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*Original Article*

# Expression of GHRL and GHSR in embryonic development of rats with ethylene thiourea-induced anorectal malformations

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**Abstract:** Since anorectal malformations (ARM) were associated with an extensive neuromuscular maldevelopment of the lower gastrointestinal tract and anorectum, the aim of this study was to find out ghrelin (GHRL) and growth hormone secretagogue receptor (GHSR) expression patterns during anorectal development in normal and ARM embryos, in an effort to establish the possible role of GHRL and GHSR in ARM pathogenesis. ARM was induced by ethylene thiourea (ETU) on the 10th embryonic day of rats, and cesarean was performed to harvest the hindgut. The changes of mRNA and proteins of GHRL and GHSR were evaluated in normal group (n=20) and ARM group (n=20) from E15d to E21d using quantitative real-time PCR, western blot, and immunohistochemical staining, respectively. The result display, in the normal group, the protein and mRNA expressions for GHRL and GHSR showed time-dependent changes from E15d to E21d, during which time high expressions were found on E15d and E17d and it gradually decreased after E19d and E21d. While in the ARM group, low expressions were observed on the same periods which also decreased after E19d and E21d. These data implied that in ARM embryos, an imbalance in the spatiotemporal expression of GHRL and GHSR was noted during anorectal morphogenesis from E15d to E17d, which may shed light on understanding the role of GHRL and GHSR in the development of normal and ARM embryos.

**Keywords:** Anorectal malformations, GHRL gene, GHSR gene, embryogenesis, rat

## Introduction

Anorectal malformations (ARM) are common congenital anomalies, occurring in approximately 1:1,000 and 1:5,000 newborns [1]. The defects are invariably detected and treated in infancy or early childhood [2]. Children with this anomaly may not develop symptoms in childhood or may continue to live with fecal incontinence until adulthood. In spite of technical advances for surgical treatment of ARM, some patients with intermediate-type and high-type ARM still suffer from postoperative anal dysfunctions. The etiology of abnormal defecation is multifactorial, which includes: (1) sacral malformations, (2) altered rectosigmoid motility, and inherent abnormalities of the enteric nervous system (ENS) of the rectosigmoid colon, (3) sphincteric insufficiency, and (4) secondary psychological problems [3]. However, due to the complexity of ARM, its etiology, embryology, and pathogenesis were poorly understood and remained controversial, so it is of great significance to look into the developmental mechanism of normal and abnormal anorectal organogenesis. In previous studies, certain signaling molecules were also found to be involved in the embryogenesis and development of ARM: such as bmp, hox, wnt, notch and hedgehog [4-9]. Embryogenesis is regulated by a number of complex signaling cascades, which are critical to normal development. Expression patterns of corresponding genes during various stages of gastrulation may help to elucidation the molecular basis of this condition.

Ghrelin (GHRL), a 28-amino acid peptide, is isolated from the P/D1 cell of stomach as an endogenous ligand for the GHSR. The human GHRL gene is located at the chromosomal locus 3p26-p25, and the prepro-hormone is encoded by four extrons and three introns [10]. GHRL has also been shown to be constitutively expressed by influencing their growth [11].

**Table 1.** The relative quantity of GHRL mRNA in normal group and ARM group

| Case | GHRL | | GHRL | | GHRL | | GHRL | | β-actin | | Times of gene (compared to normal group) | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N15 | E15 | N17 | E17 | N19 | E19 | N21 | E21 | Normal | ARM | | |
| 1 | 33.11 | 29.97 | 31.25 | 29.97 | 30.27 | 27.01 | 30.21 | 28.03 | 24.41 | 20.08 | GHRL (15 d*)=2.14 fold | 15 d=0.008 |
| 2 | 33.63 | 30.85 | 32.14 | 28.76 | 28.17 | 25.50 | 29.14 | 25.15 | 25.01 | 21.92 | | |
| 3 | 31.37 | 28.44 | 32.97 | 28.05 | 29.31 | 27.93 | 28.33 | 26.33 | 22.04 | 19.58 | GHRL (17 d*)=3.85 fold | 17 d=0.004 |
| 4 | 34.25 | 30.55 | 33.24 | 29.99 | 30.06 | 27.08 | 29.76 | 26.17 | 23.28 | 20.83 | | |
| 5 | 33.08 | 30.62 | 32.17 | 29.11 | 29.24 | 27.23 | 28.84 | 26.86 | 26.98 | 26.84 | GHRL (19 d*)=1.45 fold | 19 d=0.252 |
| 6 | 33.11 | 29.96 | 33.06 | 28.27 | 29.63 | 27.48 | 29.13 | 27.03 | 22.41 | 21.55 | | |
| 7 | 32.63 | 28.88 | 32.63 | 28.31 | 30.24 | 27.64 | 29.45 | 27.16 | 21.01 | 20.95 | GHRL (21 d*)=1.09 fold | 21 d=0.767 |
| 8 | 32.37 | 27.87 | 33.81 | 27.24 | 27.81 | 25.55 | 27.11 | 26.13 | 23.74 | 23.31 | | |
| 9 | 34.25 | 30.84 | 32.61 | 27.24 | 28.68 | 26.22 | 27.85 | 25.79 | 25.28 | 23.14 | | |
| 10 | 34.08 | 31.81 | 33.49 | 28.68 | 27.61 | 25.55 | 27.01 | 25.06 | 24.98 | 22.86 | | |
| 11 | 33.11 | 30.28 | 33.91 | 29.03 | 29.03 | 27.96 | 28.73 | 26.56 | 25.41 | 22.42 | | |
| 12 | 34.63 | 30.52 | 32.73 | 29.55 | 30.49 | 27.35 | 29.15 | 27.31 | 23.01 | 20.07 | | |
| 13 | 33.37 | 30.43 | 31.31 | 27.42 | 28.55 | 26.59 | 28.33 | 26.67 | 22.04 | 21.22 | | |
| 14 | 34.25 | 31.26 | 33.87 | 29.14 | 31.91 | 28.91 | 30.25 | 27.83 | 22.28 | 20.27 | | |
| 15 | 34.08 | 30.66 | 32.51 | 28.95 | 29.42 | 27.11 | 28.91 | 26.41 | 21.98 | 20.33 | | |
| 16 | 34.11 | 31.81 | 32.08 | 27.01 | 27.73 | 26.02 | 27.01 | 25.74 | 24.41 | 20.94 | | |
| 17 | 33.63 | 30.95 | 33.48 | 27.93 | 29.14 | 27.04 | 28.09 | 26.62 | 26.01 | 22.92 | | |
| 18 | 33.37 | 29.75 | 32.55 | 27.53 | 30.31 | 27.56 | 29.67 | 27.11 | 25.11 | 20.94 | | |
| 19 | 34.25 | 30.88 | 33.55 | 28.64 | 29.95 | 27.35 | 29.14 | 27.87 | 23.98 | 21.81 | | |
| 20 | 33.08 | 29.16 | 32.35 | 29.22 | 28.87 | 27.60 | 28.11 | 26.83 | 22.98 | 21.22 | | |

*d: day.

Moreover, these data support a role that both exogenously and endogenously produced GHRL can mediate signaling and functioning for a wide variety of immune cell types with GHRL-specific receptors [12]. Since its discovery in 1999, a number of publications have reported the role of GHRL in various proinflammatory diseases both in human and animal models [13]. Increased GHRL levels in serum or plasma have been reported in a number of inflammatory conditions including ulcerative colitis (UC), Crohn's disease (CD), ankylosing spondylitis, sepsis, pancreatitis, colitis, and possibly rheumatoid arthritis [14-18].

GHRL molecules, as have been discovered, exert their biological actions via binding to the GHSR. GHS-R type Ia (GHS-R1a), the classical GHSR, is one of the transcripts produced by the GHS-R gene (chromosome 3q26.2) [19]. GHS-R type 1b is even more widely distributed in central and peripheral tissues, but its function is still unknown. Besides, there are probably other GHS-R subtypes as well. Whether GHRL is the sole ligand or one of a number of ligands activating the GHS-R1a, it is still under investigation. On the other hand, more evidence are needed to clarify whether the GHS-R used for GHRL isolation is the sole receptor, or one of a group of receptors for one or more ligands [20].

Previous studies have demonstrated that GHRL and GHSR are involved in the growth and development of embryo. However, the expression patterns of GHRL and GHSR have not been described previously in the embryogenesis of ARM. Our observations reveal a key role that GHRL and GHSR played during embryo growth and development, and indicate that defective GHRL and GHSR may lead to dysplasia of anorectum. Moreover, the ethylene thiourea (ETU)-induced ARM rat model has been used for the study of ARM and related abnormalities. To determine the pattern of expression of GHRL and GHSR and the possible role of GHRL and GHSR in anorectal embryogenesis development, in the current study, we analyzed the distribution of GHRL and GHSR mRNA and protein in rat anorectum at different developmental stages (E15d, E17d, E19d and E21d).

## Materials and methods

### *Animal model and tissue collection*

Ethical approval was obtained from the China Medical University Animal Ethics Committee

**Table 2.** The relative quantity of GHSR mRNA in normal group and ARM group

| Case | GHSR | | GHSR | | GHSR | | GHSR | | β-actin | | Times of gene (compared to normal group) | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N15 | E15 | N17 | E17 | N19 | E19 | N21 | E21 | Normal | ARM | | |
| 1 | 33.25 | 29.86 | 31.12 | 28.76 | 30.02 | 27.56 | 29.56 | 26.89 | 23.42 | 21.87 | GHSR (15 d*)=2.63 fold | 15 d<0.001 |
| 2 | 34.27 | 31.15 | 31.38 | 29.68 | 28.21 | 25.21 | 28.85 | 25.37 | 24.13 | 22.86 | | |
| 3 | 33.16 | 29.54 | 32.45 | 28.87 | 28.79 | 25.45 | 27.69 | 25.12 | 22.75 | 20.22 | GHSR (17 d*)=2.58 fold | 17 d<0.001 |
| 4 | 33.07 | 30.09 | 32.56 | 28.69 | 29.11 | 26.32 | 28.55 | 25.06 | 22.87 | 20.96 | | |
| 5 | 33.67 | 30.11 | 30.41 | 27.34 | 28.96 | 26.12 | 28.53 | 25.33 | 24.65 | 21.08 | GHSR (19 d*)=1.09 fold | 19 d=0.574 |
| 6 | 33.23 | 30.17 | 32.87 | 28.61 | 29.21 | 27.08 | 28.67 | 26.79 | 24.67 | 21.58 | | |
| 7 | 33.41 | 29.52 | 31.53 | 27.56 | 29.87 | 26.97 | 29.12 | 26.67 | 23.52 | 20.84 | GHSR (21 d*)=1.05 fold | 21 d=0.762 |
| 8 | 33.67 | 28.44 | 33.09 | 28.72 | 28.57 | 25.79 | 28.02 | 25.85 | 24.61 | 22.15 | | |
| 9 | 34.61 | 29.63 | 31.91 | 27.13 | 29.43 | 26.13 | 28.23 | 25.62 | 24.73 | 21.14 | | |
| 10 | 33.78 | 29.76 | 32.65 | 27.54 | 28.33 | 26.14 | 27.42 | 25.53 | 24.98 | 23.41 | | |
| 11 | 33.69 | 29.06 | 33.15 | 30.12 | 29.45 | 27.17 | 28.23 | 26.14 | 24.64 | 21.22 | | |
| 12 | 34.51 | 30.21 | 32.61 | 27.49 | 29.87 | 26.44 | 28.46 | 25.99 | 22.99 | 20.61 | | |
| 13 | 33.79 | 29.52 | 32.64 | 28.13 | 29.04 | 26.78 | 27.87 | 25.45 | 24.17 | 22.92 | | |
| 14 | 33.96 | 30.78 | 32.53 | 28.47 | 30.35 | 27.76 | 29.66 | 26.24 | 23.63 | 21.17 | | |
| 15 | 34.22 | 29.43 | 32.67 | 28.14 | 29.18 | 26.09 | 27.55 | 25.86 | 23.19 | 21.26 | | |
| 16 | 34.57 | 30.57 | 31.98 | 27.75 | 28.81 | 26.72 | 28.03 | 25.91 | 23.83 | 20.41 | | |
| 17 | 33.79 | 29.63 | 32.32 | 27.12 | 28.32 | 26.39 | 27.24 | 26.13 | 24.89 | 22.12 | | |
| 18 | 32.99 | 28.57 | 30.77 | 28.03 | 29.12 | 27.24 | 28.92 | 25.51 | 24.62 | 21.04 | | |
| 19 | 33.63 | 29.47 | 31.76 | 27.23 | 29.46 | 26.85 | 28.21 | 26.26 | 24.81 | 22.77 | | |
| 20 | 34.55 | 28.88 | 32.21 | 28.37 | 28.21 | 26.57 | 27.73 | 25.47 | 24.96 | 22.31 | | |

*d: day.

prior to the start of the study (no. 2013PS07K). Mature Wistar rats (body weights: 250 to 300 g) were provided by the Medical Animal Center, Shengjing Hospital of the China Medical University. The procedures for generating ARM in fetal rats are described in earlier reports [21]. Overall, 80 time-mated pregnant Wistar rats were randomly divided into two groups: ETU-induced group, in which 35 pregnant rats were gavage-fed a single dose of 125 mg/kg of 1% ETU (2-imidazolidinethione, Aldrich Chemical Co., Germany) on E10d (E0 sperm in vaginal smear after overnight mating), and control group-the rats that received distilled water alone, without the addition of ETU. The embryos were harvested via cesarean delivery on E15, E17, E19, and E21, respectively.

The presence of ARM can be determined under the light microscope prior to staining. Then embryos were divided into normal tail (normal group) and short tail (ARM group) tissues. The harvested samples were immediately frozen in liquid nitrogen to prepare for quantitative real-time PCR (qRT-PCR) and western blot analysis. In addition, approximately one-third of the normal and ARM tissues were fixed in 4% paraformaldehyde for 12-24 h, depending on their size. The tissues from each age group were dehydrated, embedded in paraffin, and performed serial sectioning at a thickness of 4 μm for immunohistochemical staining.

*RNA isolation and qRT-PCR*

Total RNA was extracted from the normal and ARM tissues of rat embryos using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Purity of extracted total RNA was determined by the *260/280*-nm ratio with expected values between 1.8 and 2.0 and then RNA was stored at -80°C for later use. The total RNA was reverse transcribed into complementary DNA (cDNA) using TaKaRa RNA PCR kit (Takara Biotechnology, Shiga, Japan) as per manufacturers' instructions. qRT-PCR was accomplished with SYBR Premix Ex Taq (Takara Biotechnology, Shiga, Japan) on the 7900HT fast Real-time PCR system (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 54°C for 40 s. β-actin was used as the reference gene. The amplification process was followed by a melting curve analysis and CT value was recorded. The average CT value was the extreme CT

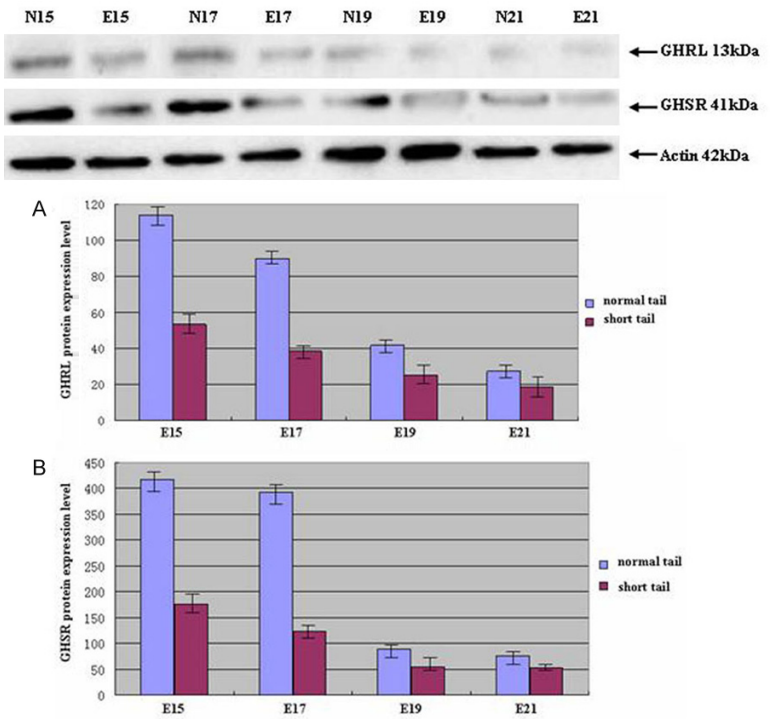


**Figure 1.** Western blot analysis of GHRL and GHSR in normal rat group and ARM group. GHRL and GHSR were detected as an approximately 13-kDa and 41-kDa band on western blots of proteins extracted from both the normal and ARM tissues analyzed. Immunoblots showed a strong signal for GHRL and GHSR proteins in the normal group, but a weak signal in the ARM group. β-actin protein is used as an internal control. A and B histogram showing the trends of GHRL and GHSR expression at each time-point. A peak can be noted on E15 and E17.

value of the sample. The expression difference of the gene was calculated by the $2^{-\Delta\Delta ct}$ method [22]. Experiments were repeated in triplicate. The primers of qRT-PCR were as follows: GHRL forward, 5'-CGG AAG ATG GAG GTC AAG-3', reverse, 5'-TGG CTG TGC TGC TGG TAC-3' (112 bp); GHSR forward, 5'-GGT CCT CTA CAG TCT CAT CG-3', reverse, 5'-ACA CCA CTA CAG CCA GCA-3' (122 bp); β-actin forward, 5'-GGA GAT TAC TGC CCT GGC TCC TA-3', reverse 5'-GAC TCA TCG TAC TCC TGC TTG CTG-3' (139 bp).

*Protein preparation and western blot*

The protein preparation was performed as previous description [23]. Protein extracts (50 μg) were heated at 90°C for 10 min and size-fractionated on Bis-TRIS sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Millipore, Billerica, Mass., USA). After being blocked with 5% fat-free milk in TRIS-buffered saline (1 h, room temperature), the extracts were subsequently incubated with rabbit anti-GHRL (bs-1375R-Bio, 13 kDa, Beijing Biosynthesis Biotechnology Co., Ltd, China) (1:200), rabbit anti-GHSR (bs-11529R-Bio, 41 kDa, Beijing Biosynthesis Biotechnology Co., Ltd, China) (1:200), and anti-actin mouse monoclonal antibody (AA128, 42 kDa, Beyotime Institute of Biotechnology, Beijing, China) (1:1000), respectively, at 4°C overnight. Membranes were washed and incubated with the secondary antibody (Goat anti-rabbit IgG, HRP conjugate; Lot#I10318, Beijing TransGen Biotech Co., Ltd. China) (1:2000) for 2 h at room temperature. Following another wash, membranes were developed by using a chemiluminescent substrate kit (Pierce, Pierce, Rockford, Ill., USA). Densitometry analysis was performed using the GEL-PRO 4.0 software (Media Cybernetics, LP, Waltham, MA, USA). Protein levels in each lane were normalized to those of β-actin as an internal standard.

*Immunohistochemical staining*

Immunohistochemical stains were performed as described previously [24]. Paraffin-embedded tissue sections (4-5 μm) immerse the slides in Xylene two times for 10 minutes each. Immerse the slides in 100% alcohol (denatured) two times for 10 minutes each. Immerse the slides in 95% alcohol once for 5 minutes. Immerse the slides in 70% alcohol once for 5 minutes. Rinse the slides in deionized water. Rehydrate the slides in PBS for 10 minutes. Incubate the sample with 1-3 drops of Serum Blocking Reagent for 15 minutes. Incubate the sample with 1-3 drops of Avidin Blocking Reagent for 15 minutes. Rinse the sample with PBS. Incubate the sample with 1-3 drops of Biotin Blocking Reagent for 15 minutes. Rinse the sample with PBS. Incubate the sample with primary antibody diluted in Incubation Buffer. The samples were incubated overnight at 4°C 16 h with rabbit anti-GHRL and rabbit anti-GHSR (diluted 1:50). Rinse the sam-

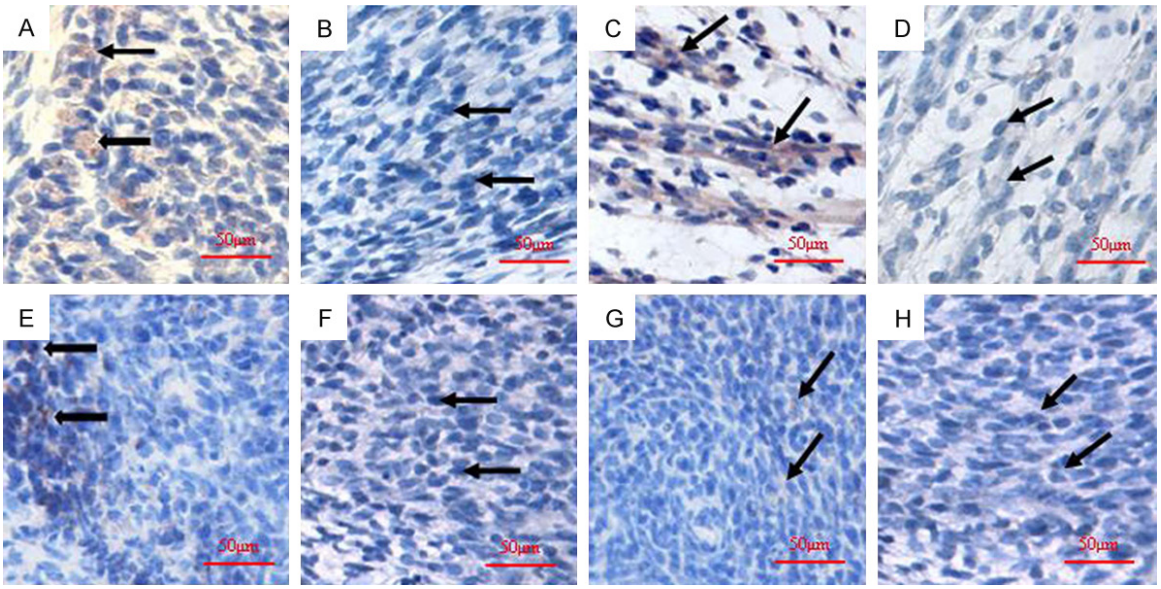


**Figure 2.** Expression of GHRL and GHSR detected by immunostaining in the normal group and the ARM group. A, C, E and G indicated the normal group: on E15 and E17 in the normal group, GHRL-labeled cells and GHSR-labeled were observed on the epithelium of the anorectum (black arrowheads: positive cells were brown yellow depositions); on E19 and E21 in the normal group, GHRL-labeled cells and GHSR-labeled were observed on the epithelium of the anorectum (black arrowheads: positive cells were punctiform). B, D, F and H indicated the ARM group; on E15, E17, E19 and E21 the GHRL-labeled cells and GHSR-labeled were observed on the epithelium of the anorectum (were no positive cells). (original magnification ×400, the bar=50 μm).

ple with PBS and then wash 3 times in PBS for 5 minutes each. Slides then were coated in a solution of goat anti-rabbit antibody (1:2000; Dako) for 30 min. Rinse the sample with PBS and then wash 3 times in PBS for 15 minutes each. Finally, transferred to a solution of streptavidin-horseradish peroxidase (LSAB2 System; Dako) at 10 min. Then the sections were counterstained with hematoxylin for 1 min. Monitor the intensity of tissue staining under a microscope. Rinse the sample with PBS and then wash in PBS for 10 minutes. Rinse the sample with distilled water. Examine the slides under a microscope. The density of the positively stained area was calculated at 400× magnification.

*Statistical analysis*

The Statistical Program for Social Sciences, version 13.0 (SPSS, Chicago, IL), was used for statistical analysis. A t-test was used to compare the GHRL and GHSR levels between the normal and ARM group. All results were presented as mean ± standard deviation (S.D.), where *P* values of 0.05 or less were considered statistically significant.

## Results

*General observations*

In this study, no malformations were observed in the 167 embryos of normal rats. Among the ETU-treated embryos, all 288 embryos had short or absent tails, and 9 of the embryos died in utero. The incidence of ARM in ETU-treated embryos was 66.3% (191/288). Embryos for qRT-PCR, western blot, and immunohistochemical staining in the normal group and the ARM group (E15d, E17d, E19d, and E21d) are 20 cases, respectively.

*qRT-PCR*

The OD value of RNA calculated by *A260/A280* was from 1.8 to 2.0. To test whether there were any changes at the transcriptional level of GHRL and GHSR, we were compared the mRNA levels by performing qRT-PCR. The mRNA levels of GHRL and GHSR of E15d were 2.14-fold and 2.63-fold higher in normal group than in ARM group ($n$=20, $P$=0.008; $n$=20, $P$<0.001). The mRNA levels of GHRL and GHSR of E17d were 3.85-fold and 2.58-fold higher in normal group than in ARM group ($n$=20, $P$=0.004; $n$=20,

*P*<0.001), respectively. The mRNA levels of GHRL and GHSR of E19d were 1.45-fold and 1.09-fold higher in normal group than in ARM group (*n*=20, *P*=0.252; *n*=20, *P*=0.574). The mRNA levels of GHRL and GHSR of E21d were 1.09-fold and 1.05-fold higher in normal group than in ARM group (*n*=20, *P*=0.767; *n*=20, *P*=0.762), respectively (**Tables 1**, **2**). Basing on these data, it should be noted that the different expression levels of GHRL and GHSR were significant in normal group and ARM group on E15d and E17d (*P*<0.05), however, there were no statistical significance in normal group and ARM group on E19d and E21d (*P*>0.05).

*Western blot analysis*

Western blot analysis specific for GHRL and GHSR were conducted to quantify the proteins expression in the embryonic development of normal tail and short tail. GHRL and GHSR were detected as an approximately 13-kDa and 41-kDa band on western blots with proteins extracted from both normal and ARM tissue analyzed. Each protein band was normalized by a corresponding β-actin band. On E15d and E17d, the key period of anus formation, the expressions of GHRL and GHSR reached estimated optimal level in normal group, while in ARM group, the GHRL and GHSR protein expressions were faint. The western blot showed that, the proteins levels of GHRL and GHSR were higher in normal group (113.79±23.14 vs and 417.65±51.27 vs) than in ARM group (53.48± 11.09 and 175.24±32.11) on E15d and were higher in normal group (89.94±19.22 vs and 391.84±47.33 vs) than in ARM group (38.68± 15.46 and 123.97±30.52) on E17d, respectively. It should be concluded that the difference between expression levels of GHRL and GHSR was significant in normal group and ARM group on E15d and E17d (P<0.05). It also indicated that, there was no difference between normal group (41.61±13.27 vs and 88.73±26.15 vs) and ARM group (25.08±11.13 vs and 53.52± 20.43 vs) in terms of protein levels of GHRL and GHSR on E19d, and there was also no statistical significance when comparing normal group (27.13±0.92 vs and 74.99±23.67 vs) with ARM group (18.62±0.35 and 51.77±17.28) on E21d (P>0.05) (**Figure 1**).

*Immunohistochemistry analysis*

To further investigate the distribution and expression of GHRL and GHSR proteins in normal group and ARM group, we performed immunohistochemistry staining. Expressions of GHRL and GHSR were mainly found in the epithelium of the anorectum on E15d and E17d (brown yellow depositions, **Figure 2A**, **2C**); however, positive cells of GHRL and GHSR saw an obvious decrease on E19d and E21d (**Figure 2E**, **2G**). In ARM group, no positive cells of GHRL and GHSR were detected in the epithelium of the anorectum on E15d, E17d, E19d and E21d (**Figure 2B**, **2D**, **2F**, **2H**).

## Discussion

GHRL is a newly discovered hormone, mainly produced in the stomach, and has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHSR). Although GHRL is essentially a gastric hormone, it is expressed ubiquitously, although at low level [25]. However, it has been shown recently that both GHRL and des-acyl GHRL inhibit apoptosis in cardiomyocytes and endothelial cells [26], indicating that GHRL may also act through a novel, yet to be identified receptor, which is distinct from GHSR-1a. In a previous study, it was reported that the GHRL and GHSR gene could restrict and induce the mesenchymal and epithelium cell differentiation and proliferation [27, 28].

Previous studies have reported that both GHRL and des-acyl GHRL bind to a common receptor in some cancer cells. In these cells, GHRL and des-acyl GHRL inhibit cell proliferation, whereas, GHSR-1a appears to mediate a proliferative signal [29, 30]. Although most survival factors also exhibit a proliferative activity, members of the transforming growth factor-β family inhibit both cell growth and cell death in myoblasts. Thus, we may speculate that the GHRL and GHSR gene product, in addition to its endocrine activity based on some cancer cells and cardiomyocytes cells form, may also be a trophic local factor acting in the epithelium of the anorectum.

Based on these observations, we raised the hypothesis that GHRL and GHSR may have a direct effect on the epithelium cells of anorectum. However, the exact role of the GHRL and GHSR genes in the development of ARM remains to be elucidated. In this study, the most important finding was that GHRL and GHSR expression, which occurred in the ano-

rectum of normal rat embryo, reached estimated optimal level on E15d and E17d, but decreased after the anus formed. In contrast, expression levels of GHRL and GHSR in ARM embryos remained low and unchanging on E17d and E21d, suggesting that GHRL and GHSR might play an essential role in the embryogenesis of the anorectum, and may also promote development of ARM. In this study, expressions of GHRL and GHSR genes showed differences between normal and ARM embryos in the spatial distribution of anorectum. In normal embryos, the positive cells of GHRL and GHSR were mostly centered on epithelium cell of the anorectum on E15d and E17d. Nevertheless, in ARM embryos, only sporadic GHRL and GHSR staining were noted on this region, and the intensity of the immunohistochemistry of GHRL and GHSR expression in the ARM is lower than that in normal embryos on E15d and E17d. Therefore, there was a relatively spatial imbalance between the normal and ARM embryos during the embryogenesis of the anorectum. Meanwhile, for both normal and ARM embryos, a immunohistochemical diagraming of GHRL and GHSR was evident (**Figure 2**). This suggests that, GHRL and GHSR may play a vital role in the development of anorectal epithelium cells. GHRL and GHSR, as a molecular messenger, acts in a dose-dependent and diffusible manner in the organizing patterns of early vertebrate development; and it is likely that the ectopic expression of GHRL and GHSR and their physical distance from the responding tissue may interfere with normal development. Accordingly, GHRL and GHSR proteins located in an unusual region might lead to a disruption in the patterning of epithelium in the local microenvironment, inducing a further maldevelopment of structures.

In addition, based on the results of western blot analysis and qRT-PCR, GHRL and GHSR expressions in normal embryos at the critical time of anorectal development (E15d and E17d) were at the highest level, suggesting that it may play an important role in the development of anorectum. However, at the same stage, the expression levels of GHRL and GHSR in ARM embryos were lower (E15d and E17d), implying that these special down regulation of GHRL and GHSR expressions may reduce the signals from endoderm to mesoderm and affect the transition from endoderm to intestinal epithelium, which eventually lead to ARM. Additionally, with the anal opening on E19d, GHRL and GHSR protein and mRNA expressions decreased gradually, suggesting that GHRL and GHSR may play an essential role in initial morphogenesis of the anorectum, whereas their role in subsequent development might be less important. These findings demonstrated that GHRL and GHSR expressions showed time-dependent changes in the anorectal development.

The genetics mechanism of ARM is an extremely complex process. Up to now, there are no reports concerning GHRL and GHSR genes that mediate the development of ARM. Combining with previous studies, we conclude that GHRL and GHSR genes may be to extremely important for the development of ARM embryos. However, this study was unable to substantiate whether GHRL and GHSR genes were the initial event/initial factors that lead to ARM, and numerous genes have recently been shown to be involved in the different phases of the development of ARM. So further studies are required to confirm the gene regulation mechanism of ARM during embryonic development, and to clarify the underlying molecular mechanisms that mediate the mal development of ARM.

## Acknowledgements

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## Disclosure of conflict of interest

None.

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