Downregulation of P2Y2 and HuD during the development of the enteric nervous system in fetal rats with anorectal malformations

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Abstract. Certain patients with anorectal malformations (ARMs) continue to suffer from postoperative dysphoria. The enteric nervous system (ENS) is closely associated with defecation. The purinergic receptor P2Y2 (P2Y2) and Hu antigen D (HuD) proteins contain multiple motifs that enable their activation and direct coupling to integrin and growth factor receptor signaling pathways; thus, they may serve as key points in ENS development. The aim of the present study was to investigate the expression pattern of P2Y2 and HuD proteins during anorectal development in ARM embryos. The embryogenesis of ARM in rats was induced by ethylenethiourea (ETU) on the 10th gestational day. The expression patterns of P2Y2 and HuD proteins were evaluated by immunohistochemistry and western blot analysis in normal, ETU and ARM rat embryos on embryonic days E17, E19 and E21; their mRNA levels were assessed via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of the distal rectum of fetal rats. Immunohistochemistry of the distal rectum demonstrated that on E17, the expression levels of the two proteins were not different between the three groups. On E19, the expression of HuD was significantly decreased in the ARM group. On E21, the two proteins were significantly decreased in the ARM group. Additionally, the expression levels of the two proteins on E17 were significantly lower than on E21 in the ARM group. Western blotting and RT-qPCR also revealed that the P2Y2 and HuD proteins and mRNA expression levels were significantly decreased in the ARM groups when compared with the normal group on E17 and E21 (P<0.01). Thus, the present study demonstrated that downregulation of P2Y2 and HuD may partly be related to the development of the ENS in ARM embryos.

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Introduction

Anorectal malformations (ARMs) are frequently encountered anomalies that represent an important component of pediatric surgery practice. The incidence rate ranges from 1:1,500 to 1:5,000 live births (1). There also appears to be a wide spectrum of ARM epidemiology ranging from isolated cloacal, supralevator and infralevator anal atresia (with and without fistula) to ectopic anus malformation, and have variable clinical presentations ranging from mild forms that may require only minor surgical interventions to more complicated cases that require management with multi-staged operations (2,3). Despite the continuous progression of ARM surgical treatments, certain patients still have anal dysfunctions following surgery, which seriously affects their quality of life (4). The cause of ARM is unknown, although arrest of the descent of the urorectal septum towards the cloacal membrane between the 4th and 8th weeks of gestation was previously considered the basic event leading to ARM (5). Since the molecular deteminants during blastogenesis are overlapping for many body systems, and these elements are closely related in timing and spacing, thus defects in blastogenesis often involve two or more progenitor fields (6). Several studies have reported on the mechanisms of enteric nervous system (ENS) development in embryos, and P2Y receptors have been revealed to primarily regulate muscle relaxation (7-10). Mulè *et al* (11), revealed that the P2Y receptor antagonist suramin inhibited ATP-induced muscle relaxation, confirming that P2Y receptors participate in muscle relaxation. P2Y receptor deficiency could lead to dysfunction in intestinal relaxation, resulting in intestinal spasticity (12). Our previous study indicated that purinergic receptor P2Y2 (P2Y2) may be one of the basic factors leading to ENS dysplasia at the end of the rectum of fetal rats with ARM in day 21 embryos (13). Another study demonstrated that P2Y2 is involved in the direct regulation of ENS, smooth muscle contractility and control of intestinal peristalsis (14).

Hu antigen D (HuD) is considered to be expressed specifically in neurons, while the other member of the Hu protein family, HuR, is ubiquitously expressed (15). Hu proteins have three RNA recognition motifs through which they associate with mRNAs bearing specific sequences that are often AU- and U-rich. HuD binds to and stabilizes the 3'-untranslated

region of target mRNAs, including p21, tau and GAP-43 mRNAs (15). HuD regulates the expression of neuron-specific genes and serves important roles in the growth, development and differentiation of neurons. It is a necessary protein for the formation and regeneration of nervous processes (16). It also modulates target mRNA translation. HuD demonstrates aberrant expression in diseased intestinal canals of children with Hischsprung's disease, which indicates that HuD has a close relationship with the development of the ENS (17). Our previous study revealed that the HuD protein is aberrantly expressed in the nerve plexuses of the intestinal wall of the terminal rectum of ARM embryonic 20-day rats, which suggests that HuD may participate in the development and maturation of the ENS in ARM embryonic rats (18). However, a previous study investigating different tissues unexpectedly observed HuD expression in ENS with ARMs (19); it was not clear whether P2Y2 and HuD continued to participate in the development of the ENS prior to the emergence of ARMs. To provide insights into the pattern of P2Y2 expression and the possible role of HuD during ENS development, the present study examined the expression of P2Y2 and HuD in normal and ARMs model rat embryos at 17, 19 and 21 days.

Materials and methods

Animal model. A total of 120 Sprague Dawley (SD) rats at 10-12 weeks of age (210-260 g) were obtained from the Experimental Animal Center at the Daping Hospital of the Third Military Medical University (Chongqing, China). Ethical approval was obtained from the Zunyi Medical College Animal Ethics Committe (no. 20150820014) prior to the commencement of the study. Mating was performed at night with male and female rats at a 3 to 1 ratio. In the early morning, vaginal smears were obtained from female rats; if sperm or vaginal suppositories were discovered under a light microscope, it was recorded as embryonic day zero (E0). A total of 40 mated pregnant SD rats were randomly divided into the following two groups: Ethylenethiourea (ETU)-treated (n=30) and normal groups (n=10). The animals were maintained in a temperature-controlled environment (20-24˚C), a humidity of 50-70% and a 12-h light/dark cycle. Solid laboratory chow and water were available *ad libitum*. In the ETU-treated group, 30 pregnant rats were administered a single dose of 125 mg/kg of 1% ETU (CAS no. 03940-I00G; Sigma-Aldrich; Merck KGaA) by oral gavage on E10 to induce the ARM model (ARM group). Rats in the normal group were treated the same as the ETU group, however, they received corresponding doses of ETU-free saline on E10 instead of ETU. Embryos were harvested by cesarean delivery at E17, E19 and E21. The presence of ARMs was determined by light microscope. Then, the embryos were divided into normal, ETU and ARM groups.

Tissue handling. The pregnant rats were anesthetized with chloral hydrate by intraperitoneal injection (0.3 ml/100 g body weight; Sinopharm Chemical Reagent Co., Ltd.). The uterine horn was incised through the abdominal wall. Embryos were obtained by cesarean section on E17, E19 and E21. Subsequently, pregnant rats were sacrificed with an excessive dose of sodium pentobarbital. The embryos were then divided into the normal, ETU (therapeutic rats that exhibited

no deformities) and ARM groups. One-third of the embryos, based on their weight, were fixed in 4% paraformaldehyde for 12 to 24 h. Then the embryos of all age groups were dehydrated and embedded in paraffin. Immunohistochemical staining was performed on serial and sagittal sections of up to 4-μm thickness. The presence of ARMs was determined under a light microscope. Under a magnifying glass, the distal rectum of the remaining two-thirds of the embryos was dissected and removed from the surrounding tissues. The distal rectum was immediately frozen in liquid nitrogen for western blot analysis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Hematoxylin and eosin (H&E) staining. The paraffin-embedded terminal rectum tissues were consecutively cut to a thickness of 3 μm. Following conventional H&E staining, the structure of the distal rectum, and the number and morphology of the intermuscular and submucosal nervous plexus of the distal rectum were observed under an optical microscope.

Immunohistochemistry. Immunohistochemical staining was performed as previously described (9,13). Sections were incubated overnight at 4˚C with the primary rabbit anti-mouse P2Y2 (1:125; cat. no. sc-518091) and mouse anti-rat HuD (1:80; cat. no. sc-28299; both from Santa Cruz Biotechnology, Inc.) polyclonal antibodies. Following primary antibody incubation, the sections were washed and incubated with biotinylated goat anti-rabbit secondary antibody A (cat. no. A0456; dilution 2 μg/l; ZSGB-BIO; OriGene Technologies, Inc.) for 20 min at room temperature. Following incubation, the sections were incubated with reagent B (ZSGB-BIO; OriGene Technologies, Inc.) for 30 min at room temperature. Immunoreactivity was visualized under an ordinary light microscope following the addition of 3'3-diaminobenzidine (ZSGB-BIO; OriGene Technologies, Inc.) and the chromogenic degree was observed to subsequently adjust the incubation time. Sections were stained with hematoxylin for 3 sec at room temperature. Negative controls received PBS instead of primary antibodies. Human cerebral cortex tissues from tissue bank (cat. no. 201324; Research Center for Medical & Biology Tissue Bank, Zunyi Medical University) were used for the P2Y2 positive control, while human breast tissue (cat. no. 201324; Research Center for Medical & Biology Tissue Bank; Zunyi Medical University) was used for the HuD positive control. All sections were photographed using an optical microscope (magnification, x400). The target images were collected and compiled with the Leica QWin image analysis system. The integral optical density was calculated using Image Pro Plus 6 image analysis software (Media Cybernetics, Inc.).

Protein preparation and western blot analysis. Protein preparation was performed as previously described (20). Total protein was extracted from the distal rectum collected from the normal, ETU and ARM groups on days E17, E19 and E21, and frozen at -80˚C. The Enhanced Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology) was used for protein quantification according to the manufacturer's protocol. Protein extracts (50 μg/lane) were mixed with 5X SDS-PAGE (Beyotime Institute of Biotechnology) loading buffer (40 μl), heated at 90˚C for 5 min, transferred to polyvinylidene fluoride

Table I. Distribution of embryos in the various age and treatment groups.

E(d)/group	Normal				ETU				ARM			
	H&E	IHC	WB	PCR	H&E	IHC	WB	PCR	H&E	IHC	WB	PCR
17	7	7	7	7	7	7	7	7	7	7	7	7
19	7	7	7	7	7	7	7	7	7	7	7	7
21	7	7	7	7	7	7	7	7	7	7	7	7
Total	21	21	21	21	21	21	21	21	21	21	21	21

E, embryonic; ETU, ethylenethiourea; ARM, anorectal malformations; H&E, hematoxylin and eosin; IHC, immunohistochemical; WB, western blotting; PCR, real-time PCR.

membranes and blocked with 5% fat-free milk in Tris-buffered saline for 3 h at room temperature. Membranes were incubated with primary rabbit anti-mouse antibodies against P2Y2 (1:400; cat. no. sc-518091; Santa Cruz Biotechnology, Inc.) and primary mouse anti-rat antibodies against HuD (1:200; cat. no. sc-28299; Santa Cruz Biotechnology, Inc.), and β-actin (1:400; mouse monoclonal; cat. no. 610154; Wuhan Boster Biological Technology, Ltd.). The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies anti-mouse (1:4,000; cat. no. sc-516102; Santa Cruz Biotechnology, Inc.) or anti-rabbit (1:6,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The membranes were developed using an ECL substrate kit (cat. no. tf-2012215; Pierce; Thermo Fisher Scientific, Inc.) and densitometric values were analyzed using the Enhance Chemiluminescence Plus detection system (EMD Millipore). Protein levels were normalized to β-actin.

RNA isolation and RT-qPCR. Total RNA was isolated from the rat distal rectum of the normal, ETU and ARM groups using RNAiso Plus (TRIzol; Takara Biotechnology Co., Ltd.), following the manufacturer's instructions. The A260/A280 OD value of the total RNA ranged from 1.8 to 2.0. The extracted RNA was diluted to a concentration of 1 µg/µl, and its equivalents were stored at -80°C. Single-strand cDNA was reverse transcribed with the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.). Primers used for RT-qPCR included the following: P2Y2 forward 5'-TGCTCTACTTTGTCACCA CCA-3' and reverse, 5'-CTTGTCATCCCGTCAATGG-3'; HuD forward, 5'-ACCAGGCTCAAAGATTCAGG-3' and reverse, 5'-CTTGTCATCCCGTCAATGG-3'; and β-actin (used as an endogenous control) forward, 5'-GGAGATTAC TGCCCTGGCTCCTA-3' and reverse, 5'-GACTCATCGTAC TCCTGCTTGCTG-3'. RT-qPCR was conducted with SYBR Premix Ex Tap (cat. no. DRR081S; Takara Biotechnology Co., Ltd.) on a 7900HT fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following thermocycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The results of qPCR were analyzed with LightCycler 1.5 System software (Roche Diagnostics GmbH). Melting curves were generated during the dissociation process to determine the specificity of the amplification. Relative levels of gene expression were determined as ΔCq=(Cq gene)-(Cq reference),

and the fold change in gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Statistical analysis. SPSS version 16.0 (SPSS Inc.) was used for statistical analyses. The data are consistent with normal distribution, with homogeneity of variance and one-way analysis of variance and Tukey's post hoc test used to analyze the levels of P2Y2 and HuD expression between the normal, ETU and ARM model rat groups. All results were expressed as the mean ± standard deviation, and P<0.05 was considered to indicate a statistically significant difference.

Results

General observation. In the present study, no anomalies were identified in the 142 normal model rat embryos examined. A total of 104 ETU-treated fetal rats were normal and 146 ARM model rat embryos were obtained from the 256 ETU-treated rat embryos. All rats produced from ETU-treated embryos exhibited a short or no tail, and 6 perished during the Cesarean section. Spinal bifida and/or meningocele were also observed externally. The incidence of ARMs (manifesting as rectoure-thral fistula or common cloaca) in ETU-treated embryos from E17 to E21 was 57.3%. The anal opening position was normal in the control and ETU groups, and the anal opening was a closed blind end in the ARM group (Table I).

H&E staining. At E17, the nerve plexus count in the submu-cousal and intermuscular clusters was not different between the three groups (Fig. 1). On E19, the nerve plexus count in the ARM group was greater than that at E17 (P<0.05; Fig. 1). On E21, the nerve plexus count in the ETU group was significantly increased compared with that at E19 (P<0.01; Fig. 1). There was decreased development of the distal rectum nerve plexus count in the E19 and E21 ARM groups compared with the normal and ETU groups. Within the ARM group, the nerve plexus count was significantly different between the E17 and E19 embryos (P<0.01; Fig. 1).

Immunohistochemical results. P2Y2 and HuD were mainly expressed in the distal submucosa and intermuscular plexus of the fetal rats, and were primarily expressed in the cytoplasm. There was a small amount of HuD expression in the nucleus. As the embryo developed, the color corresponding to

Table II. Expression of P2Y2-positive rate at the distal rectum in the three groups.

E(d)	Normal group/ETU group/ARM group (n=10)					Negative rate (%)	Strong-positive rate (%)
	-	+	++	+++	Total		
17	2/3/3	8/7/7	0/0/0	0/0/0	10/10/10	20/30/30	0/0/0
19	0/0/3	7/6/6	3/4/1	0/0/0	10/10/10	0/0/30	0/0/0
21	0/0/1	2/3/7	3/3/1	5/4/1	10/10/10	0/0/10	50/40/10

(-): negative staining; (+): weakly-positive staining, stained yellowish; (++ and +++): positive staining to strong-positive staining, stained yellowish brown to yellow brown. ETU, ethylenethiourea; ARM, anorectal malformations.

Table III. Expression of HuD positive rate at the distal rectum in the three groups.

E(d)	Normal group/ETU group/ARM group (n=10)					Negative rate (%)	Strong-positive rate (%)
	-	+	++	+++	Total		
17	2/3/4	8/7/6	0/0/0	0/0/0	10/10/10	20/30/40	0/0/0
19	0/0/2	8/8/6	2/2/2	0/0/0	10/10/10	0/0/20	0/0/0
21	0/0/0	1/2/7	5/4/2	4/4/1	10/10/10	0/0/0	40/40/10

(-): negative staining; (+): weakly positive staining, stained yellowish; (++ and +++): positive staining to strong-positive staining, stained yellowish brown to yellow brown. ETU, ethylenethiourea; ARM, anorectal malformations.
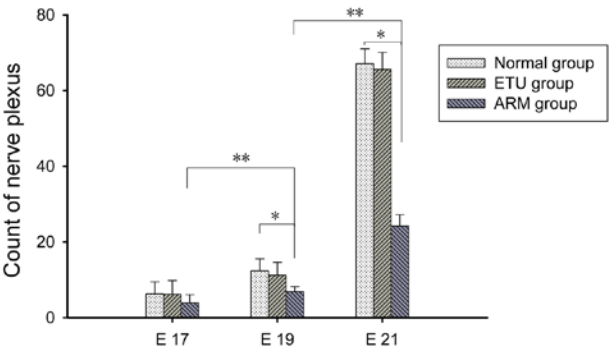
Figure 1. Hematoxyling and eosin staining analysis of nerve plexus count. At E17, the nerve plexus count in the submucousal and intermuscular clusters was not different among the three groups. At E19, the nerve plexus count in the ARM group was increased compared with E17. At E21, the intermuscular and submucosal nerve plexus count between the normal and ETU group was significantly increased compared with E19. There was a decreased nerve plexus count in the ARM group at E19 and E21 compared with the normal group. Within the ARM group, there was a significant difference in the nerve plexus count between the E17 and E19 embryos. Data are expressed as the mean ± SD. *P<0.05; **P<0.01. ETU, ethylenethiourea; ARM, anorectal malformations.

protein expression changed from yellowish to yellowish brown to yellow brown; the range of expression also increased. At E21, the expression of P2Y2 strong-positive rate was 50% in the normal group, 40% in the ETU group, and 10% in the ARM group; the expression of HuD strong-positive rate was 40% in the normal group, 40% in the ETU group, 10% in the ARM group. The nerve plexus in the ARM group was small compared with the normal and ETU groups (Tables II and III).

P2Y2 protein quantitative analysis. On E17, a small amount of P2Y2 protein with yellowish staining was observed at the distal rectum of the three groups. Respectively 8, 7, and 7 cases in the normal, ETU and ARM groups were observed with weak-positive staining. The expression of P2Y2 was low, and there was no significant difference between the three groups (P>0.05). At E19, the normal and ETU groups were stained yellowish, while some areas appeared yellowish brown. Respectively 7, 6, and 6 cases in the normal, ETU and ARM groups were observed with weak-positive staining; and 3, 4, 1 cases in the normal, ETU and ARM groups, respectively, were observed with positive staining. The degree of staining increased in intensity when compared with the staining in E17, however the expression was still low; there was no difference between the three groups. At E21, the distal rectum in the normal and ETU groups appeared yellowish brown or dark brown, with a marked increase in intensity; yellow staining could be clearly observed in the intermuscular and mucous membrane. Respectively 2, 3, and 7 cases in the normal, ETU and ARM groups were observed with weak-positive staining; 3, 3, and 1 in the normal, ETU and ARM groups respectively, were observed with positive staining; and 5, 4, and 1 in the normal, ETU and ARM groups, respectively, were observed with strong-positive staining. There was a significant difference between the E21 and E19 embryos (P<0.01). The expression reported in the ARM group was significantly lower than that of the normal group (P<0.01). There was no significant difference between the E17 and E19 ARM groups, while a significant increase in expression was observed in the E21 ARM group when compared with the E17 ARM group (P<0.01; Figs. 2A and 3).
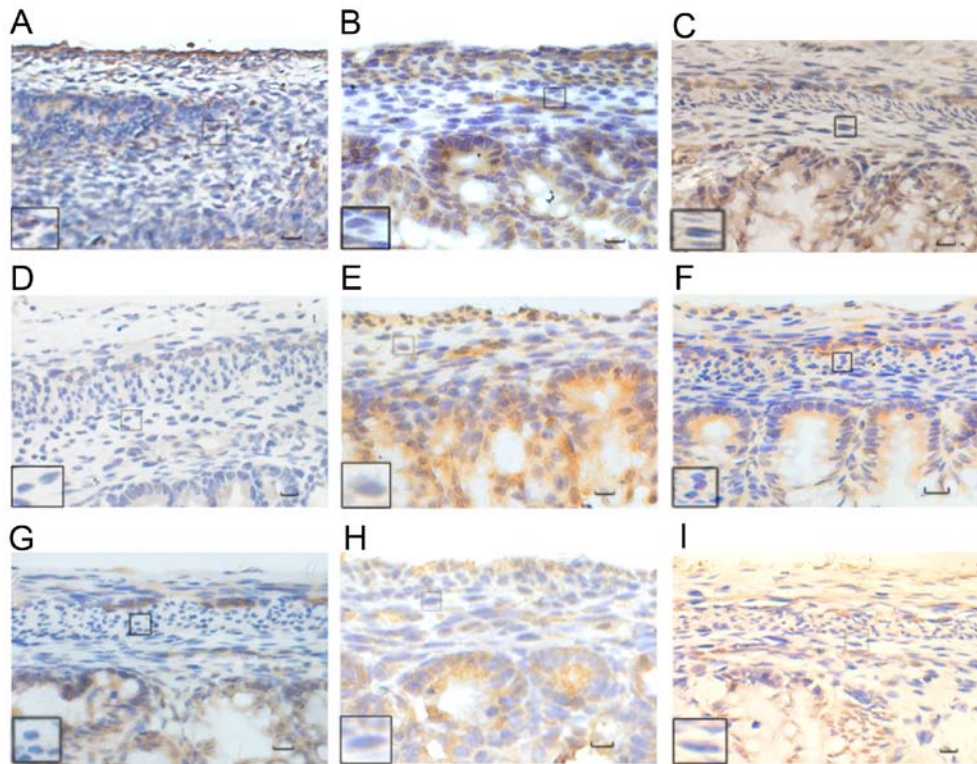
Figure 4. Immunohistochemical analysis of HuD protein. The normal group at (A) E17, (B) E19, and (C) E21. The ETU group at (D) E17, (E) E19, (F) E21. The ARM group at (G) E17, (H) E19, (I) E21. HuD was mainly expressed in the cytoplasm, with a small amount observed in the nucleus, and in the distal submucosa and intermuscular plexus of the fetal rat. The color of the expressed proteins changed from yellowish to yellowish brown to yellow brown as the embryo developed, with an increase in the range of expression also observed. Black rectangles reveal images at a higher magnification. Scale bar, 25 μm; magnification, x400. HuD, Hu antigen D; ETU, ethylenethiourea. ARM, anorectal malformations.
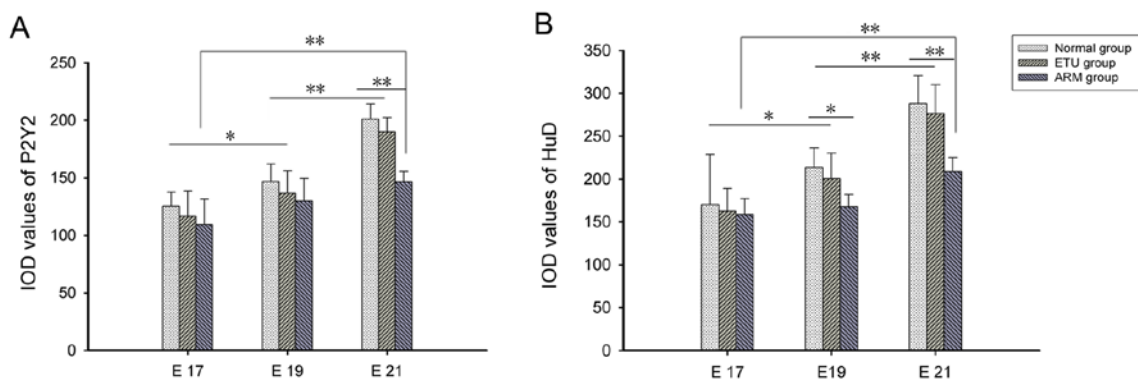

Figure 2. Immunohistochemical analysis of P2Y2 and HuD protein expression levels in the normal, ETU and ARM fetal rat groups. (A and B) Histogram revealing the trends in P2Y2 and HuD expression at each time-point. P2Y2 and HuD protein expression increased gradually over time in the three groups; the levels of HuD protein expression were significantly lower in the distal rectum from the ARM model rats at E19 and E21 when compared with the other groups. However, the levels of P2Y2 protein expression were significantly lower in the distal rectum from ARM model rats at E21 when compared with the other groups. Data are presented as the mean ± standard deviation (analyzed by one-way analysis of variance test). *P<0.05, **P<0.01. IOD, Integral optical Density; ETU, ethylenethiourea; ARM, anorectal malformations; P2Y2, purinergic receptor P2Y2; HuD, Hu antigen D.

HuD protein quantitative analysis. At E17, respectively 8, 7, and 6 cases in the normal, ETU and ARM groups were observed with weak-positive staining, a small amount of HuD protein with yellowish staining was observed at the distal rectum and there was no significant difference between the three groups (P>0.05). At E19, respectively 8, 8, and 6 cases in the normal, ETU and ARM groups were observed with weak-positive staining; and 2, 2, and 2 in the normal, ETU and ARM groups, respectively, were observed with positive staining. The normal and ETU groups were stained yellowish and brown in some areas, which was more pronounced than at E17 (P<0.01). At E21, respectively 1, 2, and 7 case(s) in the normal, ETU and ARM groups were observed with weak-positive staining; 5, 4, and 2 in the normal, ETU and ARM groups, respectively, were observed with positive staining; and 4, 4, and 1 in the normal, ETU and ARM groups, respectively, were observed with strong-positive staining. The distal rectum was stained yellowish brown or dark brown in the normal and ETU groups, with a marked increase in expression.
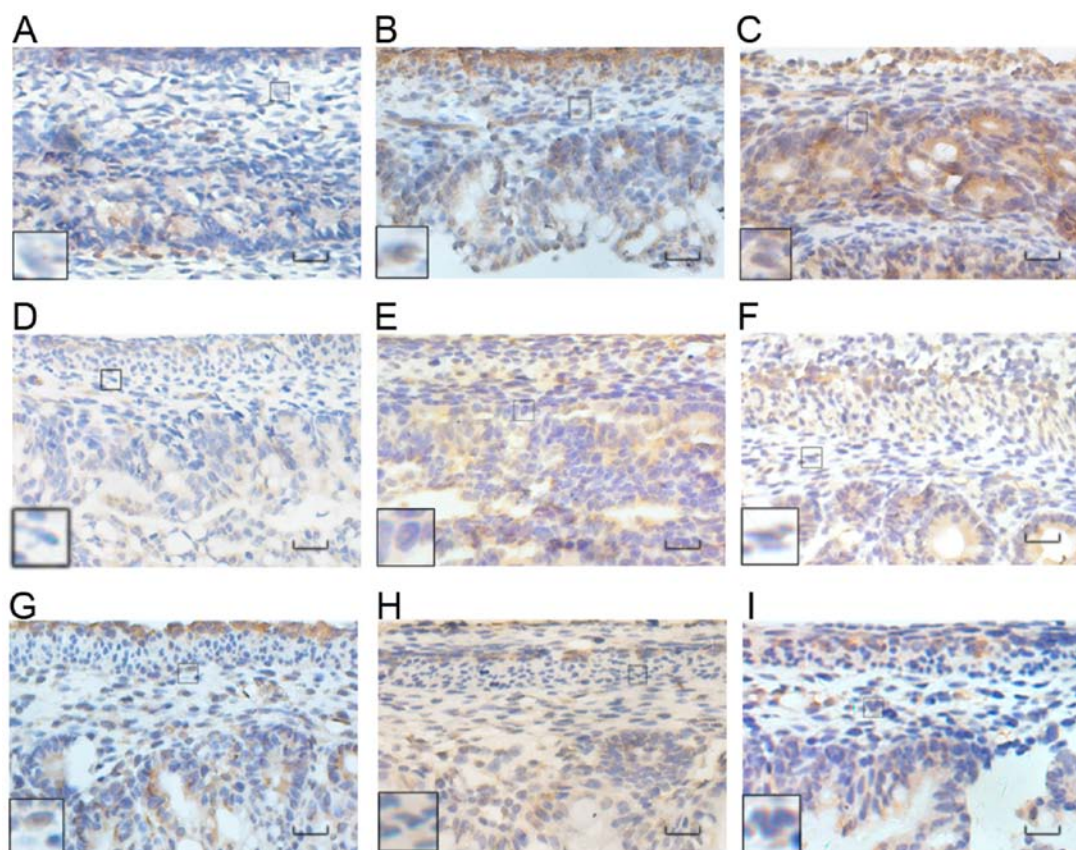
Figure 3. Immunohistochemical analysis of P2Y2 protein. The normal group at (A) E17, (B) E19 and (C) E21. The ETU group at (D) E17, (E) E19 and (F) E21. The ARM group at (G) E17, (H) E19 and (I) E21. P2Y2 was mainly expressed in the cytoplasm and in the distal submucosa and intermuscular plexus of the fetal rat. The color of the expressed proteins changed from yellowish to yellowish brown to yellow brown as the embryo developed, and an increase in the range of expression was also observed. Black rectangles reveal images at a higher magnification. Scale bar, 25 μm; magnification, x400. P2Y2, purinergic receptor P2Y2; ETU, ethylenethiourea; ARM, anorectal malformations.

The yellow dye could be clearly observed between the muscles and the mucosa, and there was a significant difference between the E21 and E19 embryos (P<0.01). The HuD expression level in the ARM group was significantly decreased when compared with the normal group (P<0.01); the expression increased with the development of the embryo in the ARM group. There was no significant difference between the E17 and the E19 ARM groups; however, the expression level of the E21 ARM group was significantly higher than the E17 ARM group (P<0.01; Figs. 2B and 4).

Western blot analysis. The expression levels of the P2Y2 and HuD proteins were evaluated by western blot analysis during ENS development in the normal, ETU and ARM group model rats. P2Y2 and HuD bands were normalized to the corresponding β-actin band. The expression of P2Y2 and HuD proteins increased with time in the three groups. Significantly decreased HuD expression was detected in the distal rectum of the ARM group at E19 and E21 when compared with the other groups (P<0.05); in addition, the decreased expression of P2Y2 protein was detected in the distal rectum of ARM model rats at E21 when compared with the other groups (P<0.05; Fig. 5).

RT-qPCR analysis. The expression levels of P2Y2 and HuD mRNA were normalized to β-actin in the same specimen. The results obtained were consistent with those of western blotting; the expression levels of P2Y2 and HuD mRNA increased

with time in the normal and ARM groups. Subsequently, the expression levels of P2Y2 mRNA in the distal rectum of the ARM model rats at E21 were significantly lower than those of the other groups (P<0.05), while the levels of HuD protein in the distal rectum of the ARM group at E19 and E21 were significantly lower when compared with the other groups (P<0.05; Fig. 6).

Discussion

ARMs represent a wide spectrum of defects and are some of the most common digestive tract anomalies encountered in pediatric surgery. In the past, many surgical techniques to repair ARM have been described. These included endorectal dissection (1-3), an anterior perineal approach to a recto-urethral fistula (4), and many different types of anoplasties. However, children with ARMs often have more complications at the postoperative stage, including constipation, diarrhea and feces incontinence, which seriously affect patient quality of life following surgery (4). At present, the key to addressing this disease is to devise a treatment method to reduce postoperative complications. A previous study has revealed that ARMs are often accompanied by ENS dysplasia (22). When the ENS is absent (aganglionosis) or defective, children may develop constipation, vomiting, abdominal pain and growth failure, and some may even perish (23,24). However, whether P2Y2
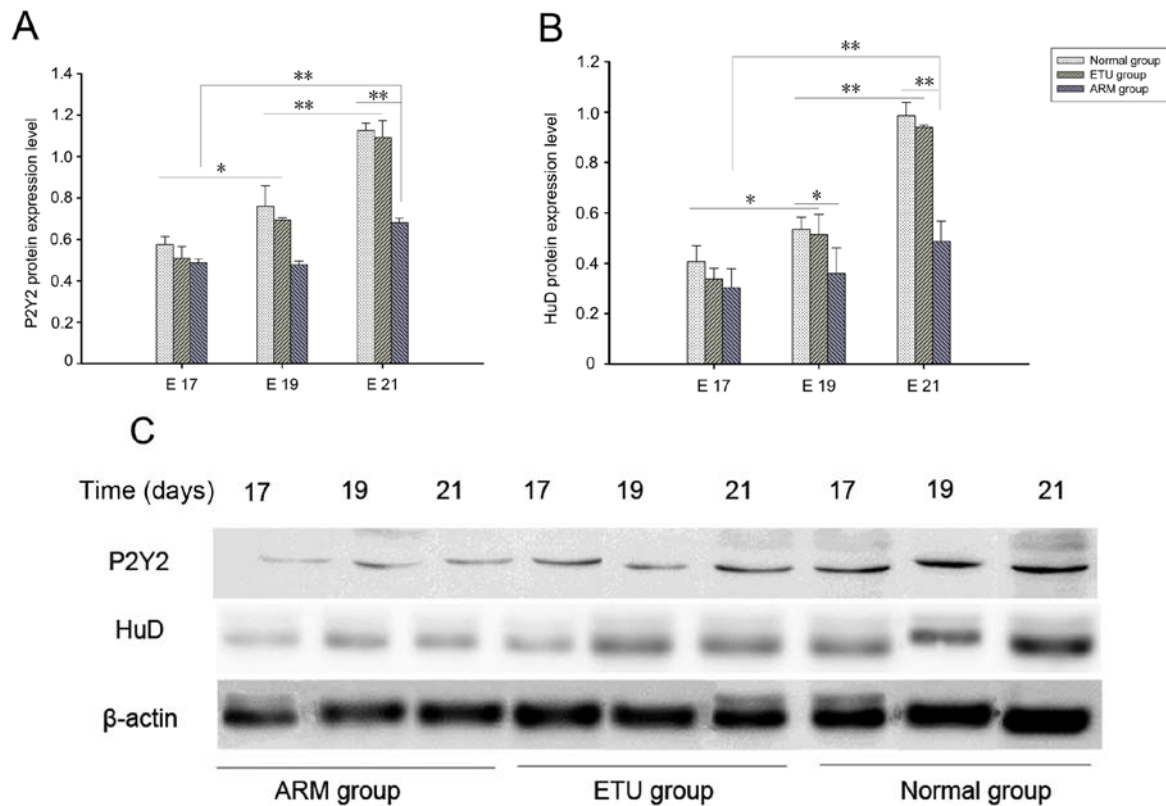
Figure 5. Western blot analysis of P2Y2 and HuD protein expression levels in the normal, ETU and ARM fetal rat groups. (A and B) Histogram revealing the trends of P2Y2 and HuD expression at each time-point. P2Y2 and HuD protein expression gradually increased over time in the three groups, with the levels of HuD protein expression significantly lower in the distal rectum from the ARM model rats at E19 and E21 compared with the other groups. However, the levels of P2Y2 protein were significantly lower in the distal rectum from ARM model rats at E21 when compared with other groups. Data are presented as the mean ± standard deviation (analyzed by one-way analysis of variance test). *P<0.05, **P<0.01. (C) P2Y2 and HuD were detected with bands of ~25 and 50 kDa, respectively; β-actin protein was used as an internal control. Expression levels were detected by western blot analysis. P2Y2, purinergic receptor P2Y2; HuD, Hu antigen D; ETU, ethylenethiourea; ARM, anorectal malformations.
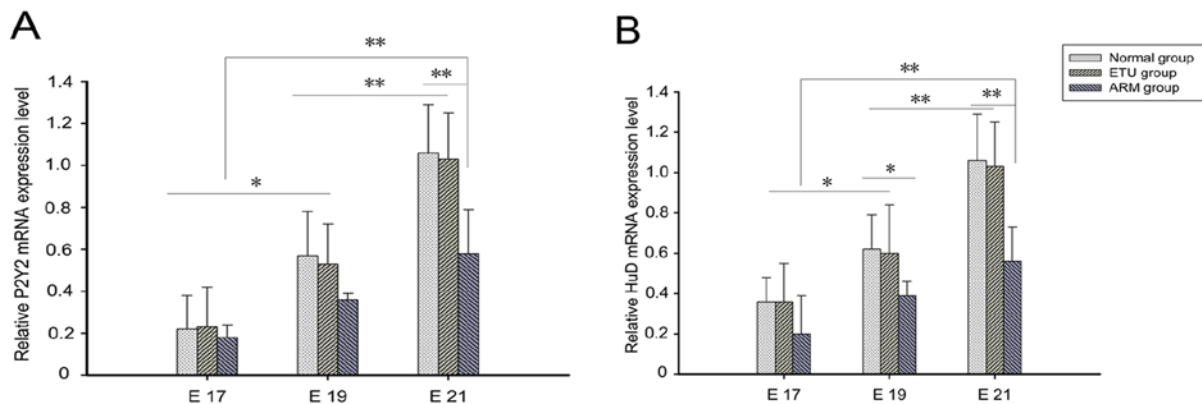

Figure 6. Reverse transcription-quantitative polymerase chain reaction analysis of P2Y2 and HuD mRNA expression levels in the normal, ETU and ARM fetal rat groups. (A and B) Histogram presenting the trends of P2Y2 and HuD expression at each time-point. P2Y2 and HuD mRNA expression gradually increased over time in the three groups. The levels of HuD mRNA expression were significantly lower in the distal rectum from ARM model rats at E19 and E21 when compared with the other groups, but the levels of P2Y2 mRNA expression were significantly lower in the distal rectum from ARM model rats at E21 when compared with other groups. Data are presented as the mean ± standard deviation (analyzed by one-way analysis of variance test). *P<0.05, **P<0.01. P2Y2, purinergic receptor P2Y2; HuD, Hu antigen D; ETU, ethylenethiourea; ARM, anorectal malformations.

and HuD have parallel developments with the ENS of ARMs remains to be clarified (25,26). The present study was designed to investigate the potential role of P2Y2 and HuD during ENS development by examining the expression patterns of P2Y2 and HuD mRNA and protein in normal and ARM model rats at different embryonic developmental stages.

P2Y receptors (P2YRs) are typical 7-channel transmembrane receptors and are heterotrimeric G protein-coupled (27,28). Activation of P2Y2R by ATP or UTP can induce the phosphorylation of growth factor receptors (29,30), which increases the activities of the mitogen-activated protein kinases, extracellular signal-regulated kinase-1/2 and the

associated adhesion focal tyrosine kinase; these go on to regulate the ENS or directly regulate the contractility of intestinal smooth muscle to control intestinal peristalsis (31,32). In our previous study, the glial cell factor, glial cell-derived neurotrophic factor and its signal receptor RET had an effect on the development of the ENS (10). Wullaert *et al* (32) used upstream agonists and inhibitors to control the purine receptor. It was revealed that ATP could promote axonal growth through the P2Y2 receptor (33). In addition, Arthur *et al* (12) also demonstrated that ATP activates the G protein-coupled receptor following activation P2Y2, which acts on the regionalization and association of tyrosine receptor A and P2Y2 receptors, promoting the growth, differentiation and migration of neurons. In addition, the endogenous ATP, which is used for the first time in the human intestinal tract, acts on the P2Y receptor located in the intestinal smooth muscle and regulates intestinal relaxation; this effect can be blocked by P2YR (34). By combining the aforementioned results with our own previous research, it appears that there is a difference between the P2Y2 receptor and the distal end of the rectum in ARMs and normal fetal rats. It was suggested that P2Y2 may partly be related to the development of the intestinal nervous system.

HuD has been revealed to promote neuronal differentiation and axonal outgrowth in neurons in culture and *in vivo* (15). HuD can be used as a supplement to P2Y2 for the accurate counting of intestinal neurons and analysis of neuron types. In a study that used an anti-Hu antibody, anti-PGP9.5 and anti-neuron specific enolase for triple staining in cultured intestinal neurons, HuD protein was markedly expressed on all of the neurons in the intramuscular clusters of the small intestine, indicating that HuD has distinct specificity (35). The expression of HuD in Hirschsprung disease was significantly lower than in the normal segment (36,37). However, there are few reports of HuD in ARM-associated diseases.

The present experimental study revealed that at E17, normal fetal rats had completely ruptured the anal membrane to form the anus, while the ETU malformation group did not form an anus, had developed multiple deformities and the development of the nerve plexus was poor. P2Y2 and HuD were mainly expressed in the distal submucosa and intermuscular plexus of the fetal rat and were primarily expressed in the cytoplasm. The expression of P2Y2 and HuD in the rectum was lower, and there was no difference in the three groups at E17. These results indicated that there was a relative imbalance in expression between the normal and ARM model embryos during ENS development. While ETU can cause ARMs in embryos, it had no marked effect on the expression of P2Y2 and HuD. At E21, the expression levels of P2Y2 and HuD in the ETU group were significantly lower than the ARM group, suggesting that P2Y2 and HuD may be responsible for the abnormal development of the ENS. The changes in these two microenvironmental factors hindered the development and migration of the intestinal nervous system. The expression of P2Y2 and HuD from E17 to E19 changed more slowly than that observed between E19 to E21. It has been suggested that E19 is the critical period of ENS formation.

In conclusion, the results from the present study, and those of previous studies, suggested that the downregulation pattern of P2Y2 and HuD may be important for ENS development in the anorectum of fetal rats with ETU-induced ARMs. Since many signaling molecules have been revealed to be expressed and function differently during different phases of ENS development, the present study was unable to determine whether P2Y2 and HuD were the primary factors that led to ENS anomalies. Further studies are required to define the two signaling molecules that are involved in regulating ENS formation during embryonic development and to clarify the specific roles of molecular mechanisms mediating the maldevelopment of the ENS, and thus help to improve our understanding of postoperative defecation disorder and patient quality of life.

Availability data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ and BC performed the majority of experiments, contributed equally to the work, authored or reviewed drafts of the paper, and approved the final draft. ZJ performed the experiments, authored or reviewed drafts of the paper, and approved the final draft. MG, CT, YM, and YQ analyzed the data, authored or reviewed drafts of the paper, and approved the final draft. YL conceived and designed the experiments, contributed the reagents/materials/analysis tools, authored or reviewed drafts of the paper, and approved the final draft and all authors agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Ethical approval was obtained from the Zunyi Medical College Animal Ethics Committee (no. 20150820014). Animal care and handling procedures for *ex vivo* studies performed in China were as per the protocol approved by the Institutional Animal Care and Use Committee of Zunyi Medical College. Animal handling procedures for *in vivo* studies conducted in China were approved by the Institutional Animal Care and Use Local Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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