The impact of HuD protein on the intestinal nervous system in the terminal rectum of animal models of congenital anorectal malformation

MENG KONG[1,2*], YURUI WU[1*] and YUANMEI LIU[2*]

[1]Department of Pediatric Surgery, Qilu Children's Hospital of Shandong University (Jinan Children's Hospital), Jinan, Shandong 250022; [2]Department of Pediatric Surgery, Affiliated Hospital of Zunyi Medical College, Zunyi, Guizhou 563003, P.R. China

Received January 20, 2016; Accepted January 19, 2017

DOI: 10.3892/mmr.2017.7204

Abstract. Patients with congenital anorectal malformation (ARM) often present with different degrees of defecation dysfunction severity following corrective operations. Therefore, studies on how to improve the postoperative defecation function of patients with ARM are of clinical importance. The present study investigated the expression of the HuD protein in the terminal rectum of ARM embryonic rats and explored the effect of HuD expression on the development of the intestinal nervous system. Pregnant Sprague Dawley rats were randomized into a control or ARM (induced by ethylene thiourea) group. The terminal rectums of the embryonic rats were obtained during pregnancy (20 days). The histological changes of the terminal rectum were observed using hematoxylin and eosin staining. The expression of the HuD protein was assessed by immunohistochemistry and western blot analysis. In the control group, the histological structure of the terminal rectum was well-defined and a large number of submucosal and intermuscular neurons with a rich cytoplasm and strong neuritis were observed. In the ARM group, the histological layers were ill-defined and the number of neurons was small. Immunohistochemistry and western blot analysis demonstrated that the concentration of the HuD protein in the ARM group was significantly lower compared with the control group (312.90±53.40:456.40±57.13; 0.24±0.05:0.45±0.06, $P<0.05$). HuD was abnormally expressed in the terminal rectum of the ARM embryonic rats and may be involved in the development and maturation of the enteric nervous system. The present study may provide a useful theoretical reference for the treatment of postoperative defecation dysfunction in patients with ARM.

Correspondence to: Professor Yuanmei Liu, Department of Pediatric Surgery, Affiliated Hospital of Zunyi Medical College, 201 Dalian Road, Zunyi, Guizhou 563003, P.R. China
E-mail: liuymcn_3@163.com

*Contributed equally

Key words: malformation, intermuscular neuron, immunohistochemistry, protein

Introduction

Congenital anorectal malformation (ARM) is one of the more common digestive tract abnormalities in children with an incidence of ~1/1,500-1/5,000 (1). With advances in surgical techniques, the ARM recovery rate has improved. However, certain patients continue to suffer from defecation dysfunctions, including fecal soiling and constipation, with an incidence of ~20-30% due to the multifactorial pathogenesis mechanisms and complex pathological changes associated with ARM. However, morphological abnormalities can be corrected following surgery (2), which greatly influence the quality of life of patients (3). Previously, these dysfunctions were thought to be associated with a postoperative megacolon or megarectum or postoperative rectal weakness. It is suggested that maldevelopment of features, including the perianal muscle groups and self-enteric nervous system (self-ENS), causes a change in enteric dynamics, which is closely associated with the development of postoperative defecation dysfunction in patients with ARM (3). Patients with ARM present with no anus however additionally with noticeable enteric nervous maldevelopment, which is particularly associated with the maldevelopment of the ENS in the terminal rectum (4).

Originating from neural crest cells, the ENS is a complex, self-created somatic nervous system. Subsequent to entering the intestinal tract and phases of gradual migration, proliferation and agglomeration, it develops into gangliocytes and colloid cells (5). During this process, the disturbance of any step can affect the development of gangliocytes, resulting in ENS maldevelopment. The ENS forms different layers of nerve plexuses in the digestive tract, including the myenteric nervous plexus (MP) and submucosal nervous plexus (SP). The MP is located between the circular muscle and longitudinal muscle layers, while the SP lies between the stratum mucosum and the circular layer. The MP and SP are responsible for processing the signals from the enteric cavity and intestinal wall and regulating the contraction, secretion and blood supply of the

intestinal tract. Therefore, they are of great significance for the formation of the defecation reflex (6). Maldevelopment of the ENS in the terminal rectum reaches up to 60% in cases of anal atresia (7). Kong *et al* (8) identified that there are no noticeable intestinal glands distributed throughout the rectal cecum; however, proliferated squamous epithelial tissue and thickened muscular layers are present in ARM embryonic rats and the number of gangliocytes in the terminal rectum of ARM rats were significantly decreased compared with that in the normal group. The ENS is a critical system for regulating the functions of the intestinal tract. Defecation function is closely associated with the developmental condition of the ENS in the terminal rectum (4). Therefore, recovery of the normal function of the ENS in the terminal rectum has become a primary focus in the treatment of ARM in recent years.

HuD is a member of the highly conserved, neuron-specific RNA-binding Hu family, which is homologous to the ELAV protein in *Drosophila*. Following transcription, HuD regulates the expression of neuron-specific genes and serves important roles in the growth, development and differentiation of neurons (9-11). It is a necessary protein for the formation and regeneration of nervous processes (12-15). HuD demonstrates abnormal expression in diseased intestinal canals of patients with a congenital megacolon, which indicates that HuD has a close association with the development of the ENS. The intestinal canal in a congenital megacolon and the terminal rectum of ARM children demonstrate gangliocyte maldevelopment (16). ARM tends to be accompanied by a megacolon (17). How HuD is expressed in the terminal rectum of the ARM embryonic rat model and whether its expression influences the development of ENS in the terminal rectum of the model remains to be elucidated.

Based on the aforementioned studies, an ARM animal model was established in the present study using ethylenethiourea (ETU) (18). The expression of HuD in the terminal rectum of the embryonic rats with ARM was assessed by immunohistochemistry and western blotting. Its role in the development of the nervous system in the intestinal wall was then explored.

Materials and methods

ARM model establishment and grouping. A total of 40 healthy, nulliparous Sprague Dawley rats aged 6-8 weeks (comprising 30 females and 10 males, specific pathogen-free grade) and weighing 200-250 g were supplied by the animal center of the Third Military Medical University (Chongqing, China). The animals were caged at a female-male ratio of 3:1 for mating. At 8:00 a.m. on the second day, a vaginal smear test was performed. The smears were evaluated using dark field microscopy (x100 magnification). An observation of sperm was noted as pregnancy day 0. Each pregnant rat was weighed at day 0 and 10. Animals without a noticeable weight increase were excluded from the study to exclude the possibility of abortion or phantom pregnancy. A total of 20 pregnancy-confirmed female rats were then randomized to the experimental or control group. Each group contained 10 rats. At day 10, the experimental group was subjected to a lavage with an injection of 1% ETU (1504-I00G; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a dose of 125 mg/kg. The control group was administered physiological saline at the same dose. At day 20, 10% chloral hydrate (NanJing SenBeiJia Biotechnology Co., Ltd., Nanjing, China) was given at 0.4 ml/100 g to the animals and then the intrauterine embryonic rats were removed. Abnormalities were judged by an experienced chief physician using a blinding method. A section of the terminal rectum was stored at 80˚C and the remaining specimen fixed in 4% formaldehyde for 12-36 h. Routine dehydration and paraffin wax embedding were then performed.

All the procedures were approved by the Institute of Animal Ethics of the Affiliated Hospital of Zunyi Medical College (Zunyi, China).

Hematoxylin and eosin (HE) staining. Paraffin wax-embedded pelvic perineal tissue and terminal rectum tissue were serially sectioned at 4 mm thickness. Routine HE staining was then performed. The structures of the terminal rectums from the two groups were observed under an optical microscope. A total of 10 sections were then obtained from each group and 10 fields were randomly selected in each section to calculate the average number of intermuscular and submucosal neurons.

Immunohistochemistry. A total of 10 paraffin-embedded sections were randomly selected from each group. They were stored at 60˚C for 2 h and then brought to room temperature. Deparaffinization was performed with xylene. The specimens were then blocked in 3% H_2O_2 at room temperature for 15-30 min. A citrate antigen-retrieval solution (pH 6.0) was used for room-temperature cooling of the microwave-processed specimens (heated to boiling for 6 min at a power level of 100 W). The specimens were incubated with a rabbit anti-mouse HuD antibody (1:200; cat. no., 300065; CapitalBio, Beijing, China) at 4˚C overnight and then washed with PBS. A goat anti-rabbit secondary antibody (cat. no. 400087; OriGene Technologies, Inc., Beijing, China) was applied for 20 min at room temperature. Hematoxylin counterstaining, dehydration, mounting and 3,3'-diaminobenzidine coloration were then performed. Cells with cytoplasm and nuclei stained yellow-brown were considered HuD-positive. The expression of HuD was analyzed with a Leica QWin relative optic density analytical system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Image-pro plus version 6.0 image analysis system (Media Cybernetics, Inc., Rockville, MD, USA) and an average integral optical density (IOD) was calculated.

Western blot analysis. A total of 10 normal specimens were randomly selected from each group. The total protein was extracted according to the protocol provided with the bicinchoninic acid (BCA) protein concentration assay kit (P0009; Beyotime Institute of Biotechnology, Nantong, China). Protein content was determined using the BCA method.

Following 10% SDS-PAGE, each protein sample (60 μl) was electro-transferred to a PVDF membrane. Following antigen blocking with 5% non-fat milk, each membrane was incubated with rabbit anti-mouse HuD (1:200; cat. no., BYK-11319R; Shanghai Long Island Antibody Diagnostica, Inc., Shanghai, China) and β-actin (1:1,000; cat. no., BYK-12521R; Shanghai Long Island Antibody Diagnostica, Inc.) antibodies at 4˚C

for 12 h and then with 800 CW-labelled goat anti-rabbit IgG antibody (1:10,000; cat. no. 926-32211). Images were obtained with an Odyssey Two-Color infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) for density analysis of the protein content. The protein expression was denoted by a relative grey scale value.

Statistical analysis. All data were processed with SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA) and presented as the mean ± standard deviation. A t-test was used to compare two independent samples. P<0.05 was considered to indicate a statistically significant difference.

Results

Observational outcomes. In the control group, a total of 108 embryonic rats were born with a survival rate of 100%. No abnormalities were observed. In the experimental group, 94 rats were born with a survival rate of 93.6%. Among the surviving embryonic rats in the experimental group, the ARM incidence was 96.8%. In addition to no anus, other abnormalities, including limb malformation, spina bifida, spinal meningoceles, celoschisis and acromphalus, were observed.

HE staining. In the control group, the opening of the rectum at the anal position was normal. The profiles of the anal and perianal muscles were sharp. The layers of the rectal wall were well-defined and lined with normal mucous membranes. A small amount of meconium was observed in the enteric cavity. In the ARM group, the terminal rectum was a closed cecum without anal structure. The cecum had proliferated squamous epithelial tissue and a thickened muscular layer. The proximal end of the rectum had degenerated (Fig. 1A and B). In the control group, the submucosal and myenteric nerve plexuses in the terminal rectum were densely distributed and ran along the intestinal canal. The histological morphology was normal. The cells were rich in cytoplasm with large and noticeable nuclei. In contrast, in the ARM group, the histological layers of the terminal rectum were ill-defined and the muscular layers were poorly developed. The volumes of the submucosal and myenteric nerve plexuses were small. The gangliocytes were poorly developed and sparsely distributed with small and pycnotic nuclei. The amount of submucosal and intermuscular neurons was significantly deceased compared with that in the control group (143.60±14.93 vs. 335.90±10.46; P<0.01; Fig. 1C and D and Table I).

Immunohistochemistry. In the control group, the HuD protein in the mucous membranes of the intestinal wall and neurons in the submucosal and intermuscular nerve plexuses exhibited notable staining with the cytoplasm and nuclei of the neurons stained. In contrast, in the ARM group, the HuD protein stain was weakly positive (light yellow-stained). The IOD value of the ARM group was significantly lower compared with the control group (P<0.01; Table II and Fig. 2).

Western blot analysis. The relative expression of HuD in the terminal rectum of the ARM group was significantly lower compared with the control group (P<0.05; Table II and Fig. 3).

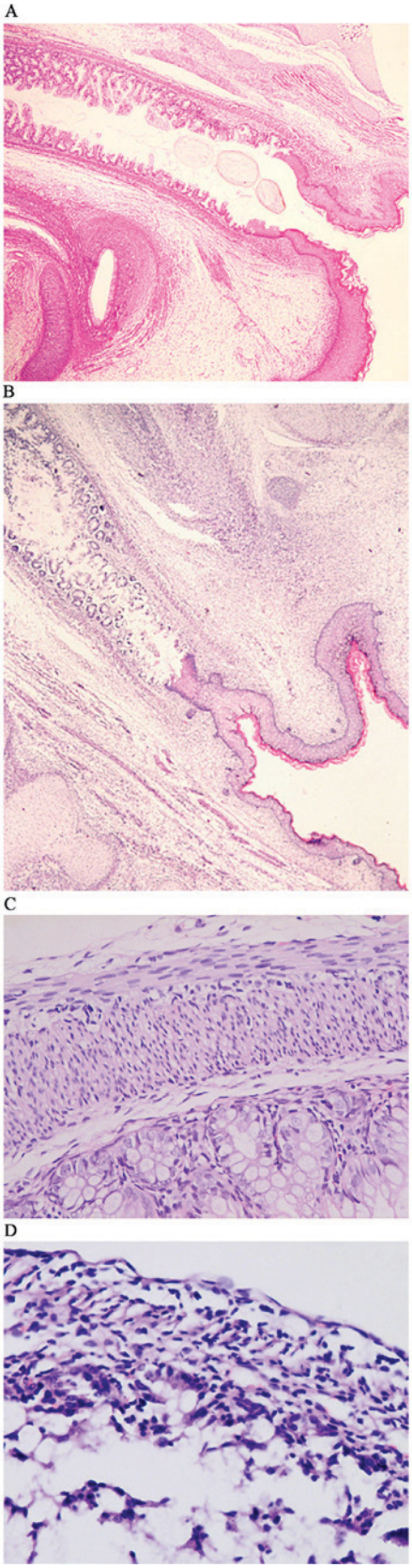

Figure 1. The HE results of the pelvic perineal tissue and terminal rectum. (A) The sagittal plane of tissue from the control group: The anus has a normal opening with a sharp profile (x40 magnification). (B) The sagittal plane of tissue from the ARM group: The terminal rectum was a closed cecum (x40 magnification). (C) The layers of the intestinal wall of the terminal rectum demonstrate good differentiation in the control group (x400 magnification). (D) The histological layers were ill-defined and the number of submucosal and intermuscular neurons was small in the ARM group (x400 magnification). HE, hematoxylin and eosin; ARM, anorectal malformation.

Table I. Comparison between the amounts of submucosal and intermuscular neurons in the terminal rectum (mean ± standard deviation, n=10).

Group	Number of submucosal and intermuscular neurons
Control	335.90±10.46
ARM	143.60±14.93[a]
t-value	10.5
P-value	<0.01

[a]P<0.01 vs. control. ARM, anorectal malformation.

Table II. The relative expression and IOD of HuD in the terminal rectum (mean ± standard deviation, n=10).

Group	IOD	Relative expression
Control	456.40±57.13	0.45±0.06
ARM	312.90±53.40[a]	0.24±0.05[a]
t-value	5.80	-8.19
P-value	<0.01	<0.01

[a]P<0.05 vs. control. ARM, anorectal malformation; IOD, integral optical density.

Discussion

Although the ARM recovery rate has improved with advances in surgical techniques, the incidence of postoperative defecation dysfunction among patients with ARM is as high as 20-30%. Defecation dysfunction following ARM surgery can be caused by multiple factors, including self-ENS maldevelopment (19). Apart from no anus, patients with ARM additionally exhibit ENS maldevelopment, particularly in the rectum (20). The ENS is an essential factor in regulating the functions of the intestinal tract. Its maldevelopment can lead to digestive tract and enteric dynamic abnormalities. Therefore, promoting the maturation process of intestinal neurons and maximally repairing the function of the ENS may become a promising method for the recovery of postoperative defecation dysfunction in ARM patients.

The HuD protein is a marker of neurons (21,22). It is a member of the Hu family, a family of highly conserved, neuron-specific RNA binding proteins. The Hu family includes HuA, HuB, HuC and HuD, although only HuC and HuD specifically exist in animal neurons (23,24). HuD regulates the expression of neuronal specificity following transcription and stabilizes the functions of cellular transcription factors, including C-Myc, neuroserpin and GAP-43, serving as trans-acting factors that serve a determinative role in the development of neurons (25). The molecular weight of HuD is between 42 and 46 kDa. It is involved in numerous processes of neural development, including regulating the proliferation of neural stem cells, maintaining the survival of the referred neurons by neural stem cells and promoting the differentiation

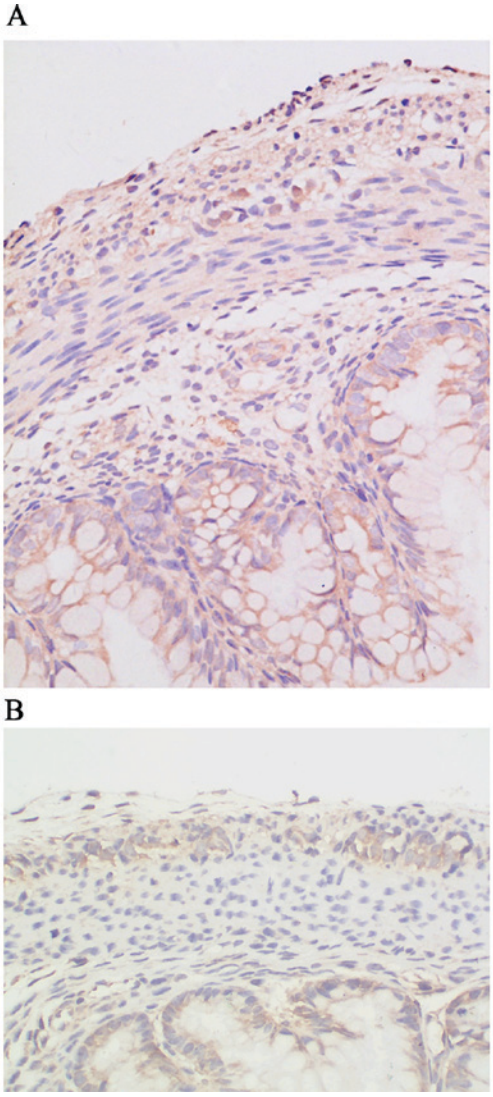

Figure 2. The expression of HuD based on immunochemistry (x400 magnification). (A) HuD is highly expressed in the control group. (B) HuD expression is decreased in the ARM group. ARM, anorectal malformation.

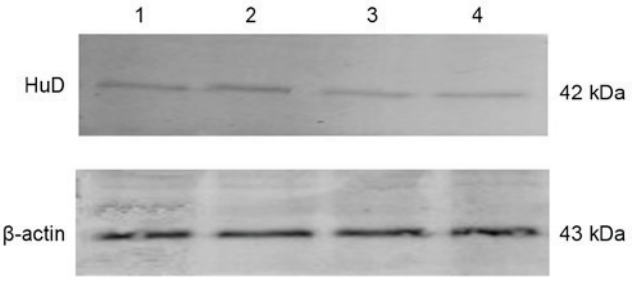

Figure 3. The quantitative results of HuD and β-actin. Lanes 1 and 2, control group; lanes 3 and 4, ARM group. ARM, anorectal malformation.

of neurons following mitosis. Therefore, it serves important roles in the growth, development and differentiation of neurons.

The results of the current study demonstrated that the submucosal and myenteric nerve plexuses in the terminal rectum were small and sparsely distributed. There were few or congenitally absent ganglion cells in the ARM group. Immunochemistry demonstrated that the expression of HuD in

the mucous membranes of the intestinal wall and the number of neurons in the submucosal and intermuscular nerve plexuses of the ARM group were significantly lower compared with those in the control group. There was a markedly positive expression of HuD protein in the ARM group, however weakly positive expression in the control group. The western blot analysis additionally demonstrated that the relative expression of HuD protein in the ARM group was significantly lower compared with the control group. It has been reported (26) that anti-HuD antibodies can cause the apoptosis of neurons, which results in intestinal neuronal abnormalities and intestinal dynamic disturbance. Akamatsu *et al* (27) identified that the neurites of certain central neurons cannot form at an embryonic stage in HuD knock-out mice. Furthermore, Akamatsu *et al* (27) identified that 70-80% of these knock-out mice at 4-6 weeks postnatal age presented abnormalities in limb extension: Their limbs reflexed toward the abdomen when they were suspended in the air by their tails. The results of Akamatsu *et al* (27) indicated that HuD serves important roles in the differentiation, neurite formation and post-maturation survival of neurons in mice. Du *et al* (16) identified that the HuD protein is not expressed in the convulsion segment of the intestinal canal in patients with a congenital megacolon and that its expression in the migration segment was noticeably lower compared with that in the expansion and normal segments, indicating that HuD protein is abnormally expressed in the diseased intestinal canal. The results of the present study are consistent with those reported in the literature (16) and demonstrate the presence of neurodevelopmental abnormalities in the terminal rectum of ARM embryonic rats. The nerve tissue in the terminal rectum serves an important role in regulating the relaxation and contraction of the smooth muscle and internal sphincter muscle of the anorectum. The HuD protein regulates the expression of neuronal genes primarily at the transcriptional level. Therefore, the decrease in the expression of HuD may affect the development, maturation and stable expression of intestinal neurons. It may serve an essential role in the development of the rectal ENS in ARM and may also serve a regulating role in the development of the nerves and muscles of the intestinal tract. These presumptions may aid in understanding the mechanisms underlying the development of postoperative defecation dysfunction in patients with ARM.

The decrease in or congenital absence of the HuD protein may influence the development and maturation of intestinal neurons by the following mechanisms: i) HuD protein influences the process of intestinal neural stem cells entering the mitotic cycle; ii) the decreased expression of the HuD protein cannot maintain the survival and development of matured neuronal cells; iii) the decrease in the expression of HuD protein causes a decreased expression of neural development-associated proteins (e.g. neuroserpin and GAP-43), which leads to a disturbance in nervous process formation (28); and iv) the HuD protein serves a critical role in regulating the expression of AchE mRNA following transcription.

However, there are still certain issues that require elucidation. A deeper understanding of the association between the HuD protein and ENS will be gained by answering questions including the following: What is the role of the HuD protein in gene regulation? Why is the HuD protein abnormally expressed in ENS development? In what phase of ENS development does the abnormal expression occur? Is the expression of the HuD protein in animal models consistent with that in patients with ARM?.

In summary, the HuD protein is abnormally expressed in the nerve plexuses of the intestinal wall of the terminal rectum of ARM embryonic rats, which suggests that HuD may participate in the development and maturation of the ENS in ARM embryonic rats. The results of the present study may be a useful experimental and theoretical reference for the treatment of ARM in clinical practice. Furthermore, the results of the present study provide new insights into the potential treatment of ARM, including the injection of exogenous HuD to improve the development of ENS in patients with ARM and prevent postoperative defecation dysfunction.

Acknowledgements

The current study was supported by the National Natural Science Foundation of China (grant no. 81360067) and the Key Project on Social Development by Department of Science and Technology of Guizhou Province [grant no. (2009) 3079].

References

1. Levitt MA, Kant A and Pena A: The morbidity of constipation in patients with anorectal malformations. J Pediatr Surg 45: 1228-1233, 2010.
2. Hamid CH, Holland AJ and Martin HC: Long-term outcome of anorectal malformations: The patient perspective. Pediatr Surg Int 23: 97-102, 2007.
3. Gariepy CE and Mousa H: Clinical management of motility disorders in children. Seminars Pediatr Surg 18: 224-238, 2009.
4. Wang H, Hughes I, Planer W, Parsadanian A, Grider JR, Vohra BP, Keller-Peck C and Heuckeroth RO: The timing and location of glial cell line-derived neurotrophic factor expression determine enteric nervous system structure and function. J Neurosic 30: 1523-1538, 2010.
5. Young HM and Newgreen D: Enteric neural crest-derived cells: Origin, identification, and differentiation. Anat Rec 262: 1-15, 2001.
6. Gao MJ, Liu YM, Mao YC, Li YH, Wang LM, Li SJ and Shen Q: Expression of nerve growth factor and its receptor in distal rectum of children with anorectal malformations. Chin J Pediatr Surg 35: 698-702, 2014.
7. Meier-Ruge WA and Holschneider AM: Histopathologic observations of anorectal abnormalities in anal atresia. Pediatr Surg Int 16: 2-7, 2000.
8. Kong M, Liu YM, Mao YC, *et al*: The expression of GDNF in distal rectum in fetal rats with anorectal malformations. Chin J Pediatr Surg 35: 62-65, 2014.
9. Pascale A, Gusev PA, Amadio M, Dottorini T, Govoni S, Alkon DL and Quattrone A: Increase of the RNA-binding protein HuD and posttranscriptional up-regulation of the GAP-43 gene during spatial memory. Proc Natl Acad Sci USA 101: 1217-1222, 2004.
10. Borgeson CD and Samson ML: Shared RNA-binding sites for interacting members of the Drosophila ELAV family of neuronal proteins. Nucleic Acids Res 33: 6372-6383, 2005.
11. Hayashi S, Yano M, Igarashi M, Okano HJ and Okano H: Alternative role of HuD splicing variants inneuronal differentiation. J Neurosci Res 93: 399-409, 2015.
12. Wakamatsu Y and Weston JA: Sequential expression and role of Hu RNA-binding proteins during neurogenesis. Development 124: 3449-3460, 1997.
13. Deschenes-Furry J, Belanger G, Perrone-Bizzozero N and Jasmin BJ: Post-transcriptional regulation of acetylcholinesterase mRNAs in nerve growth factor-treated PC12 cells by the RNA-binding protein HuD. J Biol Chem 278: 5710-5717, 2003.
14. Bolognani F, Merhege MA, Twiss J and Perrone-Bizzozero NI: Dendritic localization of the RNA-binding protein HuD in hippocampal neurous: Association with polysomes and upregulation during contextual learning. Neurosci Lett 2004: 152-157, 2004.

15. Saito K, Fujiwara T, Katahira J, Inoue K and Sakamoto H: TAP/NXF1, the primary mRNA export receptor, specifically interacts with a neuronal RNA-bingding protein HuD. Biochem Biophys Res Commun 2004: 291-297, 2004.
16. Du Y, Shi CR and Pan J: Study on the expression of HuD in protein Hirschsprung's disease. J Clin Pediatr Surg 4: 345-348, 2005.
17. Zhang TC, Chen CF, Chen YJ, *et al*: The diagnosis and treatment of intractable constipation in children with repaired anorectal malformation. Chin J Pediatr Surg 32: 103-105, 2011.
18. Garcia-Barceló MM, Chi-Hang Lui V, Miao X, So MT, Yuk-yu Leon T, Yuan ZW, Li L, Liu L, Wang B, Sun XB, *et al*: Mutational analysis of SHH and GLI3 in anorectal malformations. Birth Defects Res A Clin Mol Teratol 82: 644-648, 2008.
19. Jia H, Zhang K, Zhang S, Yuan Z, Bai Y and Wang W: Quantitative analysis of sacral parasympathetic nucleus innervating the rectum in rats with anorectal malformations. J Pediatr Surg 42: 1544-1548, 2007.
20. Yuan ZW, Lui VCH and Tam PK: Deficient motor innervation of the sphincter mechanism in fetal rats with anorectal malformation: A quantitative study by fluorogold retrograde tracing. J Pediatr Surg 38: 1383-1388, 2003.
21. Ohwada A, Nagaoka I, Takahashi F, Tominaga S and Fukuchi Y: DNA vaccination against HuD antigen elicits antitumor activity in a small-cell lung cancer murine model. Am J Respir Cell Mol Biol 21: 37-43, 1999.
22. Wang G, Chen QM, Zhu Y, Ke D, Qian W and Song W: The value of specific neuronal anti-Hu antibody in the early diagnosis of small cell lung cancer. Zhonghua Jie He He Hu Xi Za Zhi 25: 732-735, 2002 (In Chinese).
23. Park-Lee S, Kim S and Laird-Offringa IA: Characterization of the interaction between neuronal RNA-binding protein HuD and AU-rich RNA. J Biol Chem 278: 39801-39808, 2003.
24. Ross RA, Lazarova DL, Manley GT, Smitt PS, Spengler BA, Posner JB and Biedler JL: HuD, a neuronal-specific RNA-binding protein, is a potential regulator of MYCN expression in human neuroblastoma Cells. Euro J Cancer 33: 2071-2074, 1997.
25. Smith CL, Afroz R, Bassell GJ, Furneaux HM, Perrone-Bizzozero NI and Burry RW: GAP-43mRNA in growth cones is associated with HuD and ribosomes. J Neurobiol 61: 222-235, 2004.
26. De Giorgio R, Bovara M, Barbara G, Canossa M, Sarnelli G, De Ponti F, Stanghellini V, Tonini M, Cappello S, Pagnotta E, *et al*: Anti-HuD-induced neuronal apoptosis underlying paraneoplastic gut dysmotility. Gastroenterology 125: 70-79, 2003.
27. Akamatsu W, Fujihara H, Mitsuhashi T, Yano M, Shibata S, Hayakawa Y, Okano HJ, Sakakibara S, Takano H, Takano T, *et al*: The RNA-binding protein HuD regulates neuronal cell identity and maturation. Proc Natl Acad Sci USA 102: 4625-4630, 2005.
28. Sanna MD, Quattrone A, Mello T, Ghelardini C and Galeotti N: The RNA-binding protein HuD promotes spinal GAP43 over expression in antiretroviral-induced neuropathy. Exp Neurol 261: 343-353, 2014.