

Histopathologic and immunohistochemical findings in congenital anorectal malformations

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Abstract

It remains controversial whether the distal rectal pouch should be either resected or used for reconstruction in anorectoplasty for the treatment of anorectal malformations (ARMs). Hence the aim of this study was to investigate whether ARMs were associated with a global neuromuscular maldevelopment of the terminal rectum specimens.

There were 36 cases of ARMs (25 recto-bulbar fistula and 11 recto-prostatic fistula) and 10 healthy controls. The hematoxylin and eosin and Masson trichrome stain were used to conduct the histologic examination. The immunohistochemistry (IHC) and Western blot were conducted to analyze the neuron-specific enolase (NSE), S-100 protein, interstitial cells of Cajal marker (C-kit) within the rectal specimens in control group and ARM group.

The most frequently observed histologic findings in mucosa were inflammation, congestion, eroded, and hemorrhage in the ARM cases. Submucosal inflammation and congestion were the most common submucosal findings in the ARM cases. Disrupted muscularis propria was observed in 60% of ARM cases. Mature ganglionic cells were reduced and muscularis propria showed reduced and patchy positivity for NSE, S-100, and C-kit protein in ARM group compared to that in control group according to IHC. Western blotting showed the expression levels of NSE, S-100, and C-kit were lower in the ARM group than that in the control group ($P < .01$).

Histopathologic and IHC findings suggest that the distal rectal pouch has distinct defects in the neuromusculature. So it suggested that ARMs are abnormally developed tissue and need to be resected for better functional outcomes of the remaining gut.

Abbreviations: ARMs = anorectal malformations, ENS = enteric nervous system, H&E = hematoxylin and eosin, ICC = interstitial cells of Cajal, IHC = immunohistochemistry, NSE = neuron-specific enolase, PBS = phosphate-buffered saline, TBST = tris-buffered saline-Tween.

Keywords: anorectal malformations, C-kit, immunohistochemistry, neuron-specific enolase, S-100

1. Introduction

Although surgical techniques have improved significantly in the last decades, complete anatomic and functional restoration of congenital anorectal malformations (ARMs) cannot be achieved. Patients with ARMs often present with varying severity of defecation dysfunction (constipation, incontinence, and fecal soiling) following corrective operations. Constipation is one of the most frequent complications after correction of ARMs, occurring in 30% to 60% of patients.^[1,2] However, the detailed mechanism remains unclear. Enteric nervous system (ENS), smooth muscle layer, and interstitial cells of Cajal (ICC) participate in both nitrergic and cholinergic neurotransmission,

as well as in the initiation and regulation of the electrical activity which play a key role in maintaining normal functioning of gastrointestinal tract.^[3-6] It is reported that the postoperative bowel function depends not only on the type of malformation and the muscular and neurologic condition of the pelvic floor musculature, but also on the histology of the blind pouch. To the best of our knowledge, there is a paucity of literature describing the histopathologic abnormalities of the distal rectum or distal pouch.^[7,8] Hence our aim is to study the histomorphologic changes and various immunohistochemistry (IHC) markers (neuron-specific enolase [NSE], S-100, and ICC marker [C-kit]) in distal rectal specimens to assess neuronal dysfunction in a small cohort of ARM children, which could be responsible for soiling constipation, or incontinence after corrective procedure in ARMs.

2. Materials and methods

2.1. Specimen collection

Ethical approval was obtained for specimen collection from the institutional review boards of both centers involved in the study. Informed written consent for participation in the study was obtained from parents/guardians of all children preoperatively. All procedures carried out in the study period conformed to the principles of the Declaration of Helsinki. A total of 36 patient specimens with a clinical diagnosis of ARMs and 10 control specimens were included in the study. The 10 control rectal specimens came from autopsy specimens from 5 patients who died from nongastrointestinal disease, 2 resection specimens for traumatic rectal injury, and 3 specimens from resections due to necrotizing enterocolitis. The tissue specimens were the rectum

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that 2 to 4 cm above the dentate line in the control group. The tissue specimens in the ARM group consisted of 0.5 to 2.0 cm of the most distal part of the rectal pouch. Full-length rectal specimens were obtained fresh intraoperatively from patients who underwent laparoscopic surgery for ARMs and transferred to chilled phosphate-buffered saline (PBS) solution for dissection. For hematoxylin and eosin (H&E) staining and immunohistochemical studies, the specimens were fixed overnight in 4% paraformaldehyde/0.1 mol/L PBS at 4°C, then embedded in paraffin in a routine manner. Specimens were sectioned sagittally at 4 μ m thickness. For Western blot analysis, the specimens were dissected and immediately frozen in liquid nitrogen to prepare for Western blot analysis.

2.2. Immunocytochemistry

The resected specimens were fixed in 4% formalin solution and observed grossly as well as microscopically. For gross examination, the mucosal and serosal surfaces were examined. For microscopic examination, 5 sections were taken from the resected pouch arbitrarily. They were stained with H&E and Masson trichrome stain. IHC staining was done on 4 μ m-thick sections of formalin-fixed paraffin-embedded tissue. The sections were incubated overnight at 4°C with the primary antibody at dilutions of 1:200 for NSE, S-100, and C-kit (Mouse monoclonal;

Abcam Co, Shanghai, China codes ab39369, ab868, ab32363). After 3 washes in PBS, sections or slice cultures were incubated 2 hours at room temperature with the secondary antibody (molecular probes; Invitrogen, Paisley, UK) diluted 1:500 with 0.05% digitonin and 10% normal goat serum in PBS.

2.3. Western blotting

Total protein was extracted from tissues of the rectum specimens by using whole cell lysates (Beyotime, China) and protein concentrations were assessed by using the pierce BCA Protein Assay Kit (Beyotime, China). For each sample, 40 μ g protein was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel, transferred electrophoretically onto a polyvinylidene difluoride membrane, and blocked in 5% milk in 0.1% Tris-buffered saline-Tween 20 (TBST) at 4°C overnight. After 3 washes in TBST, membranes were incubated overnight at 4°C with the appropriate dilution of primary antibodies against NSE, S-100, and C-kit (AF645; R&D Systems, China Co., Ltd). The membranes were then washed with 1XTBST and incubated with secondary antibody (Cell Signaling Technology Inc, Danvers, MA). Signals were performed with ECL chemiluminescence kit (Boster, Wuhan, China) and exposed to X-ray films. Alfa-glycerolaldehyde phosphate dehydrogenase (α -GAPDH) immunoblotting was used as an internal control.

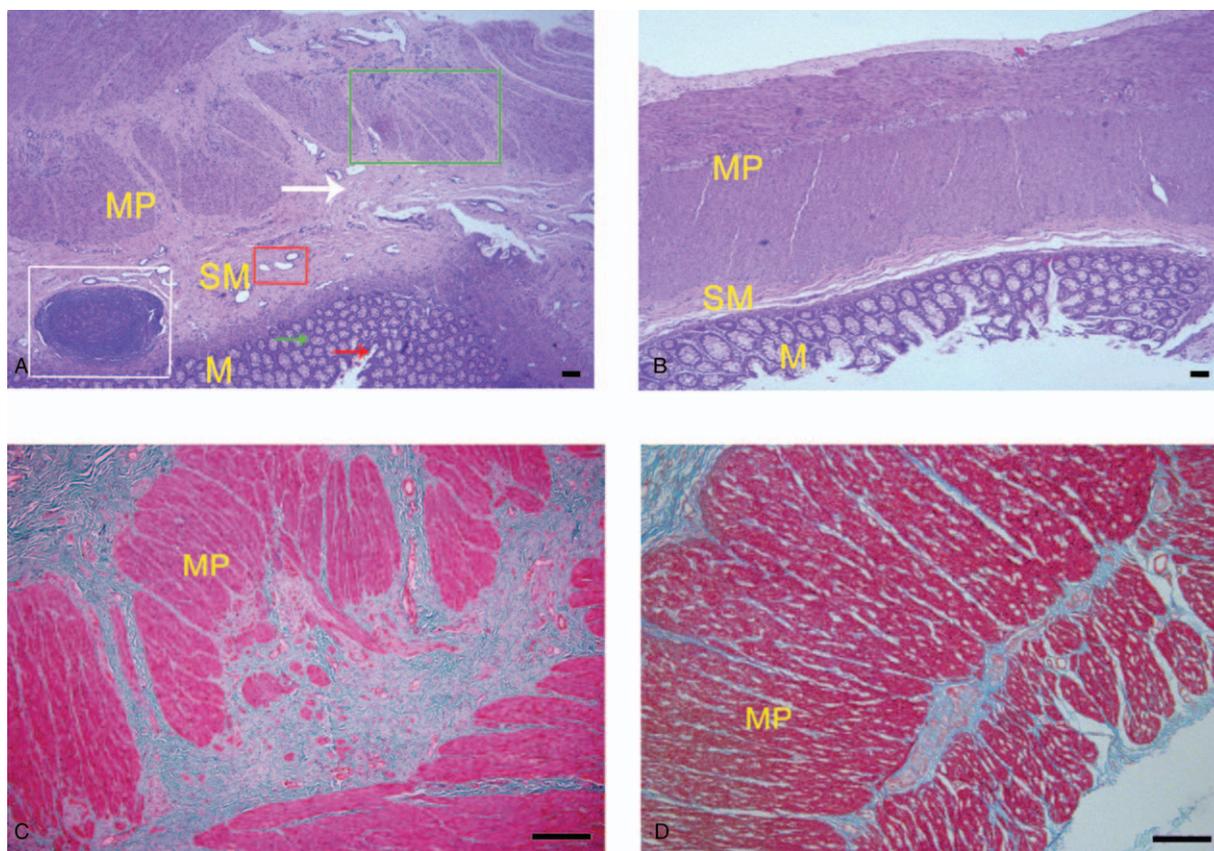


Figure 1. Comparison of the morphology of each layer of the intestinal wall between the anorectal malformation (ARM) and control group. (A) Erosion (red arrow) and inflammation (green arrow) in the mucosa, congestion (red rectangular) and lymph follicle (white rectangular) in the submucosa, and disorganized muscle bundles (green rectangular) and fibrosis (white arrow) in the muscularis propria in ARM specimens. Hematoxylin and eosin (H&E), $\times 40$. (B) Microscopic examination of the control specimens showed normal mucosa and submucosa, and a continuous, undisrupted muscle layer. H&E, $\times 40$. (C) Masson trichrome staining in a control specimen showing equal and homogenous staining of both muscle layers. Masson trichrome, $\times 100$. (D) Masson trichrome staining in a ARM specimen showing unequal staining in the muscularis propria and fibrosis in the muscularis propria, dividing it into various bands. Masson trichrome, $\times 100$. M = mucosa, SM = submucosa, MP = muscularis propria.

2.4. Image and statistical analysis

Five medium power fields were picked randomly for each section. Qwin 3000 biologic image analysis software was used to measure the percentage of the average area of the examined medium power fields. The data were analyzed using SPSS 21.0 package. The numerical data are presented as the mean \pm standard deviation. Statistical analysis was performed using the 2-sample *t* test. $P < .05$ was considered to be statistically significant.

3. Results

3.1. The demographic characteristics of the ARM and control group

A total of 36 cases of ARM and 10 controls were included in the study. The age of the patients with ARM ranged from 2 to 4 months, and there were 10 males and 26 females. About 25 patients had recto-bulbar fistulas and 11 patients had recto-prostatic fistulas, and no patients had associated congenital anomalies such as partial sacral agenesis or tethered cord. The age of the control group ranged from 2 to 7 months, and the ratio of female:male is 2:3.

3.2. H&E and Masson trichrome staining in ARM and control groups

Histopathologic examination of ARM cases showed abnormalities in the mucosa, submucosa, muscularis propria, and serosa (Fig. 1A). The most common mucosal finding seen was inflammation, along with muscularis mucosae disruption and fibrosis. There were several submucosal changes in the ARM group including congestion, submucosal widening, hemorrhage, inflammation, and the presence of lymphoid follicles, fibrosis, and edema. Microscopic examination of the control specimens showed normal mucosa and submucosa, and a continuous, undisrupted muscle layer (Fig. 1B). On Masson trichrome staining, the ARM group showed disruption and division of muscle fibers into bands and fibrosis in the muscularis propria indicated by fibers that stained blue in between those that stained red (Fig. 1C). The control group revealed equal and continuous staining of the muscle fibers (Fig. 1D).

3.3. H&E staining showed the development of ganglion cells and nerve plexus between the 2 groups

Nerve plexi were sparse and neurons within these plexi were concentrated into clumps in the ARM submucosal and muscular layers (Fig. 2A, C), while normal number and appearing ganglion

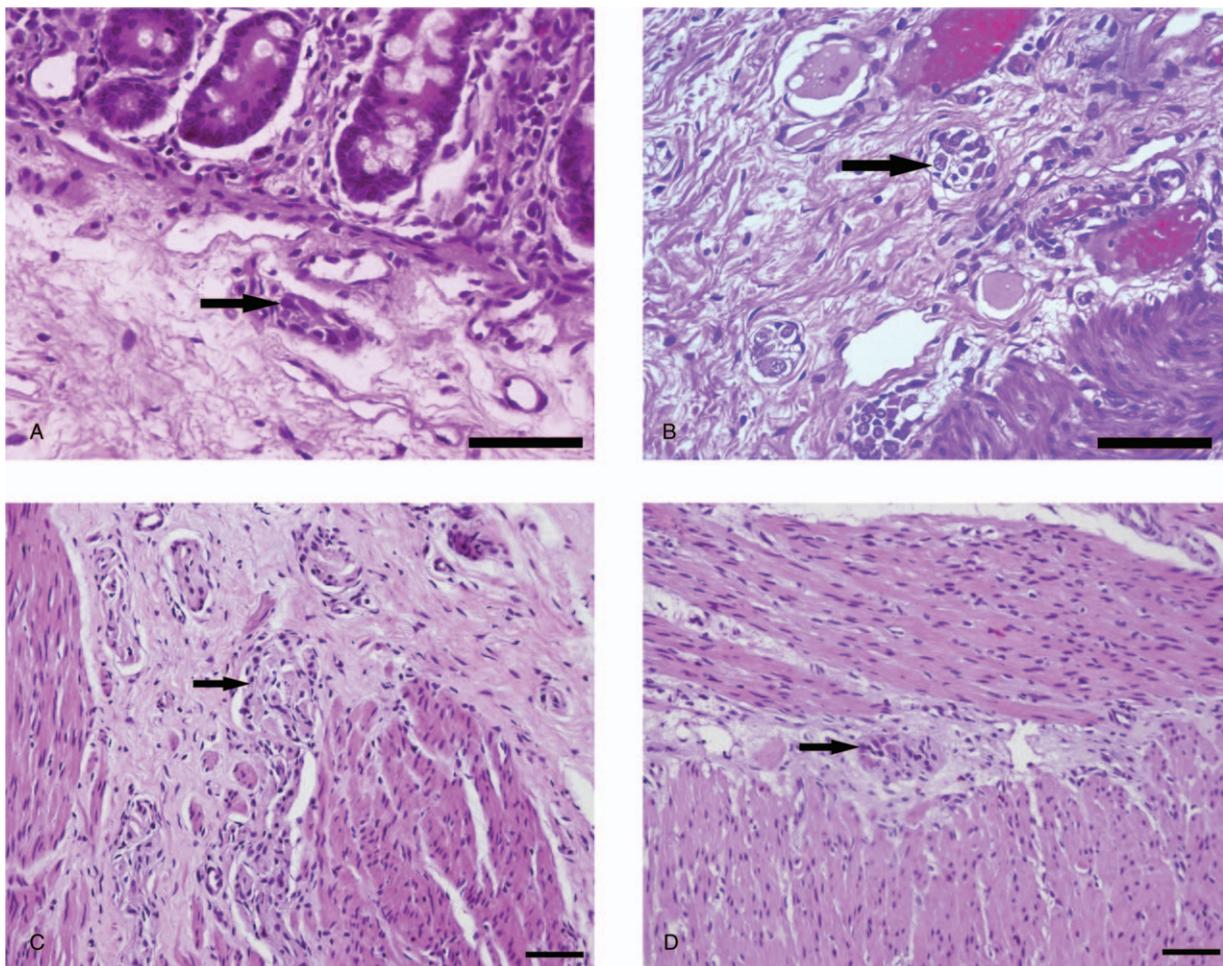


Figure 2. Hematoxylin and eosin (H&E) staining for comparison of ganglion cell development between the anorectal malformation (ARM) and control group. (A) Ganglion cells in the ARM submucosa. H&E, $\times 400$. (B) Ganglion cells in the normal-appearing submucosa. H&E, $\times 400$. (C) Ganglion cells in the ARM muscularis propria. H&E, $\times 200$. (D) Ganglion cells in the normal-appearing muscularis propria. H&E, $\times 200$. The black arrows indicate Ganglion cells. SM = submucosa, MP = muscularis propria. Scale bar = 200 μ m.

cells and nerve plexus were in the control submucosa and muscle layer (Fig. 2B, D). The Qwin 3000 biologic image analysis software was used to count ganglionic cells per millimeter in myenteric ganglia. Mean ganglion cell number in ARM group was 1.20 ± 0.73 , whereas it was 5.30 ± 1.26 in controls. It was revealed that the ganglion cell number was significantly less than that in the control group ($P = .016$).

3.4. Immunohistochemical staining for NSE, S-100, and C-kit protein in ARM and control groups

As for the IHC staining of NSE, diffuse cytoplasmic positivity in submucosa and muscle layer was reduced in ganglionic cells in ARM group (Fig. 3A, C) compared with the control group (Fig. 3B, D). Diffuse cytoplasmic positivity for S-100 in submucosal and muscle layer astrocytes was seen in the ARM group (Fig. 4A, C) compared to the control group (Fig. 4B, D). For C-kit IHC staining, diffuse cytoplasmic positivity was reduced in ICC in ARM specimens compared to controls (Fig. 5). The percentages of positive expression areas of NSE, S-100, and C-kit in the control and ARM group are listed in Table 1.

3.5. Western blot analysis of NSE, S-100, and C-kit protein in ARM and control groups

As for the Western blotting, significantly decreased expression levels of NSE, S-100, and C-kit protein were detected in ARM specimens compared with the control specimens (Fig. 6). Each protein band was normalized to a corresponding α -GAPDH band. The bottom histogram was also shown in Fig. 6. Please see supplementary material, <http://links.lww.com/MD/C362>, <http://links.lww.com/MD/C363>.

3.6. Functional results in ARM group

All the patients in the ARM group were followed up for >2 years. The Krickenbeck classification^[9] was used to evaluate the anorectal defecation function. The median follow-up period was 39 months (range 24–50 months). The rate of voluntary bowel movement was 94.4% (34/36); free from soiling or grade 1 soiling was found in 34 (94.4%) patients, and grade 2 soiling was found in 2 (5.6%) patients. Two patients (5.6%) suffered from grade 1 constipation while 1 patient (2.8%) with grade 2.

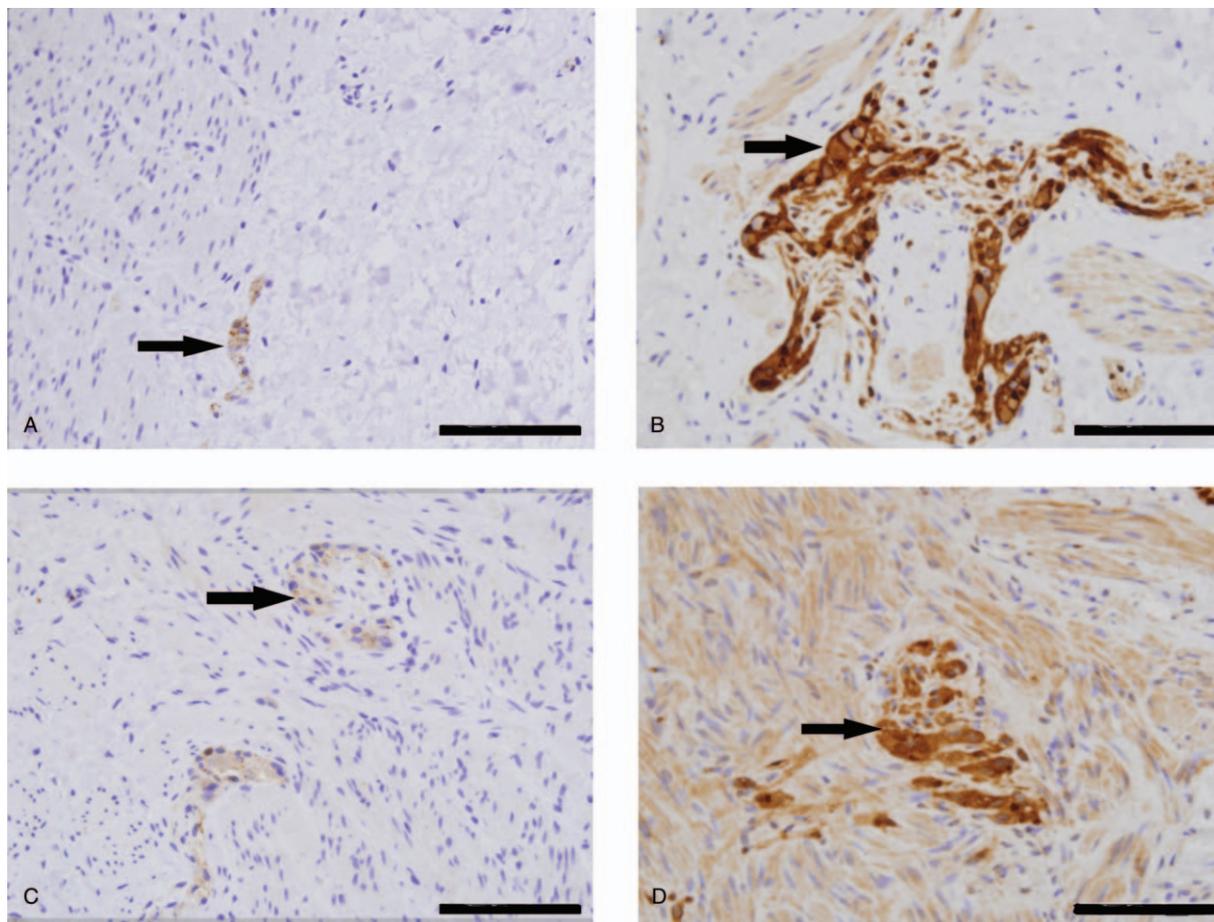


Figure 3. Immunohistochemical staining of neuron-specific enolase (NSE) between the anorectal malformation (ARM) and control group. (A) NSE expression in the ARM submucosa. IHC, $\times 400$. (B) NSE expression in the normal-appearing submucosa. IHC, $\times 400$. (C) NSE expression in the ARM muscularis propria. IHC, $\times 400$. (D) NSE expression in the normal-appearing muscularis propria. IHC, $\times 400$. The black arrows indicate NSE expression. SM = submucosa; MP = muscularis propria. Scale bar = 200 μ m.

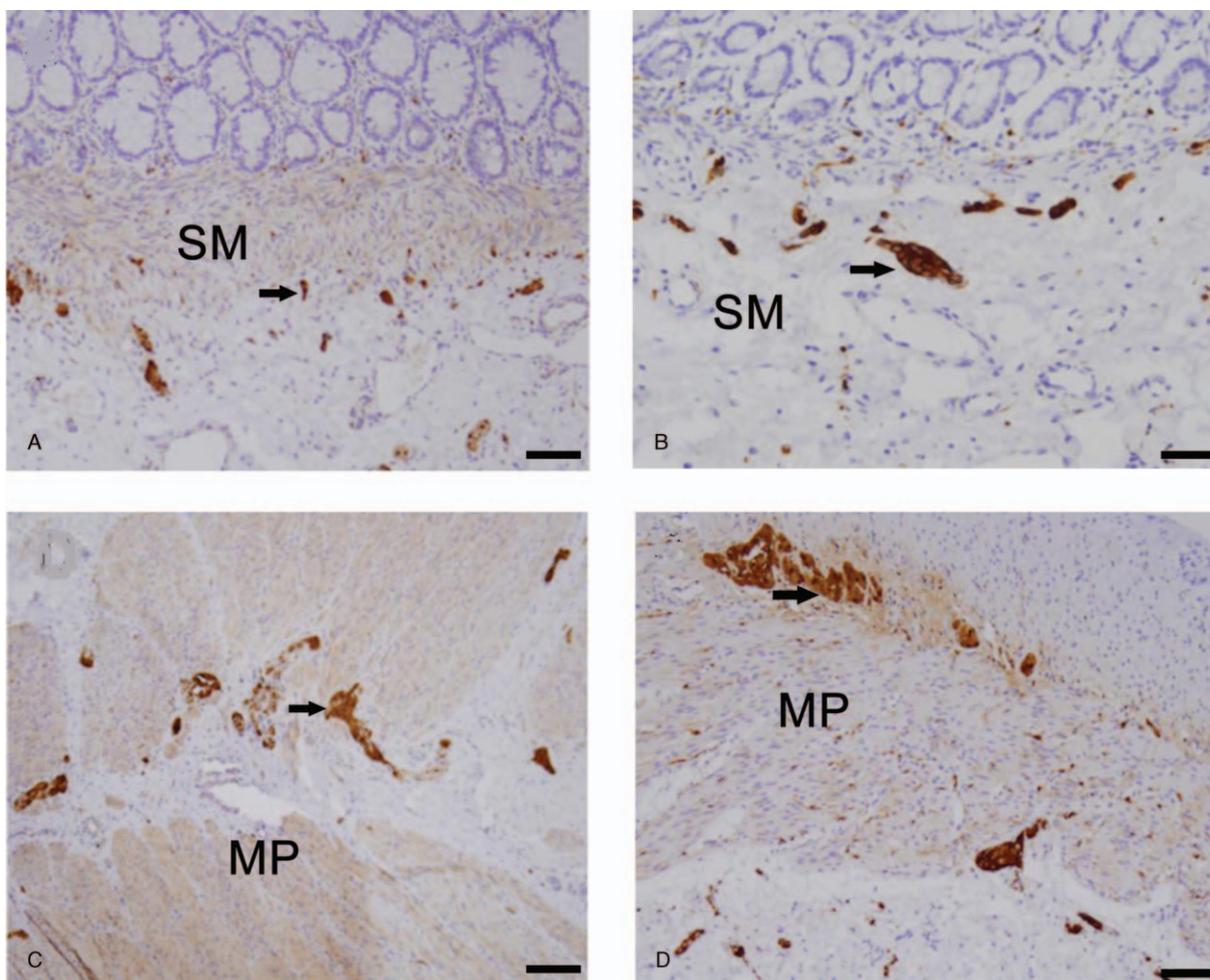


Figure 4. Immunohistochemical staining of S-100 between the anorectal malformation (ARM) and control group. (A) S-100 expression in the ARM submucosa. Immunohistochemistry (IHC), $\times 200$. (B) S-100 expression in the normal-appearing submucosa. IHC, $\times 200$. (C) S-100 expression in the ARM muscularis propria. IHC, $\times 200$. (D) S-100 expression in the normal-appearing muscularis propria. IHC, $\times 200$. The black arrows indicate S-100 expression. SM = submucosa, MP = muscularis propria. Scale bar = 200 μm .

4. Discussion

The ARMs are one of the most commonly observed congenital anomalies of the digestive system. It has been observed that the pelvic floor and the smooth muscle of the terminal rectum in ARMs remain maldeveloped. Several studies on fetal rats showed that there were abnormal innervations of neural plexus in anorectum in ARMs.^[10,11] It is noteworthy that despite advances in surgical treatments, voluntary bowel control is frequently poor, with high rates of fecal incontinence and chronic constipation after all types of reconstructive surgery.^[12–14] To evaluate for defects in neuromuscular architecture in the distal rectum of patients with ARM, we performed histomorphologic, IHC, and Western blot analysis. The histomorphologic, IHC, and Western blot analysis were done to know whether the distal rectal pouch histology in ARMs was abnormal enough to explain the potential mechanism of the poor anorectal defecation function after anorectoplasty.

Although there are many factors that may account for the constipation after the procedure of anorectoplasty such as anal stenosis, abnormalities of extrinsic intestinal innervations, rectal denervation during the surgical procedure,^[15] and abnormalities

of intrinsic intestinal innervation including aganglionosis, hypoganglionosis, and intestinal neuronal dysplasia,^[16,17] it remains controversial whether the distal rectal pouch should be used for reconstruction in anorectoplasty with the treatment of ARMs. Gans and Friedman^[18] advocated preserving the rectal blind pouch based on its histology. Yokoyama et al^[19] showed distinct thickening of the circular and longitudinal muscle layers in the distal rectal pouch in 2 neonates with high anal atresia. Meier-Ruge and Holschneider^[17] observed hypoganglionosis of the myenteric plexus proximal to the anal floor, also most commonly in intermediate ARMs, which is therefore strongly recommended to be excised for reconstruction. Lombardi et al^[20] realized that the resection of distal rectum structural abnormalities maybe helpful to permit better functional results during radical treatment.

To identify the histomorphologic changes and abnormal intrinsic neuromuscular structure in distal rectum specimen, not only the H&E stain and the Masson trichrome stain were conducted, but various IHC markers (NSE, C-kit, and S-100) were also used to reveal the abnormal neuronal structures in this study. Through the H&E staining, the focal erosion, hemorrhage,

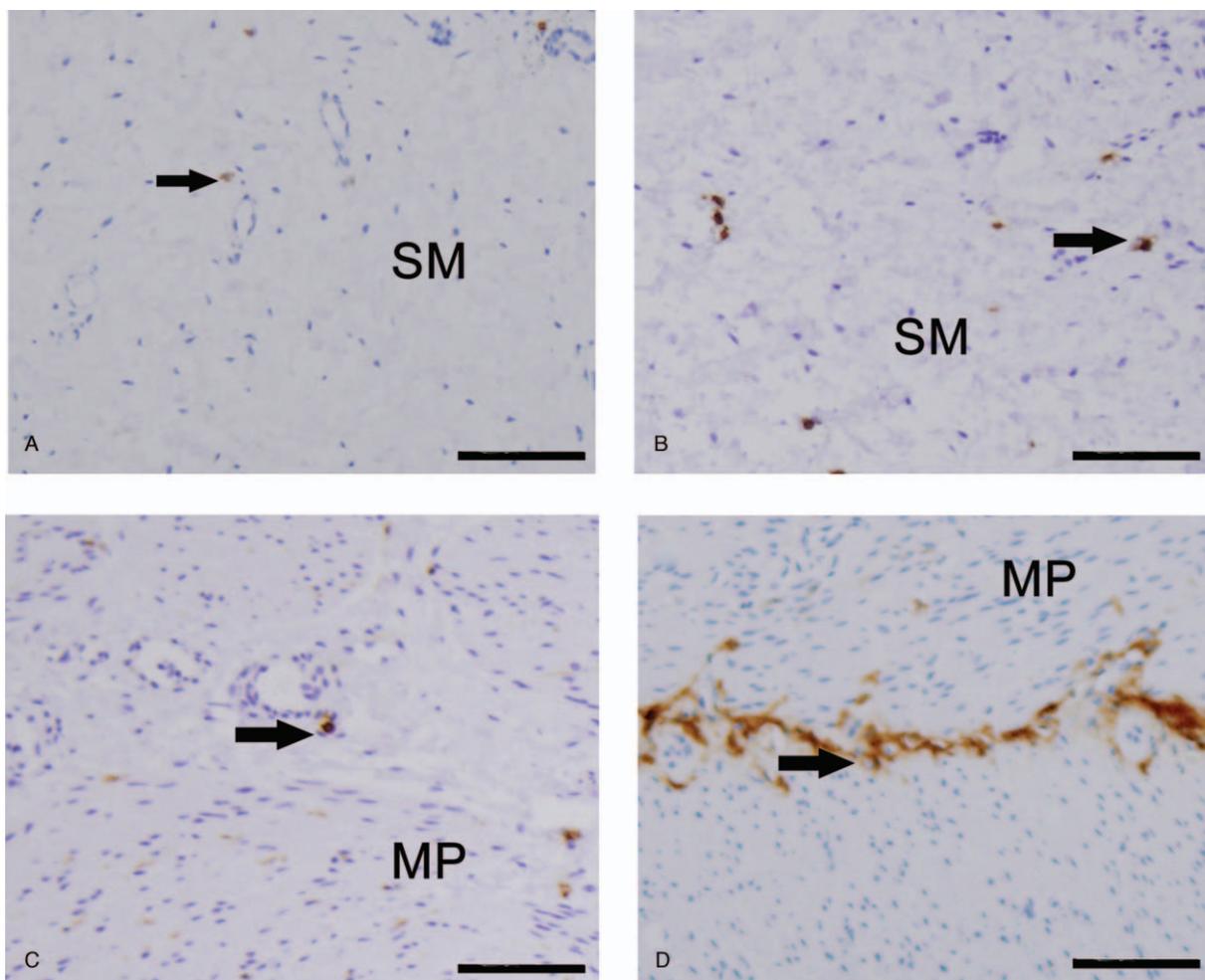


Figure 5. Immunohistochemical staining of C-kit between the anorectal malformation (ARM) and control group. (A) C-kit expression in the ARM submucosa. Immunohistochemistry (IHC), ×400. (B) C-kit expression in the normal-appearing submucosa. IHC, ×400. (C) C-kit expression in the ARM muscularis propria. IHC, ×400. (D) C-kit expression in the normal-appearing muscularis propria. IHC, ×400. The black arrows indicate C-kit expression. SM = submucosa, MP = muscularis propria. Scale bar = 200 μm.

congestion, and lymph follicle were more common in the mucosa and submucosa in the ARM group as compared to the control specimens. In addition, the fibrosis in muscular layer was present in majority of the ARM specimens, and it was obviously located between the inner circular layer and outer longitudinal layer of the ARM specimens. The muscularis propria was disrupted and

divided into bands due to fibrosis. The presence of fibrosis in the muscle bundles indicates an irreversible damage to the muscular wall that may contribute to intestinal dysmotility in ARM cases. It was known that NSE and S-100 proteins are nerve tissue-specific proteins. NSE is localized specifically within neuronal perikarya, dendrites, and axons of both the central and peripheral

Table 1

Quantitative analysis of positive expression areas of NSE, S-100, and C-kit in the ARM and control group.

IHC stains	Patients	Total	Normally positive reaction	Markedly or mildly reduced positive reaction	P
NSE*	ARM	36	14 (38.9%)	22 (61.1%)	.00064
	Control	10	10 (100%)	0	
S-100†	ARM	36	10 (27.8%)	26 (72.2%)	.000045
	Control	10	10 (100%)	0	
C-kit‡	ARM	36	11 (30.6%)	25 (69.4%)	.000087
	Control	10	10 (100%)	0	

ARM = anorectal malformation, NSE = neuron-specific enolase.

* The quantity of the positive expression of NSE in control group was significantly higher than that in ARM group ($P = .00064$).

† The quantity of the positive expression of S-100 in control group was significantly higher than that in ARM group ($P = .000045$).

‡ The quantity of the positive expression of C-kit in control group was significantly higher than that in ARM group ($P = .000087$).

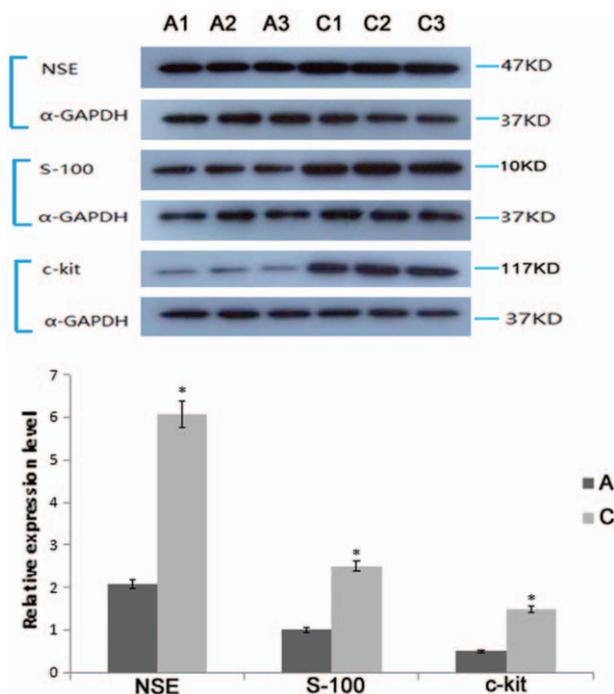


Figure 6. The expressions of neuron-specific enolase (NSE), S-100, and C-kit were evaluated by Western blotting in anorectal malformation (ARM) and control group. Each protein band was normalized by a corresponding alpha-glyceraldehyde phosphate dehydrogenase band. Significant decrease expression of NSE, S-100, and C-kit protein was detected in ARM group compared with the normal group ($P = .013, .028, .041$, respectively). A = ARMs group, C = control group. Results are presented as mean \pm standard deviation, significant differences are marked with asterisks.

nervous systems.^[21–23] S-100 that a nerve sheath marker is used to identify the presence or absence of nerve hypertrophy in various cases of intestinal obstruction.^[22] Schwann cells showed cytoplasmic positivity for S-100 and the ganglion cells were negatively stained, so in the technique of IHC staining, the NSE and S-100 were combined used to identify ganglion cells. The anti-C-kit antibody was used to identify the ICC because CD 117 staining is typically cytoplasmic, with stronger accentuation along the cell membrane.^[23,24] ICC are the pacemaker cells in gastrointestinal muscles. They also mediate or transduce inputs from enteric motor nerves to the smooth muscle syncytium. ICCs act as intermediaries between enteric motor neurons and smooth muscle cells, modulating inhibitory and excitatory signals from the ENS.^[5] They achieve this by acting as the primary electrical pacemaker cells in the gut, generating slow-wave electrical activity that propagates throughout the smooth muscle layers of the colon, giving rise to peristaltic waves.^[24] Abnormalities of ICC distribution have been observed in a range of gastrointestinal disorders including inflammatory bowel disease, idiopathic slow-transit constipation and necrotizing enterocolitis.^[25]

In this study, we observed that there was reduced IHC activity for NSE and S-100 proteins in the submucosal plexus and muscularis propria in ARM group as compared to the control group. In addition, the significant decrease in the density and distribution of ICC was also observed in ARM group. The numbers of NSE-immunoreactive cells were markedly reduced in ARM specimens. The reduction in the number of cell bodies per ganglion and sparsity of ganglia in the myenteric plexuses in

ARMs confirmed the inherently abnormal intramural nervous system, along with the alteration in density and distribution of ICC, and these changes may lead to reduced contractility of the distal rectal pouch and bowel dysfunction after definitive surgery.

Bhatia et al^[26] reported that the distal rectal pouch in ARM show various specific and nonspecific histomorphologic changes, and the postoperative dysmotility can be attributed to the significantly alteration in density and distribution of ICCs. In addition, several studies also reported that the density and distribution of ganglia gradually decreased distally along the rectum, consistent with reports by others.^[27,28] Kenny et al^[29] observed alteration in density and distribution of ICC in most of their cases with 16% of cases showing complete absence of these cells. In our study, the expression of C-kit in ARM group is significantly decreased compared with that in control group, and there exists significant alteration in density and distribution of ICC in ARM group.

Holschneider et al^[8] reported that the higher incidence of chronic constipation in ARMs after corrective surgery maybe associate with the consequence of hypoplastic hypoganglionosis or other anomalies of the ENS. In light of the results of reported studies about histology of distal rectum pouch, the ARMs should no longer be considered as simple and short anomalies. It can be concluded that there exists complex structural abnormalities in several centimeters of the distal anorectal canal. The hypoplasia frequently observed in the internal anal sphincter in ARMs in many cases contradicts the recommendation to use the rectal pouch and proximal fistula for the reconstruction during the procedure of anorectoplasty.^[28,30] In fact, according to most of the pediatric surgeons, the correction of malformations requires a minimal dissection and limited resection of the fistula tissue. However, no standard criteria were drafted about the correct length of distal pouch should be excised. If the resection of distal abnormal tissue is not adequate enough, the potential abnormal of hypoplastic hypoganglionosis, dysplasia fibrous tissue, decreased density of ICC, and anomalies of ENS may contribute to the high incidence of postoperative constipation.

The present study not only confirmed the histomorphology changes such as focal erosion, hemorrhage, congestion, and lymph follicle in the mucosa and submucosa layer, but also verified the inherently abnormalities of myenteric plexuses and reduction in density and distribution of ICCs in ARM group. The number of cell bodies within the ganglia and the density of ganglia and nerve fibers in the myenteric plexuses are markedly reduced in ARMs. Based on these results, the severe anomalies of the fibrotic tissue embedded in muscularis propria, along with the abnormalities of myenteric plexuses and reduction in density and distribution of ICCs may be responsible for the soiling constipation, incontinence after corrective procedure in ARMs.

Limitations of this study are mainly caused by its retrospective nature and relatively small sample size. As the period of follow-up is relatively short, it was not possible to evaluate the bowel function accurately. A large and prospectively randomized study and long-term follow-up studies would be conducted in clinical to demonstrate the significant differences about the histomorphologic and neuronal dysfunction associated with ARM.

5. Conclusion

The present study found that the distal rectal pouch in ARMs was abnormal structure with severe impairment in distal segment. This study sorts the excision of distal segment in the definitive procedure for better functional outcome. However, further

detailed studies on the histopathology, contractile function, electrophysiology, and IHC involving more number of ARM cases are required for better understanding and management of these problems.

Author contributions

Hui Xiao carried out the entire procedure including the literature search, data extraction, performed the statistical analysis, drafted the manuscript, revised submitted the manuscript. Long Li, Mei Diao conceived of the study, coordinated and participated in the entire process of drafting and revised the manuscript. Rui Huang, Xiao-Dai Cui, and Ping Xiao contributed to statistical analysis and revision the manuscript. Hui Xiao and Long Li contributed to the revisions of the manuscript. All authors have contributed significantly. All authors read and approved the final manuscript.

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Software: Hui Xiao, Ping Xiao.

Supervision: Mei Diao, Long Li, Dai Xiao Cui.

Visualization: Mei Diao.

Writing – review & editing: Rui Huang.

References

- [1] Rintala RJ. Fecal incontinence in anorectal malformations, neuropathy, and miscellaneous conditions. *Semin Pediatr Surg* 2002;11:75–82.
- [2] DeVries PA, Peña A. Posterior sagittal anorectoplasty. *J Pediatr Surg* 1982;17:638–43. PMID: 7175658.
- [3] Young B, O'Dowd G, Woodford P. Young B, O'Dowd G, Woodford P. *Gastrointestinal tract. Wheater's Functional Histology: A Text and Colour Atlas 6th ed.* Churchill Livingstone, Philadelphia, PA:2104;251–75.
- [4] Farrugia G. Interstitial cells of Cajal in health and disease. *Neurogastroenterol Motil* 2008;20(Suppl 1):54–63.
- [5] Benarroch EE. Enteric nervous system: functional organization and neurologic implications. *Neurology* 2007;69:1953–7.
- [6] Sanders KM. Rhythmic electrical activity and regulation of gut motility. *Eur Rev Med Pharmacol Sci* 2008;12(Suppl 1):129–33.
- [7] Holschneider AM, Pfrommer W, Gerresheim B. Results in the treatment of anorectal malformation with special regard to the histology of the rectal pouch. *Eur J Pediatr Surg* 1994;4:303–9.
- [8] Holschneider AM, Ure BM, Pfrommer W, et al. Innervation patterns of the rectal pouch and fistula in anorectal malformations: a preliminary report. *J Pediatr Surg* 1996;31:357–62.
- [9] Holschneider A, Hutson J, Pena A, et al. Preliminary report on the International Conference for the Development of Standards for the Treatment of Anorectal Malformations. *J Pediatr Surg* 2005;40:1521–6.
- [10] Faussonne-Pellegrini MS, Fociani P, Buffa R, et al. Loss of interstitial cells and a fibromuscular layer on the luminal side of the colonic circular muscle presenting as megacolon in an adult patient. *Gut* 1999;45:775–9.
- [11] Faussonne-Pellegrini MS. Comparative study of interstitial cells of Cajal. *Acta Anat* 1987;130:109–26.
- [12] Ong NT, Beasley SW. Long-term continence in patients with high and intermediate anorectal anomalies treated by sacroperineal (Stephens) rectoplasty. *J Pediatr Surg* 1991;26:44–8.
- [13] Rintala R, Lindahl H, Marttinen E, et al. Constipation is a major functional complication after internal sphincter-saving posterior sagittal anorectoplasty for high and intermediate anorectal malformations. *J Pediatr Surg* 1993;28:1054–8.
- [14] Hamid CH, Holland AJ, Martin HC. Long-term outcome of anorectal malformations: the patient perspective. *Pediatr Surg Int* 2007;23:97–102.
- [15] Holschneider AM, Koebeke J, Meier-Ruge W, et al. Pathophysiology of chronic constipation in anorectal malformations. Long-term results and preliminary anatomical investigations. *Eur J Pediatr Surg* 2001;11:305–10.
- [16] Martucciello G, Torre M, Prato AP, et al. Associated anomalies in intestinal neuronal dysplasia. *J Pediatr Surg* 2002;37:219–23.
- [17] Meier-Ruge WA, Holschneider AM. Histopathologic observations of anorectal abnormalities in anal atresia. *Pediatr Surg Int* 2000;16:2–7.
- [18] Gans SI, Friedman N. Some new concepts in the embryology, anatomy, physiology and surgical correction of imperforate anus. *West J Surg Obstet Gynaecol* 1961;69:34–7.
- [19] Yokoyama J, Hayashi A, Ikawa H, et al. Abdomino-extended sacroperineal approach in high-type anorectal malformation and a new operative method. *Z Kinderchir* 1985;40:150–7.
- [20] Lombardi L, Bruder E, Caravaggi F, et al. Abnormalities in “low” anorectal malformations (ARMs) and functional results resecting the distal 3 cm. *J Pediatr Surg* 2013;48:1294–300.
- [21] Musa ZA, Qasim BJ, Ghazi HF, et al. Diagnostic roles of calretinin in hirschsprung disease: a comparison to neuron-specific enolase. *Saudi J Gastroenterol* 2017;23:60–6.
- [22] Holland SK, Ramalingam P, Podolsky RH, et al. Calretinin immunostaining as an adjunct in the diagnosis of Hirschsprung disease. *Ann Diagn Pathol* 2011;15:323–8.
- [23] Blair PJ, Rhee PL, Sanders KM, et al. The significance of interstitial cells in neurogastroenterology. *J Neurogastroenterol Motil* 2014;20:294–317.
- [24] Huizinga JD, Chen JH. Interstitial cells of Cajal: update on basic and clinical science. *Curr Gastroenterol Rep* 2014;16:363.
- [25] Burns AJ. Disorders of interstitial cells of Cajal. *J Pediatr Gastroenterol Nutr* 2007;45(Suppl 2):S103–6.
- [26] Bhatia Y, Singh S, Rattan KN, et al. Anorectal malformations: histomorphological and Immunohistochemical evaluation of neuronal dysfunction. *J Neonatal Surg* 2017;6:29.
- [27] Pandey A, Pandey P, Singh SP, et al. Histology with immunohistochemistry of the fistula region in female anorectal malformation: can it be used for neo-anus reconstruction? *J Paediatr Child Health* 2018;54:177–82.
- [28] Gangopadhyay AN, Upadhyaya VD, Gupta DK, et al. Histology of the terminal end of the distal rectal pouch and fistula region in anorectal malformations. *Asian J Surg* 2008;31:211–5.
- [29] Kenny SE, Connell MG, Rintala RJ, et al. Abnormal colonic interstitial cells of Cajal in children with anorectal malformations. *J Pediatr Surg* 1998;33:130–2.
- [30] Frenckner B. Use of the recto-urethral fistula for reconstruction of the anal canal in high anal atresia. *Z Kinderchir* 1985;40:312–4.