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

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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.

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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).

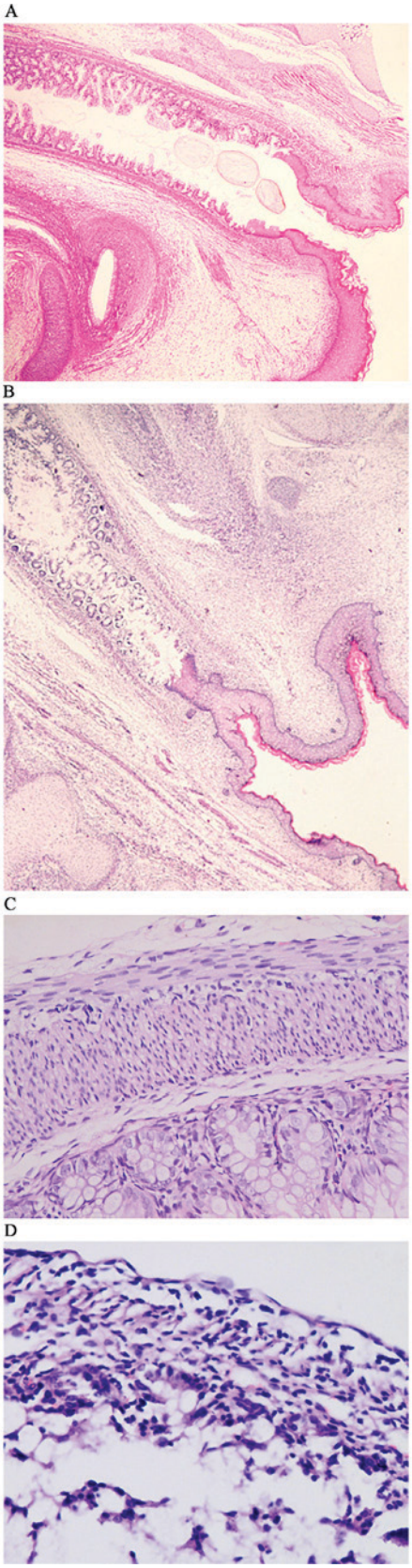


Figure 1. The HE results of the pelvic perineal tissue and terminal rectum. (A) The sagittal plane of tissue from the control group: The anus has a normal opening with a sharp profile (x40 magnification). (B) The sagittal plane of tissue from the ARM group: The terminal rectum was a closed cecum (x40 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

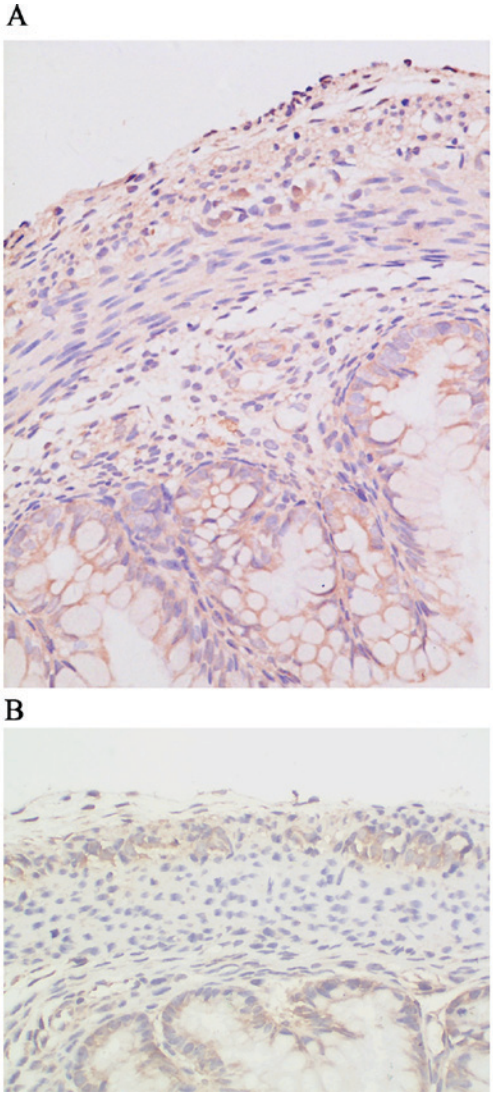


Figure 2. The expression of HuD based on immunochemistry (x400 magnification). (A) HuD is highly expressed in the control group. (B) HuD expression is decreased in the ARM group. ARM, anorectal malformation.

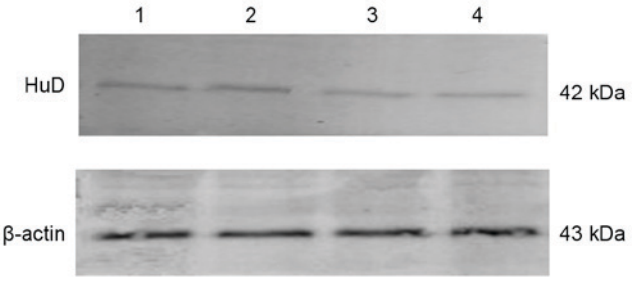


Figure 3. The quantitative results of HuD and β-actin. Lanes 1 and 2, control group; lanes 3 and 4, ARM group. ARM, anorectal malformation.

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.

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