



Abnormal serum vitamin A levels and retinoic acid receptor α expression patterns in children with anorectal malformation

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Abstract

Background Anorectal malformation (ARM) is known to be associated with maldevelopment of the enteric nervous system (ENS), and vitamin A (VA) and its metabolite retinoic acid (RA) play important roles in ENS development. Thus, our aim was to investigate serum VA levels in ARM newborns and RA receptor (RAR) expression in the rectum of ARM patients and animal models.

Methods Serum VA concentrations were detected in newly diagnosed ARM neonates ($n=32$) and neonates with non-alimentary tract malformations ($n=30$). Intestinal specimens were divided into three groups: rectum from ARM patients ($n=30$), colon from a stoma ($n=30$) and rectum from controls ($n=4$). RAR mRNA expression was evaluated by RT-qPCR. Rectum specimens from ARM patients were divided into two groups by postoperative pathology: the normal and lesion ganglion cell groups. Immunohistochemistry and Western blot were employed to detect RAR α protein expression in rectum specimens. In addition, the ARM mouse model was induced by all-trans retinoid acid (ATRA), and the expression levels of RAR α and the neuronal marker NeuN in the rectum of mice on embryonic day 16.5–18.5 (E16.5–18.5) were investigated.

Results The serum concentration of VA in ARM neonates was lower than that in control neonates ($P<0.0001$), and RAR α mRNA expression was lower in the rectum specimens from ARM patients than in the colon specimens from a stoma and the rectum specimens from controls ($P<0.05$); there was no significant difference between the colon from a stoma and the rectum from controls. RAR α protein was expressed in the nucleus of ganglion cells and nerve fibers, and RAR α protein expression in the lesion ganglion cell group was significantly lower than that in the normal ganglion cell group ($P<0.01$). Compared with the control mice, ARM mice at E16.5–18.5 showed decreased fluorescence intensity of RAR α and NeuN in the rectum. RAR α and NeuN mRNA expression in the rectum on E16.5–18.5 was lower in ARM mice than in control mice ($P<0.05$).

Conclusion Serum VA concentration and the RAR α expression pattern are abnormal in the rectum in ARM and may contribute to the ENS maldevelopment in ARM.

Keywords Anorectal malformation · Enteric nervous system · Vitamin A · Retinoic acid receptor

Introduction

Anorectal malformation (ARM) is one of the most common congenital abnormalities in newborns and occurs at a frequency of 1 per 2000–5000 live births [1, 2]. Although many advances have been made in the medical care of ARM patients, postoperative defecation problems still affect the long-term quality of life of many patients [3]. Previous studies have shown that poor postoperative anorectal function may be caused by abnormal innervation in the distal rectum

[4, 5]. Moreover, an abnormal enteric nervous system (ENS) has been found in both ARM patients and animal models [5–8].

Vitamin A (VA), an essential nutrient, is a precursor to retinoic acid (RA), which plays many significant roles in organ development. RA binds to RA receptors (RARs) and retinoid X receptors (RXRs) that heterodimerize and then bind RA response elements to regulate target genes and influence tissue morphogenesis [9]. Recent research suggests that both excess RA and RA deficiency cause abnormal ENS development [10, 11]; in addition, excess RA can induce a classical rodent ARM model [12]. However, it remains unknown whether ARM is associated with abnormal VA/RA signaling. The present study was, therefore, designed

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to investigate serum VA levels in ARM newborns and RAR expression in the rectum of ARM patients and animal models.

Materials and methods

Patients

Following ethics approval by our institution (#053/2014), surgically resected intestine specimens from 30 ARM patients were collected at the Children's Hospital of Chongqing Medical University from February 2015 to February 2017; this cohort included 18 males and 12 females with rectourethral bulbar fistula ($n=8$), no fistula ($n=7$), vestibular fistula ($n=6$), rectourethral prostatic fistula ($n=3$), cloaca ($n=3$), rectovesical fistula ($n=2$), or perineal fistula ($n=1$). All ARM patients underwent three-stage repair (colostomy, anoplasty, and stoma closure), during the anoplasty, the most distal tissues of rectal blind pouch (within 2 cm) were obtained from ARM patients at a median age of 202.5 days (151–485 days), and were divided into 2 groups by postoperative HE and Calretinin immunohistochemical staining: the normal and lesion ganglion cell groups [13, 14]. Transverse colon tissues (about 2 cm) were obtained from patients during the stoma closure at a median age of 352 days (253–635 days). Rectum tissues from 4 infants (median age 214 days; range, 165–351 days) with non-alimentary tract malformation were obtained as the control group.

ARM mouse model

The day on which a vaginal plug was observed as a sign of successful mating was designated embryonic day 0.5 (E0.5). Pregnant C57BL/C mice were administered 80 mg/kg ATRA via gavage on E8.5 and killed on E16.5–E18.5.

Measurement of serum vitamin A levels

The serum concentration of VA in ARM neonates (5.4 ± 1.5 days, $n=32$) was determined by HPLC, and healthy neonates (6.2 ± 2.6 days, $n=30$) served as the

control; there was no significant difference in age between the two groups. All neonates are breast-fed or formula-fed without other vitamin supplements.

RT-qPCR

Total RNA was extracted from clinical specimens and fetal rectum samples by TRIzol (Invitrogen, USA), and RNA was reverse transcribed to cDNA using the PrimeScript™ RT Reagent Kit (Takara, Japan) according to the manufacturer's instructions. The primers are listed in Table 1, and RT-qPCR was performed using the CFX96 system (Bio-Rad, USA).

Immunohistochemistry and immunofluorescence staining

Clinical tissues were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, and sectioned at 4- μ m thickness. After dewaxing, rehydration and antigen retrieval, the sections were incubated for 30 min with 5% BSA to prevent nonspecific binding and then overnight at 4 °C with primary goat anti-RAR α polyclonal antibody (1:200 dilution, Abcam, USA); after being washed, the sections were incubated with secondary antibody (ZSGB-BIO; China) for 15 min at room temperature, followed by a 1–2-min incubation with DAB (Zsbio, China). Images were captured using a Nikon E800 digital microscope.

Mouse rectum samples were immersed in 20% sucrose, embedded and frozen in OCT compound and sectioned at 10- μ m thickness. Then, the sections were incubated with PBS containing 1.0% Triton X-100 (Sigma, Ireland) for 20 min, subsequently blocked with 5% BSA for 30 min at room temperature, and then incubated with primary antibodies against RAR α and NeuN (1:100 dilution, Millipore, Germany) overnight at 4 °C. On the next day, the sections were washed and then incubated with the corresponding secondary antibodies (1:200 dilution, chicken anti-goat IgG Alexa Fluor 488 or chicken anti-rabbit Alexa Fluor 594, Invitrogen, USA) for 1 h at room temperature. Nuclear staining was performed with DAPI for 15 min. All sections were scanned with a fluorescence microscope (Nikon, Japan).

Table 1 Sequences of primes used for real-time PCR

Gene	Forward primer	Reverse primer
RAR α (human)	CTGTTTGCTCCCAGAGAAGG	AGGGCTGGGCACTATCTCTT
RAR β (human)	GGAACACAGCAGACAG	ACGAGTGGTGACTGACTGAC
RAR γ (human)	CGCCGAAGCATCCAGAAGAAC	GCGATTCTGGTCACTTGTG
β -actin (human)	GTGAAGGTGACAGCAGTCGGTT	GAGAAGTGGGGTGGCTTTAGGA
RAR α (mice)	CCCTGAACCGACTCAGAT	AGGTCCTGTCGGTCTCCAC
NeuN (mice)	CGGTCGTGTATCAGGATGGA	TAAGGATCAGCAGCGGCATA
β -actin (mice)	AGGGAAATCGTGCGTGAC	CGCTCGTTGCCAATAGTGA

Western blot

Total protein was extracted from clinical rectum tissues using a Total Protein Extraction Kit (Keygen Biotech, China) according to the manufacturer's instructions. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, USA), which were incubated at 4 °C overnight with primary antibodies against RAR α (1:800 dilution, Abcam, USA) and β -actin (1:1000 dilution, Proteintech, USA). On the next day, the membranes were washed with TBST and incubated with a secondary antibody (1:2000 dilution, Sizhengbo, China). The blots were visualized using ECL reagent (Bio-Rad, USA). Image acquisition and band density analysis were performed using a G:BOX chemiluminescence imager (Synoptics, UK).

Statistical analysis

All values are presented as the mean \pm standard deviation. Comparisons between groups were carried out using GraphPad version 5.0. Statistical significance was defined as $P < 0.05$.

Results

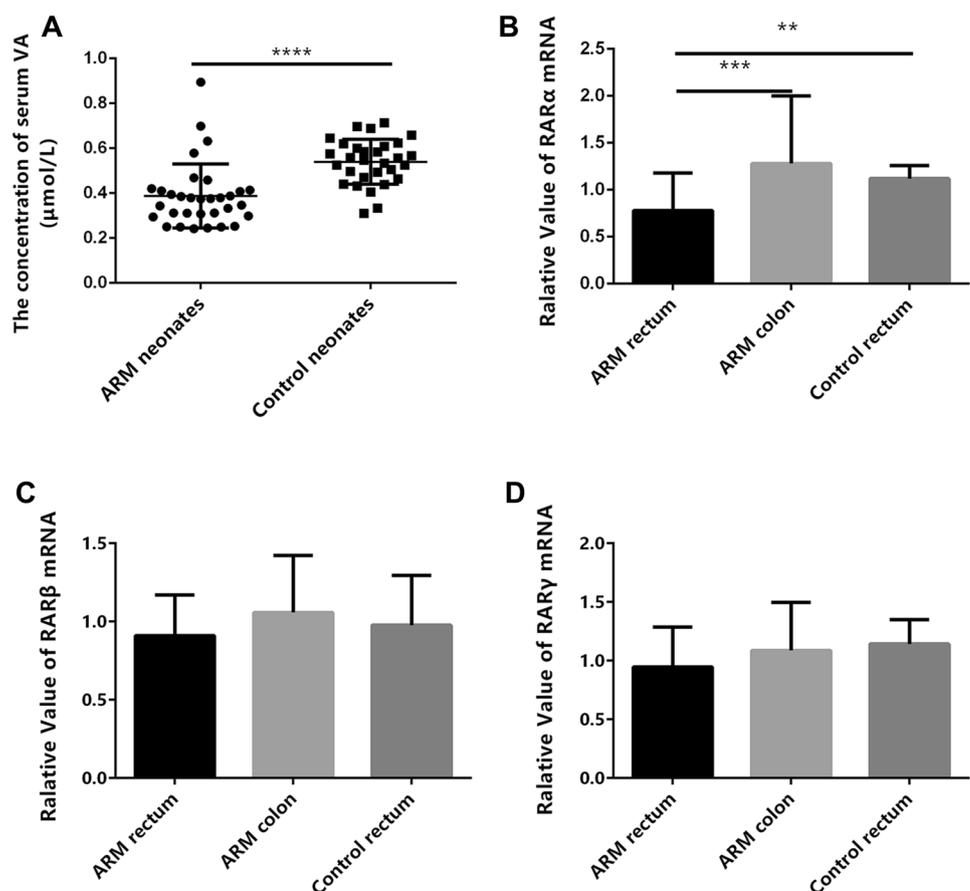
Serum VA levels in control and ARM neonates

The serum VA concentration in ARM neonates ($0.40 \pm 0.14 \mu\text{mol/L}$, $n = 32$) was significantly lower than that in control neonates ($0.54 \pm 0.10 \mu\text{mol/L}$, $n = 30$, $P < 0.0001$, Fig. 1).

Relative RAR mRNA expression levels in intestine specimens

As evidenced by qRT-PCR, relative RAR α mRNA expression levels were significantly lower in ARM rectum samples than in ARM colon samples or in control rectum samples ($P < 0.001$ and $P < 0.01$, respectively), but there was no difference between ARM colon and control rectum samples ($P > 0.05$). Moreover, no significant difference was found in relative mRNA expression of RAR β and RAR γ among the groups ($P > 0.05$, Fig. 1).

Fig. 1 Serum VA (a) concentration in ARM neonates and control neonates. Relative RAR α (b), RAR β (c) and RAR γ (d) mRNA expression levels in intestine specimens. Data analyzed using Mann–Whitney U test and Kruskal–Wallis test followed by Dunns multiple comparison test. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$



Immunofluorescence staining of RAR α and NeuN in the rectum of ARM patients

Positive staining for RAR α was mainly concentrated in the nerve plexus and partially overlapped with the region of NeuN protein expression. There was markedly lower RAR α colocalization with NeuN protein expression in the lesion group than in the normal group (Fig. 2).

Immunohistochemistry staining of RAR α in the rectum of ARM patients

Immunohistochemistry staining revealed markedly diminished RAR α expression in the myenteric plexus and submucous plexus in the lesion ganglion cell group than in the normal ganglion cell group (Fig. 3).

Western blot

Relative RAR α protein expression in the lesion ganglion cell group was significantly lower than that in the normal ganglion cell group (0.77 ± 0.08 vs 1.03 ± 0.06 , $P < 0.01$, Fig. 4).

Immunofluorescence staining of RAR α and NeuN in the fetal mouse rectum

RAR α and NeuN protein expression was detected in the rectum on E16.5–18.5, and positive staining for RAR α was mainly concentrated in the intestinal muscle region. Compared with the control fetuses, the ARM fetuses at E16.5–18.5 showed decreased fluorescence intensity of RAR α and NeuN (Fig. 5).

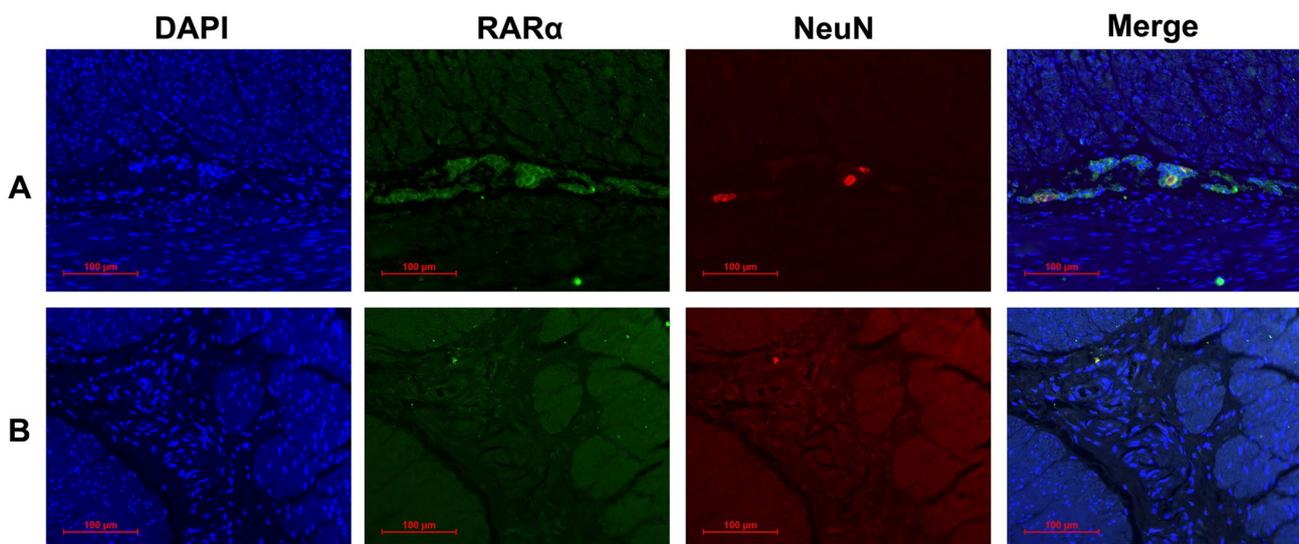


Fig. 2 RAR α and NeuN expression in the rectum of ARM patients. **a** Normal ganglion cell group, **b** Lesion ganglion cell group. Scale bars 100 μ m

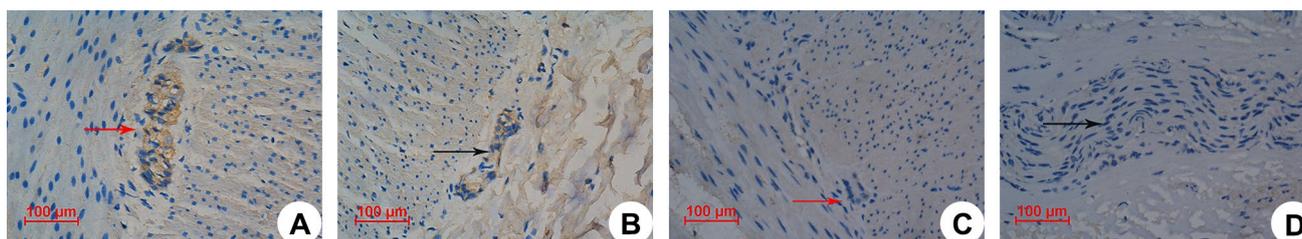
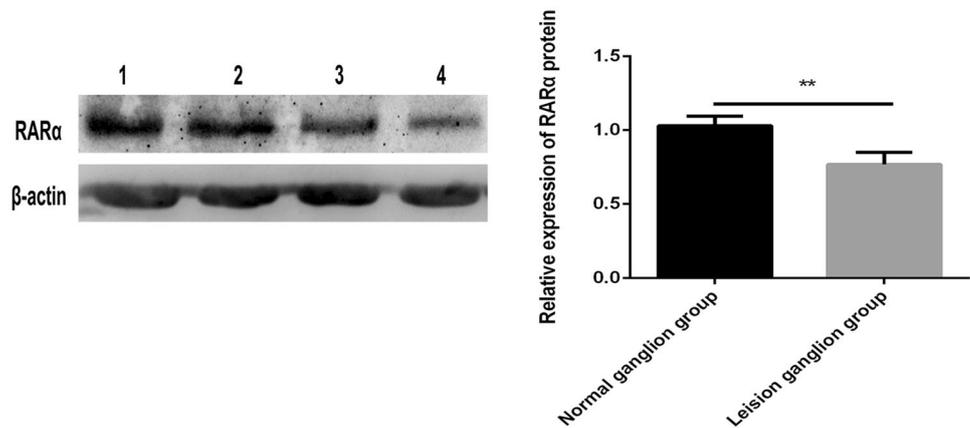


Fig. 3 RAR α expression in the rectal plexus of ARM patients. **a** Myenteric plexus of normal ganglion group, **b** submucous plexus of normal ganglion group, **c** myenteric plexus of lesion ganglion group, **d** submucous plexus of lesion ganglion group. Scale bars 100 μ m

Fig. 4 Western blot show decreased expression of RAR α protein in lesion ganglion cell group. (1, 2) Normal ganglion cell group, (3, 4) Lesion ganglion cell group. RAR α expression was normalized to β -actin expression. Data analyzed using student's *t* test



Relative RAR α and NeuN mRNA expression levels in the fetal mouse rectum

Consistent with the results obtained from immunofluorescence staining, relative mRNA expression levels of RAR α and NeuN were significantly lower in the rectum of ARM fetuses than in that of control fetuses on E16.5–18.5 ($P < 0.05$, Fig. 6).

Discussion

Anorectal malformation encompasses a broad spectrum of congenital defects. It is well recognized that children with ARM often experience ongoing problems with anorectal function after surgical repair, such as fecal accumulation, constipation, soiling and incontinence. Associated sacrum, spinal cord and neuromuscular defects have been thought to contribute to postoperative anorectal function [15, 16]. In addition, many studies have reported that children with ARM have abnormal innervation in the distal rectum [17]. Meier-Ruge reported that anomalies of the ENS were diagnosed in 60% of patients with ARM [7], but the pathogenesis of ENS maldevelopment in distal ARM has not been studied in detail.

The ENS is a complex network of neurons and glia within the bowel that controls most aspects of intestinal function; the cells that comprise the ENS are derived from vagal and sacral neural crest cells (NCCs). NCCs originate in the vagal, sacral and upper thoracic neural tubes before migrating extensively, proliferating and then differentiating to form the ENS [18]. Recent data have shown a failure of vagal NCC migration in the terminal hindgut in ARM mice [11]. VA is a lipid-soluble vitamin that plays an important role in tissue development and differentiation. RA, the main derivative of VA, binds to RARs and RXRs, members of the nuclear receptor superfamily, to regulate gene transcription. VA/RA is known to be essential for normal ENS

development. A recent study showed that VA deficiency from E7.5–14.5 impaired NCC colonization of the distal bowel, leading to distal bowel aganglionosis in vivo [19]. Furthermore, RA could promote NCC migration and proliferation in vitro [20–22]. Nevertheless, the lack of data prompted us to analyze VA/RA signaling in ARM.

In the current study, we first investigated serum VA levels in ARM and control newborns to reduce dietary interference; a significantly lower serum VA concentration was found in ARM newborns. Because maternal and neonatal concentrations of vitamin A compounds are highly correlated and because maternal VA that crosses the placenta is the major retinoid source for embryos of placental species [9, 23], we hypothesized that mothers of ARM newborns may undergo VA deficiency during pregnancy.

There are three subtypes of nuclear RARs, α , β , and γ , which play critical roles in a variety of biological processes, including development, reproduction, organogenesis and homeostasis. Previous studies have shown that RARs are expressed in the regions of the developing bowel and ENS [24]; in addition, murine embryos with ARM showed an impaired distribution of RARs in the hindgut–tailgut region [25]. Here, we analyzed the expression pattern of RARs in rectum samples from ARM patients. RT-qPCR showed that relative RAR α mRNA expression was decreased in the ARM rectum compared with the control rectum. RAR α protein was detected in the neural plexus and exhibited a partially overlapping pattern with the neuronal marker NeuN protein in ganglion cells. Furthermore, immunohistochemistry and Western blot showed that RAR α levels were dramatically decreased or even absent in the pathological lesion ganglion cell group of ARM rectum samples, suggesting that RAR α expression in the intestine is closely related to the development of the ENS. We also investigated the expression of NeuN and RAR α in the rectum of ATRA-induced ARM mice. Similar to what was observed in ARM patients, RAR α and NeuN expression was detected in the muscular layer of the embryonic rectum, and we showed that RAR α and NeuN

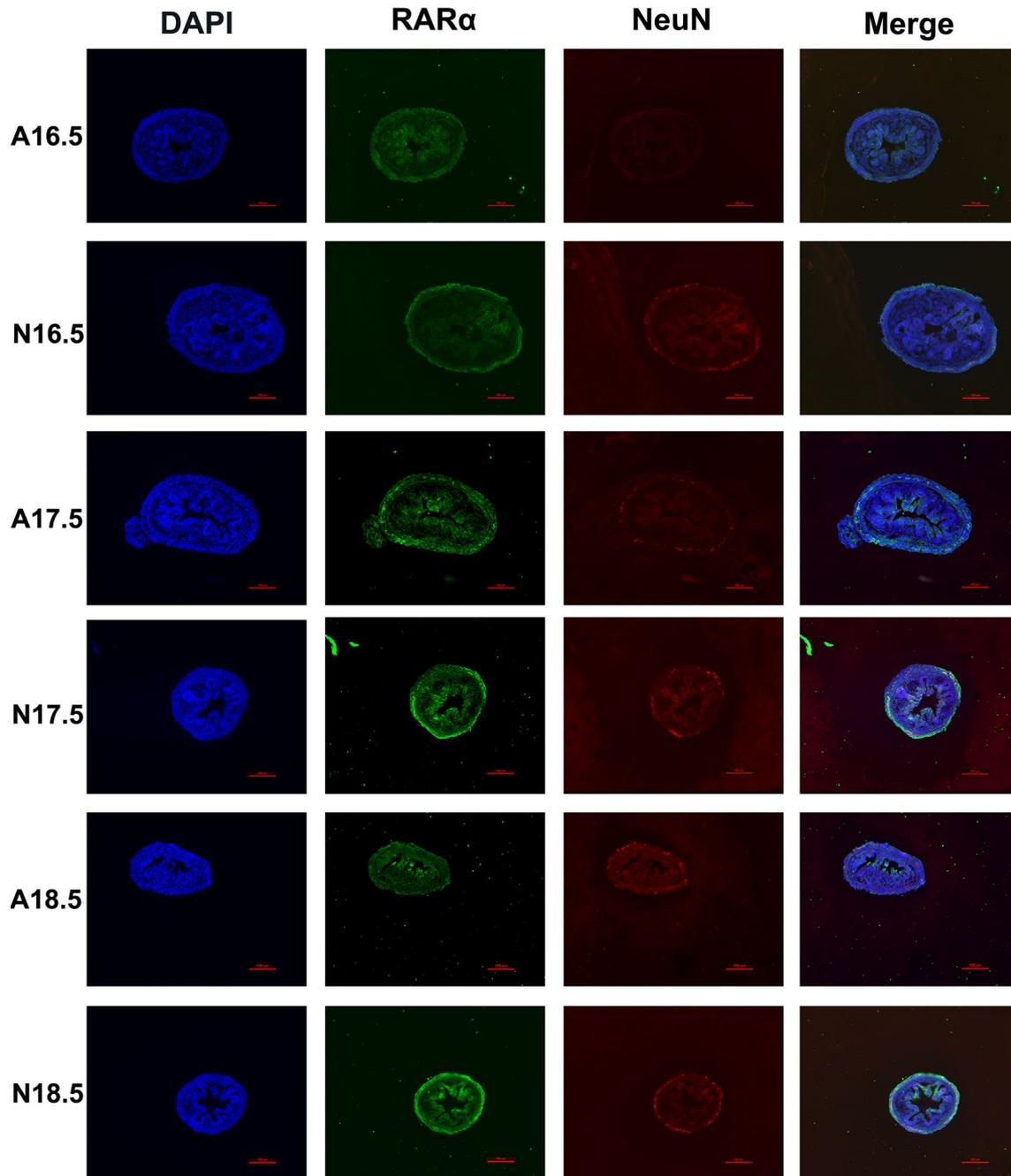


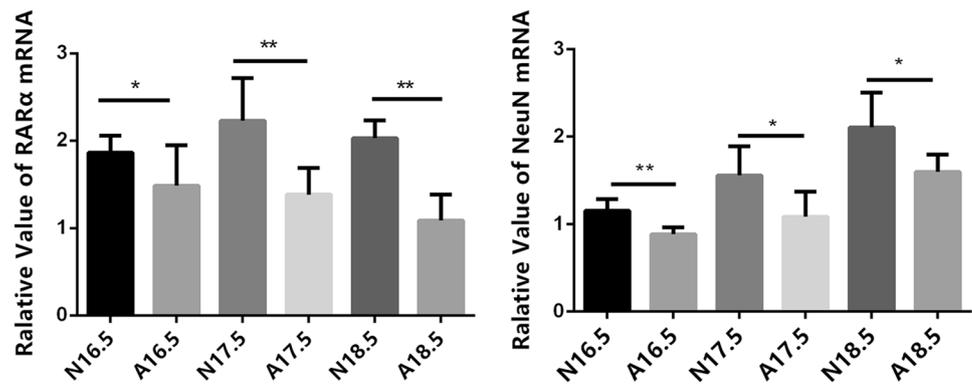
Fig. 5 Visualizing the rectum of E16.5–18.5 mice embryos using fluorescence microscopy, (A) ARM group, (N) control group. Scale bars 100 μ m

expression levels were decreased from E16.5–18.5 in ARM embryos compared with normal embryos.

Interestingly, a previous study reported that RAR inhibitor can rescue ARM in mice induced by RA [26], suggesting that the teratogenic effects of RA may be directly mediated through RARs. Meanwhile, Lee et al. showed that teratogenic dose of RA at E9.0 resulted in the reduction of RA levels in the embryo after administration, which caused

a spectrum of malformations in mice (defects of eye, ear, heart, kidney, cleft palate and ARM). In addition, some of the malformations induced by RA could be prevented by supplementation with low dose of RA, but did not include ARM. This suggests the teratogenic effects of RA via two nonmutually exclusive modes: direct action on progenitor of target tissues, and indirection action by the resultant prolonged local RA deficiency after the excess RA is removed

Fig. 6 Relative RAR α and NeuN mRNA expression levels in the rectum of mice fetuses on E16.5–18.5. (A) ARM group, (N) control group. Data analyzed using student's *t* test. **P* < 0.05, ***P* < 0.01



[27]. Therefore, our study found the decreased expression of RAR α in the rectum tissues of ARM neonates and fetal mouse may be related to the local VA deficiency.

The current study provides evidence of decreased VA levels and RAR α expression in patients and imbalanced spatiotemporal expression of RAR α during the development of the terminal rectum in ARM mouse embryos; these abnormalities may contribute to ENS development in ARM, and therefore, our study enhances the understanding of the mechanism of ENS maldevelopment in ARM. However, it must be emphasized that we have not proven how VA/RA signaling directly regulates ENS development in ARM. Although many candidate genes related to the pathogenesis of ARM and ENS have been identified, further studies are necessary to better understand the molecular mechanism of postoperative defecation problems in ARM patients.

Conclusion

Serum VA concentrations and RAR α expression patterns are abnormal in the rectum in ARM and may contribute to the ENS maldevelopment in ARM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Consent to publish All authors have read and approved the manuscript.

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