

Original Article

Expression analysis of protein gene product 9.5 and S-100 protein in the rectal terminal tissue of patients with congenital anorectal malformations

Yanlei Huang, Shan Zheng

Department of Pediatric Surgery, Children's Hospital of Fudan University, Shanghai, China

Received May 3, 2016; Accepted September 17, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Objective: To study the development of the enteric nervous system (ENS) in rectal terminal tissue of patients with anorectal malformation (ARM) and identify possible pathological mechanisms of postoperative defecation dysfunction. Methods: 78 ARM specimens (11 cases of high position malformation, 28 cases of intermediate position malformation, and 39 cases of low position malformation) were collected. Immunohistochemical analysis was performed to assess the expression levels of protein gene product 9.5 (PGP9.5) and S-100 protein in the specimens. Results: PGP9.5 and S-100 expression levels in high position ARM (ARM_{hi}) were significantly lower than in intermediate position ARM (ARM_{int}) (PGP9.5: $P = 0.0226$; S-100: $P = 0.0401$), and low position ARM (ARM_{lo}) (PGP9.5: $P = 0.0331$; S-100: $P = 0.0436$). No statistically significant difference was found between the ARM_{int} and ARM_{lo} groups. Expression levels of PGP9.5 and S-100 in terminal rectal tissue of single-stage surgery ARM_{int} were significantly lower than that of staged surgery ARM_{int} (PGP9.5: $P = 0.0128$; S-100: $P = 0.0048$). In the ARM_{lo} group, no statistically significant difference in PGP9.5 and S-100 expression in rectal terminal tissues was observed between the male and the female patients. Conclusion: ENS development of the rectal terminal tissue in ARM patients was closely related to the position of the malformation, the higher the position of ARM, the poorer the ENS development in the rectal terminal tissue.

Keywords: Anorectal malformation, enteric nervous system, protein gene product 9.5, S-100 protein

Introduction

Congenital anorectal malformation (ARM) is a common major gastrointestinal malformation in newborns. Since the late 1980s, application of the Peña procedure (posterior sagittal anorectoplasty) has minimized damage to the anal sphincter, with a much clearer anatomy that is more conducive to functional reconstruction of the anus. However, postoperative fecal incontinence, constipation, and urinary dysfunction still persist in some children with an incidence of up to 10%-73% [1]. Reportedly, postoperative defecation dysfunction of congenital ARM may be associated with postoperative megacolon, megarectum, or rectal weakness [1], but significant expansion of the colon and rectum was not observed in the majority of patients in this study during postoperative barium enema [2]. Therefore, it is speculated that defecation disorders may be influenced by the domination

and integration of the enteric nervous system (ENS) in rectal terminal tissue. Currently, it is known that ENS forms different levels of nerve plexus in the digestive tract and the submucous plexus (Meissner's plexus) and myenteric plexus are of great significance for defecation reflex formation, both of which are receptors within the intestinal wall, with the former mainly involved in intestinal mucosa sensory formation and the latter mainly stimulates intestinal smooth muscle motility. ENS dysplasia can lead to malformations and functional abnormalities of the corresponding digestive tract. Meier-Ruge et al. [3] reported that the incidence of ENS abnormalities in the rectal terminal tissue of the anus atresia patients is as high as 60%. Holschneider et al. [4] also reported non-ganglion or intestinal nerve dysplasia-like changes to the nerve plexus in the distal rectal wall in anus atresia, which was associated with the formation of postoperative constipation. Studies by

Wang et al. [5] and Xu et al. [6] reported abnormal distribution of ENS cells in the blind end rectum of children with anal atresia with a significantly decreased number of adrenergic nerve fibers as compared with normal children. In recent years, with a deeper understanding of the developmental process and mechanisms of ENS, more and more neural markers of morphological characteristics in ENS have been identified. At present, various markers expressed by neurons and glial cells after ENS differentiation are mainly used to study the etiology and pathological diagnosis of Hirschsprung disease and associated disorders, but are seldom reported for ARM.

Protein gene product 9.5 (PGP9.5) is a structural and functional protease expressed by neurons and glial cells differentiated from ENS, which exists in all levels of cells in the central and peripheral nervous system and neuroendocrine cells, demonstrated as a brown lumpish plexus scattered among dark brown ganglion cells. S-100 protein is an acidic protein with low solubility and high molecular weight that is specific to neural glial cells, which shows positive staining innerve plexus nerve fibers, Schwann cells, and surrounding cells, with a characteristic morphology shadowed by "blank" areas observed in the nerve plexus with positive staining. Immunostaining for the PGP9.5 and S-100 proteins is useful to study the morphological and structural characteristics of ENS, while staining with S-100 antibody is negative in ganglion cells. Therefore, the combined use of PGP9.5 and S-100 antibodies is complementary and improves the sensitivity and specificity of ENS. In the current study, combinational immunohistochemical methods using PGP9.5 and S-100 antibodies were employed to observe expression levels in the rectal terminal tissue of ARM children with the aim to elucidate ENS development in the rectal terminal tissue in various types of ARM, and to help further the clinical understanding of postoperative defecation dysfunction in patients with various types of ARM.

Material and methods

Research subjects

Terminal tissue specimens (namely, full wall thickness of the posterior rectal wall at 2 cm above the rectal end, 0.5×0.5 cm in size) col-

lected from 78 children with congenital ARM (57 males and 21 females; male-to-female ratio, 2.7:1) through surgical resection from January 2001 to December 2005 were assessed. The 78 ARM patients were divided into three groups of high, intermediate, and low position ARM (ARM_{hi}, ARM_{int}, and ARM_{lo}, respectively) depending on the position of the rectal end, as revealed by inverted X-ray radiography and intraoperative diagnosis: 11 cases of ARM_{hi} (seven cases of rectourethral prostatic fistula and four cases of rectobladderneck fistula), 28 cases of ARM_{int} (20 cases of rectourethral bulbar fistula, six cases of rectovestibular fistula, and two cases of cloacal malformation with a common channel length < 3 cm), and 39 cases of ARM_{lo} (all rectoperineal fistula; 26 males and 13 females). All patients in the ARM_{hi} group underwent transverse colostomy immediately after birth, followed by abdominal and sacral perineal posterior sagittal anorectoplasty within 3 to 6 months. The rectal specimens were obtained at the age of 3.4 to 7.6 months (average, 5.62 ± 0.29 months). Among the 28 cases of ARM_{int}, 21 were treated with the same procedures as for ARM_{hi} (colostomy, radical surgery, and colostomy closure), with the rectal specimens collected at age 3.2-6.8 months (average, 5.47 ± 0.22 months). The other seven cases underwent single-stage modified posterior sagittal anorectoplasty (i.e., modified Peña procedure, while retaining of the integrity of the levatorani muscle and puborectalis ring joint intraoperatively) by the sacral perineal method after birth [7]. The rectal specimens were collected from the age of 25 h to 9 days (average, 0.08 ± 0.05 months). Among the 39 patients with ARM_{lo}, the specimens were collected from the age of 27 h to 18 days (average, 0.11 ± 0.08 months) from the 26 male patients and 2 days to 8.4 months (average, 6.15 ± 0.29 months) from the 13 female patients. Above these specimens were all collected during the anorectoplasty procedures without no difficulties in obtaining or technical aspects.

The rectal samples of another 10 normal infants (six males and four females; age range, 3.5-8 months; average age, 5.91 ± 0.31 months) without gastrointestinal malformations that were obtained in our hospital from full thickness biopsy of the posterior rectal wall at 3 cm above the dentate line from cases with constipation from April 2003 to December 2005 were used as controls. No sample in the

Immunohistochemistry of ARM

Table 1. Gestational ages and birth weights of children with various types of anorectal malformation by using the chi-square test or one-way analysis of variance

| Group | Cases | Gestational age (week) | Birth weight (Kg) |
|--------------------|-------|------------------------|-------------------|
| ARM _{lo} | 39 | 38.13 ± 0.20 | 3.04 ± 0.07 |
| ARM _{int} | 26 | 38.12 ± 0.25 | 3.01 ± 0.09 |
| ARM _{hi} | 11 | 37.78 ± 0.38 | 2.94 ± 0.14 |
| Control | 10 | 38.21 ± 0.22 | 3.02 ± 0.07 |
| P-value | | > 0.05 | > 0.05 |

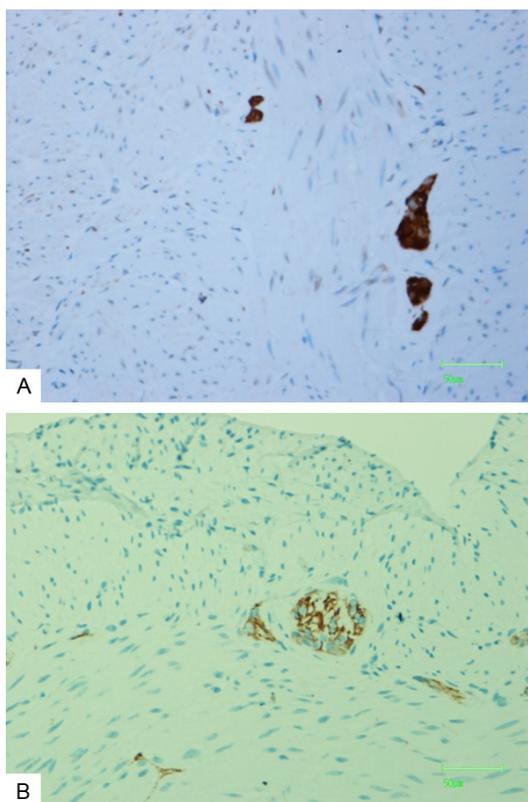


Figure 1. Immunohistochemical staining results of ARM. A. Strong positive expression of protein gene product 9.5 was observed in the cytoplasm and nuclei of enteric nervous cells of rectal terminal tissue in anorectal malformation patients. B. Expression of S-100 protein on the cell membrane of the intestinal wall was more significant, showing characteristic cell-like “blank” areas. Bar = 50 µm.

control group had obvious abnormalities during anorectal manometry or barium enema examination, while the possibility of Hirschsprung disease was also excluded through clinical follow-up and rectal biopsy. The average gestational age and birth weight of each group were recorded simultaneously.

Experiments were approved by the hospital ethics committee. Parents of all the subjects were fully informed of the objectives of the study and the procedures involved, and gave their informed, written consent.

Methods

Specimen preparation: All specimens (0.5×0.5 cm) were fixed in 4% polyformaldehyde solution, dehydrated in graded alcohol, embedded in xylene transparent paraffin, and then sectioned into 4-5 µm consecutive coronal slices, which were mounted on glass slides pretreated with 0.1% polylysine. At the same time, a tissue slice was stained with hematoxylin and eosin for tissue localization.

Immunohistochemical staining: A two-step polymer method was employed using the EnVision™ + System (Dako Denmark A/S, Glostrup, Denmark). The primary antibodies rabbit anti-human PGP9.5 polyclonal antibody (dilution, 1:50) and rabbit anti-human S-100 polyclonal antibody (S-100; dilution, 1:400) were purchased from Dako Denmark A/S. Antigen retrieval, processing of control tissues, and diaminobenzidine color development were performed according to the manufacturer's instructions. The presence of brown yellow granules observed in all positive cells was measured qualitatively. Under normal circumstance, PGP9.5 was expressed in the cytoplasm and nuclei of ganglion and nerve fibers, while S-100 was expressed on the neuronal membrane.

Imaging and statistical analysis

For every section, five high-magnification fields (20×10 mm) were randomly selected, with the average positive area percentage of the visual fields determined using biological image analysis software. All statistical analyses were performed using SPSS 14.0 software (IBM-SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation. Between-group differences were analyzed using the chi-square test or one-way analysis of variance. A probability (*p*) value < 0.05 was considered statistically significant.

Results

There were no significant differences in the average gestational age or birth weight of children with various types of ARM, as compared to

Immunohistochemistry of ARM

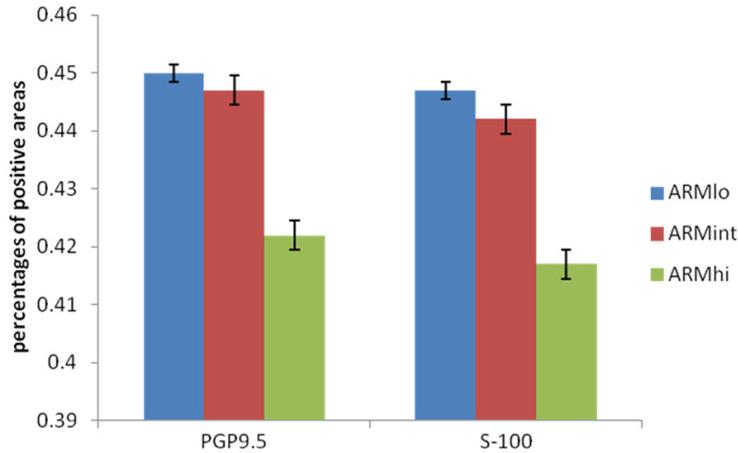


Figure 2. Expression levels of the two antibodies in rectal terminal tissue of patients with various types of anorectal malformation.

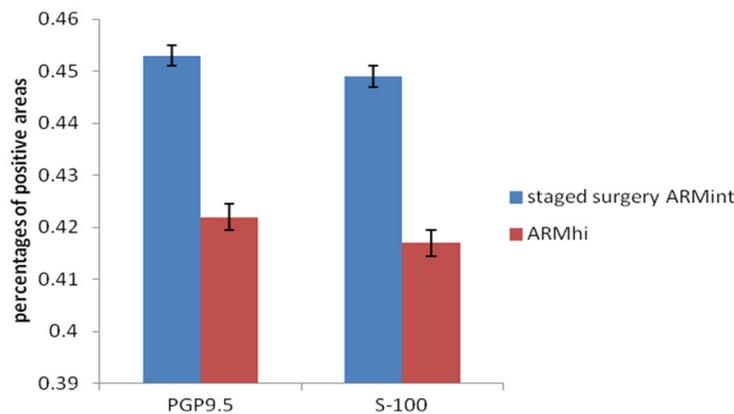


Figure 3. Expression levels of the two antibodies in the rectal terminal tissue of same-age patients in the anorectal malformation (ARM) high (ARM_{hi}) and intermediate (ARM_{int}) groups.

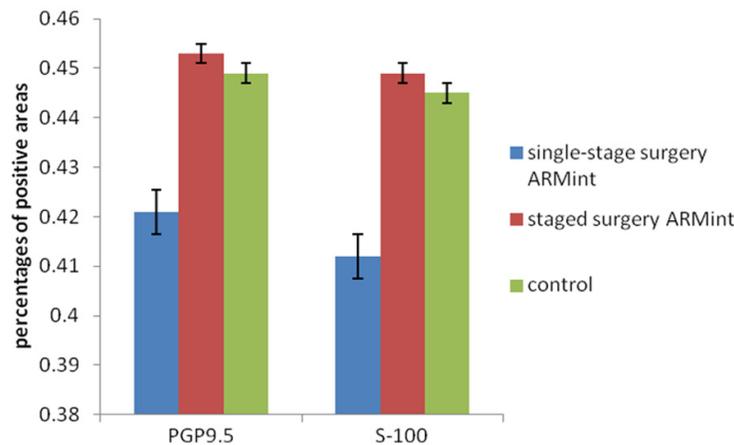


Figure 4. Expression levels of the two antibodies in the rectal terminal tissue of intermediate anorectal malformation (ARM_{int}) patients in different age groups.

those of the normal control group ($P > 0.05$, **Table 1**).

Immunohistochemical staining results

In the rectal terminal tissue of each group, PGP9.5 expression in the cytoplasm and nuclei of ganglion and nerve fiber cells was strongly positive (**Figure 1A**). S-100 on the ganglion cell membrane was more obvious and showed characteristic cell-shaped “blank” areas (**Figure 1B**).

PGP9.5 and S-100 were expressed at different levels in the rectal terminal tissue of patients with various types of ARM (**Figure 2**). The expression levels in the ARM_{hi} group were significantly lower than in the ARM_{int} group (PGP9.5: 0.422 ± 0.008 vs. 0.447 ± 0.006 , $P = 0.0226$; S-100: 0.417 ± 0.009 vs. 0.442 ± 0.007 , $P = 0.0401$) and ARM_{lo} group (PGP9.5: 0.422 ± 0.008 vs. 0.450 ± 0.009 , $P = 0.0331$; S-100: 0.417 ± 0.009 vs. 0.447 ± 0.010 , $P = 0.0436$), while there was no significant difference between the ARM_{lo} and ARM_{int} groups ($P > 0.05$).

There was no significant difference in age at the time rectal blind end specimens were collected between the ARM_{hi} and staged surgery ARM_{int} groups ($n = 21/28$, including 15 cases of rectourethral bulbar fistula, four cases of rectovestibular fistula, and two cases of cloacal malformation with a common channel length < 3 cm) (5.62 ± 0.29 vs. 5.47 ± 0.22 months, respectively; $P > 0.05$). However, expression levels of PGP9.5 and S-100 were significantly higher in the ARM_{int} group with staged surgery than that of ARM_{hi} patients at the same age ($P < 0.01$, **Figure 3**).

Table 2. Expression levels of the two antibodies in the rectal terminal tissue of low anorectal malformation (ARM_{lo}) patients at different ages by using the chi-square test or one-way analysis of variance

| Group | Age at the time of rectal terminal tissue collection (month) | PGP9.5 | S-100 |
|--------------------------------------|--|---------------|---------------|
| Male in ARM _{lo} (n = 26) | 0.11 ± 0.08* | 0.442 ± 0.005 | 0.440 ± 0.006 |
| Female in ARM _{lo} (n = 13) | 6.15 ± 0.29 | 0.453 ± 0.008 | 0.450 ± 0.007 |
| Control (n = 10) | 5.91 ± 0.31 | 0.449 ± 0.009 | 0.445 ± 0.009 |

Note: *P < 0.0001.

The average age at the time of rectal terminal tissue collection in the ARM_{int} group following single-stage surgery (n = 7/28, including 5 cases of rectourethral bulbar fistula and two cases of rectovestibular fistula) was 0.08 ± 0.05 months, which was significantly less than that of the ARM_{int} with staged surgery group (n = 21/28) (5.47 ± 0.2 months) (P < 0.0001), while PGP9.5 and S-100 expression in rectal terminal tissue of younger patients in the single-stage surgery ARM_{int} group was significantly impaired (PGP9.5: 0.421 ± 0.010 vs. 0.453 ± 0.006, P = 0.0128; S-100: 0.413 ± 0.010 vs. 0.449 ± 0.006, P = 0.0048). There were no significant differences in patient age at the time of rectal terminal tissue collection (5.47 ± 0.22 vs. 5.91 ± 0.31 months, respectively) and expression levels of the two antibodies (PGP9.5: 0.453 ± 0.006 vs. 0.449 ± 0.009, P > 0.05; S-100: 0.449 ± 0.006 vs. 0.445 ± 0.009, P > 0.05) between the ARM_{int} with staged surgery group and the control group (Figure 4).

The average age at the time of rectal terminal tissue collection of the 26 male patients in the ARM_{lo} group was significantly less than that of the 13 female patients within the same group (0.11 ± 0.08 vs. 6.15 ± 0.29 months, respectively; P < 0.0001). However, there was no significant difference in the rectal terminal tissue expression of PGP9.5 and S-100 between the male and female patients, or compared with those of the control group (P > 0.05, Table 2).

Discussion

Congenital ARM is the most common disease of the digestive tract during the neonatal period and is characterized by extremely complicated pathological mechanism changes. With the recognition and protection of the peripheral

anorectal muscle group during surgery, the incidence of anal incontinence, a severe surgical complication, has been significantly decreased. However, other postoperative complications, such as different degrees of defecation dysfunction (i.e., fecal soiling, constipation, etc.) can affect

the quality of postoperative life of children with congenital anorectal atresia.

Previous studies confirmed that the development of ENS of rectal terminal tissue was closely related to good defecation function [3-6]. PGP9.5 and S-100 protein are specifically expressed in the neurons and astrocytes of the intestinal wall [8]. The immunohistochemical results of the current study showed that expression levels of PGP9.5 and S-100 in rectal terminal tissue were significantly lower in the ARM_{hi} group, as compared to that of the ARM_{int} and ARM_{lo} groups. Moreover, the distributions of neurons in the rectal terminal tissue of patients with various types of ARM differed, and the number of neurons and ganglion cells in the rectal terminal tissue of patients with ARM_{hi} was significantly reduced. Therefore, such pathological features might be associated with the significantly higher incidence of postoperative incontinence among patients in the ARM_{hi} group, as compared with those in the ARM_{int} and ARM_{lo} groups [9]. When comparing the age at the time of rectal terminal tissue specimen collection, expression levels of PGP9.5 and S-100 in rectal terminal tissue were found to be significantly lower in same-age patients in the ARM_{hi} group than that of the staged surgery ARM_{int} group, indicating that ENS development of rectal terminal tissue was closely related to the position of the malformation. The higher the position of ARM, the poorer the ENS development in the rectal terminal tissue. While, in the ARM_{int} group, expression levels of the two antibodies in the rectal terminal tissue of patients with staged surgery were significantly higher than those after single-stage surgery, suggesting that along with age, ENS development of the rectal blind end in patients with ARM_{int} can also gradually mature, which may be

related to the gradual thickening muscle layer of the distal rectal intestinal wall along with age. These findings also implied that although the rectal terminal tissue was not utilized for a period of time (range, 3.2-6.8 months; average, 5.47 ± 0.22 months) during colostomy in ARM_{int} patients with staged surgery, it does not affect continuous ENS development of the rectal terminal tissue. However, for the ARM_{hi} group, with the increased thickness of the muscle layer of the intestinal wall of the rectal terminal tissue, ENS development in the muscle layer was still not significantly improved, which may account for the characteristic high incidence of postoperative defecation disorders in ARM_{hi} patients, which is difficult to treat. Kenny et al. [10] reported that PGP9.5 expression in the rectal terminal tissue of ARM patients was associated with postoperative refractory constipation. Also, Xu et al. [6] recommended to not retain too much rectal terminal tissue during the Peña procedure, since retaining too much rectal tissue will significantly increase the incidence of postoperative constipation in patients with ARM_{int} and ARM_{lo} [9], which is directly related to the degree of preoperative distension of the rectum [11]. But, for ARM_{hi} patients, excessive resection of the rectal terminal tissue will increase the incidence of postoperative fecal incontinence. Regarding ARM patients with an intact internal sphincter and good ENS development, resection of the expanded rectum can achieve great defecation control [12]. In addition, the results of the current study also showed that, although the age at the time of rectum specimen collection significantly differed between male and female children in the ARM_{lo} group, there was no significant difference in the expression levels of two antibodies in the rectal terminal tissue between male and female patients, or compared with that of the normal control group, suggesting that a lower ARM position is associated with smaller differences in ENS development in rectal terminal tissue, which was closer to normal infants.

Despite the above results, there were some limitations in the current study. Due to the lack of large samples, gender analysis was not done in ARM_{hi} and ARM_{int}-staged group. In addition, although no significant differences in gestational ages and birth weights were observed among the three ARM groups followed-up in the study, larger numbers of cases and long-term

follow-ups are needed to determine whether gestational age and birth weight are factors that affect ENS development in rectal terminal tissue. Moreover, the “control group” in the study is not the true sense of the normal control group, but relatively normal control group children with constipation, therefore might not be interpreted as healthy children because there might be a bias in their levels of PGP9.5 and S-100. But due to the difficulty of collecting specimens of healthy children, otherwise it would be more interesting to compare the levels with children with normal bowel habits.

In conclusion, through comparison of ENS development in rectal terminal tissue among different types of congenital ARM, the current study primarily explored that ENS development of the rectal terminal tissue in ARM patients was closely related to the position of the malformation, and the higher the position of ARM, the poorer the ENS development in the rectal terminal tissue. Whether such pathological features might be associated with the significantly higher incidence of postoperative incontinence will require further investigation.

Acknowledgements

We acknowledge the work of pathologists involved in the study (Drs Lian Chen and Hongyi He). Supported by National Natural Science Foundation of China (81401243), Shanghai Health Bureau ‘Scientific research Foundation’ (2014-2016), Shanghai Fudan University “Excellent Study Plan” (2015-2018).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shan Zheng, Department of Pediatric Surgery, Children’s Hospital of Fudan University, 399 Wan Yuan Road, Shanghai 201102, China. Tel: +86-21-64931007; Fax: +86-21-6493-1901; E-mail: szheng@shmu.edu.cn

References

- [1] Rintala R, Lindahl H, Marttinen E and Sariola H. Constipation is a major functional complication after internal sphincter-saving posterior sagittal anorectoplasty for high and intermediate anorectal malformations. *J Pediatr Surg* 1995; 28: 1054-1058.

Immunohistochemistry of ARM

- [2] Bliss DP Jr, Tapper D, Anderson JM, Schaller RT Jr, Hatch EI, Morqan A, Hall DG and Sawin RS. Does posterior sagittal anorectoplasty in patients with high imperforate anus provide superior fecal continence? *J Pediatr Surg* 1996; 31: 26-32.
- [3] Meier-Ruge WA, Holschneider AM. Histopathologic observations of anorectal abnormalities in anal atresia. *Pediatr SurgInt* 2000; 16: 2-7.
- [4] Holschneider AM, Koebke J, Meier-Ruge W, Land N, Jesch NK and Pfrommer W. Pathophysiology of chronic constipation in anorectal malformation long-term results and preliminary anatomical investigations. *Eur J Pediatr Surg* 2001; 11: 305-310.
- [5] Wang W, Zhang Z, Li Z and Wang L. Changes of adrenergic innervations of the rectal pouch in anorectal malformation. *Chin J Clin Pediatr Surg* 2003; 2: 36-38.
- [6] Xu K, Yuan J, Feng J, Liu G and Sun X. Preliminary study of constipation after posterior sagittal anorectoplasty. *Chin J Pediatr Surg* 2004; 25: 62-64.
- [7] Zheng S, Xiao X, Huang Y. Single-stage correction of imperforate anus with a rectourethral or a rectovestibula fistula by semi-posterior sagittal anorectoplasty. *Pediatr Surg Int* 2008; 24: 671-676.
- [8] Krammer HJ, Karahan ST, Sigge W and Kühnel W. Immunohistochemistry of markers of the enteric nervous system in whole-mount preparations of the human colon. *Eur J Pediatr Surg* 1994; 4: 274-278.
- [9] Peña A. Anorectal malformations. *Semin Pediatr Surg* 1995; 4: 35-47.
- [10] Kenny SE, Connell MG, Rintala RJ, Vaillant C, Edqar DH and Lloyd DA. Abnormal colonic interstitial cells of Cajal in children with anorectal malformations. *J Pediatr Surg* 1998; 33: 130-132.
- [11] Peña A and Levitt MD. Imperforate anus and cloacal malformations. *Pediatric Surgery*. Edited by Ashcraft KW, Holcomb GW and Murphy JP. Philadelphia: Elsevier Saunders; 2005. pp. 496-517.
- [12] Keshtgar AS, Ward HC, Richards C and Clayden GS. Outcome of excision of megarectum in children with anorectal malformation. *J Pediatr Surg* 2007; 42: 227-233.