GDNF) family ligands, such as GDNF, NRTN (neurturin), ARTN (artemin), PSPN (persephin), and co-receptors named GDNF-family receptor-α (GFRα1-4) (34). *RET* plays a pivotal role in both isolated and syndromic HSCR. The *RET* locus was first identified as a susceptibility locus for HSCR through linkage analyses in multiplex HSCR families (35, 36) facilitated by the finding of deletion of the proximal long arm of chromosome 10 in patients with isolated HSCR (37, 38) and the co-occurrence of HSCR and multiple endocrine neoplasia type 2 (MEN2) (39, 40). This was followed by the identification of numerous *RET* mutations, including missense, splicing variants, and short insertions and deletions (indels), across the whole spectrum of patients with HSCR occurring both as *de novo* and inherited events (10–13, 41–52). Damaging coding mutations in *RET* are identified in approximately 50% of familial and 15–20% of sporadic HSCR cases (1, 10–13), and it gradually becomes evident that rare coding variants in *RET* appear to play a less prominent role in sporadic and S-HSCR compared to the familial and L-HSCR (51, 53). Furthermore, somatic *RET* mutations have also been reported occasionally—occurring either as transmission of mosaic germline mutation from unaffected parent to affected patient or as somatic mutation in patients (54–57).

Subsequent to the discovery of *RET* in HSCR pathogenesis, mutation screening was performed on interacting partners and other key members of RET signaling pathway with reference to known biological processes and animal models. Damaging rare

TABLE 1 | Genes reported with rare damaging protein-altering variants in patients with HSCR.

Gene	Phenotype	Frequency[a]	References
RET	Isolated HSCR	50% familial 15–20% sporadic	(10–13)
	MEN2A with HSCR		
	MEN2B with HSCR		
	FMTC with HSCR		
GDNF	Isolated HSCR	Rare	(14–16)
NRTN	Isolated HSCR	Very rare	(17)
ARTN	Isolated HSCR	Very rare	(17)
PSPN	Isolated HSCR	Very rare	(17)
GFRA1	Isolated HSCR	Rare	(16, 18)
EDNRB	Isolated HSCR	Rare	(16, 19)
	Shah-Waardenburg syndrome		
EDN3	Isolated HSCR	Very rare	(18)
	Shah-Waardenburg syndrome		
ECE1	HSCR with cardiac, craniofacial, and autonomic defects	Very rare	(20)
SOX10	Isolated HSCR	Very rare	(21)
	Shah-Waardenburg syndrome		
PHOX2B	Haddad syndrome (Congenital Central Hypoventilation Syndrome with HSCR)	Very rare	(22)
	Neuroblastoma with HSCR	Very rare	
	HSCR with dysmorphic facial features	Very rare	
ZEB2	Mowat–Wilson syndrome	Very rare	(23)
KIAA1279 (KIFBP)	Goldberg–Shprintzen syndrome	Very rare	(24–26)
NRG1	Isolated HSCR	Rare	(27)
ERBB2	Isolated HSCR	Rare	(16)
SEMA3C/D	Isolated HSCR	Rare	(18)
IHH	Isolated HSCR	Very rare	(28)
GLI1	Isolated HSCR	Very rare	(29)
GLI2	Isolated HSCR	Very rare	(29)
GLI3	Isolated HSCR	Very rare	(28, 29)
L1CAM	X-linked hydrocephalus	Very rare	(30)
ITGB4	Isolated HSCR	Rare	(16)
PTK2	Isolated HSCR	Rare	(16)
DENND3	Isolated HSCR	Very rare	(31)
NCLN	Isolated HSCR	Very rare	(31)
NUP98	Isolated HSCR	Very rare	(31)
TBATA	Isolated HSCR	Very rare	(31)
VCL	Isolated HSCR	Very rare	(32)
BACE2	Isolated HSCR	Rare	(16)
ACSS2	Isolated HSCR	Rare	(18)
ENO3	Isolated HSCR	Rare	(18)
SH3PXD2A	Isolated HSCR	Rare	(18)
UBR4	Isolated HSCR	Rare	(18)

Table is updated and adapted from (2) and (33).
[a]*Rare: Variants detected in 1–7% of patients with HSCR screened and reported.*
Very rare: Variants detected in <1% of the patients with HSCR screened and reported.

variants in all four ligands of RET have been detected in patients with HSCR, yet *RET* still contributes to the vast majority of rare variants reported in genes in this core HSCR pathway (13–15, 17, 28, 51, 58–62). Mutations in these GDNF family ligands typically co-occurred with variants in other major HSCR genes, particularly in *RET*, except in a few instances (59, 61). As for those exceptions, the patients may also carry variants in other HSCR genes not yet discovered by that time. This is exemplified by a recent whole exome sequencing (WES) study by Sribudiani et al. (28) where the authors showed that a *de novo* in-frame deletion in *GDNF* was, together with rare inherited variants in *IHH* (Hedgehog signaling pathway, described later) and its mediator, *GLI3*, responsible for HSCR in a branch of a multigenerational HSCR family. Similarly for the co-receptors, no confirmed high-penetrant pathogenic variant has yet been described in patients except a marginal increase in burden of rare damaging protein-altering variants in *GFRα1* that has been detected in a recent whole-genome sequencing (WGS) study on 443 East Asian S-HSCR cases compared to 493 controls (16, 18, 63). Altogether, these findings suggest that, in the majority of cases where variants are found in GDNF family ligands and co-receptors, they alone are not sufficient to cause HSCR and rather act as modifiers together with other risk variants (rare damaging or common regulatory risk variants) in a digenic/oligogenic pattern.

EDNRB Signaling

EDNRB (Endothelin receptor type B) gene encodes a non-specific G protein-coupled receptor for endothelins (EDN1, EDN2, and EDN3) (2). After synthesis, endothelins are converted into a shorter active form by endothelin converting enzyme (ECE-1) (2). Similar to *RET*, the contribution of *EDNRB* to the risk of HSCR was discovered by family-based studies. A HSCR susceptibility locus was first mapped to 13q22 by identity-by-descent and linkage analysis in a large, inbred, Mennonite kindred that manifested high incidence of HSCR and pigmentary disorders, the Waardenburg syndrome type 4 (WS4) (64). A follow-up study identified p.Trp276Cys mutation in *EDNRB* that showed incomplete penetrance and dosage effect in addition to being absent in some patients (65). This was followed by several reports of identification of *EDNRB* mutations in patients with HSCR and WS4 syndrome (11, 66–70). However, rare damaging variants of *EDNRB* account for only 3–7% of isolated HSCR cases (2).

Subsequent search for rare variants in genes in EDNRB pathway reported pathogenic mutations in *EDN3* and *ECE1* in patients with HSCR. In general, homozygous mutations of *EDNRB* and *EDN3* are more commonly associated with WS4 and heterozygous mutations are more commonly associated with isolated HSCR (11, 66–73). Thus far, only one heterozygous *ECE1* variant has been reported in a syndromic patient with HSCR (20).

Although rare damaging variants in EDNRB pathway genes are encountered in only a small fraction of patients with HSCR (∼5%), they generally exhibit higher penetrance and confer higher risk than variants in RET signaling pathway genes. For example, in a WES study by Tilghman et al. on European HSCR cases and controls (18), pathogenic variants in EDNRB pathway genes (seven in *EDNRB* and one in *EDN3*) were

exclusively present in HSCR cases. In addition, pathway-based odds ratio (OR) for EDNRB was considerably higher [OR = 69.03; 95% confidence interval (CI): 8.68–547.92] than that of the RET signaling pathway (OR = 16.03; 95% CI: 5.21–49.28). Consistently, *EDNRB* variants showed an overall higher risk than *RET*, for S-HSCR cases in East Asian WGS analysis (16).

Transcription Factors Critical for ENS Development

SOX10 encodes a transcription factor that is a key regulator of ENS development (74) and is implicated in Waardenburg syndrome with varying spectrum of features with or without HSCR (75–77). Although *SOX10* variants were initially thought to cause HSCR as a part of WS4 (2), recent studies identified several isolated HSCR cases with *SOX10* mutations—both in its coding region (21, 78) and in its enhancers (78–80).

PHOX2B also encodes a transcription factor required for normal development of the ENS (81). Genetic defects in *PHOX2B* have been described primarily with congenital central hypoventilation syndrome (CCHS) (82), which occur with HSCR in 15–20% of cases (83) and in some reports with neuroblastoma (84, 85). These strongly suggest a pathogenic role of *PHOX2B* in HSCR. In terms of rare coding variants, a *de novo* in-frame deletion in *PHOX2B* in a female patient with L-HSCR and other anomalies but without clinical manifestations of CCHS or neuroblastoma has been described (22). However, unlike *SOX10*, *PHOX2B* pathogenic variants in isolated HSCR are yet to be found. In addition to rare variants, common *PHOX2B* polymorphisms were shown to be moderately associated with HSCR in a Chinese population (86, 87) and interaction between *RET* and *PHOX2B* polymorphisms was demonstrated to significantly increase HSCR risk (88).

ZEB2, previously known as *SIP1* or *ZFHX1B*, encodes a transcription factor that is crucial to direct the formation, migration, and specification of neural crest cells (89). Variants in this gene have been described with many patients with Mowat–Wilson syndrome, which includes HSCR in its phenotypic spectrum in approximately half of the cases (23). However, *ZEB2* pathogenic variants have not been described in isolated HSCR yet. No enrichment for rare variants in *ZEB2* was found in S-HSCR cases compared to controls, indicating that it is likely to be a very rare contributor to isolated HSCR (16).

Genes Related to Cytoskeleton

Homozygosity mapping followed by sequence analysis in a consanguineous family with multiple members having Goldberg–Shprintzen syndrome (GOSHS) identified homozygous truncating mutations in *KIAA1279 (KIFBP)* (24). HSCR has been reported in 64% of patients with GOSHS (25). Thus far, 16 different variants in this gene have been described in patients with GOSHS (25). *KIAA1279* encodes for KIF-binding protein (KIFBP), which interacts with microtubule and actin filaments and plays a role in neurite outgrowth, neuronal development, and differentiation (90, 91). Mice null for this protein show delayed gut colonization by the neural crest-derived cells (92). KIFBP was shown to interact with several kinesins and SCG10 (a microtubule destabilizing protein), implicating the potential role of cytoskeleton/microtubule-related defects in HSCR pathogenesis (90). Related to this, it should also be noted that a slightly lower intensity of immunoreactivity of Microtubule-Associated Protein 5 (MAP5) was noted in the intestinal tissue of the aganglionic segment of patients with HSCR compared to the normoganglionic segment of the same patients or intestinal tissue of control individuals (93). However, aside from the *KIAA1279* variants in patients with GOSHS, no variant has been reported in *SCG10* (94) or other related genes yet in patients with HSCR.

THE ERA OF GWAS: LARGE CONTRIBUTION OF COMMON VARIANTS IMPLICATING NEW DISEASE PATHWAYS AND EMERGING GENETIC LANDSCAPE

Altogether, the rare damaging variants identified in RET and EDNRB pathways explain only a small fraction (<30%) of sporadic HSCR cases. The inability to detect rare broadly Mendelian variants in considerable proportions of patients, along with the variable expressivity and disease severity among carriers, implied that more complex genetic etiologies are likely to underlie HSCR. Other genetic factors or epistatic regulation of *RET*, either by variants within the locus or with other unlinked genes, must exist to explain the missing heritability.

Contribution of common variants to HSCR was first indicated by (i) the variable penetrance of a damaging *RET* missense mutation (*RET* c.1859G>C;p.Cys620Ser) under different haplotype backgrounds of common variants in a multiplex family (95) and (ii) subsequent reports of overrepresentation of certain *RET* haplotypes in patients with HSCR across populations (96–98). Later, a study by Emison et al. pinpointed a common functional *RET* intron 1 enhancer variant (RET+3; rs2435357 T/C) that largely increases risk of HSCR (OR∼5). The risk allele (T) of RET+3, present in ∼60% of European and ∼80% of Asian cases, significantly reduces *RET* expression and confers higher risk for HSCR than rare alleles collectively (99). It is interesting to note that the risk allele frequency is substantially higher in the general population in East Asians than Europeans (47 vs. 23%), which could explain the higher incidence of HSCR in Asia (53). In contrast to rare damaging variants, contribution of this common enhancer variant is considerably higher in the major subtype of sporadic and isolated S-HSCR than the severe forms (53). Furthermore, another *RET* intron 1 risk variant (rs2506004 A/C), which is in near complete LD with rs2435357 ($r^2 = 0.99$ in Asians and 1 in Europeans) and separated by only 217 base pairs from it, was also identified (100, 101). The risk allele (A) alters *RET* expression by interfering with the binding of transcription factors NXF, ARNT2, and SIM2 to this locus exemplifying that more than one variant may be relevant to disease pathogenesis in the same disease-associated haplotype (101). Altogether, these findings implied that common variants can predispose to HSCR in a low penetrance manner by modifying the phenotypic expression, which opened up a new area of genetic research on HSCR, including family-based and population-based association studies

by detecting transmission disequilibrium of common single-nucleotide polymorphisms (SNP) from parents to proband and comparing frequencies of SNPs in cases vs. controls, respectively (53, 102–110). However, earlier association studies still relied on prior biological knowledge to detect association of variants in candidate region(s) and, with this candidate gene approach, no confident association beyond the *RET* locus could be detected or replicated.

The advances of high-throughput SNP array, assaying hundreds of thousands of SNPs simultaneously, permitted the transition to a hypothesis-free approach for a powerful detection of association in genome-wide scale, termed GWAS. Thus far, five array-based GWAS have been published on HSCR, which unraveled two novel, highly replicable GWAS significant loci—*NRG1* and *SEMA3C/D*—in addition to *RET* (shown in **Table 2**) (102–108, 110). Transethnic meta-analysis of GWAS discovered universal as well as ancestry-specific risk alleles, highlighting the heterogeneity of the genetic architecture of this disease (111). Other common SNPs that were found with moderate associations in GWAS includes SNPs in or nearby *PHOX2B*, *JAG1*, and *VRK2*. Epistatic interaction within and between *RET* and other loci were discovered from these GWASs as well. Novel insights and pathways previously not linked to the genetic etiology of HSCR but aroused from findings of GWAS are summarized below.

Neuregulin Signaling

Neuregulins (NRGs) are a family of growth factors that stimulate receptor tyrosine-protein kinase erbB (ERBB receptors) and are important regulators of neuronal migration and glia formation (112). Strong association of common SNPs (rs7835688 and rs16879552) lowering expression of *NRG1* was first identified in a GWAS on Chinese population and was widely replicated across Asian studies (102, 104, 105, 113–115). Interestingly, the association was also reported in some Caucasian studies albeit with smaller and variable effect size (108, 110, 111). Following the GWAS discovery, patient-specific rare damaging coding variants were found in both Chinese and European sporadic HSCR cases. Functional analyses of these variants showed aberrant expression and uneven intracellular distribution of the mutant NRG1 proteins, proving that not only common variants but also rare coding variants in *NRG1* can increase HSCR risk (27, 106). Besides *NRG1*, genome-wide copy number variation (CNV) analysis discovered the association of intronic deletions and duplications in *NRG3*, a paralog of *NRG1*, with HSCR on the same Chinese SNP array dataset (116). The recent population-based WGS study further identified a fourfold increase in the number of damaging rare variants in *ERBB2*, encoding the receptor for NRG1, in patients with HSCR compared to the controls (16). Altogether, these findings firmly established NRG-ERBB as one of the core HSCR pathways with significant contribution of both common and rare variants to disease pathogenesis.

Semaphorin Signaling

Semaphorins are extracellular signaling molecules that can directly bind to several receptor protein families particularly plexins and neuropilins. Semaphorins are important regulators of axon guidance and neural crest cell migration (117). Their involvement in HSCR pathogenesis has only been brought to focus with the identification of GWAS significant association signals within the intergenic region of *SEMA3* gene cluster from the European and Danish GWASs (108, 110). Comparing the temporal and spatial localization with Ret in mouse ENS, *SEMA3D* and *SEMA3C* were considered to be the most likely candidate gene targets of the association in the cluster. Their roles in HSCR were further supported by the plausible interaction with *RET* such that co-knockdown of both *sema3c/d* and *ret* gave a more severe, aganglionic phenotype in zebrafish model. Recently, a twofold excess of rare deleterious variants in *SEMA3C* and *SEMA3D* was also observed in patients from a WES study (18).

Hedgehog and Notch Signaling

Hedgehog (Hh) signaling is mediated by transmembrane proteins Patched (PTCH1) and Smoothened (SMO) and the downstream effector GLI transcription factors. Notch receptors (NOTCH1 and NOTCH2) are transmembrane proteins activated by their ligands (DLL1 and DLL3). They are crucial regulators of ENS development (29), and key members of these pathways were suspected to be involved in HSCR pathogenesis. Using the Chinese GWAS data (102), a significant epistatic interaction between genes of these pathways (particularly driven by *PTCH1* and *DLL3* SNPs) conferring higher risk to HSCR was discovered (29). Functional analysis validated the interaction between the two signaling pathways and the Hedgehog/Notch interaction was shown to coordinate the proliferation and differentiation of ENCCs. Another study on an independent set of Chinese patients also suggested that common variants in *PTCH1* may predispose to HSCR (118). Later, using targeted sequencing in 20 Chinese sporadic patients with HSCR devoid of *RET* coding sequence mutations, four rare coding variants in *GLI1*, *GLI2*, and *GLI3* were identified, and these mutations were demonstrated to enhance GLI transcriptional activity (119). Finally, as mentioned earlier, inherited pathogenic variants of *IHH* and *GLI3* genes, together with a *de novo GDNF* in-frame deletion, led to the manifestation of HSCR phenotype in one branch of a Dutch multiplex family, whereas the *RET* coding variant and intron-1 variant were responsible for the disease manifestation in another branch (28). These findings suggest that, although the reported variants in Hedgehog and Notch signaling pathway genes in patients with HSCR contribute to a small fraction of patients at present, more patients may be expected to be reported in the future especially with the increasing application of NGS and under oligogenic model harboring multiple variants in these genes.

Epistasis (Genetic Interaction) Is an Important Part of the Genetic Architecture of HSCR

Summarizing findings of GWAS studies, it becomes increasingly apparent that epistasis is an important component of the genetic architecture of HSCR, which contributes substantially to the modified penetrance of disease causal variants. In particular, the common *RET* enhancer variant (rs2435357) plays a pivotal

TABLE 2 | Major GWAS loci associated with HSCR.

Gene	SNP	Population	Relevant information	Reference
Common variants (MAF > 0.05) with strong association				
RET	rs2505998 (A/G)[a]	• Asian • European • Danish • Swedish • Finnish	• Strong effect: OR[b] = 3.57–7.49 in different populations • Predicted to affect the expression of *RET*	(108, 111)
NRG1	rs7005606 (G/T)[c]	• Asian • European	• Moderate effect: OR = 2.12 (95% CI: 1.70–2.63; $p = 1.11 \times 10^{-11}$) in Asians, OR = 1.64 (95% CI: 1.25–2.15; $p = 4.0 \times 10^{-4}$) in Europeans • Predicted to affect the expression of *NRG1*	(111)
Low-frequency variants (MAF 0.01–0.05) with strong association				
RET	rs9282834 (A/G)	Asian	• Exonic missense but predicted to be benign • Strong effect: 10-fold increase of HSCR risk when the risk allele exists in compound heterozygous with the risk allele of rs2435357 • May interrupt *RET* expression or function	(111)
RET	rs144432435 (T/C)	• Danish • Swedish	• Strong effect: OR = 6.6 ($p = 7.7 \times 10^{-10}$) • Mechanism of independent effect is unknown	(108)
SEMA3C/D	rs80227144 (A/C)[d]	• European • Danish • Swedish • Finnish	• Moderate to strong effect: OR = 1.88 to 5.2 in different population • May modulate activity of transcription factors GATA6 and SOX7 • Monomorphic and no association in Asians	(108, 110, 111)

[a] *The risk allele A of rs2505998 is in LD with risk allele T of rs2435357 ($r^2 = 0.98$).*
[b] *OR, odds ratio.*
[c] *The risk allele G of rs7005606 is in LD with risk allele G of rs7835688 ($r^2 = 0.71$ in East Asians and $r^2 = 1$ in Europeans of the 1000 Genomes Project).*
[d] *The effect allele A of this SNP is in phase with the common risk allele C of rs11766001 in the original European GWAS by (110). The GWAS array in the study by (108) did not include rs80227144. The lead SNP in the study was rs117617821 (C/T), which is in high LD with rs80227144 ($r^2 = 0.97$).*

role in epistatic interaction within and between HSCR genes and represents the main contributor to the sensitized genetic background of patients.

Within the *RET* locus, synergistic interaction between rs2435357 and other common/low-frequency/rare variants was observed. As reported in the transethnic meta-analysis, the low-frequency, Asian-specific missense variant (rs9282834) encoding RET D489N has no effect on HSCR risk alone (OR = 1.1); however, when it occurred *in trans* with rs2435357 (OR = 3.2), D489N increased risk of HSCR by at least 10-fold (OR = 16.7/26.7 in Chinese/Korean, respectively) (111). In addition, in the Chinese S-HSCR WGS study, the genetic effects of other rare *RET* coding mutations were largely modified to different degrees depending on the predicted pathogenicity in the presence of the common risk alleles. Again, no increase in disease risk was detected for individuals carrying only a single *RET* missense damaging or benign mutation; however, for individuals heterozygous with rs2435357, damaging missense *RET* mutations conferred ∼5-fold increase in risk of HSCR on top of the effect of rs2435357. Similarly, for individuals with at least two high-risk alleles occurring *in trans* (i.e., rs2435357 TT or compound heterozygous for rs2435357 T allele and rs9282834 A allele), a non-damaging *RET* missense mutation also conferred a twofold increase in disease risk additionally. On the other hand, three *cis*-acting regulatory variants in three distinct enhancers of *RET* [rs2506030 (G/A), rs7069590 (T/C), and rs2435357 (T/C)] were shown to increase HSCR risk synergistically in patients of European ancestry (109). Functional studies have provided valuable biological insights underlying the genetic interaction. These risk alleles might disrupt the binding sites of RARB, GATA2, and SOX10, respectively, and their combined effect significantly dysregulated the expression of *RET* and other functionally related genes in the gene regulatory network through positive and negative feedback. These results altogether demonstrated how the *RET* enhancer allele may modify the penetrance of other coding variants or amplify its effect in conjunction with other enhancers to affect the phenotypic expression.

Moreover, the effect of variants in other HSCR genes may also depend on the *RET* genetic background. Interestingly, both of the new GWAS significant loci were shown to interact with *RET*. A genetic interaction between *RET* and *NRG1* was observed (interaction $p = 0.0095$), in which the odds ratio increased by twofold for the *RET* rs2435357 risk genotype (TT) in the presence of *NRG1* rs7835688 heterozygote. Such genetic interplay was later confirmed functionally by showing that Nrg1 inhibited the Gdnf-induced neuronal differentiation and Gdnf negatively regulated Nrg1 signaling by downregulating the expression of its receptor, ErbB2 (120). Likewise, the frequency of *RET* rs2435357 was higher for subjects with the *SEMA3C/D* rs12707682 risk allele, which is in line with the synergistic effects on gut innervation observed by co-knockdown of *sema3c/d* and *ret* in the zebrafish

model. In mice, the reduced dosage of *Ret* or *Ednrb*, the second most mutated HSCR gene, independently yielded no obvious ENS phenotype, albeit combining the two oligogenic-null heterozygote models gave rise to aganglionic phenotype (121). In summary, these findings implied that the penetrance of mutations/functional variants in other HSCR susceptibility genes may also be modulated by *RET* variants and that a sensitized background of aberrant *RET* expression might be necessary for other genetic factors to act upon for disease manifestation (122).

POST-GWAS: IDENTIFICATION OF HSCR-ASSOCIATED RARE VARIANTS AND GENES BY UNBIASED APPROACH USING NGS TECHNOLOGY

The decreasing cost and hence increased adoption of NGS is expected to lead to a new era in genetic analysis of HSCR. WES and WGS studies investigating HSCR-associated rare variants in an unbiased manner are emerging and new biological insights not limited to differentiation, proliferation, and migration properties of the ENCCs begin to unveil.

Aberrant Extracellular Matrix (ECM) Composition Involving Focal Adhesion/ECM–Receptor Interaction

During the ENS development, ENCCs migrate from the neural tube and enter the foregut, and then migrate along the gastrointestinal tract in a rostrocaudal direction. The directed cell migration requires a coordinated interaction with the microenvironment including the ECM. Theoretically, genetic defects perturbing the interaction between cell and ECM may affect the migration and colonization of the ENCCs. In a recent WGS study on the severe forms of HSCR, genes with rare *de novo*, recessive or digenic variants in patients with L-HSCR/TCA were shown to be enriched for ECM–matrix receptor interaction (19). Furthermore, a significant enrichment of damaging rare variants in genes encoding cell-adhesion proteins, *ITGB4* (Integrin beta-4) and *PTK2/FAK* (Focal adhesion kinase), was identified in an independent cohort of patients with S-HSCR (16). Integrins are a large family of cell surface receptors that connect ENCCs to the ECM. Upon activation, integrins undergo conformational change to recruit signaling molecules such as FAK and vinculin (VCL), a membrane-cytoskeletal protein in focal adhesion plaques, for the cell–matrix adhesions. Interestingly, a mutation in *VCL* (M209L) was also found in a Chinese patient with S-HSCR from integrative WES and transcriptomic analysis (32). Subsequent CRISPR/Cas9-mediated correction of the mutation in patient-specific induced pluripotent stem cells (iPSCs) efficiently rescued the differentiation and migration defects of the iPSC-derived ENCCs. In fact, abnormalities in the ECM composition in the affected bowel of patients with HSCR were noted decades ago (123). ECM proteins, such as laminins, collagens, tenascin, and fibronectin, were functionally shown to be involved in ENS development (124–126). The hypothesis that genetic defects affecting ECM composition underlie HSCR is further supported by the mouse models in which (1) loss of β1 integrin in ENCC resulted in colonic aganglionosis (127) and (2) lineage-specific upregulation of *Col6a4* in Holstein mouse model increased total collagen VI protein levels in the ECM and resulted in slower migration of ENCCs and decreased extent of bowel colonization (128).

Neuronal Death Involving the BACE1–APP–BACE2 Pathway

Another novel finding from the NGS study was the stark excess of rare protein-altering variants in β-secretase 2 gene (*BACE2*) identified from patients with S-HSCR (16). *BACE2* is a homolog of *BACE1* encoding a protease that cleaves the amyloid precursor protein (APP) in the beta amyloid (Aβ) region and prevents its formation. Accumulation of Aβ induces neuronal death, representing the underlying cause of Alzheimer's disease. Using the iPSC platform, a patient-specific *BACE2* rare variant was demonstrated to significantly reduce the APP processing activity of BACE2 and resulted in accumulation of Aβ and thereby apoptosis of enteric neurons. Similarly, correction of the mutation using a genome editing approach ameliorated the apoptotic phenotype. Together with the marginal association of common variants of *PLD1* that may negatively regulate Aβ formation (16, 129, 130), these findings shed light on the important role of APP processing in HSCR pathogenesis.

Other New Findings From NGS Studies

Up till now, many other genes have been reported to be associated with HSCR from trio-based and case–control NGS studies, albeit these studies are more focused on the coding region. Functional characterization of all these genes remains a daunting task—even with the use of CRISPR/Cas9-mediated knockout or morpholino-mediated knockdown in zebrafish models in a relatively fast manner, not to mention elucidating the underlying disease mechanisms. Some of these new genes with functional support for their roles in ENS development include *DENND3*, *NCLN*, *NUP98*, and *TBATA* discovered from *de novo* mutation analysis of L-HSCR trios (31) and *ACSS2*, *ENO3*, *SH3PXD2A*, and *UBR4* identified from the case–control WES study (**Table 1**) (18); however, how these genes predispose or cause HSCR remains to be explored. Compared to coding variants, identifying HSCR-associated functional non-coding variants from WGS studies is even more challenging and requires the development of novel analytical methods and integration of multi-omics data to facilitate their discoveries. A new framework, named MARVEL, has recently been developed and used together with functional analysis in human stem cells to identify several novel disease-associated regulatory elements, further highlighting two HSCR candidate genes—*RASGEF1A* and *PIK3C2B* (131).

ROLE OF CNVS AND CHROMOSOMAL ANOMALIES

Besides rare coding and common variants, CNVs and chromosomal abnormalities have also been frequently reported in HSCR cases. Chromosomal anomalies have been reported in up to 12% of patients with HSCR, although Down syndrome

(trisomy 21) alone can be found in as much as 10% of HSCR cases, which increases HSCR risk by 50- to 100-fold (132, 133). In fact, these chromosomal anomalies have facilitated the discovery of several classical HSCR genes. CNVs that are beyond the resolution of conventional cytogenetics may contribute to the missing heritability of HSCR. These groups of CNVs are becoming more and more detectable with the advent of SNP arrays and NGS as well as a plethora of bioinformatic tools and algorithms for accurately detecting CNVs (134–137). Importantly, there appears to be a higher burden of rare CNVs in patients with HSCR compared to controls and larger CNVs in syndromic HSCR compared to isolated HSCR (116, 138, 139). Besides those affecting known HSCR genes (18, 19, 116, 139, 140), several other genic CNVs have also been detected in patients with HSCR lately, whose contribution to HSCR are yet to be functionally characterized—though many of these are also associated with other neurodevelopmental disorders (18). Among these, deletions in 16p11.2 appear to be particularly interesting as at least six patients with HSCR have been reported to have deletion in this locus (18, 141). However, there appears to be no common overlapping region deleted in all the reported patients with HSCR. Deletion and duplication of 16p11.2 are also implicated in intellectual disability and psychiatric disorders (142); however, none of the genes in this region have yet been characterized in animal models with respect to their roles in ENS. It is possible that one or more genes in this locus may be critical for ENS development and therefore deletion of their coding regions can result in haploinsufficiency and thus incomplete gut colonization. Alternatively, loss or disruption of their regulatory elements can lead to dysregulation of gene expression, and, when added up with the sensitized genetic background, can lead to HSCR.

DISCUSSION

From the discoveries of HSCR-associated rare variants, common variants, and CNVs, it is evident that HSCR is a complex disease with involvement of multiple genes and pathways important for ENS development and function. Technological advances in genotyping and sequencing surmount the knowledge-intensive limitations of candidate gene approach and revolutionize genetic studies on HSCR, with disease-associated variants and genes

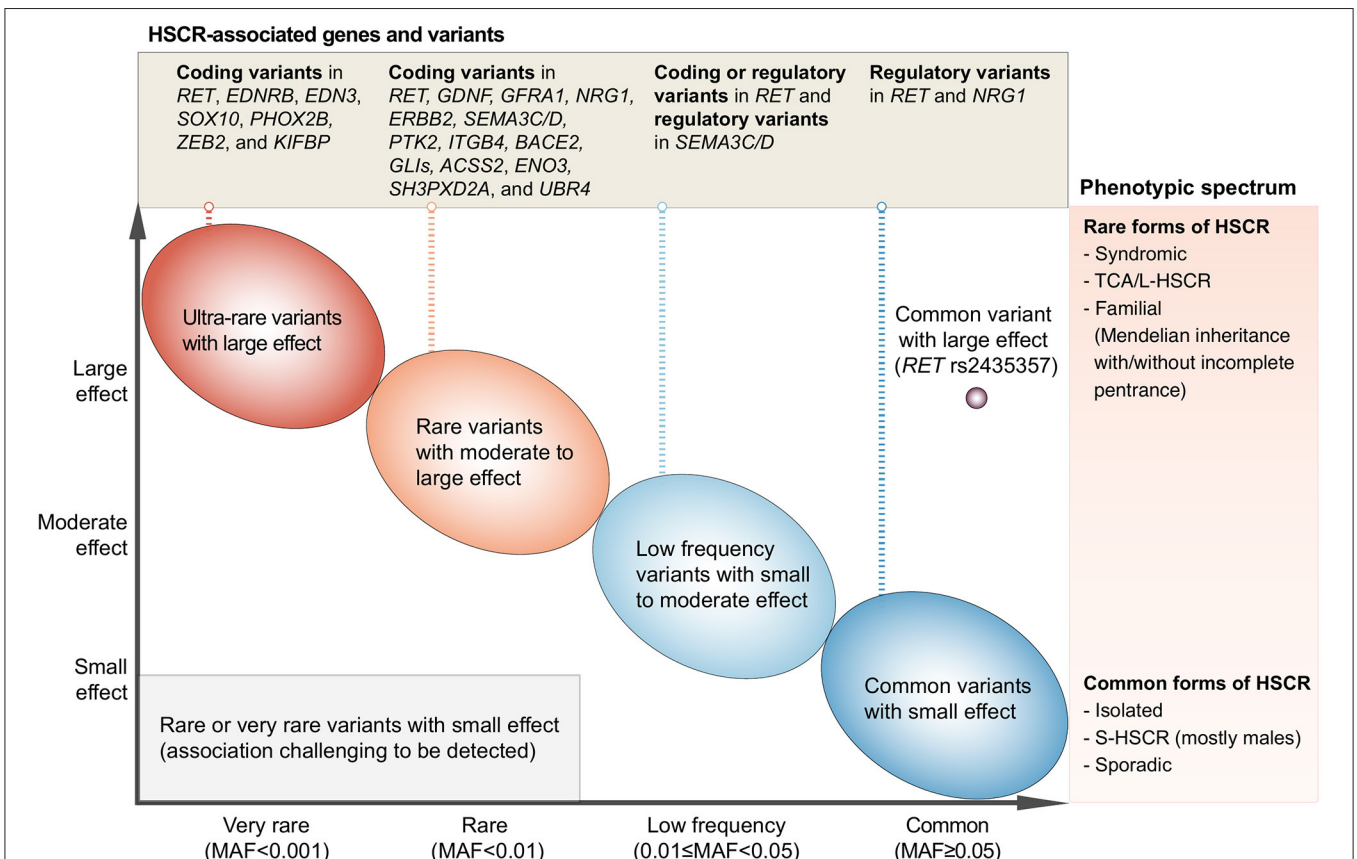

FIGURE 1 | Current understanding of the genetic architecture of HSCR. The *x*-axis denotes the minor allele frequency (MAF) of variants and the *y*-axis denotes the effect size. HSCR-associated genetic variants range from variants with extremely low MAF and very large effect size (upper-left side of the plot) to the common/low-frequency variants with small to large effect size (right side of the plot). These variants have been reported in the HSCR loci shown in the upper panel and can cause HSCR with a wide spectrum of severity as denoted in the right panel. On one end of the phenotypic spectrum lies the syndromic HSCR and TCA/L-HSCR, which are the rarer and severe form of HSCR whereas on the other end lies the commonest form of HSCR, i.e., sporadic S-HSCR.

being identified at an accelerated rate. In the past decade, the major genetic discoveries brought out by the hypothesis-free genome-wide approach have shed light into the genetic landscape and architecture of HSCR (**Figure 1**).

A varying degree of susceptibility to HSCR exists in the general population. This variation is largely due to the combinatorial effects of common, low-frequency, and rare inherited variants with increasing effect size (**Figure 1**; from right to left, from bottom to top). Indeed, the variable expressivity and phenotypic variability among carriers of the same genetic variant suggest a strong predisposition in the genetic background. The genetic findings reviewed here elegantly illustrate that common HSCR-associated variants, particularly in the major HSCR gene, *RET*, contribute not only additively but also synergistically to predisposition to HSCR. These common variants can provide a sensitized genetic background that modifies penetrance of rare disease-causing variants and, when the liability threshold is surpassed, will result in clinical manifestation. For example, a highly damaging ultra-rare loss-of-function variant in a key ENS gene (e.g., *SOX10*) is sufficient to cause the rarer form of the disease—i.e., L-HSCR/TCA—in a person with less sensitized genetic background. On the other hand, for a rare variant with moderate effect size, a highly sensitized genetic background is needed for the phenotypic expression of HSCR. Such joint and epistatic effects between and within regulatory and coding variants represent the integral component of the genetic architecture of HSCR, particularly for sporadic S-HSCR.

Another interesting point to note is that the biological pathways linked to HSCR are not independent of each other (**Figure 2**). Rather, there is considerable epistasis and crosstalk between these pathways both statistically and biologically, and a dynamic interaction of these pathways orchestrates the ENS development. A deeper understanding of the spatiotemporal crosstalk as well as the interaction between the migrating ENS precursor cells and the extracellular niche is warranted.

Despite the significant advancement in our understanding of HSCR genetics from the past three decades of research, the translation of these findings to clinical utility has yet to be established. Genetic risk prediction has potential clinical impact of assisting diagnosis, providing genetic counseling, informing treatment options, or predicting

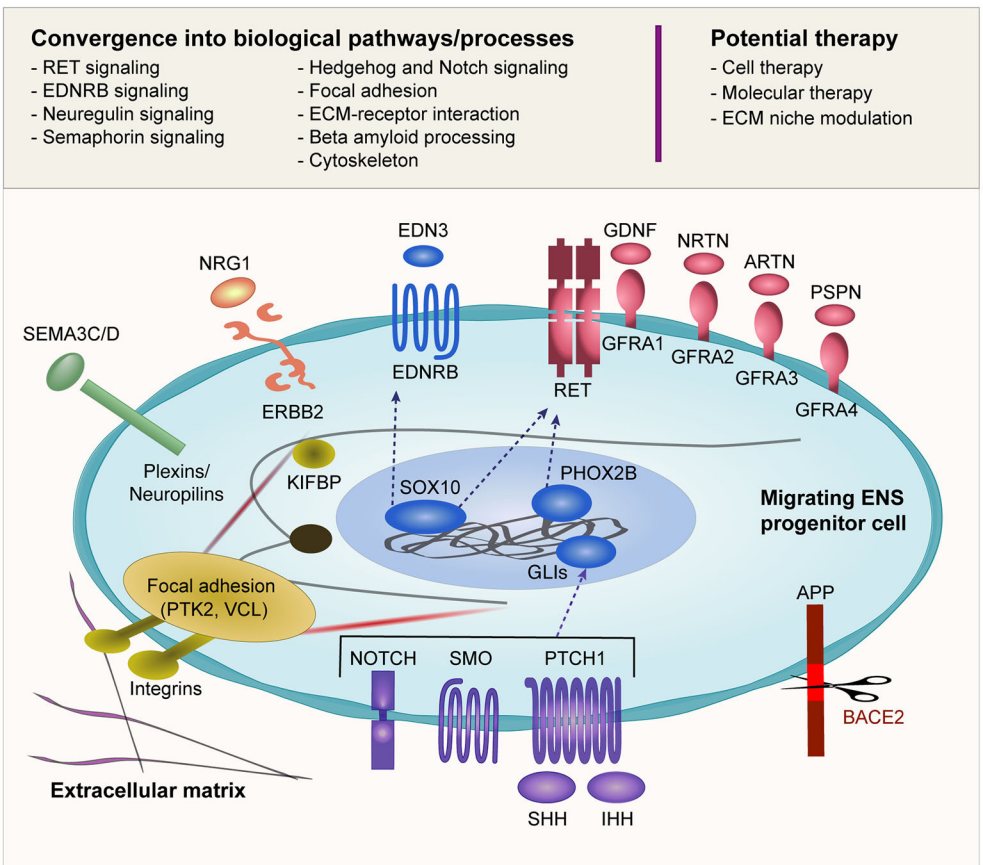

FIGURE 2 | Biological pathways implicated in HSCR. Many of the reported HSCR-associated genes converge into several biological pathways as shown in this figure. The schematic diagram illustrates an ENCC migrating through the gut and its interaction with the surrounding environment including the ECM. The ENCC receives cues from the surrounding environment through different signaling molecules that regulate the cellular machinery as well as feedback interaction with the surrounding niche. The potential therapeutic approaches that are currently under investigation are also shown.

prognosis/complication/survival. Currently, there is no evidence-based consensus guideline for genetic testing of HSCR under clinical setting. The necessary first step for the clinical implementation of genetic risk prediction can be prioritized on the HSCR patient subgroups in which the phenotypic presentation is presumed to be caused by high penetrant pathogenic genetic variants and where the incremental benefits on clinical care and decision-making are optimal. Clinical genetic testing can first be considered in the case of syndromic presentation where a genetic diagnosis of a syndrome may lead to the discovery of additional anomalies in other organs and thereby enhance clinical management to improve patient care. Another potential application of clinical genetic testing can be in multiplex family where identification of segregating pathogenic variant may assist recurrence risk prediction and therefore allow genetic counseling. However, its clinical utility on non-syndromic and sporadic patients remains to be explored. Many genetic studies have demonstrated unequivocally that genetic variants of wide frequency spectrum (coding, regulatory, and CNV/chromosomal anomalies) can capture the genetic susceptibility to HSCR albeit with low to moderate discriminative power (18). Sophisticated methods combining polygenic risk of these genetic variants may leverage the performance of risk prediction, and such polygenic model may be applied for genetic testing of the major HSCR subgroup with high genetic heterogeneity. One powerful application of polygenic risk prediction is the potential ability to predict complications; however, up till now, it remains largely unknown if there exists any gene or variant significantly associated with prognosis or complication of HSCR (e.g., enterocolitis, severity of constipation, or incontinence following surgery). Further research should be encouraged to address these knowledge gaps. With the decreasing cost of NGS, WES and preferably WGS can be used in a research setting for genetic profiling. Whenever possible, parents should be included in genetic studies to facilitate the interpretation of variants. For each patient, comprehensive profiling of coding and non-coding variants, SNPs/Indels, and CNVs/chromosomal anomalies should be carried out. Together with other omics approaches (e.g., transcriptomics) and detailed phenotyping of the patients, more HSCR-associated candidate genes and biological pathways, and their differential contribution to the disease severity and complications can be discovered. In parallel, novel therapeutic options should be explored, e.g., stem cell therapy to regenerate the ENS, molecular therapy to modulate ENS formation or ECM niche manipulation, or correction of the culprit mutation with genome editing. While these alternative therapeutic approaches are still in the rudimentary stage, we envision a future where genetic testing would impact clinical care for most of the patients with HSCR by assisting diagnosis and clinical care, predicting complications, and, more importantly, providing alternative treatment options with much better clinical outcome.

AUTHOR CONTRIBUTIONS

AK and CT wrote the manuscript. PT did the final critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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Comparison of Two Different Cut-Off Values of Scoring System for Diagnosis of Hirschsprung-Associated Enterocolitis After Transanal Endorectal Pull-Through

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Background: Hirschsprung-associated enterocolitis (HAEC) is a major contributor in the mortality of Hirschsprung disease (HSCR) patients that can occur both preoperatively and post-operatively. Several cut-off values of HAEC score have been used, i.e., ≥10 and ≥4. Here, we compared the HAEC frequency after transanal endorectal pull-through (TEPT) using two cut-offs of scoring system and associated them with the risk factors.

Methods: Cross-sectional analysis was conducted using medical records of HSCR patients who were aged ≤18 years old and underwent TEPT at our institution, Indonesia between 2009 and 2016. HAEC was determined using the scoring system with cut-off values of ≥10 and ≥4.

Results: Seventy subjects were used in the final analysis, consisting of 44 males and 26 females. There was a significant difference in one HAEC finding between the ≥10 and ≥4 cut-off groups; diarrhea with explosive stools ($p = 0.002$). The HAEC frequency was 5/70 (7.1%) and 49/70 (70%) patients using cut-off values of ≥10 and ≥4 ($p < 0.0001$), respectively. We found that patients with anemia (i.e., iron deficiency anemia) had a higher risk of HAEC after TEPT than patients with normal hemoglobin level with OR of 3.77 (95% CI = 1.28–11.1; $p = 0.027$), while no associations were found between other variables, including sex, age at diagnosis, age at definitive therapy, albumin level, and nutritional status and HAEC following TEPT ($p = 0.87$, 0.15, 0.33, 0.26, and 0.60, respectively). Also, no associations were observed between maternal education level, mother's age at pregnancy and gestational age and HAEC after definitive surgery ($p = 0.10$, 0.46, and 0.86, respectively).

Conclusions: This report is the first study comparing two different cut-off values of scoring system to evaluate the HAEC frequency after TEPT and results suggest further

using cut-off of ≥4 to expand the diagnosis of HAEC. Moreover, we also show for the first time that hemoglobin level is a strong risk factor for the HAEC development after TEPT.

Keywords: HAEC following pull-through, developing country, HAEC scoring system, transanal endorectal pull through, Hirschsprung disease, children

INTRODUCTION

Hirschsprung-associated enterocolitis (HAEC) is a major contributor of mortality for patients with Hirschsprung disease (HSCR) (1, 2). It can occur both preoperatively and post-operatively (3, 4).

The objective of surgical treatment for HSCR is to remove the aganglionic colon and to anastomose the ganglionic colon above the dentate line (1, 5). Transanal endorectal pull-through (TEPT) is the most current popular procedure in the surgical correction of HSCR since it is less invasive and has many advantages compared with the transabdominal approaches (5–9).

Various risk factors of HAEC after TEPT have been reported, such as sex, age at HSCR diagnosis and pull-through performed, however, they are still limited (10, 11). Moreover, several cut-off values of HAEC score have been used, i.e., ≥10 and ≥4 (12–15). Originally, the HAEC scoring criteria was determined using a Delphi-based consensus of experts that concluded that a score ≥10 was diagnostic of HAEC (12). Frykman et al. (16) performed a critical univariate and multivariate application on those scoring criteria, clustered some of the criteria and concluded that a score ≥4 optimized sensitivity and specificity. Therefore, they proposed a score ≥4 as diagnostic of HAEC (16). Several papers confirmed that the HAEC scoring system with cut-off of 4 increase the sensitivity, which is better for screening purposes (15, 17). Here, we compared the HAEC frequency after TEPT using two cut-offs of scoring system and associated them with the risk factors, including sex, age at HSCR diagnosis and TEPT performed, nutritional status, hemoglobin and albumin level, gestational age, mother's age during pregnancy, and maternal education level.

MATERIALS AND METHODS

Subjects

We conducted a cross-sectional analysis using medical records of patients with HSCR who were aged ≤18 years old and underwent TEPT at our institution, Indonesia. Our institution is an academic and tertiary referral hospital for patients from two provinces, i.e., Special Region of Yogyakarta and southern part of Central Java, Indonesia. Medical records with incomplete data were excluded from analysis.

This study received prior approval from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital (KE/FK/786/EC/2015, KE/FK/713/EC/2015, and KE/FK/1356/EC/2015).

TEPT

The surgical procedure was conducted based on previous reports (6, 7, 18). First, the anal mucosa was exposed by performing everting sutures throughout the anus. Subsequently, the mucosa about 0.5 cm above the dentate line was incised circumferentially. A submucosal dissection was conducted proximally about 1–2 cm, followed by full thickness of rectal wall conversion until the transition zone was determined. Frozen section was conducted to confirm the presence of the ganglion cells in the colon. Once the ganglion cells identified, as a minimum an additional 5 cm of colon was resected to make sure that the transition zone was removed along with the aganglionic colon. Final step was a colo-anal anastomosis.

HAEC Scoring System

Diagnosis of HAEC was determined by scoring system, and included patient history, physical examination, radiologic, and laboratory findings (12–15). We used scores of ≥4 and ≥10 as cut-off values for diagnosis of HAEC (13–17).

Risk Factors

We gathered data on sex, age at HSCR diagnosis, age at TEPT performed, weight and height before TEPT, preoperative albumin, and hemoglobin level.

We categorized hemoglobin and albumin level into anemia (i.e., iron deficiency anemia) and normal (19), and hypoalbuminemia and normal, respectively. The weight and height data were used to measure the nutritional status. Nutritional status of children younger than 5 years old was expressed as weight-for-age z scores (WAZ), while for children over 5 years old, it was expressed as BMI-for-age in relation to growth standards of the age and gender according to the WHO growth chart. We used WHO anthro-application to calculate the nutritional status. The scores gained were then classified as underweight-severely underweight (z <-2) and normal (+2> z >-2) (20, 21).

We also collected the gestational age, mother's age during pregnancy and maternal education level. For maternal education level, we categorized it into low (no education—elementary) and high (junior—high school—bachelor), while for gestational age and mother's age during pregnancy, we classified them into preterm and full-term and ≤35 and >35 years, respectively.

Statistical Analysis

Sample size estimation was determined using an anticipated incidence of HAEC cut-off value of ≥10 and ≥4 of 30 and 70%, respectively, a confidence level of 95% and a power of 80%. The calculated minimum size required was 46 samples. Data were presented as number and percentage for categorical variables. Univariate analysis using Chi-squared or Fisher's Exact

TABLE 1 | Baseline characteristics of HSCR patients treated with TEPT procedure in our institution.

Characteristics	*N* (%)
Sex	
Male	44 (62.9)
Female	26 (37.1)
Age at HSCR diagnosis	
Neonate (median = 20.5 [IQR, 14–26] days)	20 (28.6)
Post-neonate (median = 137 [IQR, 44–1078.25] days)	50 (71.4)
Age at TEPT performed	
Neonate (median = 21 [IQR, 15.75–26] days)	17 (24.3)
Post-neonate (median = 110.5 [IQR, 44.75–985.5] days)	53 (75.7)

HSCR, Hirschsprung disease; TEPT, transanal endorectal pull-through; IQR, interquartile range.

tests were performed for each categorical variable to evaluate the association between risk factors and HAEC incidence.

RESULTS

Baseline Characteristics

We collected 75 medical records which fulfilled the inclusion criteria and excluded 5 medical records due to incomplete data. Thus, data from 70 subjects were used in the final analysis, consisting of 44 males and 26 females. Most of our patients were diagnosed (71.4%) and operated on (75.7%) at the post-neonatal period (**Table 1**). All patients had short-aganglionosis, non-syndromic, and underwent one-stage TEPT without any prior colostomy. The median of follow-up was 131 [interquartile range (IQR), 50.5–342.5] days after TEPT.

Comparison of HAEC Frequency After TEPT Using Two Different Cut-Offs Scoring System

There was a significant difference in one HAEC finding between the ≥10 and ≥4 cut-off groups; diarrhea with explosive stools ($p = 0.002$, **Table 2**).

Next, we compared the HAEC incidence after TEPT using two cut-offs of scoring system. The HAEC frequency was 5/70 (7.1%) and 49/70 (70%) patients using cut-off of ≥10 and ≥4 ($p < 0.0001$), respectively.

Association Between Risk Factors and HAEC After TEPT

We found that patients with anemia had a higher risk of HAEC after pull-through than patients with normal hemoglobin level with OR of 3.77 (95% CI = 1.28–11.1; $p = 0.027$) when we used cut-off value of ≥4 (**Table 3**). Apart from that, there were no associations between gender, albumin level, and nutritional status with HAEC incidence following TEPT procedure ($p = 0.87$, 0.26, and 0.60, respectively).

We also observed no associations between maternal education level, mother's age at childbirth and gestational age and HAEC occurrence after TEPT ($p = 0.10$, 0.46, and 0.86, respectively).

TABLE 2 | HAEC findings in patients with HSCR after TEPT using different cut-off values.

HAEC findings	Cut-off ≥10 ($n = 5$) *N* (%)	Cut-off ≥4 ($n = 49$) *N* (%)	*p*-value
History			
Diarrhea with explosive stool	5 (100)	12 (24.5)	0.002*
Diarrhea with foul-smelling stool	5 (100)	24 (49)	0.05
Diarrhea with bloody stool	1 (20)	2 (4.1)	0.26
History of enterocolitis	3 (60)	11 (22.4)	0.10
Physical examination			
Explosive discharge of gas and stool on rectal examination	4 (80)	19 (38.8)	0.15
Distended abdomen	5 (100)	46 (93.9)	1.0
Decreased peripheral perfusion	1 (20)	3 (6.1)	0.33
Lethargy	2 (40)	17 (34.7)	1.0
Fever	5 (100)	39 (79.6)	0.57
Radiologic examination			
Multiple air fluid levels	1 (20)	2 (4.1)	0.26
Dilated loops of bowel	1 (20)	13 (26.5)	1.0
Sawtooth appearance with irregular mucosal lining	1 (20)	1 (2)	0.18
Cut-off sign in rectosigmoid with absence of distal air	0	1 (2)	1.0
Pneumatosis	0	3 (6.1)	1.0
Laboratory			
Leukocytosis	2 (40)	15 (30.6)	1.0
Shift to left	0	15 (30.6)	0.31

$p < 0.05$ was considered significant; HAEC, Hirschsprung-associated enterocolitis; HSCR, Hirschsprung disease; TEPT, transanal endorectal pull-through.

However, none of the risk factors were associated with HAEC following pull-through when we used cut-off value of ≥10 (**Table 4**).

DISCUSSION

Comparison of HAEC Frequency After TEPT Using Two Different Cut-Offs Scoring System

HAEC has remained a serious complication for patients with HSCR despite the various advancements in diagnosis and therapy. Its pathogenesis and etiology continue to be enigmatic (22). Previous studies about definitive treatment and associated risk factors of HAEC have obtained inconsistent findings (8, 9, 13). Moreover, several cut-offs of scoring system for HAEC diagnosis have been proposed (12, 13, 15–17). Our study intended to compare the HAEC frequency after TEPT between two cut-offs scoring system and identify any associated risk factors. Here, we found that the HAEC frequency after TEPT procedure in our institution was 10-times higher when using cut-off value of ≥4 (∼70%) than when using cut-off value of ≥10 (∼7%). These findings support our previous study that cut-off value of ≥4 increases the possibility to diagnose HAEC,

TABLE 3 | Association between risk factors and HAEC after TEPT using cut-off value of ≥4.

Variables	HAEC ($n = 49$)	Non-HAEC ($n = 21$)	p-value	OR (95% CI)
Sex				
Male	30	14	0.87	0.79 (0.27–2.31)
Female	19	7		Ref
Age at diagnosis				
Neonate	17	3	0.15	3.2 (0.82–12.38)
Post-neonate	32	18		Ref
Age at TEPT performed				
Neonate	14	3	0.33	2.4 (0.61–9.45)
Post-neonate	35	18		Ref
Albumin level				
Hypoalbuminemia (<3.5 g/dL)	15	3	0.26	2.65 (0.68–10.36)
Normal (≥3.5 g/dL)	34	18		
Hemoglobin level				
Anemia[ß]	32	7	0.027*	3.77 (1.28–11.1)
Normal	17	14		Ref
Nutritional status				
Under-nourished	26	9	0.60	1.51 (0.54–4.22)
Normal	23	12		Ref
Mothers' age at childbirth (years)				
≥35	7	2	0.46	1.58 (0.3–8.35)
<35	42	19		Ref
Gestational age				
Preterm	1	0	0.86	0.75 (0.03–19.21)
Full-term	48	21		Ref
Maternal educational level				
Low	13	2	0.10	3.43 (0.7–16.81)
High	36	19		Ref

$p < 0.05$ was considered significant; CI, confidence interval; HAEC, Hirschsprung-associated enterocolitis; HSCR, Hirschsprung disease; OR, odds ratio; Ref, reference; TEPT, transanal endorectal pull-through.

[ß]*Iron deficiency anemia.*

particularly the mild one (15). However, at least two novelties were noted in our current study: (1) we compared two cut-off values of HAEC scoring system in patients following transanal-approach pull-through [vs. transabdominal Soave and Duhamel pull-through (15)]; and (2) our study provided a new evidence supporting the use of cut-off value of ≥4 to increase the possibility to diagnose HAEC after TEPT from a developing country setting [vs. developed countries (16, 17)].

Frykman et al. suggested that the HAEC scoring system with cut-off of 4 [sensitivity 83.7%; specificity 98.6%, AUC 0.910 (0.85–0.97)] should be used rather than 10 [sensitivity 41.9%; specificity 100%, AUC 0.744 (0.669–0.820)] to prevent under-diagnosing patients with HAEC (16). Thus, lowering the cut-off score could double the incidence of HAEC. Here, we got the average Delphi score of 6.59 ± 2.081, which means many patients would be underdiagnosed if we had used 10 as our cut-off point.

Association Between Risk Factors and HAEC After TEPT

Interestingly, the hemoglobin level was significantly associated with HAEC incidence after TEPT ($p = 0.027$, **Table 3**). Anemia might induce dysbiosis in infant intestinal lumen which may contribute to the development of HAEC together with alterations in the intestinal barrier and impaired GI mucosal immunity (22, 23). Patients might have better nutrition and oxygen transport during an operation in a non-anemic condition which would eventually improve the surgery outcome. To the best of our knowledge, our study is the first report showing the effect of iron deficiency anemia on the incidence of HAEC after pull-through.

Our results show that the incident of HAEC after TEPT was not associated either with age at HSCR diagnosis nor age at TEPT performed ($p = 0.15$ and 0.33, respectively, **Table 3**). Previous study also reported no significant increase in post-operative HAEC following pull-through with delayed diagnosis or timing of surgery (24, 25). Another study showed that HAEC admissions decreased by 30% with each doubling of age at diagnosis. Early initial management might be more important than definitive surgery itself, for example, colonic irrigation to reduce the size of distended colon and giving probiotics to maintain intestinal integrity, and controlling cytokines' regulatory effect, which would affect the maturation and development of gut immunity (26–28).

TABLE 4 | Association between risk factors and HAEC after TEPT using cut-off value of ≥10.

Variables	HAEC (n = 5)	Non-HAEC (n = 65)	*p*-value	OR (95% CI)
Sex				
Male	4	40	0.38	2.5 (0.26–23.66)
Female	1	25		Ref
Age at diagnosis				
Neonate	0	20	0.29	0.2 (0.01–3.82)
Post-neonate	5	45		Ref
Age at TEPT performed				
Neonate	0	17	0.36	0.25 (0.01–4.8)
Post-neonate	5	48		Ref
Albumin level				
Hypoalbuminemia (<3.5 g/dL)	1	17	0.62	0.71 (0.07–6.77)
Normal (≥3.5 g/dL)	4	48		Ref
Hemoglobin level				
Anemia[ß]	3	36	0.61	1.21 (0.19–7.72)
Normal	2	29		Ref
Nutritional status				
Under-nourished	3	32	0.50	1.55 (0.24–9.88)
Normal	2	33		Ref
Mothers' age at childbirth (years)				
≥35	0	9	0.69	0.54 (0.03–10.6)
<35	5	56		Ref
Gestational age				
Preterm	0	1	0.86	3.91 (0.14–107.8)
Full-term	5	64		Ref
Maternal educational level				
Low	1	14	0.71	0.91 (0.09–8.81)
High	4	51		Ref

CI, confidence interval; HAEC, Hirschsprung-associated enterocolitis; HSCR, Hirschsprung disease; OR, odds ratio; Ref, reference; TEPT, transanal endorectal pull-through.
[ß]*Iron deficiency anemia.*

One study showed that patients with underweight status have higher proportion of colitis/enteritis after colorectal resection when compared to normal or obese patients (29). Although not statistically significant as a risk factor, malnutrition might contribute to HAEC incidence by its correlation with low hemoglobin and albumin level (29, 30).

We found that hypoalbuminemia during TEPT procedure did not correlate with HAEC incidence after TEPT procedure (**Table 3**). However, a previous study found that any decrease of albumin level from its normal level has serious outcomes for each 1 g/dl albumin level drop in colorectal surgical patients (31). This discrepancy might be due to different outcomes being measured, while we specifically looked for incident of HAEC in after TEPT procedure. Despite its insignificance as a prognostic factor for HAEC after TEPT, the level of albumin >3.5 g/dl before doing a surgery is important to be considered due to its relation to recovery time, length of stay, morbidity, and mortality in colorectal surgery (31).

Another novelty of our study is we evaluated maternal variables as possible risk factors of HAEC [vs. patient risk factors, i.e., sex, length of aganglionosis, and presence of other major anomalies (10, 11)]. Although, mothers with higher education level may have easier understanding about the acceptance of diagnostic measures, procedures, and risks, we did not find any difference in HAEC incidence between low and high education level (32).

Previous studies suggested that infants born preterm have looser tight junction, less goblet cell secretion, lesser Paneth cells number and secretory function, and lack of IgA secretion and this lack of immunity can lead to higher risk of developing HAEC (33). But, we also failed to prove the correlation and this insignificancy might be associated with the low number of preterm HAEC infants in this study, i.e., one case.

Infants born from mothers with age more than 35 years old have higher tendency to have trisomy 21, while trisomy 21 might increase the incidence of HAEC (34). It might be associated with the fact that trisomy 21 infants show less effective immune response and poorer wound healing (34). However, our study failed to show the association between mothers' age at childbirth and HAEC after TEPT.

Limitations of this study include its retrospective nature and data were subjected to different confounding variables such as variation of follow-up times and various surgeons performing

TEPT. Since the medical records were used, our study might bias toward patients with post-operative HAEC and exclude patients with fewer HAEC criteria. Moreover, further prospective multicenter study is necessary and important to clarify and confirm our results.

CONCLUSIONS

This report is the first study comparing two different cut-off values to evaluate the frequency of HAEC after TEPT and results suggest further using cut-off of ≥4 to expand the diagnosis of HAEC. Moreover, we also show for the first time that hemoglobin level is a strong risk factor for the HAEC development after TEPT.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

G, RL, BA, and FA conceived the study. RL, SK, BA, FA, RP, and G collected and analyzed the data. SK drafted the manuscript. GS, AR, GA, AD, AM, and G critically revised the manuscript for important intellectual content. G, AD, and AM facilitated all project-related tasks. All authors have read and approved the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Safety and Accuracy of Suction Rectal Biopsy in Preterm Infants

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Purpose: Most pediatric surgeons give little attention to the diagnosis of Hirschsprung disease (HD) in preterm infants. We aimed to explore the safety and accuracy of suction rectal biopsy (SRB) for diagnosing HD in preterm infants.

Methods: A retrospective review was conducted of 45 preterm patients who underwent SRB from 2015 to 2019 in our hospital. We collected the clinical characteristics and pathology results of the patients and information on follow-up. The sensitivity and specificity of SRB for HD diagnosis were calculated.

Results: The median gestational age of the patients was 35 weeks (range: 28.9–36.9 weeks), the median gestational age at biopsy was 38.6 weeks (range: 33.4–60.0 weeks), and the median weight was 2,790 g (range: 1,580–4,100 g). Fifteen patients (33.3%) were positive for HD, which was confirmed after pull-through surgery. Ganglion cells were present in 30 patients. The diagnosis of HD was excluded in 29 patients after discharge follow-up. The sensitivity of SRB ranged from 93.7 to 100%, and the specificity was 100%. No complications occurred after SRB among the patients whose biopsy age was <37 weeks (10 patients) or biopsy weight was <2,000 g (five patients).

Conclusion: SRB is accurate and safe for diagnosing HD in late preterm infants.

Keywords: suction rectal biopsy, Hirschsprung disease, preterm infants, safety, accuracy

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INTRODUCTION

Hirschsprung disease (HD) in premature infants has attracted increasing attention, as preterm infants' medical conditions have improved in recent years. Delayed passage of meconium is common in premature infants, and the incidence appears to be inversely correlated with gestational age (1). In the past, the occurrence of HD in premature infants was considered uncommon; symptoms of delayed passage were attributed to the immature development of the enteric nervous system (2). A case series in 2013 described a cohort of premature newborns with HD treated at a single center and showed that HD occurs significantly less often in premature infants than in term infants; hence, suction rectal biopsy (SRB) should be used more selectively in preterm infants (3). However, in recent years, two large population-based studies have shown that preterm HD (PHD) is comparable to term infant HD in terms of occurrence. The incidences of both are approximately 1/5,000. In addition, PHD accounts for approximately 6% of all HD. The diagnosis of HD is often delayed in premature newborns (4, 5). From the perspective of embryonic development, it is currently believed that HD is caused by the destruction of the early embryonic neuroblast migration process. The migration of neuroblasts generally occurs between 7 and 12 weeks of

gestation in human embryos (6, 7). Therefore, confirming PHD early is beneficial to clinicians so that appropriate treatment plans can be made and focus shifted to other diagnoses if a diagnosis of HD is excluded. SRB is considered the gold standard for the diagnosis of HD. However, it is often delayed until the child reaches term-adjusted gestational age due to the belief that SRB is unreliable in preterm infants. The objective of this study was to quantify the sensitivity, specificity, complications and outcomes associated with SRB in preterm infants by reviewing the data for our preterm infants suspected of having HD.

METHODS

Patients

We performed a retrospective review of preterm infants (gestational age <37 weeks) who underwent SRB from December 2015 to June 2019. We selected and documented the following data: sex, gestational age of birth, gestational age of biopsy, weight of birth, weight of biopsy, biopsy results, procedural complications, surgical procedures performed, and clinical outcome.

SRB Procedure

All SRBs were performed by the pediatric surgical fellow or attending surgeon. We used the standard techniques as described on the RBi2 website (8). Rectal irrigation was performed before the biopsy. The device with the cartridge was inserted into the anus to approximately 3 cm to 4 cm from the exit of the anal canal. At least 2 biopsies were performed posteriorly or laterally (6 o'clock or 4, 8 o'clock). Specimens were delivered to pathology in formalin. Both mucosal and submucosal layers were included in each biopsy.

The biopsies were processed and embedded using routine procedures. At least six slides were made from one tissue, with more being obtained for staining as available. Hematoxylin and eosin staining and calretinin immunohistochemical staining were performed with tissue from each patient. All pathology slides were reviewed by a pathologist with subspecialty training in pediatric pathology.

Statistical Analysis

We analyzed all the data with SPSS 23.0. Continuous variables were presented as the mean with standard deviation or median and interquartile range if the normality hypothesis test rejected the null hypothesis of normal distribution. Categorical variables were reported as counts and percentages. The sensitivity and specificity of SRB for HD diagnosis were calculated.

RESULTS

Patient Characteristics

Forty-five preterm infants underwent SRB during the study period. **Table 1** presents the demographic information of the patients in the study cohort. Males comprised 48.9% of the cohort, while females comprised 51.1%. The median age at birth was 35 weeks (range: 28.9–36.9 weeks), and the median age at biopsy was 38.6 weeks (range: 33.4–60 weeks). The median weight at biopsy was 2,790 g (range: 1,580–4,100 g). No biopsy complications were found in our cases.

TABLE 1 | Patient characteristics.

Clinical characteristic	*n* (%)
Sex	
Male	22 (48.9)
Female	23 (51.1)
Age at birth	
< 33 weeks	10 (22.2)
33–34 weeks	6 (13.3)
34–35 weeks	3 (6.7)
35–36 weeks	13 (28.9)
36–37 weeks	13 (28.9)
Age at biopsy	
<33 weeks	0
33–34 weeks	2 (4.4)
34–35 weeks	1 (2.2)
35–36 weeks	3 (6.7)
36–37 weeks	4 (8.9)
37–40 weeks	18 (40.0)
>40 weeks	17 (37.8)
Weight at biopsy	
<1,750 g	2 (4.4)
1,750–1,999 g	3 (6.7)
2,000–2,249 g	6 (13.3)
2,250–2,499 g	5 (11.1)
2,500–2,999 g	11 (24.4)
>3,000 g	18 (40.0)

Pathological Features, Treatment Strategies, and Prognosis

As shown in **Table 2**, histologic examination reports were grouped into three types. One group of patients had normal, calretinin-positive ganglion cells in the submucosa. The HD patients lacked ganglion cells with hypertrophic nerves and were calretinin negative. The third group of patients had ganglion cells in the submucosa, but some of them were hypoplastic ganglion cells and were calretinin positive. All 15 patients whose biopsy specimens lacked ganglion cells underwent surgery, namely, a pull-through operation ($n = 12$), colostomy ($n = 2$), and ileostomy ($n = 1$). The colostomy patients underwent a pull-through operation after 3 months. The ileostomy patient was confirmed to have total colonic HD. The patient's parents abandoned further treatment, so this patient had no further follow-up records. The diagnosis of HD was confirmed through demonstration of an aganglionic segment on final pathology in all 15 patients. Among the 30 patients with ganglion cells on initial biopsy, the diagnosis of HD was excluded in 29 patients after discharge follow-up. The median follow-up period was 2 years (range: 1–4 years). Five of the patients underwent ileostomy because of necrotizing enterocolitis (NEC) or bowel resection for gut stenosis after NEC. Close surgeries were performed after

TABLE 2 | Pathological features, treatment strategies, and prognosis of the patients.

Pathological diagnosis	Treatment strategies	Outcome
HD ($n = 15$)	Pull-through surgery ($n = 12$); Colostomy 2; Ileostomy (total colonic, n = 1)	Normal[a]
Normal ($n = 22$)	No surgery ($n = 17$); Surgery (NEC or gut stenosis after NEC, $n = 5$)	Normal
Hypoplasia ganglion cell partly (else normal, $n = 8$)	No surgery ($n = 8$)	One died[b], the rest were normal

[a] *Well-developed, no gastrointestinal symptoms.*
[b] *The baby's parents abandoned the treatment and went home.*

3 months. Symptoms resolved with appropriate treatment in each case. One patient died at home a few days after treatment was abandoned.

Among the 15 PHD patients (10 boys and 5 girls), 8 had rectosigmoid disease, 6 had long segment disease, and 1 had total colonic aganglionosis. The median gestational age at the operation was 38.6 weeks (range: 36.3 to 43.4 weeks), and the median weight was 2,940 g (range: 2,100 to 4,000 g). Among all the patients, 4 had a weight of <2,500 g.

The Accuracy of SRB in Preterm Infants

If we consider the patient who died after treatment abandonment whose biopsy result excluded a diagnosis of HD as a false negative since no autopsy was performed, the sensitivity of SRB was 93.7% (95% CI 67.7–99.7%), and the specificity was 100% (95% CI 85.4–100.0%) in the cohort. If the case was a true negative, the sensitivity of SRB was 100% (95% CI 74.7–99.7%), and the specificity was 100% (95% CI 85.9–100.0%).

DISCUSSION

SRB has proven to be a valuable diagnostic technique for HD since 1965, especially due to its high accuracy and minimal invasion. In recent years, it has been proven that the sensitivity and specificity of SRB are 96.8 and 99.4%, respectively, in some systematic reviews (9). SRBs are generally safe, and the most common complication is inadequate histology (10). However, another study reported that SRB and full-thickness rectal biopsy appear equivalent in their ability to provide adequate submucosa (11). The sensitivity and specificity of SRB in term-corrected infants has ranged from 46–100% and from 97–100%, respectively (12–14). Most clinicians agree that preterm patients who are suspected of having HD should not undergo SRB until reaching term-corrected age or gaining more weight. It has been confirmed that the intestinal wall muscle layer increases with age and that the intestinal wall of preterm infants is thinner than that of term infants. Therefore, in theory, the risk of bowel perforation in preterm infants undergoing rectal biopsy is greater than that of full-term or older children. Drs Meinds and Kuiper indicated that infant age influenced the accuracy of SRBs for diagnosing HD. SRB was used to identify HD in patients younger than 39 days old with significantly lower sensitivity than in older patients (50 vs. 88%). The specificity with which SRB identified infants without HD was not affected by age (average 95%) (14). However, Halleran et al. reviewed the SRB of PHD at their institute and indicated that this procedure can be performed safely in preterm infants as small as 1,590–2,000 g with high accuracy. Clinicians should not hesitate to perform a biopsy for a premature infant when clinically appropriate (15). Keyzer-Dekker found that SRB can also be reliably and safely performed in preterm-born infants. The sensitivity and specificity of SRB were 83 and 97%, respectively (13). In our cases, 10 patients were younger than 37 gestational weeks at the time of biopsy. The biopsy results suggested HD in four of them, which was confirmed after surgery. Among the remaining patients, five were cured with appropriate treatment and have developed well to date. A total of 35 patients were older than 37 gestational weeks at the time of biopsy; among them, eleven had HD, which was verified during surgery. The remaining 24 cases were cured with appropriate treatment and have developed well to date. The sensitivity of SRB ranged from 93.7 to 100%, and the specificity was 100% in our cohort.

There were a total of 16 preterm infants whose biopsy weights were <2,500 g and five infants whose biopsy weights were <2,000 g. The youngest baby was 31.5 weeks old, with a biopsy age of 33.5 weeks, and his biopsy weight was 1,580 g. His biopsy results were normal. However, it is worth noting the safety of biopsy in premature infants because of their hypoplasia. In this research, the patients were a highly selected group with a stable condition and a high pretest probability of HD. This may be one reason why we have a small cohort. In addition, before SRB for the preterm infants, we performed the procedure for the term infants hundreds of times. It is more appropriate to use a 20 ml syringe as the suction device, and pulling the piston to 6 ml is the best position for most preterm cases based on our experience. Bleeding was defined as the absence of hemorrhage <2 ml. There were no complications in the cohort. In fact, there were some complications, either bleeding or inadequacy, in our past SRB data for term infants. Very few of them needed blood transfusion therapy. The incidence of that was approximately 1/200–300. That experience ensured that we performed better for the preterm infants in the current cohort. RSBs were performed when most of the patients reached term gestational age which was related to the concept of conservative treatment (the RSB was invasive). That might be one of the reasons for the good outcomes. At the same time, nearly 40% of these infants weigh <2,500 g and the risk was similar to that of premature infants. This study demonstrates that SRB can be used to reliably diagnose PHD. Because most premature infants have delayed feces, which is a symptom similar to the manifestations of HD, it is difficult to distinguish them. The accuracy of contrast was lower in younger HD patients, while the risk of NEC may increase. From our case,

the safety and accuracy of SRB in late preterm infants were high. Early diagnosis can provide the best treatment for children in a targeted manner.

Based on the results of this study, we consider that performing SRB in premature infants with a biopsy age of older than 33 weeks is safe and accurate. Of course, more cases are needed to verify these conclusions. Investigators should evaluate the condition of the preterm infant. Bedside SRB is not universally recommended for premature infants <32 weeks old, especially those with a body weight of <1,500 g.

In addition, an experienced pathologist is very important. Immature ganglion cells may not be detected since the biopsy result is dependent on the entire process—material extraction, sectioning, staining, etc. Even if our clinicians obtain satisfactory specimens, namely, from the right region of the rectum (not taken too close to the dentate line) and of the appropriate size (at least 3 mm in diameter, and a minimum of one-third of the sample should include the submucosa according to the International Working Group of the 2009 World Congress of Gastroenterology), biopsies do not have ganglion cells on every slide. Our hospital's pathologists found that ∼6–15 slides made from each tissue were enough, while some needed to be sliced continuously or repeatedly according to the specimen. Calretinin immunohistochemical staining was also performed, and ganglion cells were identified when both the nucleus and cytoplasm were calretinin positive. However, dysplastic ganglion cells show less typical staining than normal ganglion cells, and there is no uniform definition of the developmental stages of ganglion cells, such as cell size and nucleoplasm morphology. An experienced pathologist therefore has higher accuracy when identifying those cells. According to the follow-up results of our cases, the pathological results were consistent with the clinical outcomes.

Finally, we aimed to explore the safety and accuracy of SRB in preterm infants rather than the outcomes of premature infants suspected of having HD. The patients in this study were premature infants who had distention, vomiting or other intestinal symptoms. We chose these cases retrospectively because SRB was performed. There are a number of limitations to this small, retrospective study that limit its use in a broader population. While the results are encouraging that SRB can be performed early, the complications of concern occur very infrequently, and thus, a study of this size is underpowered to identify their true incidence. For the various clinical symptoms of premature infants, the timing of biopsy still needs to be determined by clinicians.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Beijing Children's Hospital (2020-Z-082).

AUTHOR CONTRIBUTIONS

YG, KH, YoZ, and YaZ participated in the clinical work. YaZ carried out data collection, analysis of data and preparation of the manuscript. YC and JH designed the study. SY participated in the analysis of data and preparation of the manuscript. All authors read and approved the final manuscript.

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The Efficacy of Biofeedback Therapy for the Treatment of Fecal Incontinence After Soave Procedure in Children for Hirschsprung's Disease

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Introduction: Hirschsprung's disease is a common digestive tract malformation in children, and the Soave procedure is one of the classic surgical methods for Hirschsprung's disease (HD). Fecal incontinence is one of the most common postoperative complications that can cause significant distress to the patients and their family, the incidence of which is 20% in a recent series. Biofeedback therapy (BFT) can be an effective treatment for managing anorectal disorders, but there has been little report of the efficacy of BFT for the treatment of fecal incontinence after the Soave procedure, and the main objective of this study is to evaluate it.

Methods: We retrospectively analyzed postoperative fecal incontinence in 46 children who received the Soave procedure for HD and who received BFT at our institution from March 2016 to February 2020, which included 38 males and 8 females (mean age 8.1 years, from 3.7 to 14 years). Anal sphincter contraction training was performed using BFT for 10 days per session in the hospital, one time each day, and 20 min each time. BFT was performed by employing visual and verbal feedback techniques using the biofeedback instrument. Long-term functional outcomes were objectively assessed using the Rintala Bowel Function Score (RBFS), and the patients were scored according to the sum total as excellent (18–20 points, 0 case), good (11–16 points, 0 case), fair (9–11 points, 9 cases), or poor (6–9 points, 37 cases). Defecation questionnaires and anorectal manometry were completed pretreatment and after three, six, or nine sessions, and primary outcome measures of anorectal manometry were anal maximal contraction pressure (AMCP), anal longest contraction time (ALCT), rectal rest pressure (RRP), and anal rest pressure (ARP).

Results: Followed up from 6 months to 4 years, the symptoms of fecal incontinence disappeared completely in 39 (84.78%) patients. Among them, 14 (30.43%) had complete disappearance of symptoms after 3 sessions of treatment, 25 (54.34%) patients had improved symptoms after 6 sessions of treatment, symptoms completely disappeared after 6 sessions of treatment, and 7 (15.22%) cases still suffered fecal incontinence mildly. The AMCP after three and six sessions in the poor group was

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significantly increased compared with that before treatment [(85.87 ± 31.75) mmHg vs. (135.33 ± 37.69) mmHg vs. (128.41 ± 33.45) mmHg, $P < 0.05$]. The ALCT and ARP showed the same trend, while the RRP after three and six sessions were not significant ($P > 0.05$). The mean (±SD) score of the RBFS increased from 9 to 17.40 ± 0.84 in the fair group, while it increased from 7.22 ± 0.76 to 16.58 ± 1.66 in the poor group after six sessions ($P < 0.05$).

Conclusion: Biofeedback therapy is a safe and effective treatment of fecal incontinence after the Soave procedure of children for Hirschsprung's disease. It is beneficial to design the individualized treatment programs for the children with varying degrees of fecal incontinence.

Keywords: biofeedback therapy, Hirschsprung's disease, fecal incon tinence, individual treatment, child

INTRODUCTION

Hirschsprung's disease, or congenital aganglionic megacolon, is characterized by varying extent of contiguous aganglionosis extending from the anorectum proximally, which is caused by the failed migration of colonic ganglion cells during gestation. Intestinal obstruction is a typical symptom, and pull-through surgery is a typical method for treating HD in children. The Soave procedure is one of the main procedures for the treatment of HD. The outcomes of numerous reports indicate that impaired bowel function is common after surgical treatment, such as fecal incontinence and constipation (1), although surgical techniques have continuously improved in recent years (2–4). Fecal incontinence is the inability to control the passage of fecal contents through the anus and has enormous consequences on a patient's psychological, emotional, and social life (5, 6). The reported incidence of fecal incontinence after operative management of HD is broad because of varying definitions and methods of assessment (7, 8). Though BFT has been increasingly used in many clinical signs and symptoms, there is little report about the management of the fecal incontinence of HD after the Soave procedure. The main objective of this study is to evaluate the efficacy of BFT for the treatment of postoperative fecal incontinence of children for HD.

MATERIALS AND METHODS

Patients

We retrospectively analyzed postoperative fecal incontinence in 46 children who received the Soave procedure for HD and who received BFT at our institution from March 2016 to February 2020, which included 38 males and 8 females (mean age 8.1 years, from 3.7 to 14 years). Exclusion criteria included children undergoing other procedures including Swenson, Duhamel, and Rehbein, combined with other digestive tract diseases, or neurogenic fecal incontinence. Anorectal manometry is a valuable diagnostic tool. The clinical outcome was evaluated by the RBFS (**Table 1**) (9), which considers seven basic parameters: ability to hold back defecation, feels/reports the urge to defecate, frequency of defecation, soiling, accidents, constipation, and social problems. And patients were scored according to the sum total as excellent (18–20 points, 0 case), good (11–16 points, 0 case), fair (9–11 points, 9 cases), or poor (6–9 points, 37 cases). Defecation questionnaires and anorectal manometry were

TABLE 1 | Rintala bowel function score.

	Score
Ability to hold back defecation	
Always	3
Problems <1/week	2
Weekly problems	1
No voluntary control	0
Feels/reports the urge to defecate	
Always	3
Most of the time	2
Uncertain	1
Absent	0
Frequency of defecation	
Every other day to twice a day	2
More often	1
Less often	1
Soiling	
Never	3
Staining less than 1/week, no change of underwear required	2
Frequent staining, change of underwear often required	1
Daily soiling, requires protective aids	0
Accidents	
Never	3
Fewer than 1/week	2
Weekly accidents; often requires protective aids	1
Daily, requires protective aids during day and night	0
Constipation	
No constipation	3
Manageable with diet	2
Manageable with laxatives	1
Manageable with enemas	0
Social problems	
No social problems	3
Sometimes (foul odors)	2
Problems causing restrictions in social life	1
Severe social and/or psychic problems	0

completed pretreatment and after three, six, or nine sessions, and the treatment options were decided based on whether the symptoms have improved completely. The procedures are shown in **Figure 1**.

Biofeedback Therapy Protocol

Informed consent was obtained from the parents, and this project was approved by the institutional review board of the hospital. Anal sphincter contraction training was performed using BFT by

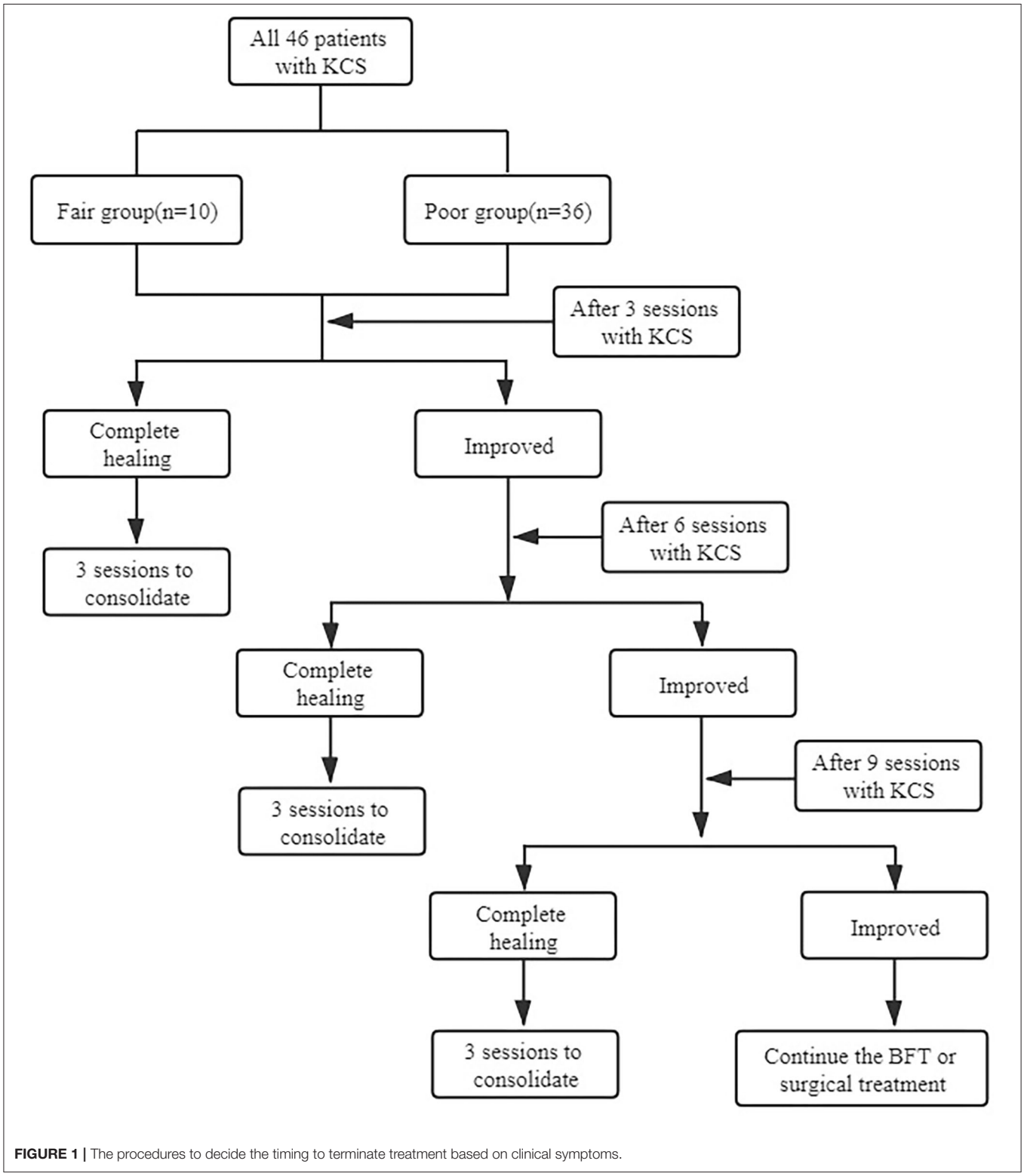

FIGURE 1 | The procedures to decide the timing to terminate treatment based on clinical symptoms.

employing visual and verbal feedback techniques and by using the biofeedback instrument [XDJ-S8G, KaiLi, HeFei, AnHui, China (**Figure 2a**)]. Children were in the supine position with a catheter (**Figure 2b**) in the anus. Anal sphincter contraction or relaxation signals could be transformed into signals and displayed on the screen. Children could watch the signal on the screen and try to adjust the strength of abdomen pressure and anal sphincter contraction or relaxation. BFT included contraction training to improve the strength and persistence of the anal sphincter contraction, relaxation training to improve the relaxation of the anal sphincter, and coordinate training to remodel the anus defecation movement and improve the defecation movement coordination, for 10 days per session in the hospital outpatient department, 1 time each day, and 20 min each time. The time in between different biofeedback sessions is 1 week. The coordinate training was the hardest part of which the goal was to produce an adequate abdominal push effort, as reflected by an increase in intra-abdominal/intrarectal pressure, which was synchronized with anal relaxation, as reflected by a decrease in the anal sphincter pressure (10). Meanwhile, patients were provided advice regarding bowel habits, exercise, laxatives, dietary fiber, and fluid intake. The therapist taught subjects how to improve their push effort by using postural and diaphragmatic breathing techniques (11). All patients completed the training regimen and were followed up for 6 months to 4 years.

Outcome Measurement

Anorectal manometry was used to evaluate the degree of fecal incontinence in all children before and after each course of treatment. Primary outcome measures were AMCP, ALCT, RRP, and ARP. The clinical outcome was evaluated by the RBFS (1, 12, 13).

Statistical Analysis

The data were analyzed using SPSS software for Windows (version 22.0). Analysis of variance was applied to evaluate the changes between baseline and post-treatment values within the BFT. Data were presented as the mean ± standard deviation (SD). Significant differences were considered when $P < 0.05$.

RESULTS

A total of 46 patients (38 males and 8 females; mean age 8.1 years, from 3.7 to 14 years; mean duration of postoperative fecal incontinence, 24.0 months) were included in this analysis. All of the patients completed the study, and the severity of the phenotype showed rectosigmoid segment (14 patients),

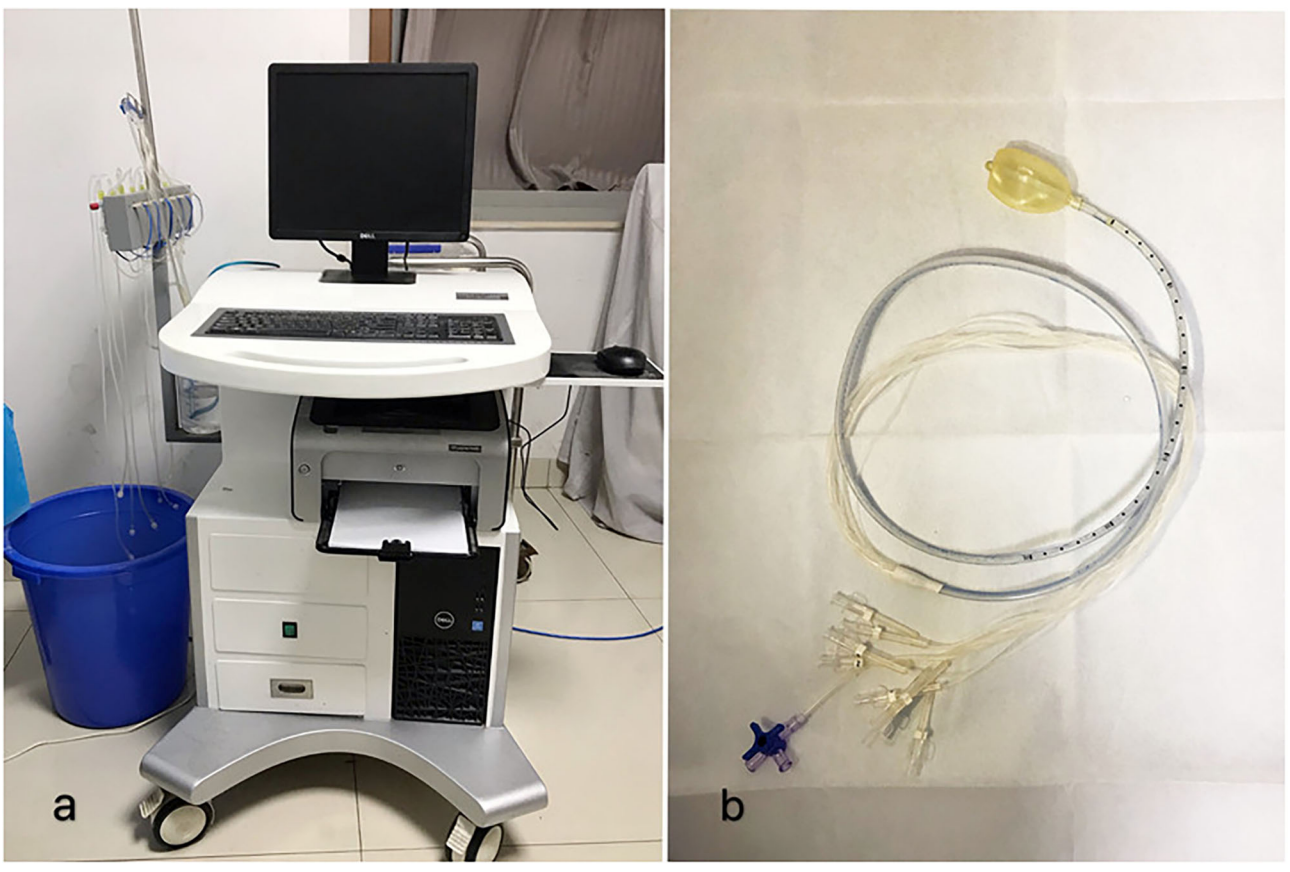

FIGURE 2 | (a) The biofeedback instrument (XDJ-S8G, KaiLi, HeFei, AnHui, China). **(b)** The BFT catheter.

long segment (27 patients), and total colonic (5 patients) aganglionosis. All of the children went through the Soave procedure at different ages (3 cases in the neonatal period, 26 cases in 3–6 months, 5 cases in 6 months to 1 year, and 12 cases after 1 year). The demographic characteristics and the clinical spectrum of the patients are shown in **Table 2**.

All 46 patients were classified in the analysis as belonging to either the excellent (0 cases), good (0 cases), fair (9 cases), or poor (37 cases) group according to the RBFS. The AMCP after three and six sessions in the poor group was significantly increased compared with that before treatment [(66.10 ± 39.17) vs. (110.65 ± 26.34) vs. (94.41 ± 31.02) mmHg, $P < 0.05$], while it showed [(85.87 ± 31.75) vs. (135.33 ± 37.69) vs. (128.41 ± 33.45) mmHg, $P < 0.05$] in the fair group. The ALCT after three and six sessions in the poor group was significantly increased compared with that before treatment [(20.99 ± 12.47) vs. (38.10 ± 8.85) vs. (47.93 ± 26.95) s, $P < 0.05$], while it had the same tendency in the fair group [(27.34 ± 12.21) vs. (45.45 ± 14.76) vs. (65.55 ± 30.13) s, $P < 0.05$]. The ARP after three and six sessions in the poor group was significantly increased compared with that before treatment [(41.41 ± 23.95) vs. (67.51 ± 26.41) vs. (58.61 ± 21.97) mmHg, $P < 0.05$], while it showed [(49.17 ± 31.88) vs. (53.03 ± 23.41) vs. (62.88 ± 29.61) mmHg, $P < 0.05$] in the fair group. There was no significant change in RRP after three or six sessions in both the fair [(6.16 ± 5.67) vs. (6.97 ± 6.21) vs. (6.48 ± 4.59) mmHg, $P > 0.05$] and poor groups [(6.54 ± 5.91) vs. (5.76 ± 4.70) vs. (6.81 ± 3.69) mmHg, $P > 0.05$]. The effect of biofeedback on the anorectal manometry parameters of the patients is shown in **Table 3**.

Different results were presented with regard to AMCP and the cure rate before and after biofeedback therapy for varying degrees of fecal incontinence (**Table 4**). After biofeedback therapy, the dyssynergic pattern of defecation was entirely corrected in 39 patients (84.8%), among which 14 cases (30.4%) were entirely corrected after three sessions of treatment, and 25 cases (54.3%) were entirely corrected after six sessions of treatment. Seven patients (15.2%) still had fecal incontinence after six sessions of treatment (**Table 5**). The seven patients had a poor prognosis after six sessions, the severity of the phenotype of which showed rectosigmoid segment (two patients), long segment (two patients), and total colonic (three patients). The ages at surgery ranged from 3 months to 1 year (six cases in 3–6 months, one case in 1 year). The ages at biofeedback treatment ranged from 3 to 14 years (**Table 6**). The mean (± SD) score of the RBFS group increased from 9 to 17.40 ± 0.84 in the fair group, while it increased from 7.22 ± 0.76 to 16.58 ± 1.66 in the poor group at the end of treatment ($P < 0.05$).

TABLE 2 | Demographic characteristics and clinical spectrum of patients.

Characteristics		Number (%)
Age	Mean	8.1
	Range	3.7–14
Gender	Male	38 (82.6)
	Female	8 (13.3)
Severity of the phenotype	Rectosigmoid segment	14 (30.4)
	Long segment	27 (58.7)
	Total colonic	5 (10.9)
	Neonatal period	3 (6.5)
Age at surgery	3–6 months	26 (56.5)
	6 months to 1 year	5 (10.9)
	After 1 year	12 (26.1)
Classification	Excellent	0 (0)
	Good	0 (0)
	Fair	9 (19.6)
	Poor	37 (80.4)

DISCUSSION

Hirschsprung's disease is a common digestive tract malformation in children, which affects about 1 in 5,000 people worldwide, with the highest incidence in Asia. Operation appears to be the most effective way to manage it. Several operating procedures have been reported for treating HD (14), and the Soave procedure is one of the classic surgical methods for HD (15). While most of the patients will be able to regain normal bowel function 3 months later after the operation, fecal incontinence is common after surgical treatment, which can cause significant distress on the quality of life and social of the patients. Biofeedback is a conditioning treatment where information about a physiologic process (contraction and relaxation of a muscle) is converted to a simple visual or auditory signal to enable the patient to learn to control the disordered function, which has been increasingly used over the last decades in many clinical signs and symptoms including rehabilitation (16), tension-type headache (17), psychiatric disorders (18), and other areas like sport (19). Engel et al. first pointed out in 1974 that biofeedback training could be used to treat fecal incontinence due to sphincter damage (20, 21).

Peña and Levitt proposed that the mechanisms needed to maintain continence are intact sensation, voluntary sphincter control, and appropriate colonic motility. Loss of any of these three mechanisms can alter the patient's ability to have voluntary bowel movements (22). Xiaobing Sun et al. believed that internal sphincter damage is one important cause for fecal incontinence after the Soave procedure. The damage of the internal anal sphincter could be caused by a lower level of dissection, vigorous anal dilation, and excessive anal canal traction during operation (23).

In recent years, it is widely believed that biofeedback therapy can enhance the contraction response and strength of the anal sphincter so as to improve the ability of defecation control (24). The efficacy could be measured by anorectal manometry. Our experience in this study shows that the ARP before treatment was at a lower level compared to baseline, which reflects the impaired function of the anal sphincter, and may be the main cause of postoperative fecal incontinence, consistent with relevant literature reports. It also shows the positive effects of biofeedback on most physiological parameters, including AMCP, ALCT, and ARP, in children with postoperative fecal incontinence, and patients with severe clinical symptoms need more courses

TABLE 3 | Effect of biofeedback on anorectal manometry parameters of patients.

	Fair				Poor			
	Pre-biofeedback	**After 3 sessions**	**After 6 sessions**		**Pre-biofeedback**	**After 3 sessions**	**After 6 sessions**	
AMCP (mmHg)	85.87 ± 31.75	135.33 ± 37.69	128.41 ± 33.45	$P < 0.05$	66.10 ± 39.17	110.65 ± 26.34	94.41 ± 31.02	$P < 0.05$
ALCT(s)	27.34 ± 12.21	45.45 ± 14.76	65.55 ± 30.13	$P < 0.05$	20.99 ± 12.47	38.10 ± 8.85	47.93 ± 26.95	$P < 0.05$
ARP (mmHg)	49.17 ± 31.88	53.03 ± 23.41	62.88 ± 29.61	$P < 0.05$	41.41 ± 23.95	67.51 ± 26.41	58.61 ± 21.97	$P < 0.05$
RRP(mmHg)	6.54 ± 5.91	5.76 ± 4.70	6.81 ± 3.69	$P > 0.05$	6.16 ± 5.67	6.97 ± 6.21	6.48 ± 4.59	$P > 0.05$

TABLE 4 | The AMCP before and after biofeedback therapy for varying degrees of fecal incontinence.

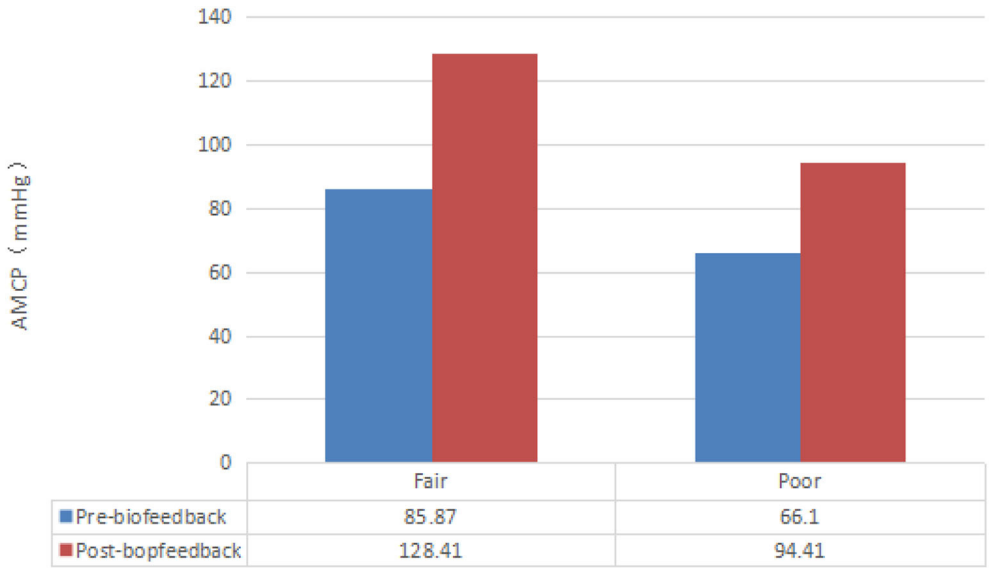

TABLE 5 | The cure rate of different degree of fecal incontinence.

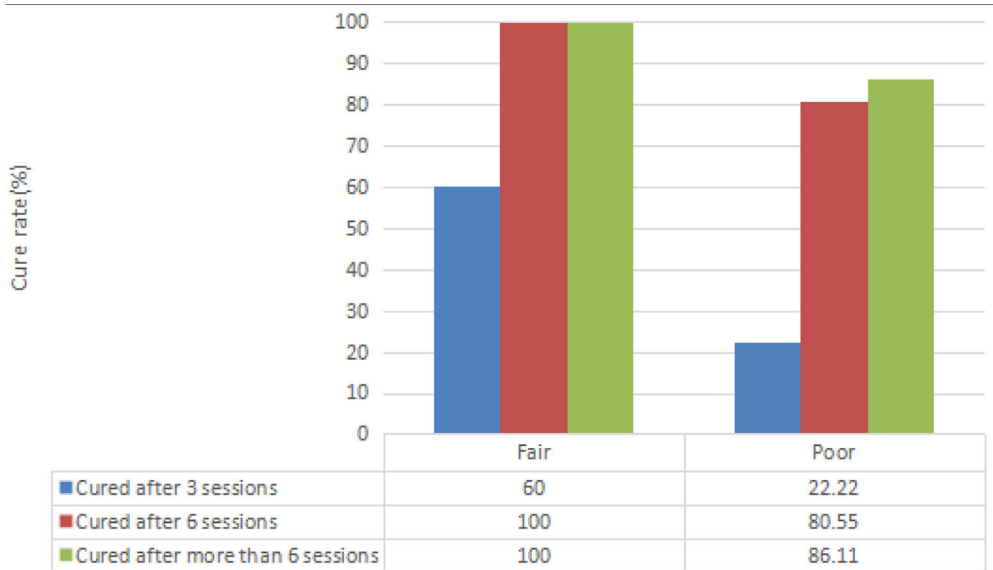

TABLE 6 | Demographic characteristics and clinical spectrum of the seven patients who had a poor prognosis after six sessions.

	Gender	Severity of the phenotype	Age at surgery	Age at BFT	RBFS before BFT	RBFS after BFT
1	Male	Long segment	4 months	3 years	6	14
2	Male	Total colonic	6 months	11 years	7	11
3	Male	Rectosigmoid segment	1 year	4 years	7	13
4	Male	Total colonic	3 months	14 years	6	13
5	Male	Long segment	3 months	5 years	7	14
6	Female	Total colonic	3 months	9 years	6	15
7	Male	Rectosigmoid segment	6 months	7 years	6	16

of treatment to recovery. As it turns out, after six sessions, AMCP and ALCT were more than double compared with that before treatment, while ARP was also increased obviously. These advances could provide adequate relief in fecal incontinence, and satisfaction with bowel movement in patients after treatment was significantly higher than before treatment. The clinical outcome was evaluated by the RBFS, of which an advantage is that it quantifies clinical outcomes, allowing us to more intuitively compare outcomes before and after treatment. The above two reflect subjective and objective indicators, respectively.

We observed a curious phenomenon in this research in which the AMCP had a trend of increasing significantly and then decreasing slightly. This brings us to an important point: the AMCP does not continue to increase with the number of sessions. We surmise that with the increase in the treatment course, patients may feel constant tiredness, so that the AMCP may decrease slightly after six sessions; but it was not statistically significant and therefore has little effect on prognosis.

It was shown that it requires different sessions of treatment according to different degrees of fecal incontinence. This paper will be improved by a data analysis of predictive factors to the biofeedback that provided the worse results. Seven patients had a poor prognosis after six sessions, the severity of the phenotype of which showed rectosigmoid segment (two patients), long segment (two patients), and total colonic (three patients). We observed that the admission to surgery or BFT of the two patients who showed rectosigmoid segment was delayed. Therefore, major factors leading to a poor prognosis may be associated with the aganglionic length, the age at surgery, as well as the age at biofeedback treatment. Biofeedback therapy requires children to understand the treatment process, cooperate actively, and insist on active exercise. Therefore, it is also important to select appropriate ages for treatment. Some children have poor autonomy and poor self-consciousness in training, which also have an impact on the treatment effect. Therefore, we believe that BFT should adhere to long-term exercise.

CONCLUSION

Postoperative fecal incontinence of children for HD can be a heavy burden for many children and their families. At our institution, BFT appears to result in a significant improvement in AMCP, ALCT, ARP, and the RBFS, so we believe that biofeedback therapy is a safe and effective treatment. It is beneficial to design the individualized treatment programs for the children with varying degrees of fecal incontinence. BFT does not have a uniform standard protocol for fecal incontinence of different types; we attempt to create a standardized protocol to reduce pain and improve the quality of life of children who suffer from fecal incontinence. Furthermore, the lack of long-term reassessment of results is a major limitation of this study, so long-term follow-up will continue in order to make the results reproducible and reliable.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee Office of the First Affiliated Hospital of Zhengzhou University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HY designed the experiment and modified the paper. YY and MX performed the experiment. MX and GW processed the data. YY wrote the paper. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Aberrant Development of Enteric Glial Cells in the Colon of Hirschsprung's Disease

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Objective: The aim of this study was to explore the development of enteric glial cells (EGCs) in different segments of Hirschsprung's disease (HSCR).

Methods: Colonic specimens from 35 children with HSCR were selected to analyze the relative expression of glial fibrillary acidic protein and S100 calcium-binding protein B using Western blotting and real-time fluorescence quantitative PCR. Immunofluorescence and immunohistochemical staining were performed to determine the distribution of myenteric EGCs and neuronal cells in different segments of HSCR.

Results: There was a trend of diminished protein and mRNA expression of glial fibrillary acidic protein and S100 calcium-binding protein B from the proximal, dilated, and transitional segments to the aganglionic segment ($p < 0.05$). Immunofluorescence and immunohistochemistry showed that the EGCs in the aganglionic, transitional, and dilated colonic muscles were morphologically abnormal, which was consistent with the dysplasia of myenteric neurons.

Conclusion: Aberrant development of myenteric EGCs was observed in the colon of HSCR, which may affect the survival of enteric neurons.

Keywords: Hirschsprung's disease, enteric glial cells, enteric nervous system, glial fibrillary acidic protein, S100 calcium-binding protein B

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INTRODUCTION

The enteric nervous system (ENS) is the second largest nervous system in the human body, and it regulates intestinal movement, nutrient absorption, immune response, and other functions (1). During the embryonic period, the ENS originates from neural crest-derived cells and finally colonizes the distal colon to differentiate into enteric neural cells (ENCs) and enteric glial cells (EGCs) (2, 3). Defects during migration of neural crest-derived cells will cause the lack of ganglia in the distal colon to form aganglionic segments, which will lead to intestinal motor dysfunction diseases, such as Hirschsprung's disease (HSCR) and Hirschsprung allied disorders (4, 5). Several genes produced by EGCs, such as *glial cell-derived neurotrophic factor*, are involved in the survival, proliferation, migration, and differentiation of myenteric neurons (6, 7). Previous studies have found abnormal changes in ENCs, interstitial cells of Cajal, and EGCs in patients with slow colonic transit (8). As for the pathogenesis of HSCR, it is currently unknown whether abnormal changes in EGCs precede the missing of neurons.

Glial fibrillary acidic protein (GFAP), an astrocyte marker, is expressed in different subtypes of EGCs. It exists as a monomer in the human body and usually appears in mature EGCs (9). S100 calcium-binding protein β (S100β), which is highly expressed in activated glial cells, is also used as an astrocyte marker (9, 10). In order to study whether myenteric EGCs have developed abnormally in HSCR and to determine their distribution relationship with ENCs and their changing trends in HSCR, we compared the expression of GFAP and S100β in different segments of children with HSCR through several molecular biology methods.

MATERIALS AND METHODS

Collection of Colon Specimens

Specimens were collected from 35 children with HSCR who underwent surgical treatment at the Pediatric Surgery Department of Qilu Hospital of Shandong University from December 2018 to December 2020, including 23 males and 12 females, with an average age of 16.15 ± 28.54 months. Among them, there were 30 cases of short-segment HSCR, 3 cases of long-segment HSCR, 1 case of total colonic type, and 1 case of extending to the small intestine. All research procedures were approved by the Ethics Committee of Qilu Hospital of Shandong University (KYLL-2018(KS)-092), and patient permission was obtained to use their specimens for research purposes only. Each colonic specimen was divided into four segments according to its lesion shape: aganglionic segment, transitional segment, dilated segment, and proximal segment. The aganglionic segment was defined to the narrow part lacking ganglia. The dilated segment was limited to the part that was significantly expanded due to feces accumulation, and the transitional segment was taken from the junction of the aganglionic and dilated parts. The proximal segment was collected from the relatively normal part of the resection margin. The aganglionic segment, transitional segment, and dilated segment were considered as diseased segments, and the proximal segment was used as control. Each part was no <500 mg. The tissue (0.5 cm) was soaked in 4% paraformaldehyde, and the remainder was stripped of the mucosa and submucosa (to eliminate the influence of hyperplastic cholinergic nerves) and stored at −80°C (11).

Western Blot

WB was used to analyze GFAP and S100β protein expression in each segment. After thawing the tissue on ice, the Minute™ Total Kit (Invent, Plymouth, MN, USA) was used to extract total protein from the colon. Twenty micrograms of loading protein was electrophoresed on a 10% SDS-PAGE gel (Vazyme, Nanjing, China) and transferred to a PVDF membrane (Millipore, Germany). After blocking with 5% BSA, the primary antibody (1:1,000) was shaken overnight at 4°C. Then, the secondary antibody (1:5,000) was incubated for 1 h at room temperature. Detection was performed using chemiluminescence (Bio-Rad ChemiDoc). **Supplementary Table 1** shows the details of the antibodies.

Real-Time Fluorescence Quantitative PCR

RT-qPCR was used to analyze the mRNA expression of the targets. The tissues were thawed in RNAlater™ (Beyotime, Shanghai, China) on ice. An RNA Extraction Kit (Fastagen, Shanghai, China) was used to extract total RNA, and A260/A280 was between 1.8 and 2.2. The SYBR (Tokyo, Japan) reaction system was prepared after reverse transcription to cDNA, and a Roche Light Cycler 480 was used for RT-qPCR. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were selected based on data published elsewhere or from the Beacon Designer software (Premier Biosoft, Palo Alto, CA, USA). The melting curve after each reaction was used to confirm specificity. The primer sequences are listed in **Supplementary Table 2**.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry (IHC) and IF were performed to compare the distribution of EGCs and ENCs in the diseased colonic muscles. The specimens were incubated in 4% paraformaldehyde and coated in 5-μm paraffin slices. After dewaxing and antigen retrieval, the slices were blocked with 5% BSA and incubated with the primary antibodies (1:500) overnight at 4°C, then incubated with IgG (H + L) or Alexa Fluor-594 and 498 secondary antibodies (Abcam, Cambridge, MA, USA) at 1:200 for 1 h at 37°C. All antibody incubation and washing steps were performed in PBS at pH 7.4. Images were acquired with an Olympus DP 72 (Tokyo, Japan) and the cellSens Dimension and Software image acquisition system. Diaminobenzidine (ZSbio, Beijing, China) staining was monitored under a microscope. Hematoxylin and 4′,6-diamidino-2-phenylindole were used for nuclear staining. ImageJ software was used for densitometric analysis of positive staining. **Supplementary Table 1** shows the details of antibodies used.

Statistical Analysis

All data are presented as means ± SD. GraphPad Prism 8.0 was used for statistical analysis and graphing. The significance of the differences between groups was calculated using one-way ANOVA, and $p < 0.05$ was considered to be statistically significant.

RESULTS

GFAP and S100β Expression Diminished in Diseased Segments

The protein and mRNA expression levels of GFAP and S100β in the different segments were examined by WB and RT-qPCR. Compared with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase, there was a trend of decreased expression of GFAP and S100β from the proximal, dilated, and transitional segments to the aganglionic segment ($p < 0.05$) (**Figure 1**).

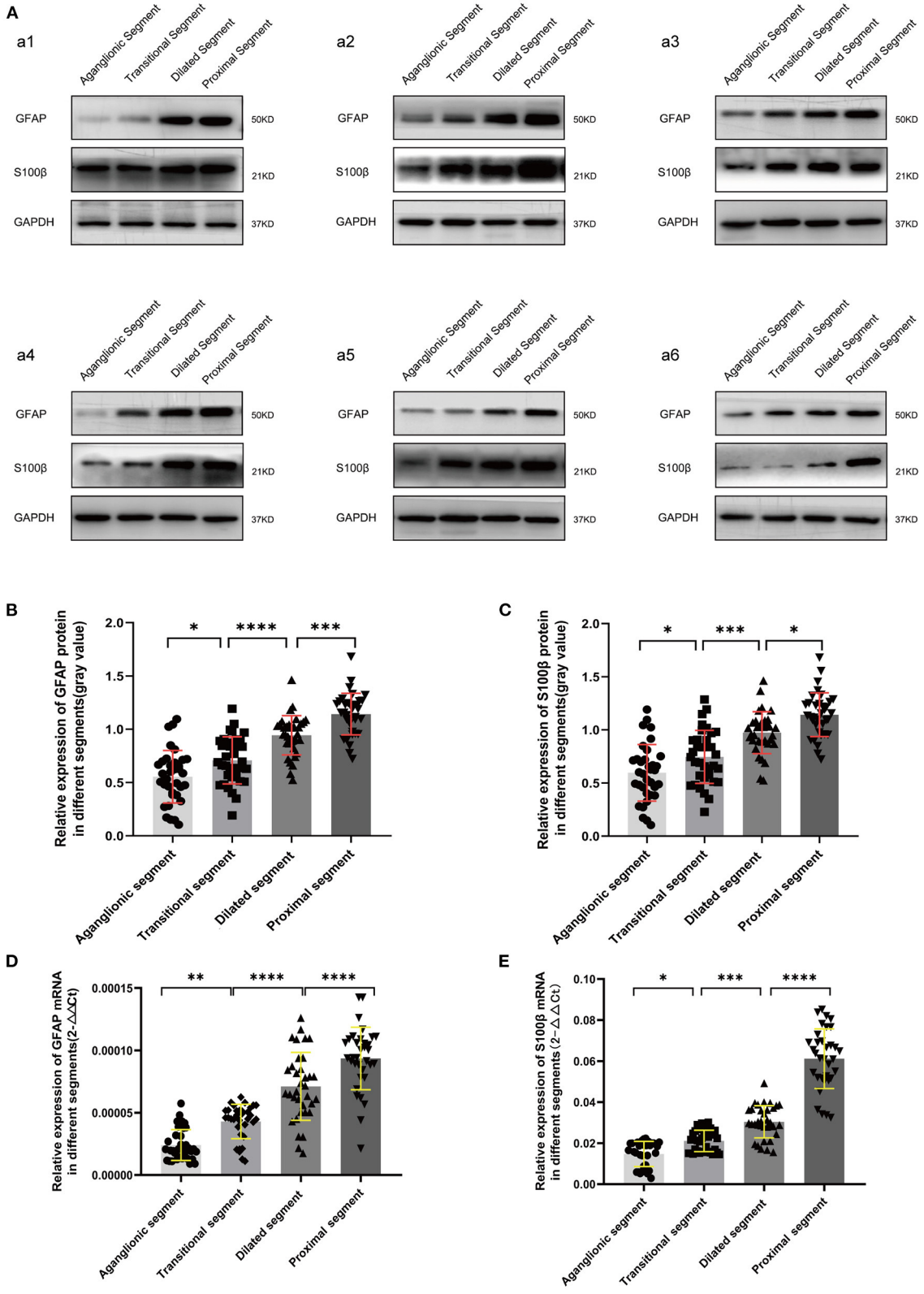

FIGURE 1 | WB **(A-C)** and RT-qPCR **(D,E)** revealed significantly decreased protein and mRNA expression of GFAP and S100β in the diseased segments, compared with proximal segments of HSCR patients. Equal loading amounts were confirmed using glyceraldehyde 3-phosphate dehydrogenase. The assays were performed in triplicates, and values are given as mean ± SD. (ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

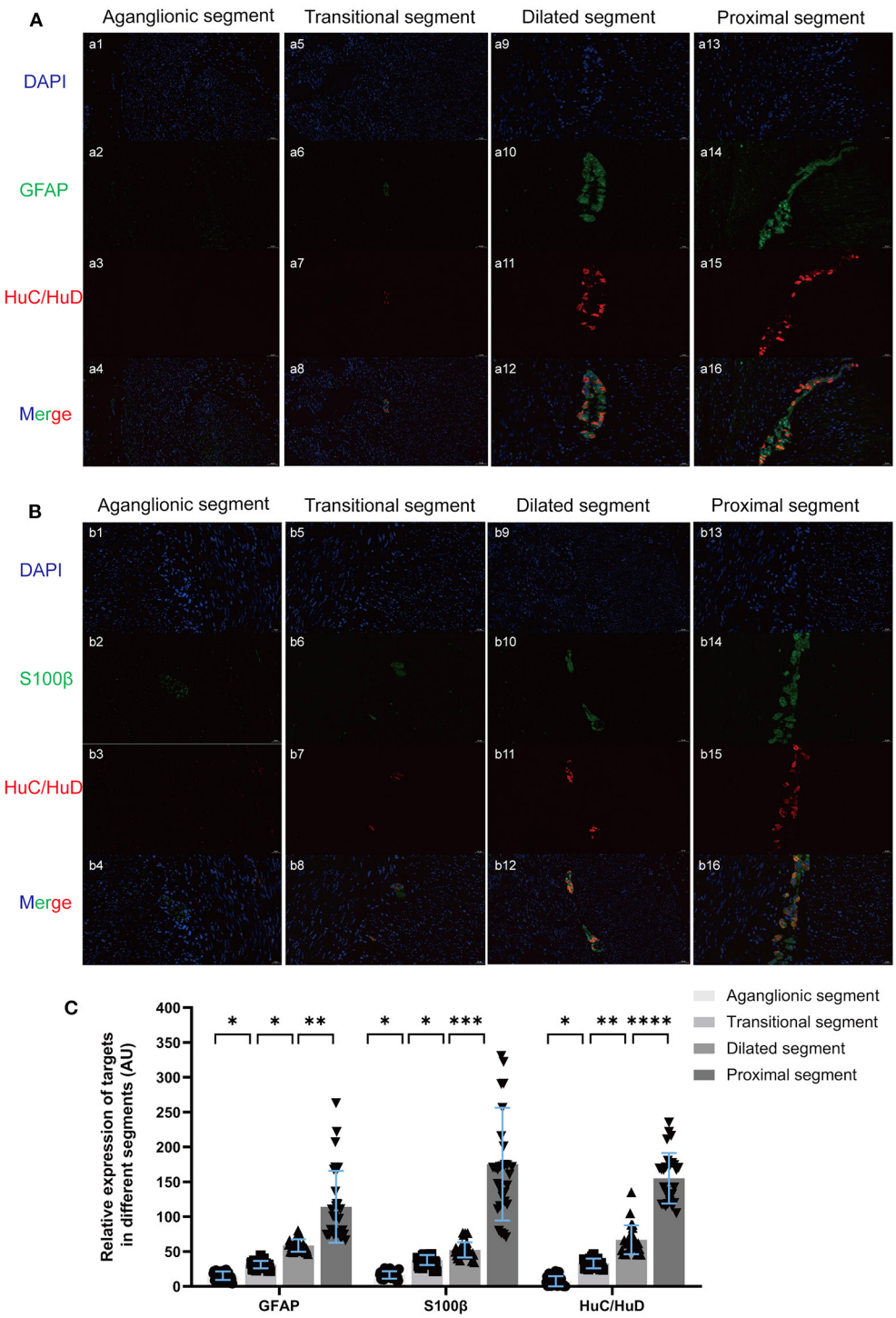

FIGURE 2 | Double-label IF of GFAP (**A** green), S100β (**B** green), and HuC/HuD (red) revealed a corresponding relationship in the location of EGCs and ENCs in myenteric ganglions, and the average fluorescence intensity of the unit area was quantified **(C)**. There was no obvious positive staining of targets in the aganglionic segment (a1-a4, b1-b4). The shapes of ganglions in the muscles in transitional and dilated segments were irregular and hypoplasic (a5-a12, b5-b12), while the ganglion in proximal segments was more intact and the EGCs were located around the ENCs (a13-a16, b13-b16). (Scale bar: 20 μm ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

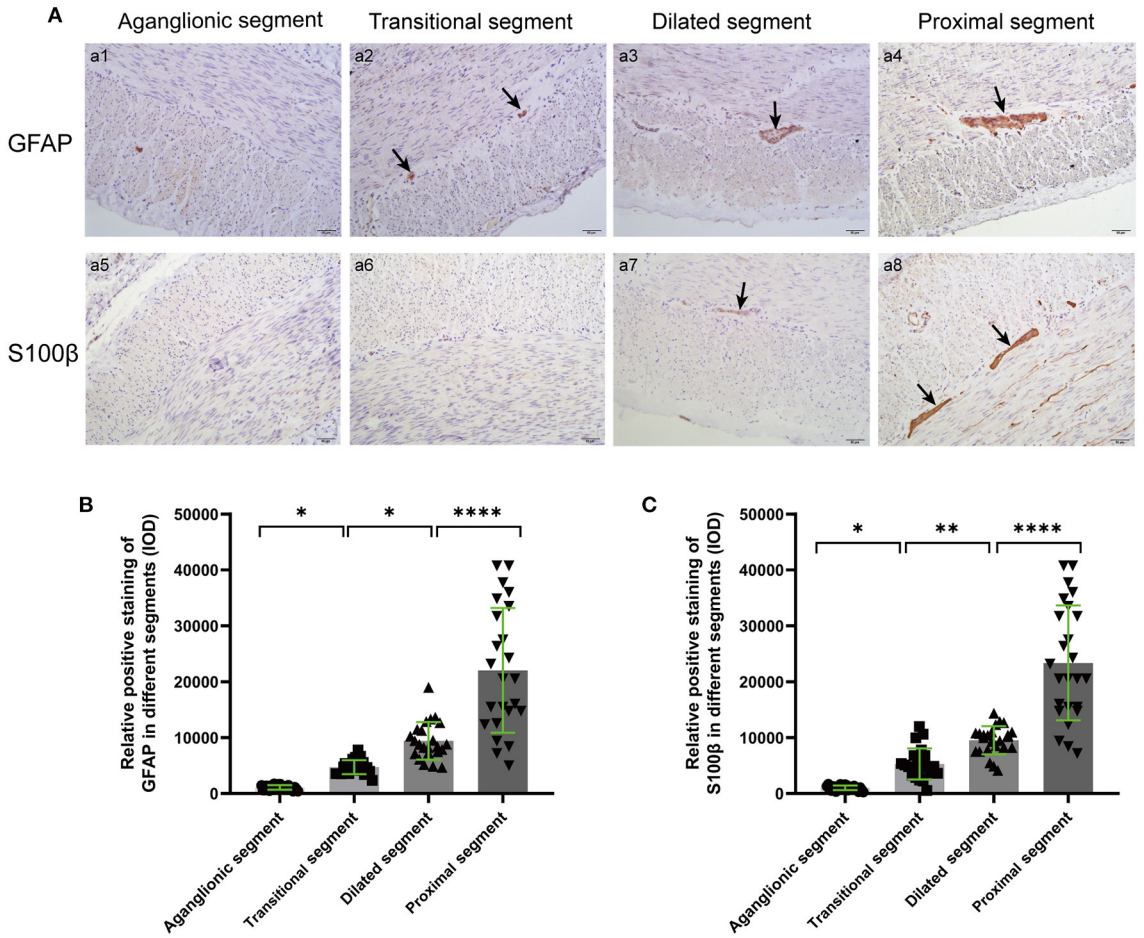

FIGURE 3 | IHC **(A)** and quantitative analysis **(B,C)** of GFAP and S100β in the different segments of colonic muscle sections. The target GFAP and S100β were not significantly stained in aganglionic segments (a1, a5), and the positive staining particles in transitional (a2, a6 arrows) and dilated segments (a3, a7 arrows) were significantly attenuated and irregularly shaped, compared with the proximal segments (a4, a8 arrows). (Scale bar: 50 μm, ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

EGC Hypoplasia and Loss of Neurons

GFAP, S100β, and the classic enteric neuron marker HuC/HuD were stained using IF, revealing a corresponding relationship between the development and location of EGCs and ENCs in myenteric ganglia (**Figure 2**). Attenuated ENCs and EGCs coexisted in diseased segments, and the EGCs of proximal segments were located around the ENCs and participated in the formation of myenteric ganglions.

Expression Position of GFAP and S100β in Different Segments

The positions of GFAP and S100β in different segments were displayed by IHC, and positive staining was quantified based on intensity and area. The myenteric ganglia showed obvious positive staining of tan particles in proximal segments, and the positive particles of the aganglionic and transitional segments were significantly attenuated (**Figure 3**).

DISCUSSION

It was thought that EGCs were used to support neurons at first, while existing studies on EGCs focused more on inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, and less on congenital diseases such as Hirschsprung allied disorders. EGCs are divided into different subtypes according to their location and function (12). On the one hand, they are similar to astrocytes in the central nervous system and can secrete a variety of neurotrophic factors to participate in the regulation of neuronal functions, such as glial-derived neurotrophic factor and S-nitrosoglutathione (6, 13, 14); on the other hand, EGCs also play a role in maintaining the balance of epithelial barrier and resist aggression through inflammatory reactions (7).

In this study, we compared the changing trends of myenteric EGCs in 35 children with HSCR and found their corresponding relationship with myenteric neurons in four segments. In line with the study by Tani et al. (15), in addition to aganglionic segments, immature EGCs appear in the proximal colon of HSCR

children compared to normal controls. As a phenomenon-level study, the results are consistent with previous functional studies on EGCs, which found that the neurotrophic factor produced by EGCs can enhance the migration ability of neural crest-derived cells and augment the size of enteric neurospheres (16). Aubé et al. (17) confirmed that the absence of EGCs in the intestinal wall can lead to impaired colonic motor function. Soret et al. (10) showed that primary EGCs cultured *in vitro* enhance the barrier function of epithelial cells and produce Ca^{2+} transients upon induction of extracellular ATP. Therefore, we assume that abnormally developed EGCs in HSCR may lose their protective support and nutritional effect on ENCs.

However, after undergoing the surgery, some patients continued to experience postoperative complications, such as constipation soiling or enterocolitis, even though we thought that sufficient lesions had been removed. The appearance of persistent symptoms after surgery may be related to abnormalities in EGCs and ENCs in the unresected proximal colon (15, 18). This experiment prompted us to further study the interactions between EGCs and ENCs. Studies have shown that GFAP is related to delayed differentiation of EGCs. Under the stimulation of certain foreign antigens such as lipopolysaccharides, EGCs can proliferate reactively, exhibit an "active state" with GFAP upregulation, produce neuroprotective factors, and enhance the protective effect against ENCs (9, 13, 19). Carvalho et al. (20) proposed a more efficient preoperative biopsy method that recommends pathology mapping of the entire colon to assess the excision range. This prompted us to assume the possibility of preserving more colon tissue in future HSCR surgery by improving the function of neurons and EGCs in dilated and transitional segments. In addition, through the detection of GFAP and S100β, the normal EGCs in the resection margin may provide a reference for the scope of surgical resection (21). Furthermore, a reasonable assessment of ENS development can complement diagnosis and significantly reduce the incidence of complications such as postoperative enteritis (22).

An increasing amount of evidence demonstrated that EGCs play a vital role in maintaining the homeostasis of ENS. However, more attention was paid to enteric neurons in HSCR, compared with EGCs. Whether and how EGCs are involved in the development and maturation of ENS and whether hypoplasia of EGCs precedes the absence of neurons remain to be further studied.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Qilu Hospital, Cheeloo College of Medicine, Shandong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TZ: design of the work, acquisition, analysis, and interpretation of data. WL, XY, ZC, WM, PH, and CR: collection of specimens and obtaining informed consent from patients. AL: drafting the manuscript or revising it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.746274/full#supplementary-material

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ORIGINAL RESEARCH
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Role of GDNF, GFRα1 and GFAP in a *Bifidobacterium*-Intervention Induced Mouse Model of Intestinal Neuronal Dysplasia

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Objective: To investigate the effects of glial cell-derived neurotrophic factor (GDNF), GDNF family receptor alpha 1 (GFRα1), and glial fibrillary acidic protein (GFAP) on colonic motility in a mouse model of intestinal neuronal dysplasia by intervention with *Bifidobacterium* and to explore the influence of *Bifidobacterium* on enteric glial cells (EGCs).

Methods: Western blotting and qRT-PCR were employed to detect the expression of GFRα1 and GFAP in colonic tissues of mice with or without Tlx2 mutations, and ELISA was used to detect the expression of GDNF in serum. IHC was used to detect the appearance of the ganglion cells. Subsequently, Tlx2 homozygous mutant ($Tlx2^{-/-}$) mice were treated with *Bifidobacterium.* Colonic motility was measured before and after intervention by measuring the glass bead expelling time. The variations in abdominal circumference and GDNF, GFRα1, and GFAP expression were measured. In addition, 16SrRNA gene sequencing was performed to detect the abundance of the intestinal microbiota.

Results: The mRNA and protein expression of GFRα1 and GFAP was decreased in the colonic tissues of $Tlx2^{-/-}$ mice and GDNF expression was decreased in serum compared with $Tlx2^{+/-}$ and WT mice. After confirming the colonization of *Bifidobacterium* by 16S rRNA gene sequencing, the expelling time and abdominal distension were ameliorated, and the expression of GFAP, GDNF, and GFRα1 was increased.

Conclusions: The expression of GDNF, GFRα1, and GFAP is associated with colonic motility. The altered expression of EGC-related factors suggested that *Bifidobacterium* may be involved in the EGC activation process. The amelioration of IND symptoms after intervention with *Bifidobacterium* prompted the elicitation of adjuvant therapy.

Keywords: intestinal neuronal dysplasia (IND), gut microbiota, *Bifidobacterium*, homozygous mutant mice of Tlx2, GDNF

INTRODUCTION

Intestinal neuronal dysplasia (IND) is a disorder of the enteric nervous system (ENS) associated with intestinal dysmotility (1). Similar to Hirschsprung's disease (HSCR), patients with IND show severe prolonged defecation time, constipation, abdominal distension, and even intestinal obstruction. However, the pathological features of IND are distinct, and include: hyperplasia of the submucosal nerve plexus, immaturity of ganglia, and hypertrophy of the nerve trunks in clinical cases (2, 3). Since the first description by Meier-Ruge in 1971 (4), the definition of IND is still a subject of controversy. Although the histopathological diagnostic criteria for IND are continuously updated, its etiology and pathogenesis have not been elucidated. Glial cell line-derived neurotrophic factor (GDNF), secreted by enteric neuroglial cells (EGCs), are regarded as one of the most important neurotrophic pathways in the formation of the ENS (5). GDNF binds to GDNF family receptor alpha 1 (GFRα1), causing phosphorylation of the tyrosine kinase receptor RE-arranged during transfection (RET). Signals through the activation of downstream signaling pathways direct the development of the ENS, including neuroblast migration and axonal outgrowth (6, 7).

Gut microbiome can affect the function and number of enteric neurons (8–10), and the early gut microbiota plays an important role in the development of the ENS after birth (11, 12). Several studies have shown that abnormalities in the myenteric plexus and a decrease of intestinal motility can be observed in early postnatal germ-free mice (11). While there are hundreds of species of bacteria in the human intestine, *bifidobacteria* is a key microbial player in the infant gut microbiota and has a beneficial effect on gut microbiota composition, leading to reduced necrotizing enterocolitis (NEC) incidence (13, 14). Furthermore, *bifidobacterium* can affect the expression of inflammatory factors in EGCs to inhibit intestinal inflammation and upregulate the expression of enteric glial cell–derived nerve growth factor (NGF) and neurotrophin 3 (NT-3) (15). These findings prompted us to determine whether colonic dysmotility of IND can be improved by the probiotic components of intestinal flora through activation with EGCs.

To verify this hypothesis, Tlx2 homozygous mutant ($Tlx2^{-/-}$) mice were chosen as the model. T-Cell Leukemia Homeobox Protein 2 (Tlx2), a member of orphan homeobox-containing transcription factor family, is also known as HOX11L1, Ncx and Enx (16). Tlx2 has been proved to be crucial to the development of ENS. $Tlx2^{-/-}$ mice show colonic dysmotility with hyperplasia of intestinal ganglion and immature enteric neurons after birth, which has been confirmed to be consistent with IND (17, 18). Glial fibrillary acidic protein (GFAP), a type III intermediate filament (IF) protein that is highly expressed in mature and activated EGCs (19–21), was chosen as a marker of EGCs. This study aimed to explore the effects of GDNF, GFRα1, and GFAP on colonic motility and the influence of *Bifidobacterium animalis* on EGCs, and to further clarify the potential pathogenesis of IND.

TABLE 1 | Detailed primer information.

Primer	Species	F sequences (5′-3′)	R sequences (5′-3′)
Tlx2	mus	TTGATGAGGCTTCTGTGGTT	AAGAGCGACGAGTTGTGC
GFAP	mus	TAACGACTATCGCCGCCAAC	CATTTGCCGCTCTAGGGACT
GFRα1	mus	CTATCGTCCCTGTGTGCTCC	CCAATCAGTCCCGAGTAGGG
GDNF	mus	GTCACCAGATAAACAAGCGGC	CTCTGCGACCTTTCCCTCTG
GAPDH	mus	TGTCTCCTGCGACTTCAACA	GGTGGTCCAGGGTTTCTTACT

MATERIALS AND METHODS

Animal and Sample Preparation

The study was approved by the Ethics Committee of Qilu Hospital of Shandong University (IACUC Issue No. Dull-2020-013). The mice were treated according to the animal use guidelines of the Animal Care and Use Committee (ACUC) of Qilu Hospital, Shandong University. Genechem (Shanghai, China) used CRISPR/Cas9 gene-targeting technology to knock out 173 bp of the second exon nucleotide sequence of the C57BL/6 mouse Tlx2 gene, resulting in $Tlx2^{+/-}$ mice. $Tlx2^{+/-}$ mice were interbred to obtain $Tlx2^{-/-}$ mice. The mice were reared under specific pathogen-free conditions. At the age of 3 weeks, different genotypes were screened out through genotype identification (WT $Tlx2^{+/-}$ and $Tlx2^{-/-}$). Colonic motilities and abdominal circumference were measured 1 d before or 1 d after the intervention. Feces were collected 1 d after the intervention and before colonic motility measurement. Blood samples and segments of the distal colon were harvested after anesthesia, and were stored at −80°C.

Genotyping

The genotype of mice was identified by Southern blotting. Then the mice were divided into three groups (group WT $Tlx2^{+/-}$ and $Tlx2^{-/-}$, nine mice for each group) according to their genotypes for experiments before probiotic intervention. Genomic DNA was isolated from mouse tails using a Mouse Tail DNA Extraction kit (CWBio, Beijing, China). The primer sequences for Tlx2 are shown in **Table 1**. Subsequently, the genomic DNA was amplified by PCR and separated by 1.2% agarose gel electrophoresis. Images were acquired using the BIO-RAD gel image acquisition system (Bio-Rad, Hercules, CA, USA).

Measure of Mouse Colonic Motility

Colonic motility was measured 1 d before or 1 d after the intervention. A small glass bead with a diameter of 2.5 mm was inserted slowly into the colon of the mice to a distance of 2 cm with a smooth glass rod (2.5 mm diameter). After checking that the glass bead was completely pushed in, the mouse was placed on a clean surgical drape, and the expulsion was recorded.

Bifidobacterium animalis Intervention

Bifidobacterium animalis (AS1.1852, biobw) was cultured in *Bifidobacterium* nutrient liquid medium (Haibo, Qingdao, China) in an anaerobic incubator at 37°C, and Spectroscopy Photometric Microplate Reader (ThermoFisher, MA, US) was

TABLE 2 | Detailed antibody information.

Antibodies	Company	Species	Dilution	Cat. No
GFAP	Affinity	Rabbit	WB 1:1000 IHC 1:500	AF6166
GFRα1	Abcam	Rabbit	1:800	Ab8026
GAPDH	Proteintech	Rabbit	1:1000	10494-1-AP
IgG (H + L)	Affinity	Goat	WB 1:5000 IHC 1:200	S0001

used to monitor the density of the bacterial colony (OD 600). The bacterial colony density was adjusted to 1×10^9 CFU/ml before intervention. Tlx2$^{-/-}$ mice with a suitable weight at the age of 3–4 weeks were divided into three groups (eight mice for each group). *Bifidobacterium animalis* group (group BB) was given live *Bifidobacterium* liquid according to the weight of each mouse by retention-enema at 0.2 ml/20 g (0.2×10^9 CFU/20 g) daily for 3 weeks. The solution should be prepared before each intervention everyday and used in time after dilution to maintain the probiotic activity. The *Bifidobacterium* nutrient medium group (group NM) was administered the same dose of nutrient medium, and the normal saline/blank control group (group NS) was administered the same dose of normal saline.

Western Blotting

Western blotting was used to analyze the relative expression levels of GFRα1 and GFAP proteins in the colonic tissue. The Minute™ Total Rapid Protein Ext Kit (Invent, MN, USA) was used to isolate total protein from the full-thickness colonic tissue. Equal amounts of protein were separated on a 10% SDS-PAGE gel and then transferred to a PVDF membrane. The PVDF membrane was incubated with primary antibodies at 4°C overnight after 5% BSA blocking. The membrane was then washed with 1 × TBST and incubated with secondary antibody for 1 h at room temperature. ECL kit (Millipore, MA, USA) was used for chemiluminescence, and the gray values were calculated. **Table 2** shows the details of the antibodies.

Quantitative Real-Time PCR

The qPCR assay was employed to investigate the relative expression of GDNF, GFRα1, and GFAP in colon tissue at the mRNA level. Total mRNA was isolated using TRIzol reagent (Ambion, USA). After concentration measurement, mRNA was reverse transcribed to cDNA using an RT-PCR kit (Toyota, Japan). The qPCR reactions were performed with SYBR Green PCR Master Mix (Toyota, Japan) on a Roche 480 real-time fluorescent PCR instrument. Primers were selected from the Beacon Designer software (Premier Biosoft, Palo Alto, California, USA). The detailed information is shown in **Table 1**. The $2^{-\Delta\Delta Ct}$ values for each group were calculated for analysis. Colon sample of each mouse was repeated three times, and a melting curve was used to confirm specificity.

Immunohistochemistry Staining

Immunohistochemistry was used to detect the appearance of ganglion and glial cells. The colon tissue was fixed in 4% paraformaldehyde for 24 h and then embedded in 5-μm paraffin. After dewaxing and antigen retrieval, the slices were blocked with goat serum and incubated with the primary antibodies (1:500) overnight at 4°C, then incubated with secondary antibodies (1:200) for 1 h at 37°C. All antibody incubation and washing steps were performed in PBS at pH 7.4. DAB (ZSbio, Beijing, China) staining was monitored under a microscope. Finally, the sections were counterstained with hematoxylin and coverslipped. The integrated optical density (IOD) of positive staining within five random fields from paraffin sections of each mouse was measured at 20× magnification using Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Bethesda, MD, USA). The average value was used for statistics.

ELISA

An ELISA kit (Boster, Wuhan, China) was used to detect the level of GDNF in serum according to the manufacturer's instructions. Serum was added to a 96-well plate (100 μl per well) for detection. The OD values were measured at 450 nm after the reaction, and the concentrations were calculated.

16S rRNA Gene Sequencing

Total bacterial DNA was extracted from fecal samples using the E.Z.N.A. Stool DNA Kit (Omega, Inc., USA). The V3-V4 region of the bacterial 16S rRNA gene was amplified using the universal primers 338F (5-ACTCCTACGGGAGGCAGCAG-3) and 806R (5-GGACTACHVGGGTWTCTAAT-3). PCR products were purified with AMPure XT magnetic beads (Beckman Coulter Genomics, Danvers, MA, USA), and quantified using Qubit (Invitrogen, California, USA). The amplicon library was prepared for sequencing, and its size and quantity were assessed using an Agilent 2100 Bioanalyzer (Agilent, CA, USA) and the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). The amplicon library was paired-end sequenced using the Illumina MiSeq platform at a commercial company (LC-Bio Technology Co., Ltd, Hang Zhou, China).

Statistics and Analysis

All statistical analyses were performed using GraphPad Prism®8 software, and the results are presented as the mean ± SD. *T*-test was used for comparisons between two independent groups, and one-way analysis of variance (ANOVA) and Tukey's test were used for comparisons between multiple groups; $P < 0.05$ was considered statistically significant.

RESULTS

Tlx2$^{-/-}$ Mice Showed Decreases in the Expression of GDNF, GFRα1 and GFAP

The allele of the wild-type was 568 bp, whereas the mutant was 395 bp. T1x2 $^{-/-}$ mice obtained by interbreeding were genotyped by Southern blot analysis (**Figure 1A**). To confirm whether the expressions of GDNF, GFRα1, and GFAP were different among WT, Tlx2$^{+/-}$, and Tlx2$^{-/-}$ mice, the colonic samples and sera of these mice were used for analysis. The protein and mRNA expression of GFRα1 and GFAP in colonic tissues of Tlx2$^{-/-}$ mice were lower than those in Tlx2$^{+/-}$ and WT mice ($P < 0.05$)

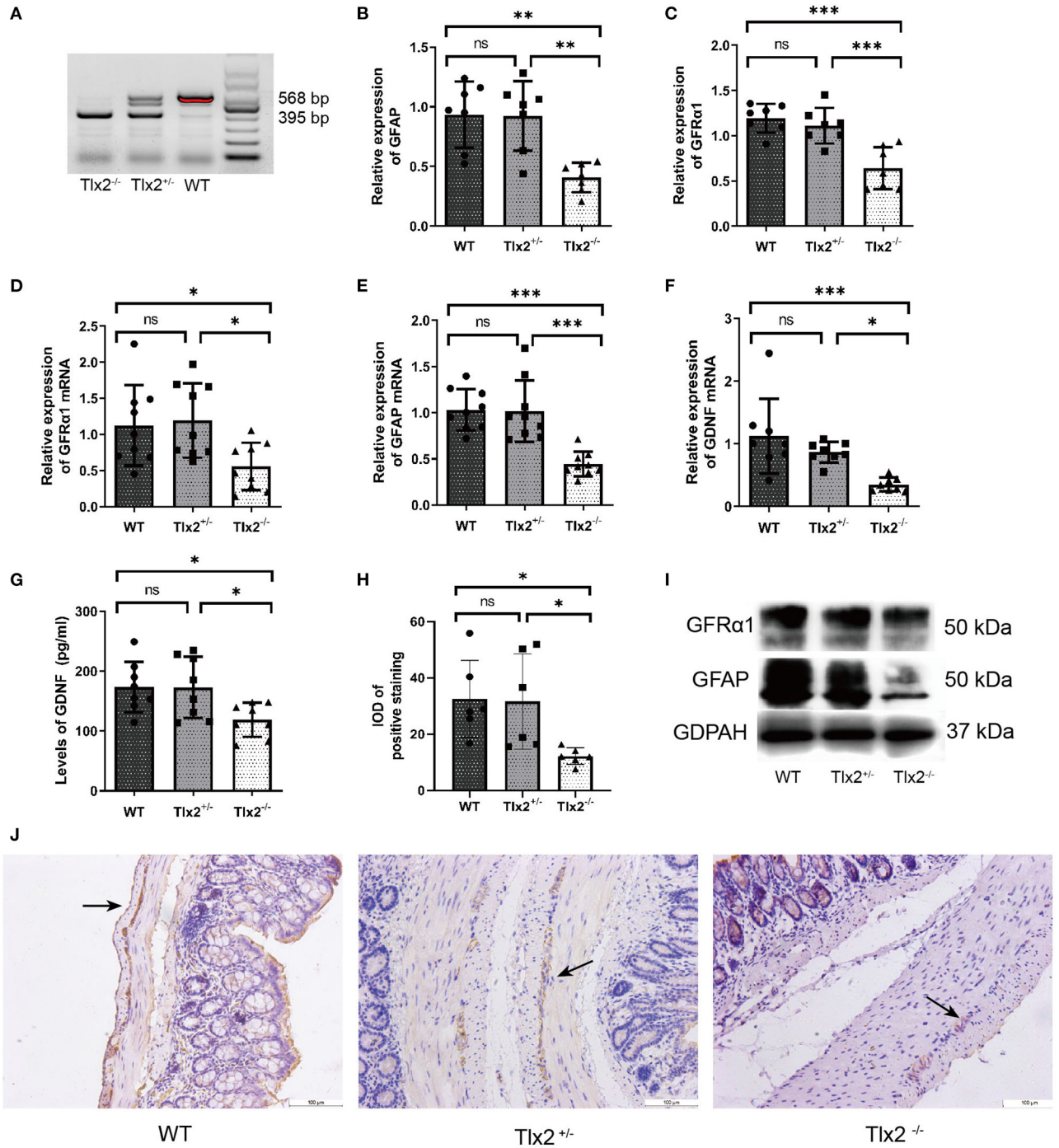
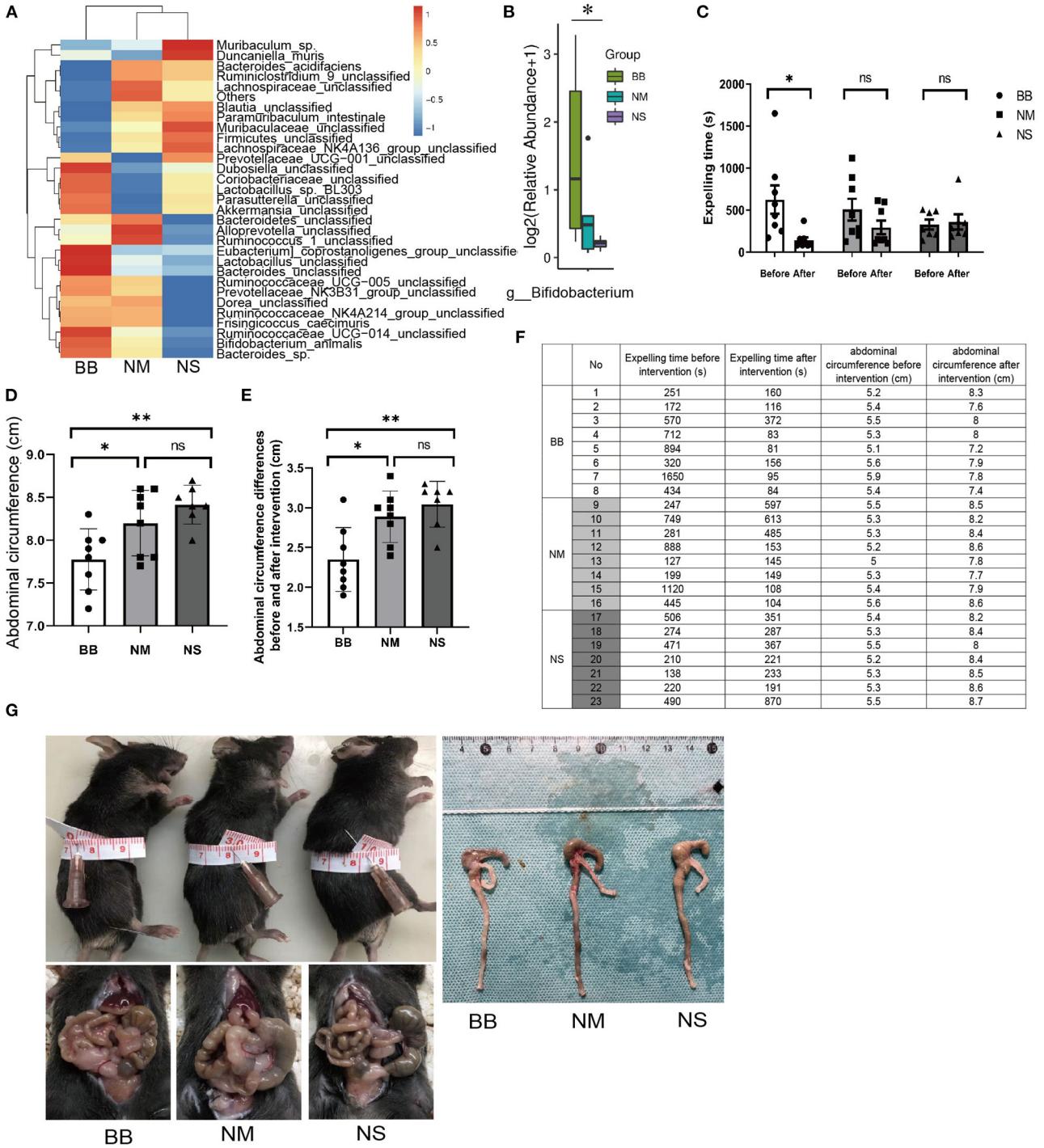


FIGURE 1 | Example genotyping and decreased expressions of GDNF, GFRα1 and GFAP in Tlx2$^{-/-}$ mice. **(A)** Genotyping; the wild-type allele is 568 bp, and the mutant allele is 395 bp. **(B,C)** Western blot indicated that the protein expressions of GFAP and GFRα1 in colonic tissues of Tlx2$^{-/-}$ mice were decreased ($n = 7$ for each group). **(D–F)** The mRNA expressions of GDNF, GFRα and GFAP were decreased in colon tissues of Tlx2$^{-/-}$ mice ($n = 9$ for each group). **(G)** The expression level of GDNF in serum of Tlx2$^{-/-}$ mice was lower than that in Tlx2$^{+/-}$ mice and WT mice ($n = 8$ for each group). **(H)** IOD of positive staining indicated that GFAP was significantly downregulated in the colonic tissues of Tlx2$^{-/-}$ mice ($n = 6$ for each group). **(I)** Representative western blot analysis and detail bands of other mice were attached in **Supplementary Figure 1**. **(J)** GFAP was mainly expressed in EGCs of the colonic myenteric plexus (ns $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; GAPDH, glyceraldehyde-3-phosphate; Scale bar: 100 μm).



	No	Expelling time before intervention (s)	Expelling time after intervention (s)	abdominal circumference before intervention (cm)	abdominal circumference after intervention (cm)
BB	1	251	160	5.2	8.3
	2	172	116	5.4	7.6
	3	570	372	5.5	8
	4	712	83	5.3	8
	5	894	81	5.1	7.2
	6	320	156	5.6	7.9
	7	1650	95	5.9	7.8
	8	434	84	5.4	7.4
NM	9	247	597	5.5	8.5
	10	749	613	5.3	8.2
	11	281	485	5.3	8.4
	12	888	153	5.2	8.6
	13	127	145	5	7.8
	14	199	149	5.3	7.7
	15	1120	108	5.4	7.9
	16	445	104	5.6	8.6
NS	17	506	351	5.4	8.2
	18	274	287	5.3	8.4
	19	471	367	5.5	8
	20	210	221	5.2	8.4
	21	138	233	5.3	8.5
	22	220	191	5.3	8.6
	23	490	870	5.5	8.7

FIGURE 2 | Measurement of the colonic motility and abdominal circumference of mice in groups BB, NM and NS. **(A)** Hierarchical clustering heatmap of the top 30 bacterial species of each group. **(B)** The relative abundance of *Bifidobacterium* among groups BB, NM and NS. **(C,F)** T After *Bifidobacterium* intervention, the expelling time in BB group was shortened compared to groups NM and NS. **(D,F,G)** The abdominal circumference after *Bifidobacterium* intervention among the three groups. **(E,F)** Differences in abdominal circumference before and after *Bifidobacterium* intervention in group BB was smaller than the other groups. (ns $P > 0.05$; *$P < 0.05$; **$P < 0.01$).

(**Figures 1B–E,I**). Compared to Tlx2$^{+/-}$ mice and WT mice, the protein expression of GDNF in serum and its mRNA expression in colonic samples of Tlx2$^{-/-}$ mice was also decreased ($P < 0.05$) (**Figures 1F,G**). Meanwhile, IHC staining showed that GFAP was mainly expressed in the EGCs of the colonic myenteric plexus (**Figure 1J**). The IOD of positive staining indicated that GFAP

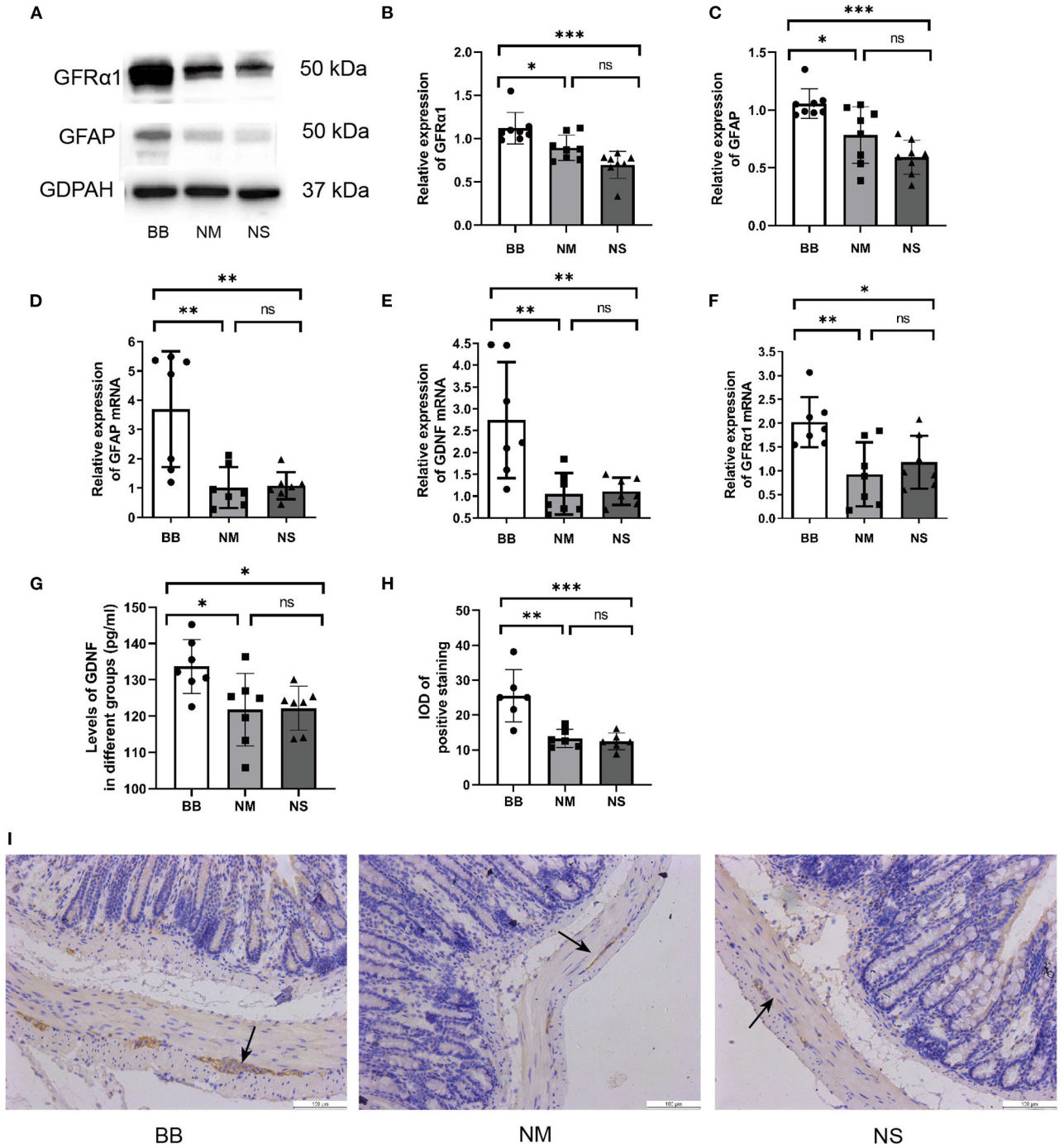


FIGURE 3 | Expression of GDNF, GFRα1 and GFAP was increased in group BB. **(A–C)** The protein expression of GFAP and GFRα in the colonic tissues of group BB was higher than those in the other two groups ($n = 8$ for each group), detail bands of other mice can be found at **Supplementary Figure 1**. **(D–F)** The mRNA expressions of GDNF, GFRα and GFAP in colon tissues of group BB were increased ($n = 7$ for each group). **(G)** The serum levels of GDNF in group BB increased compared to those in groups NM and NS after intervention ($n = 7$ for each group). **(H)** The IOD of positive staining in group BB was increased compared to that of the NM and NS groups ($n = 6$ for each group). **(I)** Representative results of IHC staining of GFAP in different groups under 20× microscope (ns $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; GAPDH, glyceraldehyde-3-phosphate; Scale bar: 100 μm).

was significantly downregulated in colonic tissues of Tlx2$^{-/-}$ mice compared to other tissues (**Figure 1H**).

The Expelling Time of Tlx2$^{-/-}$ Mice Was Shortened and the Abdominal Circumference Was Smaller After *Bifidobacterium* Intervention

To confirm that the *Bifidobacterium* intervention was effective, 16S rRNA gene sequencing was performed. The relative abundances of intestinal flora were presented in a hierarchical clustering heatmap and *Bifidobacterium* in group BB was much higher than that in groups NM and NS (**Figures 2A,B**). The time of bead expulsion in different groups of Tlx2$^{-/-}$ mice before and after enema was recorded (**Figure 2F**). The expelling time of group BB after enema was shorter than that before the intervention ($P < 0.05$). However, nutrient medium and normal saline did not affect colonic motility (**Figure 2C**). The abdominal circumference of mice in group BB after intervention was significantly smaller than that of the other two groups ($P < 0.05$) (**Figures 2D,F**) and the differences in abdominal circumference before and after *Bifidobacterium* intervention in the BB group were smaller than those in the other groups ($p < 0.05$; **Figure 2E**). The abdominal distension in the BB group was relieved (**Figure 2G**). The gross anatomy of the intestines revealed that the fecal retention of mice in group BB was mild, and the dilatation of the cecum, distal ileum, and proximal colon was relieved compared with other groups (**Figure 2G**).

The Expression of GDNF, GFRα1 and GFAP Was Increased in Tlx2$^{-/-}$ Mice After Intervention

The protein expression of GFAP and GFRα1 was significantly increased in the BB group (**Figures 3A–C**), which was consistent with the qRT-PCR results (**Figures 3D,F**). ELISA was used to detect the expression level of GDNF in the serum, confirming the upregulated expression of GDNF in the BB group (**Figure 3G**). Similarly, the qRT-PCR assay revealed that mRNA expression of GDNF increased in the colonic tissues of mice in the BB group (**Figure 3E**), and there was no statistically significant difference between the other two groups. The location and expression of GFAP are shown by IHC (**Figures 3H,I**). As can be seen, the stain-positive cells of GFAP presented tan granules. The IOD of positive staining in group BB was higher than that in groups NM and NS, which was consistent with the results of WB and PCR.

DISCUSSION

The pathogenesis of IND is unresolved; however, several mechanisms have been proposed, including secondary infections, inflammation, and developmental failures (22–24). It has been confirmed that the seriously impaired colonic motility of IND is related to abnormal innervation between ENS and intestinal smooth muscle cells (25, 26). Tlx2$^{-/-}$ mice, a verified model of IND, usually show hyperplasia of the intestinal ganglion and persistence of immature enteric neurons after birth (18, 27). In our previous studies, Tlx2$^{-/-}$ mice showed obvious abdominal distension and impaired colonic motility, and 26% (33/127) of the mice died 8 weeks after birth (25), which may be related to histopathological changes in the colonic tissues of Tlx2$^{-/-}$ mice.

The main origin of the ENS is neural crest cells (28). EGCs and enteric neurons, vital components of the ENS, show interactive influences during the maturation and function of the ENS (28), and GDNF and GFRα1 play significant roles in this process (6, 7, 29). In this study, the expression of GDNF, GFRα1, and GFAP in Tlx2$^{-/-}$ mice was lower than that in Tlx2$^{+/-}$ and WT mice, confirming that the three may participate in the pathogenesis of IND. Alternatively, we observed higher expression of GDNF, GFRα1, and GFAP in mice after intervention with *Bifidobacterium*. In addition, the expelling times in group BB were significantly shortened. All of these results indicate possible relationship between GDNF, GFRα1, and GFAP and the relief of colonic motility dysfunction, which might explain the colonic motility dysfunction resulting from the immaturity of ganglion cells.

The gut microbiota affects the activation of the immune system by modulating antigen-specific adaptive immune responses, resulting in physiological functions (8, 30). Bacteria metabolites regulate the intestinal microenvironment by modulating the release of inflammatory factors (31). *Bifidobacterium*, the main probiotic component of the gut microbiota in humans, can reduce inflammatory responses by inhibiting the NF-κB and external cell signaling pathways (32). It has been confirmed that EGCs can be regulated by *Bifidobacterium* through inhibition of inflammation and up-regulation of nerve growth factors *in vitro* (15). In our study, the *Bifidobacterium* intervention in IND model mice caused the colonization of *Bifidobacterium* and increased expression of GDNF, GFRα1, and GFAP in colonic tissues. The variations in the expression of GDNF, GFRα1, and GFAP suggested that *Bifidobacterium* may participate in EGC activation and nutritional factor upregulation, but the specific mechanism of interaction between *Bifidobacterium* and EGC should be further researched.

IND and HSCR show similar clinical features, including constipation, abdominal distension, and even intestinal obstruction. In this study, after intervention by *Bifidobacterium*, the expelling time of Tlx2$^{-/-}$ mice was shortened and abdominal distension was reduced. The recovery of the impaired colonic motility confirmed the positive influence of *Bifidobacterium* on colonic motility, which is consistent with our clinical experience. These results are also consistent with recent findings that fecal microbiota transplantation can improve intestinal function (33, 34).

As shown above, the expression of GDNF, GFRα1, and GFAP decreased in Tlx2$^{-/-}$ mice and increased after *Bifidobacterium* intervention, while the IND-related symptoms were relieved after *Bifidobacterium* intervention. We can conclude that the expression of GDNF, GFRα1, and GFAP is associated with colonic motility dysfunction and the pathogenesis of IND. The altered expression of EGC-related genes suggested that *Bifidobacterium* may be involved in the activation process of EGCs. The amelioration of IND symptoms after

intervention with *Bifidobacterium* prompted the elicitation of adjuvant therapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the dataset also forms part of an ongoing study. Requests to access the datasets should be directed to 591043945@qq.com.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Qilu Hospital of Shandong University.

AUTHOR CONTRIBUTIONS

WL and TZ: design of the work, acquisition, analysis, and interpretation of data for the work. JT, XY, CR, ZC, and PH: feeding of laboratory animal and collection of specimens. QZ and AL: drafting the work or revising it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.795678/full#supplementary-material

Supplementary Figure 1 | (A) Western blot indicated that the protein expressions of GFAP and GFRα1 in colonic tissues of $Tlx2^{-/-}$ mice were decreased. **(B)** The protein expression of GFAP and GFRα in the colonic tissues of group BB was higher than those in the other two groups.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Enterocolitis Is a Risk Factor for Bowel Perforation in Neonates With Hirschsprung's Disease: A Retrospective Multicenter Study

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Background and Aim: We evaluated the clinical features of neonatal Hirschsprung's disease (HD)-associated bowel perforation (perforated HD) and investigated risk factors related to it.

Methods: We retrospectively collected clinical data of neonates (<1 month of age) with perforated HD from multicenters in China from January 2006 to December 2019. A total of 142 patients (6.7%) with perforated HD were enrolled in the study. A 1:2 matching method was used to compare the clinical information of HD patients with and without bowel perforation during the neonatal period. The risk factors for bowel perforation were identified using univariate and multivariate logistic risk regression analyses.

Results: Perforation site was present in the proximal ganglionic bowel in 101 (71.1%) cases and the distal aganglionosis segment in 41 (28.9%) cases. Adjacent marginal tissue from the perforated intestine revealed varying degrees of inflammatory cell infiltration, and the severity of enterocolitis was higher in the proximal ganglionic bowel than in the distal aganglionosis segment ($p < 0.05$). In the univariable and multivariable logistic analyses, clinical symptoms, such as vomiting (adjusted OR = 2.06, 95% CI: 2.01–2.88, $p < 0.05$), and inflammation index in hematologic tests, such as neutrophil proportion (adjusted OR = 1.09, 95% CI: 1.05–1.33, $p < 0.05$) and CRP (adjusted OR = 2.13, 95% CI: 1.01–3.27, $p < 0.05$) were associated with increased risk for perforated HD.

Conclusion: Clinical Hirschsprung disease-associated enterocolitis (HAEC) highly correlated with perforated HD. Timely treatment of HAEC should be appropriate therapeutic approaches to prevent perforated HD.

Keywords: Hirschsprung disease, bowel perforation, enterocolitis, risk factor, neonates

INTRODUCTION

Hirschsprung's disease (HD) is a congenital intestinal malformation, characterized by the absence of ganglion cells in the submucosal and myenteric plexus of the bowel. Neonates with HD frequently present with clinical signs of abdominal distension, vomiting, constipation, and failure to pass meconium. HD is subdivided by the proximal extent of the aganglionic bowel segment into short-segment HD (rectosigmoid colon, SS-HD), long-segment HD (transverse or left colon, LS-HD), total colonic aganglionosis (TCA), and total intestine aganglionosis (TIA) (1). Bowel perforation (perforated HD), a serious complication of HD, mostly occurs during the neonatal period (2). According to a nationwide survey conducted in Japan from January 1, 2008, to December 31, 2012, perforated HD accounted for 1.8% of all neonatal perforations (3). Perforated HD is mostly associated with severe consequences, including high staged-operation rate, long parenteral feeding, and potentially worse prognosis.

Newman et al. first reviewed the perforation in neonates with HD and speculated that the mechanism of perforation could be related to increased intraluminal pressure (2). Afterward, Arliss et al. reported a neonatal appendiceal perforation diagnosed with SS-HD. Microscopic sections of the appendix demonstrated periappendicitis without transmural inflammation. Therefore, the authors suggested that both the special anatomical structure of the neonatal appendix and excessive luminal pressure could be responsible for appendiceal perforation (4). In addition, Yamamoto et al. reported a case of cecal perforation with SS-HD and suggested the cause of perforation to be ischemia, secondary to the vascular accident of the intestinal wall (5). It has been difficult to understand the true etiology of perforated HD until now due to the little systematic data related to epidemiologic findings or risk factors associated with perforated HD.

Hirschsprung disease-associated enterocolitis (HAEC) is one of the most common complications of HD, which can occur at any time from the neonatal period into adulthood. Teitelbaum et al. reported that neonates with HAEC had a mortality rate of 5% and a morbidity rate of 30% (6). Histological evidence of HAEC consists of a few features including crypt abscess, leukocyte aggregates, and ulceration in the affected intestinal wall (7). Significant clinical features associated with HAEC during the acute phase include bilious vomiting, fever, marked abdominal distension, and even bowel perforation (8). Therefore, we hypothesized that HAEC was the cause of perforation in neonates HD. The present work collected information on patients diagnosed with perforated HD from multicenters in China for over 10 years and evaluated the clinical features, aims to provide a comprehensive understanding of the risk factors associated with perforated HD.

METHODS

In this study, the medical records of neonates with HD treated between January 2006 and December 2019 at three different tertiary children's medical centers in China, were reviewed. The inclusion criteria were neonates $\leq$ 1-month-old with bowel perforation and diagnosed by HD. HD was diagnosed based on postoperative histological findings. Patients diagnosed with TIA, necrotizing enterocolitis (NEC), imperforate anus, intestinal atresia, and meconium plug syndrome were excluded. Neonates who were brought from outside the three participating hospitals were also excluded from the study.

In total, 2,119 consecutive patients with neonatal HD were admitted to our centers, of whom 142 (6.7%) were diagnosed with perforated HD. All clinical data of patients with perforated HD were recorded, including gestational age at birth, birth weight, gender, type of HD, site of perforation, site of stoma, and outcomes. Among them, there were 108 males (76.1%) and 34 females (23.9%), with a median age of 5 days at perforation (IQR: 2–18 days). All patients who developed bowel perforation were term or near-term infants, which was similar to that reported in a previous study (9). Histopathology of the adjacent marginal tissue of the perforated intestine from patients was retrospectively collected and reviewed by two pathologists independently.

A retrospective matched case–control study was conducted to assess the risk factors associated with perforated HD in neonates. For each index case suffering from neonatal HD with bowel perforation, we matched two controls, that is, neonatal HD without bowel perforation (1:2 matching method). The control population was matched for factors including age (days of bowel perforation in cases, $\pm$3 days), gestational age, the same admissible hospital, and the same year of hospitalization.

Cases and control subjects were identified by reviewing their electronic medical records that included demographic, hospital course, and outcome data for all admissions to the hospitals. The potential risk factors for perforated HD included sex, *in vitro* fertilization, gestation details, type of delivery, family history, birth weight, delayed meconium defecation (>24 h after birth), abdominal distention or vomiting after birth, feeding timing (oral feeding or not), feeding pattern (breast or formula milk, or both), complicated with multi-disorders, blood transfusion (erythrocytes, thrombocytes, or fresh frozen plasma), and the latest laboratory blood examination. The risk factor data were collected for each case and its matched control subjects from birth to the day before bowel perforation in the case subject. For example, if the patient had developed bowel perforation on day 5, data about the hospital course in the case and assigned control subjects were collected from birth through day 4. Certain variables, such as birth Apgar score, blood pressure, and pH value, were incomplete and therefore excluded from analyses. This retrospective case–control study was approved by the medical ethics committee of the Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China (No. 2019-S108). The trial was registered at Clinical Trial (No: NCT05044741). The study was performed according to the Declaration of Helsinki. All authors had access to the study data and reviewed and approved the final manuscript.

Statistical Analysis

Continuous variables are reported as mean and standard deviation (SD), or median and interquartile range (IQR) according to normal and non-normal distribution, respectively. Categorical variables are expressed as frequencies and

TABLE 1 | Demographic and outcome data in neonates with perforated HD ($n = 42$).

Characteristics	
Age (days), median (IQR)	5 (2–18)
Gestational age (weeks), median (IQR)	38.1 (37.4–40.1)
Type of HSCR	
Short-segment	78 (54.9%)
Long-segment	44 (31.0%)
Total colonic aganglionosis	20 (14.1%)
Site of Perforation, *n* (%)	
Sigmoid colon	60 (42.3%)
Descending colon	7 (4.9%)
Cecum	34 (23.9%)
Ileum	41 (28.9%)
Site of stoma, *n* (%)	
Sigmoid colon	21 (14.8%)
Transverse colon	46 (32.4%)
Ileum	75 (52.8%)

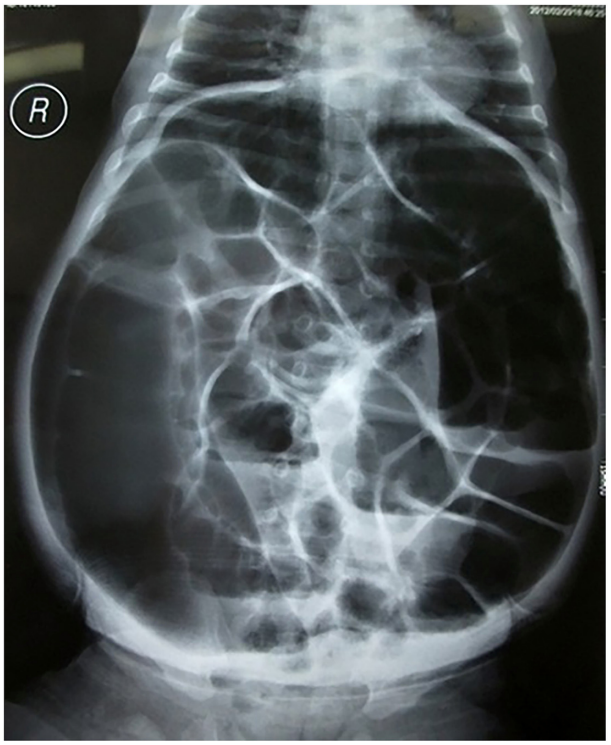

FIGURE 1 | Typical anterior-posterior plain radiographs showed extensive intestinal and colon flatulence and multiple fluid levels.

percentages. Various demographic and clinical characteristics, as well as established risk factors for bowel perforation, were compared between cases and control subjects using univariate logistic regression analyses. The results are presented as two-sided *p*-values, unadjusted odds ratios (ORs), and corresponding 95% confidence intervals (95% CIs). Patients' characteristics showing significant trends ($p < 0.05$) in association with important variables were evaluated through a multivariate conditional logistic regression analysis. Data are presented as two-sided *p*-values, adjusted OR (aOR), and corresponding 95% CI. All data were analyzed using Stata version 15. Significance in the adjusted and unadjusted analyses was established with $p < 0.05$.

RESULTS

The clinical characteristics of patients are summarized in **Table 1**. In patients with perforated HD, vomiting was reported in 108 cases (76.1%) and abdominal distension was reported in 129 cases (90.8%). In addition, 30 cases (21.1%) were hospitalized with intestinal obstruction as the major complaint, and 112 cases (78.9%) underwent bowel perforation during perinatal hospitalization. Typical orthostatism plain films showed extensive intestinal and colon flatulence and multiple fluid levels (**Figure 1**). With respect to the type of HD, 78 (54.9%) cases were diagnosed with SS-HD, 44 (31.0%) cases were diagnosed with LS-HD, and 20 (14.1%) cases were diagnosed with TCA (**Table 2**).

The site of perforation bowel was sigmoid colon in 60 (42.3%) cases, descending colon in 7 (4.9%) cases, cecum in 14 (9.9%) cases, appendix in 20 (14.1%) cases, and terminal ileum in 41 (28.9%) cases. Of these, perforation site in 101 (71.1%) cases was present in the proximal ganglionic bowel, including 70 cases with SS-HD and 31 cases with LS-HD. Perforation site in 41 (28.9%) cases was present in the distal aganglionosis segment, including 8 cases with SS-HD, 13 case with LS-HD, and 20 cases with TCA. In cases with SS-HD or LS-HD, the majority of the perforation sites (89.7 and 70.5%, respectively) were located in the proximal ganglionic bowel. However, the perforation site was located in the cecum or appendix in all TCA cases. Adjacent marginal tissue from the perforated intestine was obtained in 122 (85.9%) patients, histological examination of the adjacent marginal tissue from the perforated intestine revealed mild inflammatory cell infiltration [Grade I according to HAEC histological grading system (7)] in 37 samples (30.3% of tested specimens); severe inflammatory cell infiltration, with significant signs of variable crypt dilation (Grade II) in 31 samples (25.4% of tested specimens); and numerous crypt abscesses with the destruction of the epithelium (Grade III) in 54 samples (44.3% of tested specimens) (**Figure 2**). Concretely, in Grade II and Grade III samples, the severe inflammatory response was more common in the proximal ganglionic bowel (61 cases, 71.8% of Grade II and Grade III) than in the distal aganglionosis segment (24 cases, 28.2% of Grade II and Grade III) ($p < 0.05$).

All cases received emergency surgical enterostomy, including sigmoid colostomy in 21 cases (14.8%), transverse colostomy in 46 cases (32.4%), and ileostomy in 75 cases (52.8%). During the diversion of luminal flow, HAEC occurred in 7 cases (4.9%), and constipation after enterostomy occurred in 9 cases (6.3%), which were resolved by daily bowel irrigation until radical colectomy. Staged stoma closure and pull-through colectomy were performed 3–6 months later. Seven cases (4.9%) were lost to

TABLE 2 | A comparison of risk factors for HD bowel perforation in neonates and control subjects.

Variables	Perforated HSCR (*n* = 142)	Non-perforated HSCR (*n* = 284)	Unadjusted OR (95% CI)	*P*-value
Male gender, *n* (%)	108 (76.1%)	189 (66.5%)	1.63 (0.16–2.41)	0.49
Birth weight (kg), median (IQR)	3.1 (2.9–3.4)	3.5 (2.9–4.0)	0.35 (0.11–1.17)	0.09
Primigravid (*n*) (%)	81 (57.0)	176 (62.0%)	0.87 (0.43–5.04)	0.53
Primiparity (*n*) (%)	74 (52.1%)	155 (54.6%)	0.82 (0.35–4.19)	0.75
Cesarean delivery, *n* (%)	81 (57.0%)	148 (52.1%)	1.23 (0.24–2.78)	0.76
In vitro fertilization, *n* (%)	30 (21.1%)	80 (28.2%)	0.82 (0.87–3.71)	0.57
Complications/syndromes, *n* (%)	28 (19.7%)	76 (26.8%)	0.45 (0.07–2.76)	0.39
Transfusion history, *n* (%)	27 (19.0%)	40 (14.1%)	1.21 (0.14–3.14)	0.68
Family history, *n* (%)	7 (4.9%)	14 (4.9%)	NA	1
Meconium > 24 h, *n* (%)	128 (90.1%)	203 (71.4%)	1.46 (0.05–4.50)	0.13
Constipation after birth, *n* (%)	122 (85.9%)	256 (90.1%)	0.88 (0.24–10.60)	0.64
Fever (°C), median (IQR)	36.5 (5.8–7.4)	36.7 (6.0–7.8)	0.84 (0.32–3.61)	0.55
Abdominal distention, *n* (%)	129 (90.8%)	216 (76.1%)	1.34 (0.06–1.98)	0.23
Vomiting, *n* (%)	108 (76.1%)	102 (35.9%)	3.39 (2.65–4.77)	**<0.01**
Oral feeding, *n* (%)	115 (80.9%)	213 (75.0%)	1.25 (0.17–3.31)	0.71
Type of feeding, *n* (%)				
Breast milk	47 (33.1%)	108 (38.1%)	0.86 (0.57–1.62)	0.89
Formula	34 (23.9%)	48 (16.9%)	1.43 (0.66–3.22)	0.10
Both	34 (23.9%)	60 (21.1%)	1.25 (0.87–2.56)	0.14
Did not feed	27 (19.1%)	68 (23.9%)	0.78 (0.66–3.04)	0.23
Diagnosis, *n* (%)				
SS-HSCR	78 (54.9%)	176 (62.0%)	0.87 (0.41–1.82)	0.71
LS-HSCR	44 (31.0%)	27 (9.5%)	1.63 (0.78–3.56)	0.06
TCA	20 (14.1%)	81 (28.5%)	0.34 (0.23–1.35)	0.09
White blood cells count (10^9/L), median (IQR)	11.3 (9.6–17.9)	10 (8.9–12.1)	1.13 (0.33–2.24)	0.16
Neutrophil proportion (%), mean (SD)	82.3 ± 12.3	54.8 ± 23.5	2.34 (1.24–6.23)	**0.03**
Neutrophil count (10^9/L), mean (SD)	8.1 ± 4.5	6.7 ± 3.3	1.23 (0.73–3.24)	0.13
Red blood cell count (10^{12}/L), median (IQR)	3.76 (3.24–8.21)	4.59 (3.72–7.21)	0.21 (0.09–5.12)	0.67
Hemoglobin (g/L), mean (SD)	112 ± 34	132 ± 24	0.43 (0.25–3.11)	0.44
Platelet count (10^9/L), mean (SD)	207 ± 87	255 ± 101	0.67 (0.32–4.21)	0.32
Albumin levels (g/L), mean (SD)	43 ±12	37±11	2.41 (0.55–5.23)	0.65
C-reactive protein (mg/L), median (IQR)	14.8 (6.6–20.5)	2.5 (1.7–3.5)	3.65 (1.06–4.57)	**0.02**

Note: Values in bold are significant.

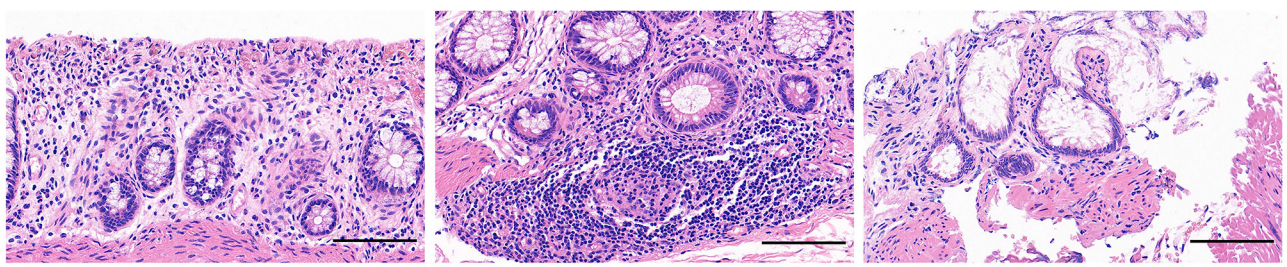

FIGURE 2 | Pathological manifestation of adjacent marginal tissue from the perforated intestine. **(A)** Mild inflammatory cell infiltration, with focal mucosal ischemic necrosis. (Grade I); **(B)** HAEC Grade II: Infiltration of a large number of inflammatory cells with crypt dilation and mucin retention. (Grade II); **(C)** Infiltration of a large number of inflammatory cells with crypt abscesses and epithelium destruction. (Grade III).

follow-up. The remaining patients survived through follow-ups with no need for re-operation after radical colectomy. No deaths were reported.

Univariable logistic analysis revealed no significant differences with respect to sex, birth weight, primigravid, primiparity, delivery type, *in vitro* fertilization, complicated

TABLE 3 | Multivariate regression analysis of risk factors for HD bowel perforation in neonates.

Variables	*P*-value	Adjusted OR	(95% CI)
Vomiting	0.04	2.06	2.01–2.88
Neutrophil percentage (%), mean (SD)	0.02	1.09	1.05–1.33
C-reactive protein (mg/L), median (IQR)	0.02	2.13	1.01–3.27

with multi-disorders, blood transfusion, family history, fever, abdominal dilation after birth, feeding practice, and HD type. Furthermore, no significant difference was observed between the groups for red blood cell (RBC) count, hemoglobin, platelet count, and albumin levels before diagnosis with perforation. The complicated with multi-disorders/syndromes in the perforation group included congenital heart disease ($n = 10$), inguinal hernia ($n = 5$), polydactyly ($n = 4$), Down's syndrome ($n = 3$), Waardenburg-Shah syndrome ($n = 2$), hypoxic-ischemic encephalopathy ($n = 1$), and ectopic pancreas ($n = 3$). Seventy-six complicated with multi-disorders/syndromes were reported in the control group: congenital heart disease ($n = 25$), inguinal hernia ($n = 14$), polydactyly ($n = 14$), syndactylia ($n = 8$), Down's syndrome ($n = 5$), Meckel's diverticulum ($n = 5$), hypoxic-ischemic encephalopathy ($n = 3$), and congenital hydrocephalus ($n = 2$).

On the day of birth, patients with perforated HD reported a higher rate of vomiting compared with the non-perforation groups (76.1 and 35.9%, respectively). In addition, they reported a significantly high risk of perforated HD (unadjusted OR = 3.39, 95% CI: 2.65–4.77, $p < 0.01$). The latest laboratory data before diagnosis of perforated HD revealed significantly increased neutrophil proportion (unadjusted OR = 2.34, 95% CI: 1.24–6.23, $p < 0.05$) and C-reaction protein (CRP) (unadjusted OR = 3.65, 95% CI: 1.06–4.57, $p < 0.05$) in the infants with perforation (**Table 2**).

Multivariable analysis revealed that vomiting (adjusted OR = 2.06, 95% CI: 2.01–2.88, $p < 0.05$), increased neutrophil proportion (adjusted OR = 1.09, 95% CI: 1.05–1.33, $p < 0.05$), and CRP (adjusted OR = 2.13, 95% CI: 1.01–3.27, $p < 0.05$) were associated with an increased risk of perforated HD (**Table 3**).

DISCUSSION

Bowel perforation is an uncommon and severe complication of HD. The rate of perforation among patients with HD has been reported to range from 3 to 6%, with variations among different centers (2, 10, 11). Most of the studies over the past decades are based on cases reports, and little information is available regarding the risk factors for bowel perforation in HD. It is essential to identify neonates HD at high risk of developing bowel perforation and provide prompt intervention.

Although associated risk factors contributing to the development of perforated HD have been considered, most of the previous studies have directly related it to increased luminal pressure caused by distal bowel obstruction (2, 11). However, certain questions remained unanswered, e.g., compared with other common neonatal obstructive diseases, such as imperforate anus (most predisposed to intestinal perforation due to high luminal pressure) (9), the high-risk period for perforation was normally between 36 and 48 h after birth (12). This is considerably earlier than that for HD perforation. Furthermore, spontaneous perforation of the imperforate anus was estimated to occur in 2% of neonates (13), which is also lower than the occurrence of HD perforation.

To elucidate the mechanism of perforated HD, we reviewed the adjacent marginal tissue from perforated intestinal of 122 (85.9%) patients. Histopathological analysis revealed varying degrees of infiltration of inflammatory cells in all samples. Furthermore, the inflammatory response was more severe in the proximal ganglionic colon than in the distal aganglionosis colon. HAEC is the most serious and potentially life-threatening complication of HD. To further understand the pathogenesis of HAEC, we have previously reported the activation of different types of inflammatory cells, especially pro-inflammatory macrophages (M1), resulting in crypt abscesses and mucosal damage, and that HAEC occurs preferentially in the proximal dilated colon of HD patients (14). This finding was consistent with the pathological results described above. The majority of the perforation occurred in the proximal ganglionic bowel, which could be ascribed to the occurrence of severe enterocolitis. Due to the limited literature reporting associated pathological HAEC with perforated HD (15), our study was the first to study inflammatory changes in the intestinal tissue in perforated HD.

To further support the hypothesis that HAEC was associated with the occurrence of perforated HD, our logistic analysis confirmed that inflammatory biomarkers in the laboratory tests of blood, including CRP, and neutrophil proportion, were significantly related to increased rates of perforated HD. Indeed, these biomarkers reflected the serum inflammatory status in these cases (16). Therefore, clinical HAEC is associated with high risks of perforated HD, in line with local and systemic inflammation.

In contrast, our regression results also showed vomiting as an independent risk factor for perforated HD (aOR: 2.06, CI: 2.01–2.88), indicating that increased luminal pressure may also contributed to gastrointestinal tract perforation. Surprisingly, certain dietary factors, including feeding pattern and feeding time, were not associated with perforated HD. As another index of increased intraperitoneal pressure, abdominal distention was insignificantly related to regression in the present study, which could be attributed to the prevalence of abdominal distention in neonates with perforated or non-perforated HD (90.8 and 76.1%, respectively) (17). However, we still lack specific evidence about excessive luminal pressure as an independent cause of perforated HD. In this regard, a prospective study to further detect dynamic changes in the intraperitoneal pressure would be beneficial.

In addition, Arliss et al. (4) have reported appendicular perforation in a 7-day-old boy diagnosed with SS-HD. Histological evidence provided by the authors showed peri-appendicitis without transmural inflammation. Therefore, authors related the special funnel-shaped structure of the neonatal appendix opening into the cecum to the potential point of maximum tension, resulting in pressure perforation in the

presence of distal obstruction (18). This speculation supplements the explanation of the phenomenon of perforation at the cecum and appendix (9.9 and 14.1% in all cases, respectively), where the most common perforation sites of TCA were located in the current study.

Given that HAEC is proved as a risk factor of perforated HD, we need to diagnose HAEC promptly and choose the best treatment strategy. When there are obvious symptoms of neonatal ileus with delay in the passage of meconium, but mechanical ileus such as imperforate anus is excluded, neonatal HD should be considered (19). The hematologic tests are routinely monitored, once the inflammatory index, such as CRP value, elevated neutrophil proportion, and white blood cells account, are unusually increased, with feeding difficulties, vomiting, lethargy, with or without fever, diagnosis of HAEC should be made and symptomatic treatment aimed at HAEC should be timely provided (20). Broad-spectrum antibiotic therapy and intestinal decompression are recommended as critical approaches in the initial management of confirmed HAEC, with additional management strategies, including fluid resuscitation and correction of electrolyte disturbances (8). Timely intestinal decompression is essential to control intestinal inflammation because distal bowel obstruction is also a strong causative factor for the HAEC episode (7). Noticeably, for fulminant neonate HAEC, especially in LS-HD or TCA, rectal washouts have a high risk of iatrogenic perforation (21). In case of failure to adequately decompress the bowel or perforation, an emergency operation with the diversion of luminal flow is required. Because perforation may still occur in the aganglionic or transitional segment, where stoma may result in persistent obstruction, the transition zone must be carefully detected to determine the appropriate location of the stoma. An intraoperative frozen section is used to ensure that the stoma is located in the ganglionic bowel, and a multipoint biopsy should be performed (15).

Limitations

Because this study was the first to explore the causes of bowel perforation in HD, it had certain limitations. First, HD patients were recruited in the study over a decade, during which treatment strategies must have changed drastically. Second, the sample size was limited and underpowered to detect slight changes between the groups. However, we limited the effect of these factors by matching HD patients and controls through a multicenter study design approach. Third, some data were not available for all patients due to the retrospective nature of the study, indicating that certain risk factors associated with perforation remained undetected. Further prospective larger-sample studies that can contribute more details would provide a multivariable model with a better fit.

CONCLUSION

In conclusion, perforated HD is more likely to occur in full-term or near-term neonates. The perforation site is more likely to be located in the proximal ganglionic bowel. Combined with a severe pathological inflammatory response, especially in the proximal dilated bowel, and independent risk factors, including high CRP value and elevated neutrophil proportion in hematologic tests, HAEC is proved to be associated with perforated HD. Timely treatment of HAEC with or without the diversion of luminal flow would be the appropriate approach to prevent perforated HD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of the Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China (No. 2019-S108). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JF designed the research study. TZ, GZ, HY, XX, and JF performed the research. TZ and XM analyzed data. TZ, JY, YN, and YH wrote the initial draft of the manuscript. HY, XX, and JF provided critical assessments during the revision process leading to the final submitted manuscript. All authors have reviewed and approved the final version of this manuscript.

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Familial Experience With Hirschsprung's Disease Improves the Patient's Ability to Cope

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Introduction: Familial occurrence of Hirschsprung's disease may have a positive effect on patients' ability to cope with the disease. The aim was to compare long-term bowel function and generic quality of life between patients with familial and non-familial Hirschsprung's disease.

Methods: This was a nationwide, cross-sectional study in which we included all 830 Hirschsprung patients of 8 years and older who had undergone surgery between 1957 and 2015. We excluded patients with a permanent stoma, intellectual disability, or an unknown or foreign address. We requested patients to complete the validated pediatric or adult Defecation and Fecal Continence questionnaire and the Child Health Questionnaire Child Form-87, or the World Health Organization Quality of Life-100 Assessment Instrument.

Results: We analyzed 336 Hirschsprung patients, 15.8% of whom were familial cases and 84.2% were non-familial cases. After adjusting for aganglionic length, sex, and age, patients with familial Hirschsprung's disease were twice more likely to suffer from constipation (OR = 2.47, 95% CI, 1.21–5.05, $p = 0.013$). The quality of life of the pediatric patients was comparable, but in adult patients the energy/fatigue, thinking/learning/concentration, and work capacity facets showed better scores in the familial patients with Hirschsprung's disease of the rectosigmoid ($p = 0.029$, $p = 0.024$, $p = 0.036$, respectively).

Conclusions: Different facets of generic quality of life are better in adult patients with familial Hirschsprung's disease of the rectosigmoid. It seems that familial experience with the disease influences patients' coping abilities positively.

Keywords: Hirschsprung, quality of life, psychosocial development, inheritance, coping

INTRODUCTION

Hirschsprung's disease is a congenital bowel disorder characterized by a lack of ganglion cells in the distal bowel (1). The aganglionic bowel usually leads to bowel obstruction and requires surgery at an early age. It is known that about 20% of the cases of Hirschsprung's disease are familial, with an overall familial recurrence risk between 4 and 8% (2–4). Furthermore, it is known that familial Hirschsprung's disease is associated with longer lengths of the aganglionic segment (2–4).

The genetics of Hirschsprung's disease have received considerable attention in the past years. Nevertheless, we still know little about the differences in bowel function between patients with and without familial Hirschsprung's disease, except for rare reports on progressive disease in siblings (4). Since modern surgery has led to substantial improvement in the survival of Hirschsprung patients, the responsibilities of pediatric physicians and surgeons regarding postoperative function and quality of life has increased accordingly (5). One can imagine that it might be easier to talk about bowel function problems, like constipation and fecal incontinence, with relatives who suffer from Hirschsprung's disease themselves. It has also been suggested that familial coping strategies have a positive influence on patients' own ability to cope (6, 7). Thus, one may expect that familial occurrence of Hirschsprung's disease may improve patients' ability to cope.

We therefore hypothesize that, differences in aganglionic length apart, the quality of life of patients with familial Hirschsprung's disease is better than that of non-familial patients. The aim of this study was to compare long-term bowel function and generic quality of life between patients with familial and non-familial Hirschsprung's disease.

MATERIALS AND METHODS

Study Design

This was a nationwide, cross-sectional study on 830 patients who had undergone surgical resection for Hirschsprung's disease in one of the six Dutch pediatric surgical centers between 1957 and 2015. We only included patients who were older than seven years at the time the study commenced. We excluded patients without a known Dutch postal address, with a permanent stoma, patients who were intellectually disabled, or deceased patients. This was based on information from the medical records or by self-report of the patients. After the patients had returned the informed consent form, we sent them two questionnaires. We asked the parents or caretakers of patients younger than 18 years to complete the questionnaire together with their children. One investigator searched the medical files for perioperative clinical data. Analyses of the data generated in the study were published previously (8, 9). For the purpose of the current analysis, we excluded 10 patients with an ultrashort aganglionic segment on account of the small number of familial and non-familial patients in this subgroup. Our study population consisted of Hirschsprung patients with aganglionosis of the rectosigmoid, long-segment, or total colon.

Questionnaires

We sent the under 18-year-old patients the Pediatric Defecation and Fecal Continence questionnaire (P-DeFeC) (10, 11) as well as the Child Health Questionnaire Child Form 87 (CHQ-CF87, **Figure 1**) (12). We sent the adult participants the Defecation and Fecal Continence (DeFeC) (10) and the WHO Quality of Life 100 (WHOQOL-100, **Figure 1**) questionnaires (13). All four questionnaires were validated for use in the Netherlands (10, 11, 14, 15).

Although the questions of the P-DeFeC questionnaire are adapted to the level of children, its contents is analogous to the questions of the adult DeFeC questionnaire. Both questionnaires incorporate various validated scoring systems of bowel and urinary function as well as demographic information, medical history, and familial occurrence of Hirschsprung's disease. Familial Hirschsprung's disease was defined as a patient's self-report on the disease in first- (the patient's parents and/or children), second- (the patient's grandparents and/or siblings), or third- or fourth-degree relatives (the patient's uncles and aunts and/or niblings and/or cousins).

Bowel Function

We assessed the prevalence of constipation in accordance with the Rome IV criteria. Patients are defined as constipated if they suffered from at least two of the six symptoms described here: more than 25% straining when defecating, sensation of incomplete defecation, sensation of anal blockage, hard/lumpy stool, a defecation frequency of less than three times per week, using manual maneuvers when defecating. In addition, also part of the Rome IV criteria, is that constipated patients are rarely able to defecate without laxatives (16). The Rome IV criteria for assessing constipation in children also include a rectal examination by a clinician. However, given the design of this study this was not possible. We therefore applied the adult Rome IV criteria instead. We also applied the Rome IV criteria to determine the prevalence of fecal incontinence (17). Fecal incontinence is defined as suffering from at least two to four incidents of involuntary loss of liquid or solid stool during a 4-week period in the past 6 months. To analyze the severity of constipation we used the validated Agachan score, which ranges from 0 to 30 (18). To analyze the severity of fecal incontinence we used the incontinence score developed by Jorge and Wexner, which ranges from 0 to 20 (19). The higher the score, the more severe the constipation or the fecal incontinence. The use of laxatives, enemas, antidiarrheals, and/or colonic irrigations was defined as usage of the treatment during the past 6 months for at least several times a month.

Generic Quality of Life in Children

To assess the generic quality of life in children we used the CHQ-CF87 questionnaire (12). It is divided into 10 domains and two single-item questions. The domain scores range from

Abbreviations: CHQ-CF, Child Health Questionnaire Child Form; DeFeC, defecation and fecal continence; P-DeFeC, pediatric defecation and fecal continence; WHOQOL-100, World Health Organization Quality of Life Assessment Instrument.

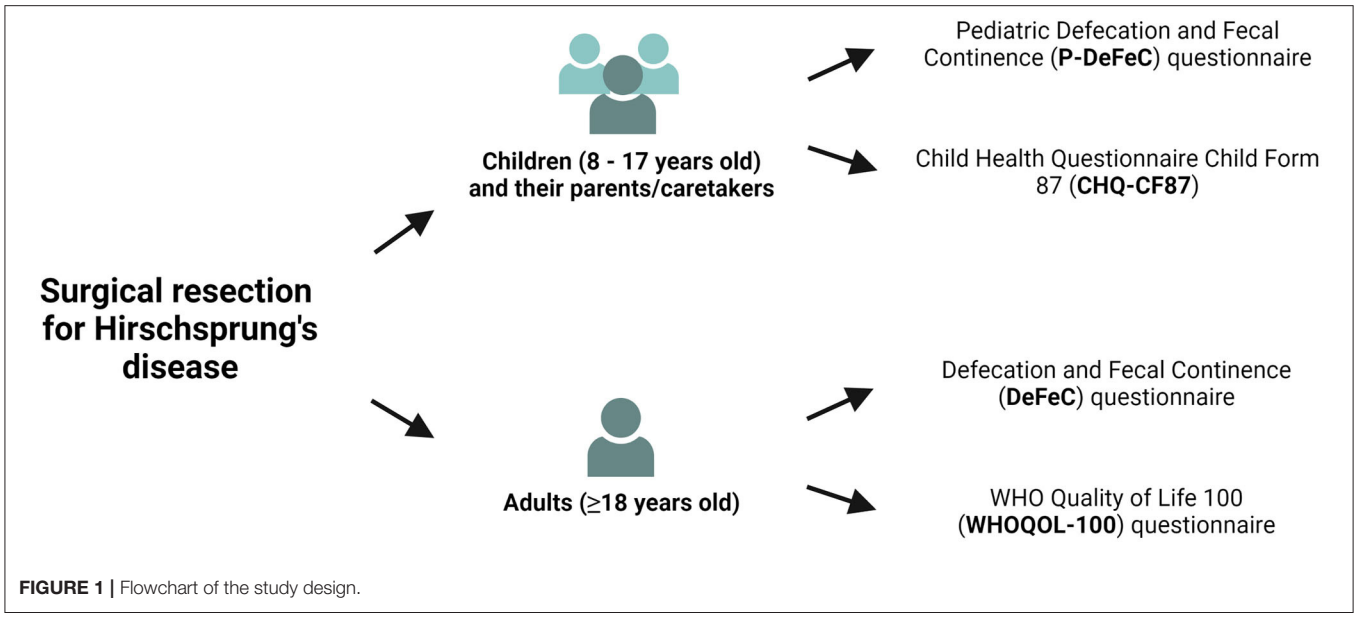

FIGURE 1 | Flowchart of the study design.

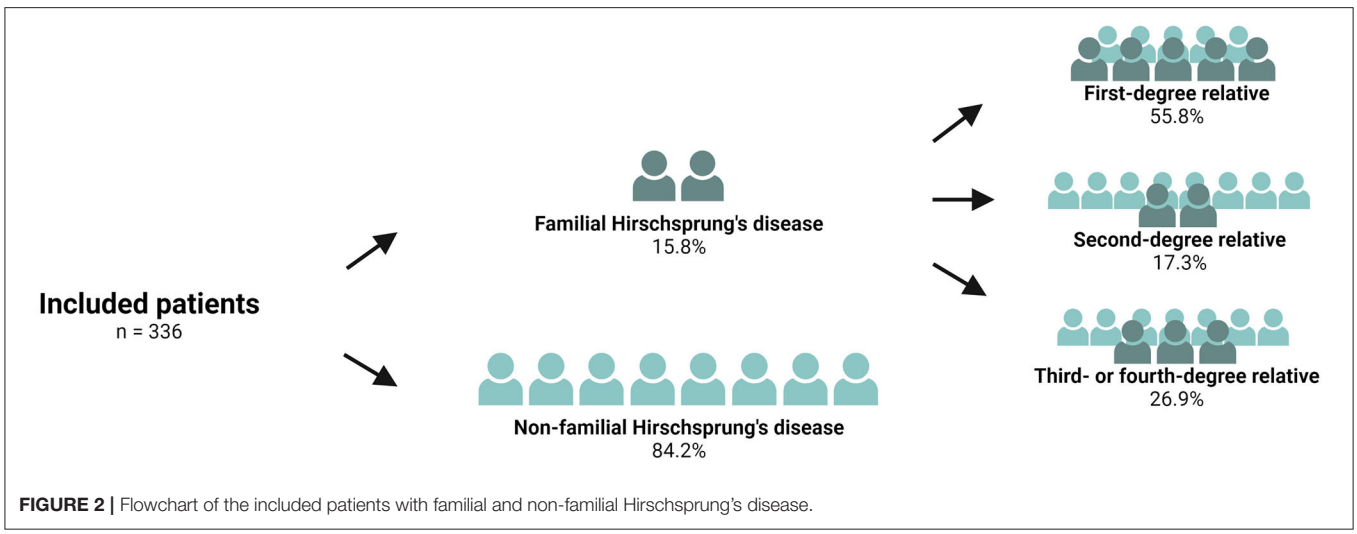

FIGURE 2 | Flowchart of the included patients with familial and non-familial Hirschsprung's disease.

0 to 100, with 100 indicating the highest quality of life. For this study we focused on the eight domains that are related most to functioning in daily life, including physical functioning, mental health, and self-esteem.

Generic Quality of Life in Adults

To assess the generic quality of life in adults we used the WHOQOL-100 questionnaire (13). It consists of multiple facets that form six domains, for example, physical, psychological, and social health domains and overall quality of life. The scores in every domain or facet range between 4 and 20 points, where a higher score equals better quality of life. For this questionnaire, all six domains were analyzed, together with selected facets that provided the best impression of functioning in daily life, for example, energy, concentration and work capacity.

Statistical Analysis

In case of a normal distribution, we present the results as means and standard deviations. For the variables with a skewed distribution, we report medians and interquartile ranges. For the quality of life data we used means and standard deviations for every analysis, because these were Likert scales. We used numbers and percentages to present categorical variables. With regard to comparisons, we used the chi-square test for categorical variables and Student's *T*-test or Mann–Whitney test for continuous variables. We considered *p*-values of less than 0.05 as statistically significant. We performed univariable and multivariable binary logistic regression analyses to determine the likelihood of constipation and fecal incontinence and to adjust for theoretical confounding variables. Potential interactions were checked. The software we used for the statistical analysis was IBM SPSS Statistics, Version 23.0 (Armonk, NY, USA: IBM Corp.).

We created the figures with Graphpad Prism, Version 9.1.0 (GraphPad Software, California, USA) and BioRender.com.

Medical Ethical Review

A local certified Medical Ethical Review Board approved this study (approval code METc 2013/226, University Medical Center Groningen, the Netherlands).

RESULTS

Patient Characteristics

The study comprised a total of 830 patients who had undergone surgery for Hirschsprung's disease. We excluded 57 patients with an unknown or foreign address, 25 patients with a permanent stoma, 86 patients who were intellectually disabled, and 43 deceased patients. Of the remaining 619 patients, 346 patients returned both questionnaires, representing a response rate of 55.9%. A comparison of the responders vs. the non-responders revealed that the responders were younger, as has been described previously (8). We excluded ten patients with an ultrashort aganglionic segment. Finally, the study population comprised 336 patients, 53 (15.8%) of whom were patients with familial Hirschsprung's disease and 283 (84.2%) were non-familial Hirschsprung patients (**Figure 2**). Of the 53 patients with familial Hirschsprung's disease, the closest family member with the same disease was a first-degree relative in 55.8% of the cases, a second-degree relative in 17.3%, and a third- or fourth-degree relative in 26.9% of the cases (**Figure 2**).

We present a comparison of demographic and clinical characteristics between familial and non-familial Hirschsprung patients in **Table 1**. The prevalence of long-segment or total colonic aganglionosis was significantly higher in the patients with familial Hirschsprung's disease than in the non-familial patients (17.0 and 13.2% vs. 7.1 and 6.4%, respectively, $p = 0.009$).

TABLE 1 | Characteristics of patients with familial vs. non-familial Hirschsprung's disease.

	Familial no. (%)	Non-familial no. (%)	*p*-value
Overall	53 (100.0)	283 (100.0)	
Male patients	42 (79.2)	224 (79.2)	0.988
Age at follow-up (years)[a]	19.0 (8.0–42.0)	17.0 (8.0–45.0)	0.270
Age at surgery (months)[a]	6.3 (0.5–160.4)	5.9 (0.3–169.4)	0.753
Length of aganglionosis			
Rectosigmoid	37 (69.8)	245 (86.6)	0.009*
Long-segment	9 (17.0)	20 (7.1)	
Total colonic	7 (13.2)	18 (6.4)	
Congenital comorbidities	5 (9.4)	26 (9.2)	0.955
Preoperative enterocolitis	6 (11.3)	38 (13.4)	0.676
Preoperative stoma	29 (54.7)	137 (48.4)	0.399
Type of reconstruction			
Duhamel	29 (56.9)	178 (63.1)	0.171
Soave	1 (2.0)	0 (0.0)	
Rehbein	13 (25.5)	56 (20.9)	
Swenson	0 (0.0)	1 (0.4)	
Transanal pull-through	8 (15.7)	44 (15.6)	
Surgical approach			
Laparotomic	35 (68.6)	207 (73.4)	0.622
Laparoscopic	8 (15.7)	31 (11.0)	
Combined transanal	8 (15.7)	44 (15.6)	
Anal sphincterotomy	6 (11.3)	11 (3.9)	0.023*
Anal dilatation	12 (22.6)	61 (21.6)	0.860
Postoperative complication	3 (5.8)	31 (11.0)	0.252
Postoperative enterocolitis	12 (22.6)	36 (12.7)	0.058
Redo pull-through	2 (3.8)	21 (7.4)	0.335

[a] *Values are expressed as medians ± ranges.*
**Statistical significance of $p < 0.05$.*

Likelihood and Treatment of Constipation

Univariable regression analysis did not show a significantly different probability of constipation in patients with or without familial Hirschsprung's disease (**Table 2**). However, when we adjusted for length of aganglionosis, sex, and age at follow-up in multivariable regression analysis, we found that patients with familial Hirschsprung's disease were more than twice as likely to suffer from constipation (OR 2.47, 95% CI, 1.21–5.05, $p = 0.013$, **Table 2**). Patients with a second- to fourth-degree relative with Hirschsprung's disease had an increased likelihood of constipation, compared to patients with a first-degree relative, following univariable regression analysis (OR 3.69, 95% CI, 1.04–13.12, $p = 0.043$). The difference was no longer significant in multivariable regression analysis.

We also compared the severity of constipation in the three categories of aganglionic length between patients with and without familial Hirschsprung's disease (**Figure 3A**). A significantly worse median Agachan score was found in patients with familial Hirschsprung's disease in the rectosigmoid category (4.0 vs. 6.0, $p = 0.024$). The Agachan score was not significantly different between patients with a first-degree relative with Hirschsprung's disease vs. a second- to fourth-degree relative (median 5.0 vs. 6.0, respectively, $p = 0.255$).

When we looked at treatments used for constipation, patients with familial Hirschsprung's disease of the rectosigmoid used laxatives twice as often as patients without familial Hirschsprung's disease of the rectosigmoid (35.1 vs. 17.1% respectively, $p = 0.010$, **Figure 4A**). For patients with long-segment or total colonic aganglionosis we found no difference in the use of constipation treatments between patients with and without familial Hirschsprung's disease (**Figure 4A**).

Likelihood and Treatment of Fecal Incontinence

In contrast to constipation, both univariable and multivariable regression analysis showed no significantly different likelihood of fecal incontinence between patients with familial vs. non-familial Hirschsprung's disease (**Table 2**). Likewise, the association between fecal incontinence and a first-degree relative with Hirschsprung's disease vs. a second- to fourth-degree relative was also not significantly different. The severity of fecal incontinence was comparable for patients with and without familial Hirschsprung's disease within the three categories

TABLE 2 | The likelihood of constipation and fecal incontinence.

	Univariable logistic regression		Multivariable logistic regression	
	Odds ratio (95% CI)	***p*-value**	**Odds ratio (95% CI)**	***p*-value**
Constipation[a]				
Familial Hirschsprung's disease				
No	Reference		Reference	
Yes	1.79 (0.93–3.46)	0.081	2.47 (1.21–5.05)	0.013*
Degrees of consanguinity with relatives with Hirschsprung's disease				
First degree	Reference		Reference	
Second to fourth degree	3.69 (1.04–13.12)	0 .043*	2.22 (0.55–8.98)	0.262
Fecal incontinence[b]				
Familial Hirschsprung's disease				
No	Reference		Reference	
Yes	0.84 (0.43–1.65)	0.612	0.92 (0.45–1.86)	0.809
Degrees of consanguinity with relatives with Hirschsprung's disease				
First degree	Reference		Reference	
Second to fourth degree	1.11 (0.31–3.91)	0.872	1.72 (0.35–8.50)	0.505

[a]*Multivariable analysis was adjusted for length of the aganglionosis, sex, and age at follow-up.*
[b]*Multivariable analysis was adjusted for length of the aganglionosis, redo pull-through, and age at follow-up.*
CI, confidence interval.
**Statistical significance of $p < 0.05$.*

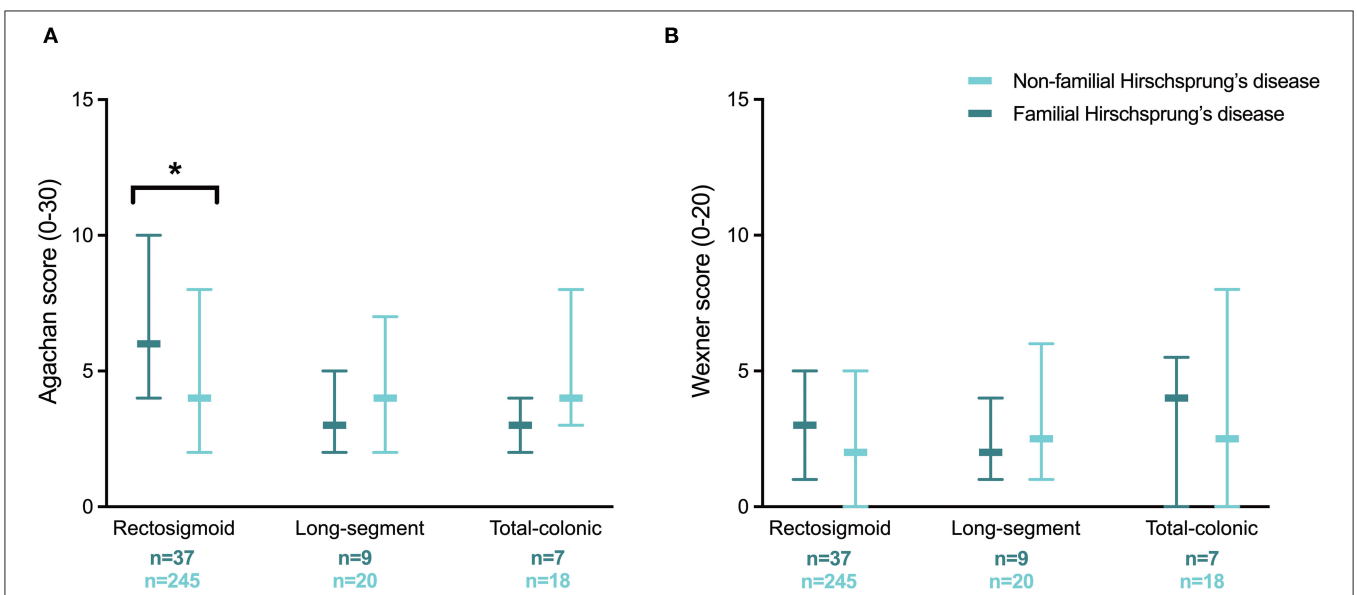

FIGURE 3 | Severity of constipation **(A)** and fecal incontinence **(B)** in patients with familial and non-familial Hirschsprung's disease for different categories of aganglionic length. Values are reported as medians with interquartile ranges. *Statistical significance of $p < 0.05$.

of aganglionic length (**Figure 3B**). The Wexner score was also not significantly different between patients with a first-degree relative with Hirschsprung's disease vs. a second-to fourth-degree relative (median 3.0 vs. 2.0, respectively, $p = 0.787$).

The prevalence of treatments related to fecal incontinence, including the use of colonic irrigations or antidiarrheals, was not significantly different between patients with and without familial Hirschsprung's disease of the rectosigmoid, long-segment, or total colon (**Figure 4B**).

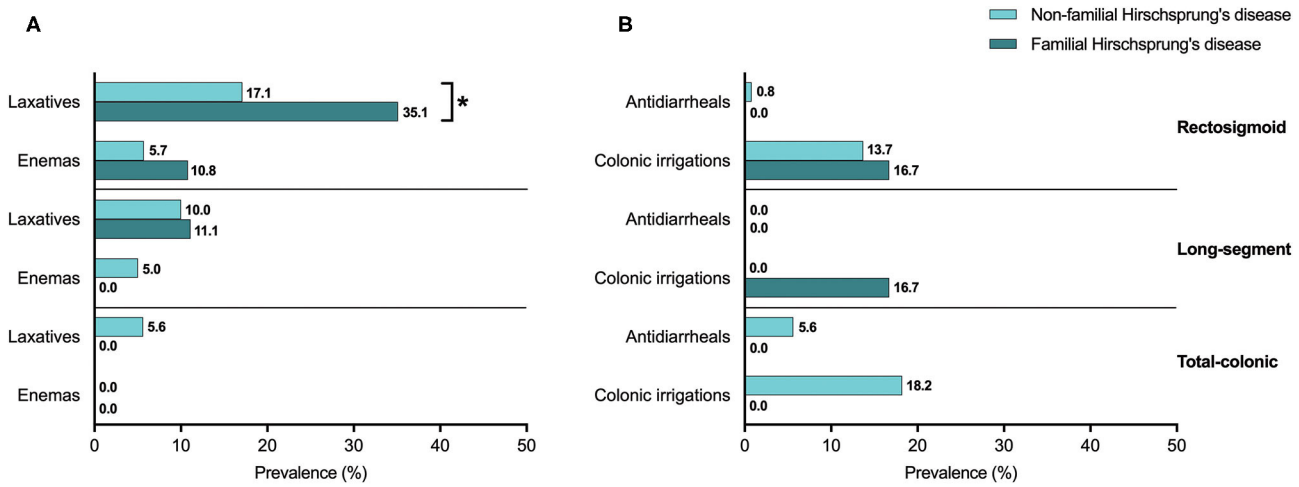

FIGURE 4 | (A,B) The use of defecation treatment in patients with familial and non-familial Hirschsprung's disease. *Statistical significance of $p < 0.05$.

TABLE 3 | Generic quality of life in pediatric patients with Hirschsprung's disease of the rectosigmoid.

Domains of the CHQ-CF87	Familial ($n = 14$)	Non-familial ($n = 110$)	*p*-value
	Mean (SD)	Mean (SD)	
Physical functioning	97.9 (4.8)	97.6 (7.9)	0.899
Bodily pain	82.9 (21.6)	80.4 (22.1)	0.691
General behavior	73.6 (20.8)	71.9 (20.5)	0.776
Mental health	77.0 (12.2)	78.3 (13.1)	0.723
Self-esteem	73.2 (13.4)	76.3 (13.5)	0.420
General health perceptions	75.1 (17.2)	73.7 (19.5)	0.794
Family activities	90.2 (16.8)	86.1 (17.9)	0.415
Family cohesion	71.4 (18.4)	74.8 (20.8)	0.567

CHQ-CF, Child Health Questionnaire Child Form.

Generic Quality of Life in Children

We determined the generic quality of life in 146 pediatric patients, among whom there were 19 (13.0%) children with familial Hirschsprung's disease. We found no significant difference for any of the investigated domain scores of the CHQ-CF87 between patients with and without familial Hirschsprung's disease of the rectosigmoid (**Table 3**). The number of patients with familial long-segment or total colonic aganglionosis was exceedingly small ($n = 3$ and $n = 2$, respectively). The only significant different score was in the domain "bodily pain" among familial vs. non-familial patients with total colonic aganglionosis (40.0 vs. 76.4, $p = 0.046$, **Supplementary Table 1**). Children with a first-degree relative vs. a second- to fourth-degree relative with Hirschsprung's disease showed a significantly higher score for "general behavior" (84.4 vs. 63.3, $p = 0.021$, **Supplementary Table 2**).

TABLE 4 | Generic quality of life in adult patients with Hirschsprung's disease of the rectosigmoid.

Domains/Facets of the WHOQOL-100	Familial ($n = 18$)	Non-familial ($n = 110$)	*p*-value
	Mean (SD)	Mean (SD)	
Physical Health	16.8 (2.9)	15.5 (2.7)	0.053
Energy and fatigue	16.3 (3.5)	14.4 (3.4)	0.029*
Psychological	16.4 (2.0)	15.5 (2.3)	0.121
Thinking, learning, and concentration	16.5 (1.5)	15.5 (2.6)	0.024*
Self-esteem	16.4 (2.1)	15.2 (2.7)	0.061
Independence level	18.3 (1.8)	17.5 (2.3)	0.157
Work capacity	18.3 (2.2)	16.9 (2.8)	0.036*
Social relations	16.2 (2.2)	15.8 (2.6)	0.474
Personal relationships	17.3 (2.0)	16.4 (2.7)	0.189
Environment	16.7 (1.4)	16.2 (2.1)	0.277
Spirituality/religion/ personal beliefs	13.3 (4.4)	12.9 (4.4)	0.681
Quality of life from the point of view of the evaluated subject	17.1 (2.3)	16.2 (2.8)	0.208

WHOQOL, WHO Quality of Life.
**Statistical significance of $p < 0.05$.*

Generic Quality of Life in Adults

In the same way, we analyzed the 155 adult patients, 26 (16.8%) of whom had familial Hirschsprung's disease. The domain scores on the WHOQOL-100 were all comparable between patients with and without familial Hirschsprung's disease of the rectosigmoid (**Table 4**). Looking at the facets, significantly higher scores for "energy/fatigue" (16.3 vs. 14.4, respectively, $p = 0.029$), "thinking/learning/concentration" (16.5 vs. 15.5, respectively, $p = 0.024$), and "work capacity"

(18.3 vs. 16.9, respectively, $p = 0.036$) were found in the familial patients vs. the non-familial patients with Hirschsprung's disease of the rectosigmoid. Once again the number of patients with familial long-segment or total colonic aganglionosis was exceedingly small ($n = 3$ and $n = 5$, respectively). No significant different domain or facet scores were found between these familial patients vs. the non-familial patients (**Supplementary Table 3**). Adults with a first-degree relative vs. a second- to fourth-degree relative with Hirschsprung's disease showed a significantly lower score for "thinking/learning/concentration" (15.6 vs. 17.2, $p = 0.033$, **Supplementary Table 4**).

DISCUSSION

Despite worse bowel function problems, patients with familial Hirschsprung's disease of the rectosigmoid show better physical and psychosocial generic quality of life upon reaching adulthood.

In our study population 15.8% of the patients reported having a relative who had also been born with Hirschsprung's disease. This percentage is comparable to the average prevalence of 20% found in the literature (2–4). It is known that longer aganglionic lengths are associated with a higher familial occurrence (2–4) and this fact corresponds with our findings. We found that patients with familial Hirschsprung's disease were twice more likely to suffer from constipation when stratified for length of aganglionosis, sex, and age. These are three well-known confounders (16), although the reported effect of aging on constipation in Hirschsprung patients varies widely between relief (20, 21) and persistence (21–23). Unfortunately, our data did not enable us to indicate the cause of the increased likelihood of constipation in patients with familial Hirschsprung's disease. Nevertheless, awareness should be raised for the possibility of more severe constipation in children with familial Hirschsprung's disease of the rectosigmoid. The fact that laxatives were used by twice as many patients with familial Hirschsprung of the rectosigmoid may either be caused by more severe disease or by more willingness to take laxatives because their environment may be more familiar with the treatment of Hirschsprung's disease.

Despite the increased likelihood of constipation in patients with familial Hirschsprung's disease, the overall and physical quality of life was comparable between the familial and non-familial patients in children and adults alike. This may be explained by the fact that not constipation but fecal incontinence is considered the main cause of impaired physical quality of life in Hirschsprung patients (7, 23–26), which was not found to be increased in familial cases. Apart from physical quality of life, previous studies found impaired psychosocial quality of life in Hirschsprung patients (26, 27). Although we found the psychosocial domains to be comparable between pediatric familial and non-familial patients, energy, concentration, and work capacity of adult patients with familial Hirschsprung's disease was better, despite their worse bowel function. That we found better quality of life in facets indicative of functioning in daily life could possibly derive from the exemplary role of a relative with the same disease. This may not only lead to realistic expectations of long-term outcomes, but also to small adaptations in how to live "a normal life" despite the disease. Previously, it was reported that there is a low ability to recognize Hirschsprung-related symptoms in adult healthcare (28). Patients with familial Hirschsprung may be at an advantage regarding the recognition of bowel function problems and may possibly find it easier to talk about these problems, which might improve their ability to cope. Based on our results, it is likely that families who are familiar with Hirschsprung's disease provide a better environment for developing good coping strategies than families new to the disease.

In addition, the fact that families who are familiar with Hirschsprung's disease experience a lower level of disease-related stress may affect the way patients experience their own condition. This assumption is justified by high levels of stress and anxiety in parents of patients with Hirschsprung's disease, as has been recognized by others (7, 24, 29, 30). Our results emphasize the need for more familial involvement in the long-term follow-up of Hirschsprung's disease, because familial coping seems to have a positive influence on the ability to cope of the patients themselves, as has been suggested before (6, 7).

This is the first study to compare bowel function and generic quality of life in patients with familial and non-familial Hirschsprung's disease. We acknowledge that this was not a longitudinal study, which precludes analysis of how bowel function and quality of life developed over time. Comparison between patients with first- vs. second- to fourth-degree relatives with Hirschsprung's disease did not reveal many differences in either bowel function or quality of life, but the number of patients per subgroup was exceedingly small. Theoretically, a close relative could play a considerable role in the patient's life and may have more influence than if such a relative were absent. Unfortunately, the rarity of Hirschsprung's disease complicates reporting on large groups of patients. For the same reason the comparison of quality of life between familial and non-familial patients with long-segment and total-colonic aganglionosis was limited. Another limitation was the exclusion of patients with a permanent stoma. This could theoretically have biased our results because patients with intractable fecal incontinence may sometimes receive a permanent stoma. However, seeing that only 25 out of 830 patients were excluded because of a permanent stoma, no substantial bias is expected from this lack of data. Finally, data about familial occurrence of Hirschsprung's disease was gathered through self-report by the patients. As a result, we may have missed cases in which patients were unaware of any relatives who also suffered from Hirschsprung's disease. Nevertheless, also in the clinical setting, familial occurrence of the disease is usually assessed by self-report. Besides, if patients are not aware of the familial occurrence of the disease, no exemplary role could stem from the relative or relatives with the same disease either.

CONCLUSION

Despite a higher prevalence and severity of constipation, different facets of generic quality of life are better in patients with familial Hirschsprung's disease of the rectosigmoid. Familial experience with the disease seems to have a positive influence on patients' own ability to cope.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Review Board of the University Medical Center Groningen. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SV, RM, MT, and PB conceived the research idea, finalized the methods, and analyzed the data. SV wrote the first draft. SV, RM, AS, CS, EH, IB, WG, MW, KV, MT, and PB contributed to data collection, interpretation of the results, and drafting and finalizing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.820976/full#supplementary-material

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Laparoscopic Complete Excision of the Posterior Muscular Cuff: Technique Refinements and Comparison With Stepwise Gradient Muscular Cuff Cutting for Hirschsprung Disease

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Objectives: Our institution had modified the Soave pull-through procedure using laparoscopic stepwise gradient muscular cuff cutting (LSGC) for Hirschsprung disease (HSCR). However, we found that a few children still suffered from obstructive symptoms and enterocolitis during the follow-up. Previous studies suggested that these symptoms might be caused by the retained muscular cuff. The purpose of this study was to employ a modified procedure of laparoscopic complete excision of the posterior muscular cuff (LCEPC) for HSCR and compare it with the laparoscopic stepwise gradient cutting muscular cuff (LSGC) procedure.

Methods: Our institution records of 83 patients with classic form HSCR who underwent LSGC or LCEPC between August 2014 and July 2018 at the Pediatric Surgery Department of Zunyi Medical University (Zunyi, China) were carefully reviewed (LSGC, $n = 52$; LCEPC, $n = 31$). In the present study, we compared the postoperative complications and defecation functions of the two groups. All patients were followed-up (1–5 years, with an average of 2 years).

Results: There were no differences regarding the operation time and the length of hospitalization between groups, while the anal dissection time in the LCEPC group (22.4 $\pm$ 4.8 min) was shorter than that of the LSGC group (45.5 $\pm$ 7.5 min) ($p < 0.001$). The postoperative complication of soiling was significantly increased in six patients (19.4%) in the LCEPC group compared with two patients (3.8%) in the LSGC group ($p = 0.021$). However, the total incidence of enterocolitis (two patients, 6.5%) was significantly decreased in the LCEPC group compared with the LSGC group (12 patients, 23.1 %) ($p = 0.050$). For anastomotic stricture, muscular cuff infection, and constipation, there were no significant differences between the two groups. No patients experienced bladder paralysis and incontinence postoperatively in this study. Anorectal manometries

presented that the anorectal resting pressure was significantly lower in the LCEPC group (14.8 ± 2.7 mmHg) than the LSGC group (22.0 ± 3.8 mmHg), ($p < 0.001$).

Conclusion: The laparoscopic complete excision of the posterior muscular cuff method was demonstrated as safe and efficient, with a decrease in the incidence of enterocolitis, although it may increase the number of soiling incidents in the short period post-surgery owing to a dissected partial internal anal sphincter.

Keywords: Hirschsprung disease, modified Soave, muscular cuff, enterocolitis, soiling, laparoscopy

INTRODUCTION

Soave's first report on the endorectal pull-through without anastomosis approach to the treatment of Hirschsprung disease (HSCR) dates back to 1963 (1). With the rapid development of laparoscopic operations in the early 1990s, Georgeson et al. (2) reported a technique utilizing laparoscopic dissection of the rectum combined with anal mucosal dissection in 1995. Subsequently, many laparoscopic approaches to modified Soave–Georgeson procedures were described, including short muscular cuff anastomosis (3), long cuff dissection, and short V-shaped partially resected cuff anastomosis (4). A common complication was found in the literatures about the modified Soave–Georgeson procedure. These patients often had recurrent obstructive symptoms. Clinical features were presented as recurrent enterocolitis, constipation, and overflow incontinence (5). The purpose of these modifications was to decrease the postoperative complications due to internal anal sphincter achalasia and rectal cuff.

The laparoscopic Soave–Georgeson procedure was designed to protect the vital nerve and blood vessels of the pelvis from injury, by performing laparoscopic rectal dissection combined with endorectal dissection, and subsequently transanal pull-through. In the original descriptions, the submucosal dissection was extended above the peritoneal reflection at about 5–6 cm (6). However, the long muscular cuff could create a tight constricting band around the pulled-through bowel, which might increase the incidence of obstructive symptoms and enterocolitis. Amin et al. (7) used a short cuff operation that retained a muscular cuff of 1–2 cm and achieved excellent outcomes. Due to our increased experience to Soave–Georgeson operation, we have modified the Soave–Georgeson procedure that developed laparoscopic stepwise gradient cutting muscular cuff procedure and shortened the muscular cuff to ∼1–2 cm in neonates and infants or 3–4 cm in children. Good results using the laparoscopic stepwise gradient cutting muscular cuff (LSGC) procedure (**Figure 1**) have been reported by Zheng et al. (8).

Although a few patients suffered from enterocolitis of the LSGC procedure, we found that the incidence of enterocolitis in patients with a 1–2 cm muscular cuff was lower than that in patients with a 3–4 cm muscular cuff. According to the above finding, we developed the laparoscopic complete excision of the posterior muscular cuff (LCEPC) procedure in July 2017. This study reports our institution's experience with two modified Soave methods for the purpose of developing an effective treatment technique for the classic form of Hirschsprung disease.

Abbreviations: HSCR, Hirschsprung disease; LSGC, laparoscopic stepwise gradient cutting muscular cuff; LCEPC, laparoscopic complete excision of the posterior muscular cuff.

MATERIALS AND METHODS

Patients

The medical records of 97 children admitted to the institution between August 2014 and July 2018 with a diagnosis of classical segment HSCR when the aganglionic segment did not extend beyond the upper sigmoid were carefully reviewed. Fourteen patients were excluded because of lost follow-up. Consequently, 83 patients were retrospectively reviewed in this study (male/female, 56:27; age, 2 months−6 years, with an average of 0.78 years). All patients were diagnosed with HSCR by barium enema, anorectal manometries, and biopsy pathologies. Ultimately, 52 patients received the LSGC operations, and 31 patients underwent LCEPC operations. The data collected included the operative time, anal dissection time, length of hospitalization, postoperative complications, and episodes of postoperative enterocolitis. A Stooling Survey was completed through a telephone interview or through scoring obtained from outpatient visit notes. Stooling scores comprised of a composite evaluation of stooling pattern (e.g., excessively loose, or explosive stooling), continence, and/or evidence of enterocolitis. The enterocolitis severity was graded using a previously designed scoring system ranging from grade I to grade III (9). The study protocols and their informed consents were reviewed and approved by the Medical Ethics Committee of Zunyi Medical University (approval no. ICUC-2014081934; Guizhou, China).

Operative Technique

After general anesthesia induction, all patients were placed in a supine position perpendicular to the operating table with legs suspended from the body. A catheter was placed to decompress the bladder. The LCEPC procedure was performed for patients with HSCR confined to the classic segment based on a preoperative barium enema examination and acetylcholinesterase staining in a rectal suction biopsy. Typically, three ports are used with a 5-mm transumbilical camera trocar, and two 3- or 5-mm operative trocars at right and left lateral abdomen, respectively. At first, several intestinal myometrial biopsies were performed to detect the ganglion cells in the myometrial plexus. The aganglionic lengths were determined by biopsy and enema results. The mesentery of the colon was

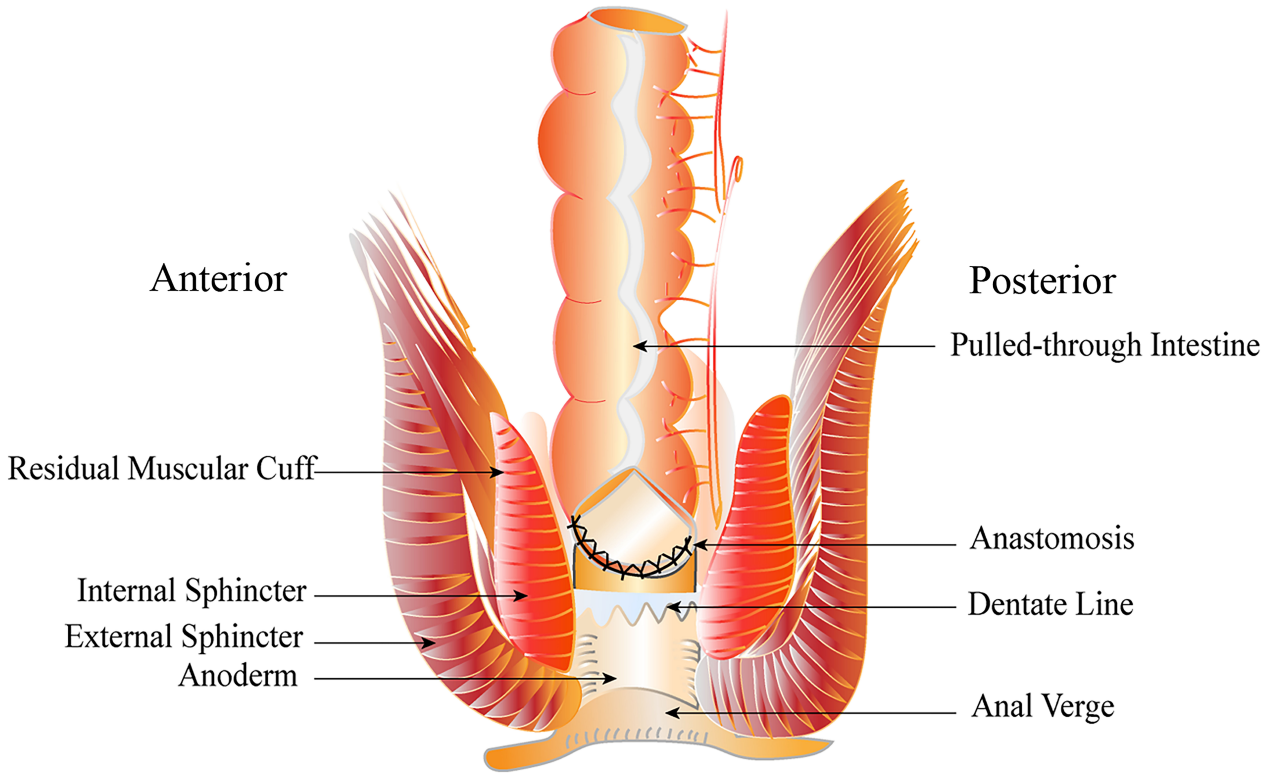

FIGURE 1 | Diagram of laparoscopic stepwise gradient cutting muscular cuff (LSGC) procedure.

separated by laparoscopy with the vessel of the pull-through bowel preserved. Under the rectal peritoneal reflection, close to the rectal wall, separated with the electric hook, the anterior wall of the rectum were separated to the bladder neck or the posterior wall of the vagina. The posterior wall of the rectum was separated down to 1 cm above the dentate line (**Supplementary Video 1**). Anal retractors were used around the anal verge to expose the anus. A 4/0 Vicryl suture was placed as a traction suture 0.5–1.0 cm above the dentate line. The perineal steps involved the dissection of the rectal mucosa and the subsequent pull-through of the colon. The transanal mucosal rectal dissection started with a mucosal incision performed 0.5–1 cm above the dentate line (**Supplementary Video 2**). The blunt approach was used to separate the mucosa under the visible identification of a submucosal plane. The mucosectomy proceeded proximally for 1–2 cm until the plane of the rectal dissection (performed laparoscopically) had been reached (**Figure 2**). At this point, the rectum "prolapsed" outside. The rectal muscular layer ("cuff") was sectioned at its distal part, and it was reintroduced into the pelvis after the section of the anterior and posterior edges. This technique was inspired by Dickie et al. for the treatment of problematic Soave cuff in HSCR (5). The posterior wall of the muscular cuff was completely removed along the left and right side, accounting for two-thirds of the whole circular muscular cuff to 0.5 cm of the dentate line edge, to avoid damaging the sphincter (**Figure 3**; **Supplementary Video 3**). One-third of the anterior wall of the muscular cuff was retained because there were afferent nerves controlling urination and defecation in the anterior wall of the muscular cuff. The next step was to pull through the colon inside the position of the remaining rectal muscular cuff until the normally ganglionic region (where there the biopsy was performed) was identified. The aganglionic bowel was resected, and Colo-anal anastomosis was performed (**Figure 4**).

The LSGC procedure was outlined by Zheng et al. (8) in a previous description. In brief, the aganglionic colon dissection by laparoscopy procedures was the same as the LCEPC procedure. The endorectal dissection was performed by a surgical incision of the stepwise gradient muscular cuff up toward the peritoneal cavity; the muscular cuff remained 1–2 cm in neonates and infants or 3–4 cm in children.

Post-surgical Care

All patients were given intravenous broad-spectrum antibiotic (Ceftazidime for injection; 50 mg/kg body weight; GlaxoSmithKline S.p.A., Research Triangle Park, North Carolina, USA) therapy for 3–5 days, and an anal supporting tube was inserted and maintained for 3 days to prevent enterocolitis. Patients without a maintained nasogastric tube can be fed a liquid diet 6 h after the operation and normal milk 1 day after the operation. All patients were discharged about 7–8 days after surgery. Two weeks later, patients received anal dilatation based

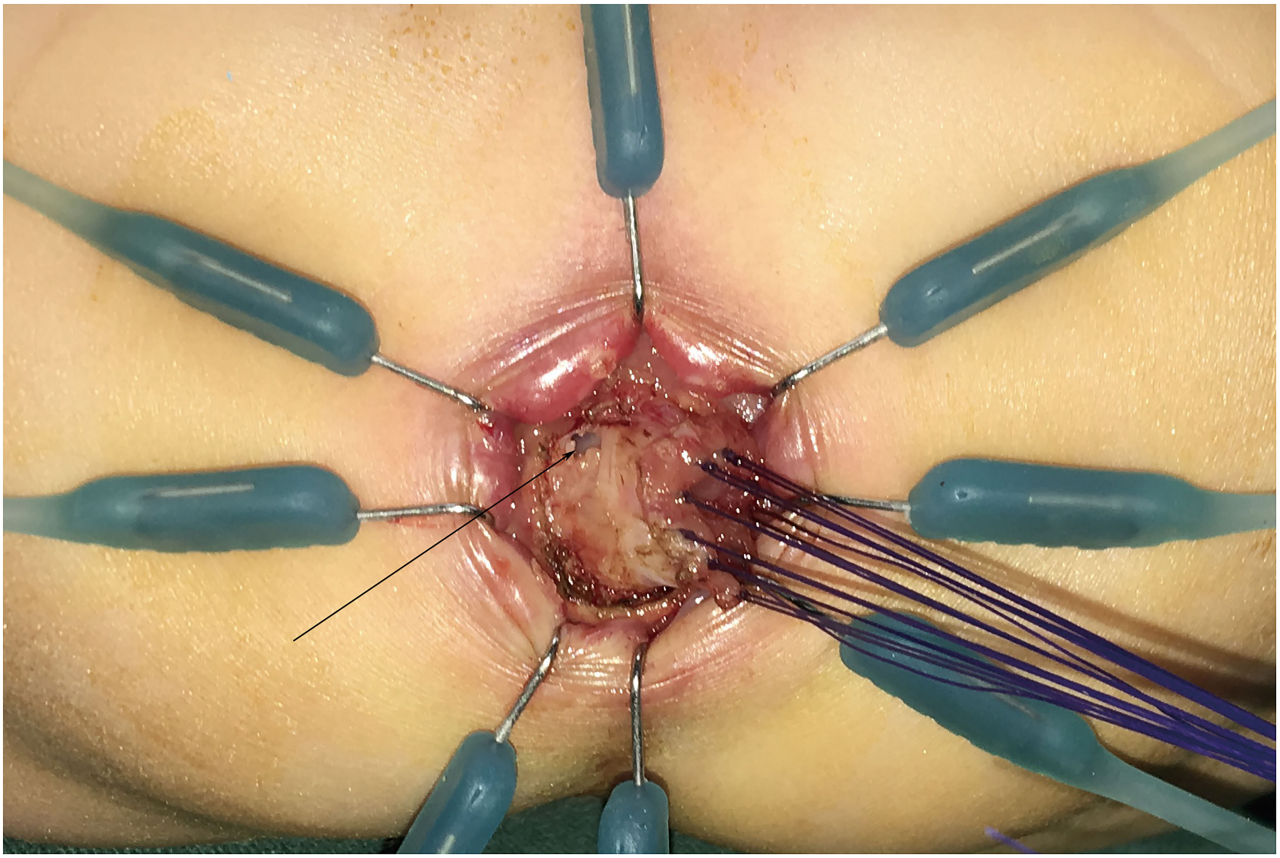

FIGURE 2 | The rectal mucosa was separated through the anus for 1–2 cm to reach the rectal plane under laparoscopy (as the black arrow).

on the anal examination results. Anorectal resting pressure were conducted by anorectal manometries 6 months post-surgery.

Statistical Analysis

Continuous data are expressed as mean ± standard deviation. A *t*-test was used to compare operative time, anal dissection, and the length of postoperative hospitalization between LSGC and LCEPC procedures. Qualitative data were presented as percentages and compared with the chi-squared test. Statistical analysis was carried out using SPSS19.0 (IBM SPSS Software, Armonk, NY, USA). A *p*-value of < 0.05 was considered statistically significant.

RESULTS

Between August 2014 and July 2018, 83 children with classic-form HSCR successfully underwent the two procedures and were routinely followed up. Patient demographics and clinical characteristics are presented in (**Table 1**). For patients in the LCEPC group, there had no significant difference in operative time (121.1 ± 9.7 min vs. 119.4 ± 13.7 min; $p = 0.544$) or length of hospitalization (8.9 ± 2.3 days vs. 9.9±3.1 days; $p = 0.107$) compared with the LSGC group. However, the anal dissection time in the LCEPC group (22.4±4.8 min) was shorter than that of the LSGC group (45.5 ± 7.5 min) ($p < 0.001$).

The postoperative complications are listed in **Table 1**. The incidence of soiling in was significantly increased, with six patients (19.4%) in the LCEPC group compared with two patients (3.8%) in the LSGC group ($p = 0.021$). Regarding constipation, no patients in the LCEPC group reported such problems, but three patients (5.8%) in the LSGC group suffered from constipation; however, no significant difference was observed between groups ($p = 0.173$). One of three patients was diagnosed with the recurrence of HSCR, which was improved by LCEPC procedure. Regarding muscular cuff infection, no patients in the LCEPC group exhibited symptoms, but four patients (7.7%) in the LSGC group did; however, the difference was not significant ($p = 0.113$). Two patients (6.5%) in the LCEPC group and eight patients (15.4%) in the LSGC group exhibited anastomotic stricture, which was relieved by anal dilatation, but no significant difference was observed ($p = 0.227$). No incontinence and bladder paralysis were reported for either groups during long-term follow-up.

The total incidence of enterocolitis was significantly decreased with 2 patients (6.5%) in the LCEPC group compared to 12 patients (23.1%) in the LSGC group ($p = 0.050$). There was one patient (3.2%) diagnosed with grade I enterocolitis in the

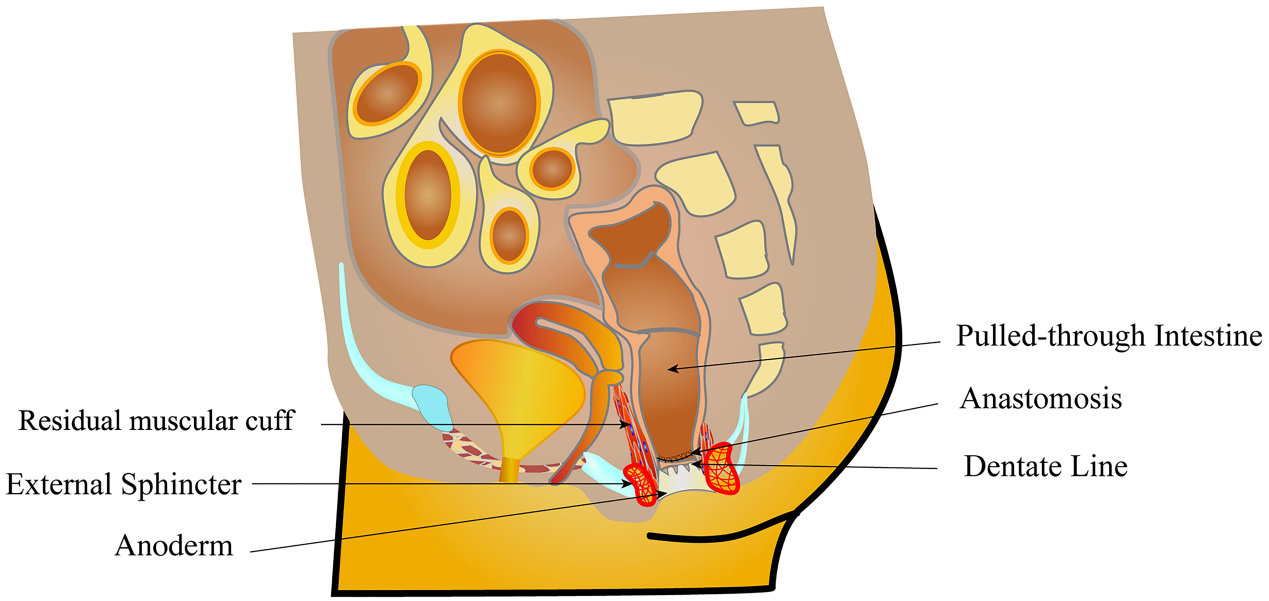

FIGURE 3 | The muscle sheath 1–2 cm from the anterior wall of rectum was retained between the posterior wall of vagina and the pull-through colon. The muscular cuff of the posterior rectal wall was completely removed.

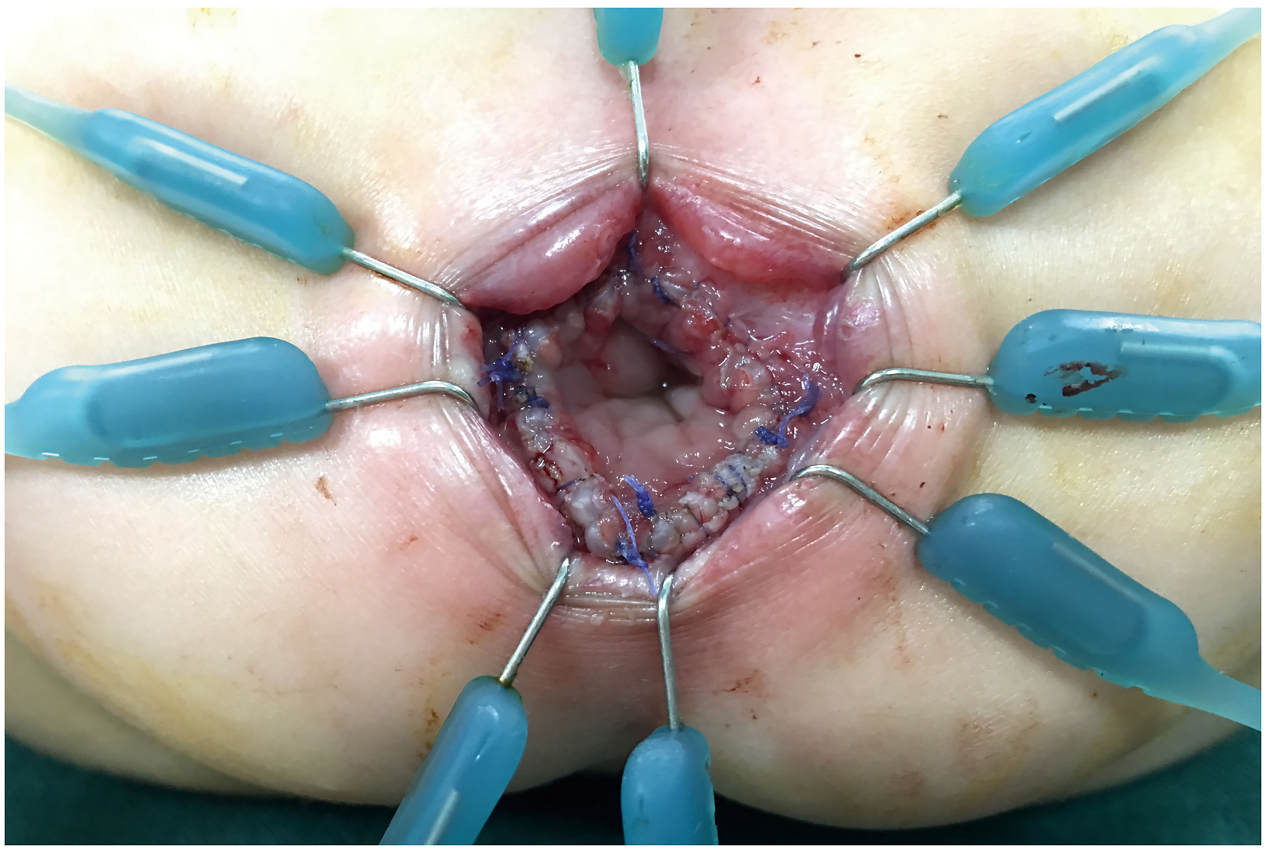

FIGURE 4 | Completed anastomosis.

LCEPC group and eight patients (15.4%) in the LSGC group ($p = 0.085$). There was one patient (3.2 %) diagnosed with grade II enterocolitis in the LCEPC group and four patients (7.7 %) in the LSGC group ($p = 0.408$). No patient experienced grade III enterocolitis in either of the two groups. The severity of these episodes was not significantly different between the two groups. Anorectal manometries indicated that the anorectal resting pressure was significantly lower in the LCEPC group (14.8 $\pm$ 2.7 mmHg) than the LSGC group (22.0 $\pm$ 3.8 mmHg) ($p < 0.001$).

DISCUSSION

The Soave approach and endorectal dissection were designed to prevent injury to structures surrounding the rectal wall, especially the nerves of the bladder and sexual function management with HSCR (9). However, Swenson (10) reported that the Soave procedure left a long aganglionic muscular cuff, which might extrinsically obstruct the pull-through bowel and influence peristalsis on the normal colon. Many studies indicated that the long aganglionic muscular cuff may be related to the postoperative obstructive symptoms, constipation, and enterocolitis in HSCR patients (6, 11). Subsequently, other surgical procedures that shortened the length of the muscular cuff were developed to relieve the above complications (4). Wester et al. (12) recommend a residual 1–2 cm muscular cuff. Short muscular cuff reduced the incidence of enterocolitis to 17.5% and also shortened the hospital stay. Yang et al. (4) reported a long cuff dissection and a short V-shaped resected cuff anastomosis procedures that reduced the incidence of anastomotic stricture and constipation. Nevertheless, these short muscular cuff procedures may increase the risk of damage to perirectal nerves and anal sphincter.

In our center, we have carried out the stepwise gradient cutting muscular cuff procedure since 2003 and shortened the muscular cuff to avoid the long aganglionic muscular cuff problem (8). We found that the shortened muscular cuff procedures could decrease the incidence of enterocolitis and obstruction symptoms. However, the incidence of enterocolitis was still higher than some other studies reported, and there were some patients who suffered from constipation during the follow-up period (3). In 2013, Levitt et al. (13) reported a novel modification of Swenson's original transabdominal dissection concept using full-thickness rectal dissection for HSCR; all the patients achieved voluntary urinary and fecal continence after operations, and the incidence of enterocolitis was only 14%. We carefully reviewed the data described by Sherman et al. (14) and adopted the Swenson operation, and since then, there had no anastomosis obstructive problem post-surgery, and the rate of post-operative enterocolitis was much lower than other resection procedures.

Based on the understanding and experience of Soave operation and full-thickness rectal dissection from our institution and other famous clinical centers (13), we modified Soave as LCEPC by the following changes: the posterior wall of the muscular cuff was completely removed along the left and right sides, accounting for two-thirds of the whole circular muscular cuff to 0.5 cm of the dentate line edge, to avoid damaging the sphincter. One third of the anterior wall of the muscular cuff was retained. The modified Soave procedure with a complete excision of the posterior muscular cuff was similar to the modified Swenson procedure, although there had been some differences between the two procedures. First, in modified Swenson procedure, the rectum below the peritoneal reflection was dissociated up to the inferior border of the levator ani muscle. This process might damage the pelvic strictures, especially the pelvic nerves and the bladder/vagina. The higher anal anastomosis was performed if the rectum was not dissociated enough by laparoscopy, which increased the risk of anastomotic leakage and recurrence of HSCR. However, the transanal mucosal rectal dissection started with a mucosal incision. The mucosectomy proceeded proximally for 1–2 cm until the plane of the rectal dissection (performed laparoscopically) had been reached in our study, which protected the structure around the rectum. Second, the full-thickness rectal dissection was performed from the Herrmann line in modified Swenson procedure, which was higher than our study. Finally, the whole aganglionic muscular cuff was removed by circumferential full thickness in modified Swenson procedure. However, the muscular cuff of the anterior wall of the rectum was retained in our study, which was the essential nerves access position that controlled the bladder and sexual function.

Laparoscopic separation of the rectum in the pelvic cavity and transanal resection of the muscular sheath of the posterior wall of the rectum were important steps in the LCEPC. To decrease the time of anal dilatation and extension in transanal procedure, the rectal dissociation by laparoscopy is required as lower as possible (11). In the LCEPC procedure, under the rectal peritoneal reflection, the anterior wall of the rectum was easily separated from the bladder neck or the posterior wall of the vagina. The posterior wall of the rectum was separated down to 1 cm above the dentate line. Some doctors considered that too much separated rectum below the peritoneal reflection might increase the risk of injury to the pelvic nerves and bladder/vagina (15). Therefore, the long muscular cuff anastomosis was a highly praised procedure for HSCR. During our practice, the posterior wall of rectum had loose knot and hoof tissue, which was convenient for us to dissociate rectum with electric hook. However, excessive bleeding would obscure the surgical vision; hemostasis should be manipulated meticulously. The anal dissection time was shorter than LSGC procedures, which was partially owing to the dissociation of rectum by laparoscopy. No bladder paralysis or vaginal and urethral complications were presented in this study.

We observed 6.5% of enterocolitis in the LCEPC procedures. Enterocolitis is the major cause of morbidity and mortality in HSCR. However, the etiology of enterocolitis is multivariable and poorly understood, and the obstructive cuff alone is likely to participate in its development (16). Enterocolitis has a widely variable incidence of between 4.6% and 54%, and no sufficient evidence shows whether the incidence correlates with the type of residual muscular cuff performed (17). In our study, the relatively low rate of enterocolitis in the LCEPC procedures may be due to the complete removal of muscular cuff at the posterior wall of the rectum and the 1–2 cm muscular cuff retained at the anterior wall of the rectum. This was carried

TABLE 1 | Clinical outcomes in HSCR children undergoing surgery repair.

Characteristic	Total	LCEPC	LSGC	*p*-value
Postoperative course				
Operative time (min) (n/mean ± SD)	83/120 ± 12.4	31/121.1 ± 9.7	52/119.4 ± 13.7	0.544[a]
Anal dissection time (min) (n/ mean ± SD)	83/36.8 ± 13.0	31/22.4 ± 4.8	52/45.5 ± 7.5	<0.001[a]
Length of hospitalization (day) (n/ mean ± SD)	83/9.5 ± 2.9	31/8.9 ± 2.3	52/9.9 ± 3.1	0.107[a]
Postoperative complication (n/%)				
Soiling	8/9.6	6/19.4	2/3.8	0.021[b]
Constipation	3/3.6	0/0	3/5.8	0.173[b]
Muscular cuff infection	4/4.8	0/0	4/7.7	0.113[b]
Anastomotic stricture	10/12	2/6.5	8/15.4	0.227[b]
Bladder paralysis	0/0	0/0	0/0	
Incontinence	0/0	0/0	0/0	
Enterocolitis	14/16.9	2/6.5	12/23.1	0.050[b]
Grade I	9/10.8	1/3.2	8/15.4	0.085[b]
Grade II	5/6.0	1/3.2	4/7.7	0.408[b]
Grade III	0/0	0/0	0/0	
Anorectal resting pressure (mmHg)	83/19.3 ± 4.9	31/14.8 ± 2.7	52/22.0 ± 3.8	<0.001[a]

LCEPC, laparoscopic complete excision of the posterior muscular cuff; LSGC, laparoscopic stepwise gradient cutting muscular cuff.
[a]*t-test.*
[b]*Chi-square test.*

out to avoid obstructive symptoms and decrease the incidence of constipation and anastomotic stricture as shown in our patients. Studies reported that 75% patients with recurrent enterocolitis following a pull-through operation had improved symptoms after a sphincterotomy (18). Some surgeons would be worried about the postoperative incontinence as a result of sphincterotomy and potential permanent injury to the sphincter (19). During our clinical practice, preserving the partial internal anal sphincter below the dental line is the key to postoperative continence. At the same time, we retained 1–2 cm of muscular cuff between the pull-through and the bladder/vagina to reduce the potential damage to the bladder/vagina and nerves of sexual function. However, sexual function needs long-term follow-up.

Compared to the previous studies, the occurrence of soiling in the LSGC procedure group was decreased (3.8%). Soiling is another postoperative complication after pull-through for HSCR. Levitt et al. (20) described that continence is related to normal anal sensation, voluntary sphincter control, and appropriate colonic motility. Some researchers reported that the muscular cuff participated in part in the function of internal sphincter and maintained the control function of defecation (21). Interruption of any of these elements leads to HSCR children being partially or totally incontinent. However, the soiling is pseudoincontinent, implying that the continence mechanism is intact. Many studies have reported that soiling improved with the follow-up time (22). In this study, we found that 19.4% of soiling in the LCEPC procedure was higher than the LSGC procedure, and the anal resting pressure was significantly lower in the LCEPC group. Tran et al. (23) reported that the internal and external anal sphincters contributed to 55.0% and 35.0% of the anal resting pressure, respectively. Therefore, the decreased anal resting sphincter pressure was related to the partial resection of the internal anal sphincter in the LCEPC procedure. In our series, patients presented with occasional soiling after breaking wind. Patients were evaluated by contrast enema and an anal examination for the integrity of the anal canal. Medical management started for those who presented with soiling 2 weeks after pull-through. Laxatives were prescribed for patients with colonic dilation, loperamide, and a special dietary regimen (constipating diet). All patients' symptoms were gradually improved by medical management 6 months post-surgery. We advocate that the complete excision of posterior muscular cuff is beneficial to both the continence or the enterocolitis.

This study has some limitations; some of the limitations and drawbacks of this study are the small sample size and the short follow-up period, limiting its generalizability. Further studies in larger patient groups, multicenter study, and long-term follow-up are needed. Another is that this observational study has some degree of heterogeneity relating to several factors that the study was not randomized controlled and certified by multiple medical institutions, with a different surgeon.

In summary, the laparoscopic complete excision of the posterior Soave cuff procedure for classic segment HSCR demonstrated safety and efficacy, with a decrease in the incidence of enterocolitis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Affiliated Hospital of Zunyi Medical University. Written informed consent to participate in

this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ZZ performed the LCEPC procedure and collected data. ZJ performed the LCEPC procedure. MG, CT, LH, and YG analyzed the data. YL conceived and designed the experiments, performed the LSGC and LCEPC procedures, contributed reagents, materials, and analysis tools. All authors reviewed drafts of the article and approved the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.578843/full#supplementary-material

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