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# A novel finding of anoctamin 5 expression in the rodent gastrointestinal tract

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ABSTRACT

Anoctamin 5 (Ano5) belongs to the anoctamin gene family and acts as a calcium-activated chloride channel (CaCC). A mutation in the Ano5 gene causes limb-girdle muscular dystrophy (LGMD) type 2L, the third most common LGMD in Northern and Central Europe. Defective sarcolemmal membrane repair has been reported in patients carrying this Ano5 mutant. It has also been noted that LGMD patients often suffer from nonspecific pharyngoesophageal motility disorders. One study reported that 8/19 patients carrying Ano5 nutations suffered from dysphagia, including the feeling that solid food items become lodged in the upper portion of the esophagus. Ano5 is widely distributed in bone, skeletal muscle, cardiac muscle, brain, heart, kidney and lung tissue, but no report has examined its expression in the gastrointestinal (GI) tract. In the present study, we investigated the distribution of Ano5 in the GI tracts of mice via reverse transcription-polymerase chain reaction (RT-PCR), Western blot and immunofluorescence analyses. The results indicated that Ano5 mRNA and protein are widely expressed in the esophagus, the stomach, the duodenum, the colon and the rectum but that Ano5 immunoreactivity was only detected in the mucosal layer, except for the muscular layer of the upper esophagus, which consists of skeletal muscle. In conclusion, our present results demonstrate for the first time the expression of Ano5 in the GI epithelium and in skeletal muscle in the esophagus. This novel finding facilitates clinical differential diagnosis and treatment. However, further investigation of the role of Ano5 in GI function is required.



## 1. Introduction

Anoctamin 5 (Ano5), also referred to as TMEM16E, belongs to the anoctamin family, which includes 10 members. The Ano family proteins display high structural homology and consist of eight hydrophobic helices that have been predicted to function as transmembrane domains and cytosolic N- and C-termini [1]. Ano1 and Ano2 act as calcium activated chloride channels (CaCCs) [2,3]. Importantly, Ano1 is highly expressed in epithelial tissue [4,5] and plays an important role in $Ca^{2+}$-dependent $Cl^-$ secretion [6]. All anoctamin proteins, including Ano5, display CaCC activity [7]. CaCCs are widely distributed in multiple tissues, especially in secretory epithelial cells, such as in the trachea [8–10] and the GI epithelium [11–13], and are involved in $Cl^-$ secretion. Epidermal growth factor up-regulates Ano1 expression in colonic epithelial cells [14]. The amino acid sequence of Ano1 displays 59% similarity to that of Ano5 [15]. However, no report has examined whether Ano5 expression in the GI epithelium is similar to that of Ano1. LGMD patients often suffer from nonspecific pharyngoesophageal motility disorders [16]. Dysphagia, a feeling that solid food items are lodged in the upper portion of the esophagus, has been reported in 8/19 patients carrying Ano5 mutations, indicating that nearly 50% of LGMD patients suffer from this upper GI tract syndrome. The high reported incidence of dysphagia is a novel phenotypic characteristic of patients carrying Ano5 mutations [17]. The CaCC Ano5 is predominantly expressed in bone and skeletal and cardiac muscle tissue and is also expressed in brain, heart, kidney and lung tissue [18]. To date, there has been no report of Ano5 expression in esophageal muscular tissue. Therefore, the aim of the present study was to investigate the distribution of Ano5 in the murine GI tract via reverse transcription-polymerase

*Abbreviations:* Ano5, anoctamin 5; CaCC, calcium-activated chloride channel; LGMD, limb-girdle muscular dystrophy; GI, gastrointestinal; RT-PCR, reverse transcription-polymerase chain reaction; DAPI, 4,6-diamino-2-phenylindole; PBS, phosphate-buffered saline.

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chain reaction (RT-PCR), Western blot and immunofluorescence analyses.

## 2. Materials and methods

### 2.1. Animals

Adult male C57BL/6J mice (25–30 g) were provided by the Laboratory Animal Services Center of Capital Medical University. All of the experiments in this study were performed in accordance with the guidelines of the National Institutes of Health (NIH, USA) and with the approval of the Animal Care and Use Committee of Capital Medical University of China. All efforts were made to minimize the suffering of the animals and the number of animals required to produce reliable scientific data.

### 2.2. Chemicals and reagents

The Ano5 antibodies (sc-169628 and or39268) were obtained from Santa Cruz Biotechnology (Santa Cruz, Dallas, USA) and Biorbyt Ltd. (Biorbyt, Cambridge, UK). The Alexa 594-conjugated anti-goat antibody (A11058) and the Cy3-conjugated anti-rabbit antibody (A0517) were provided by Life Technologies Corporation (Invitrogen™, Carlsbad, CA, USA) and Beyotime Institute of Biotechnology (Beyotime, Haimen, China). Horseradish peroxidase (HRP)-conjugated IgG antibodies (sc-2004, A0208) were obtained from Santa Cruz and Beyotime. GoScript™ Reverse Transcriptase (A5000) and GoTaq® Green Master Mix (M7122) were obtained from Promega Corporation (Promega, Madison, USA). TRIzol Reagent was provided by Invitrogen. DAPI (4,6-diamino-2-phenylindole) was obtained from Beyotime.

### 2.3. RNA isolation and RT-PCR

The primer sequences used for RT-PCR analysis were as follows: Ano5, 5′-CGTGGAGGATTTGAAGAAAGAT-3′ (sense) and 5′-TGTTGAGGATGGAAAGAAAGTG-3′ (antisense); and β-actin, 5′-GTCCCTCACCCTCCCAAAAG-3′ (sense) and 5′-GCTGCCTCAACACCTCAACCC-3′ (antisense). Total RNA was isolated from the mouse leg muscles and GI tracts using TRIzol Reagent and was quantified by assessing the optical densities at 260 and 280 nm. The RNA samples were mixed with a random primer and Oligo(dT) primer for 5 min at 70 °C and then immediately chilled in ice water for at least 5 min. RNA was reverse-transcribed using GoScript™ Reverse Transcriptase via an annealing step of 5 min at 25 °C followed by an extension step of 1 h at 42 °C. Each reaction contained 12.5 μl of GoTaq® Green Master Mix, 2.5 μl of each primer and 5 μl of cDNA. After 5 min at 95 °C to activate Taq polymerase, the cDNA was amplified using 30 cycles of 95 °C for 40 s, 54 °C for 40 s and 72 °C for 1 min followed by a final step of 72 °C for 5 min; then, the samples were held at 4 °C (MyCycler, Bio-Rad, USA). Pooled cDNA samples from all organs served as standards. To verify the accuracy of the amplification, PCR products were further analyzed on ethidium bromide-stained 1.5% agarose gels, which were visualized using a UV transilluminator. Light Cycler 480 software (Roche Applied Science) was used to analyze the data. The target expression levels were normalized to those of the reference β-actin.

### 2.4. Western blot

Tissues were isolated from the skeletal muscles and the digestive tracts of adult mice and were homogenized in RIPA buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.5% sodium deoxycholate) (Beyotime, Haimen, China) for protein extraction. The proteins were separated using 8% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were washed for 10 min with TBST (20 mM Tris–Cl, pH, 7.5, 0.15 M NaCl, 2.7 mM KCl, 0.05% Tween-20) and were blocked with 5% bovine serum albumin in TBST for 2 h at room temperature. The membranes were probed using the rabbit polyclonal anti-Ano5 antibody at 4 °C overnight. The (HRP-conjugated) goat anti-rabbit secondary antibody was applied for 2 h at room temperature. The protein bands were visualized via chemiluminescence (Beyotime) using a Bio-Rad ChemiDoc Imaging System (Bio-Rad, USA). The Ano5 protein levels were normalized to those of GAPDH in the corresponding lanes.

### 2.5. Immunofluorescence

Eight-micron-thickness cryostat sections were generated from frozen mice leg muscles and digestive tracts and were air-dried overnight at room temperature. The sections were immersed in citrate buffer (0.01 M, pH 6.0) and heated to a temperature of 95–100 °C in a microwave oven for 15 min for antigen retrieval. The sections were washed with phosphate-buffered saline (PBS) and then treated with blocking solution consisting of PBS containing 10% donkey serum for 1 h at room temperature. The sections were incubated in an anti-Ano5 polyclonal antibody at 4 °C overnight. The Ano5 protein and nuclei were stained with a second antibody and DAPI for 2 h and 5 min at room temperature, respectively. Immunofluorescence analysis was performed using a Nikon 80i fluorescence microscope equipped with a Leica DFC300 FX camera. The immunoreactivity was assessed in five animals for each marker. At least eight sections were examined for each animal.

## 3. Results

### 3.1. Ano5 expression in the mouse GI tract

To distinguish the mucosal layer from the muscular layer, we isolated the GI mucosal and muscular layers from the mouse GI tract, including the upper esophagus, the stomach, the duodenum, the colon and the rectum. We performed RT-PCR and Western blot on these tissues to examine the expression of Ano5 in the GI tract.

Total RNA extracted from the mucosal and muscular layers of the GI tract was subjected to RT-PCR. Ano5 mRNA (Fig. 1A and B) was clearly expressed in the mucosal layer in all segments of the GI tract. Alternatively, in the muscular layer, only the upper esophagus displayed Ano5 expression (Fig. 1C), and Ano5 was not detected in the stomach, the duodenum, the colon or the rectum (Fig. 1C). Ano5 mRNA expression in the quadriceps femoris muscle of mice was used as a positive control (Fig. 1D).

Similar to the collection of RNA, protein was also extracted from the mucosal and muscular layers of the GI tract, and these samples were subjected to Western blot. The Ano5 protein was widely expressed in the mucosal layer in all regions of the GI tract (Fig. 2A). However, in the muscular layer, Ano5 was only expressed in the upper esophagus (Fig. 2C), and Ano5 expression was not detected in the stomach, the duodenum, the colon or the rectum (Fig. 2C). This result was consistent with the mRNA expression data. The different regions of the GI tract displayed different Ano5 expression levels (Fig. 2B).

Ano5 protein expression in the quadriceps femoris muscle of mice was used as a positive control (Fig. 2D).

### 3.2. Ano5 immunoreactivity in the GI tract

As a positive control [13], we examined the distribution of endogenous Ano5 in the quadriceps femoris muscle, a skeletal muscle isolated from the mouse leg. The results revealed a very

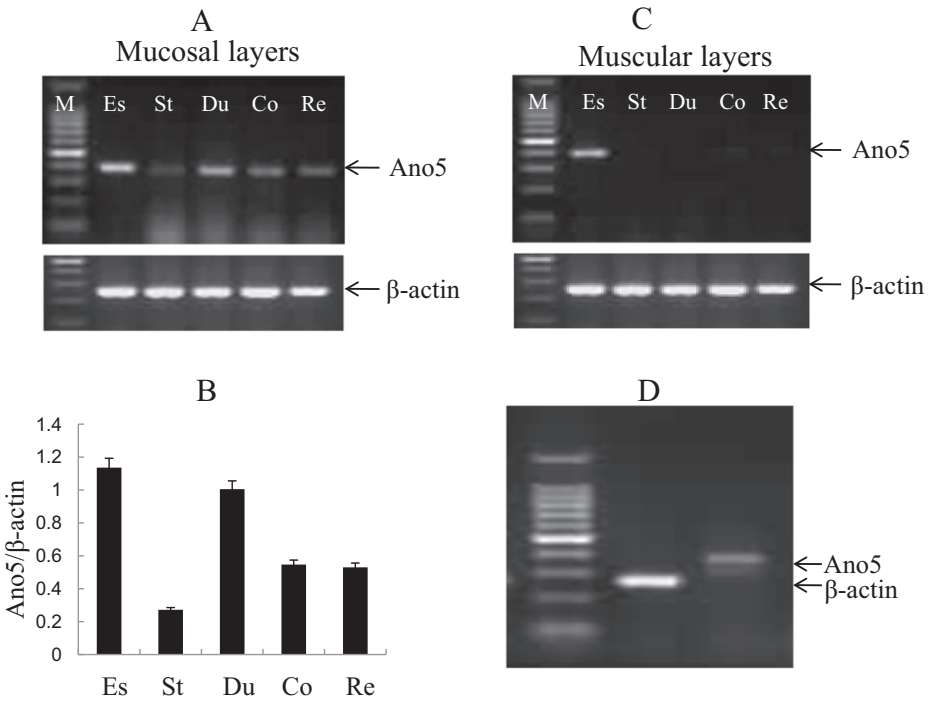


**Fig. 1.** Ano5 mRNA expression in the mouse GI tract. (A) Mucosal layer. (B) Summary histogram showing the Ano5 mRNA expression levels in the mucosal layer. (C) Muscular layer. (D) The mouse quadriceps muscle was used as a positive control. β-actin was used as a loading control. M, marker; Es, esophagus; St, stomach; Du, duodenum; Co, colon; Re, rectum.

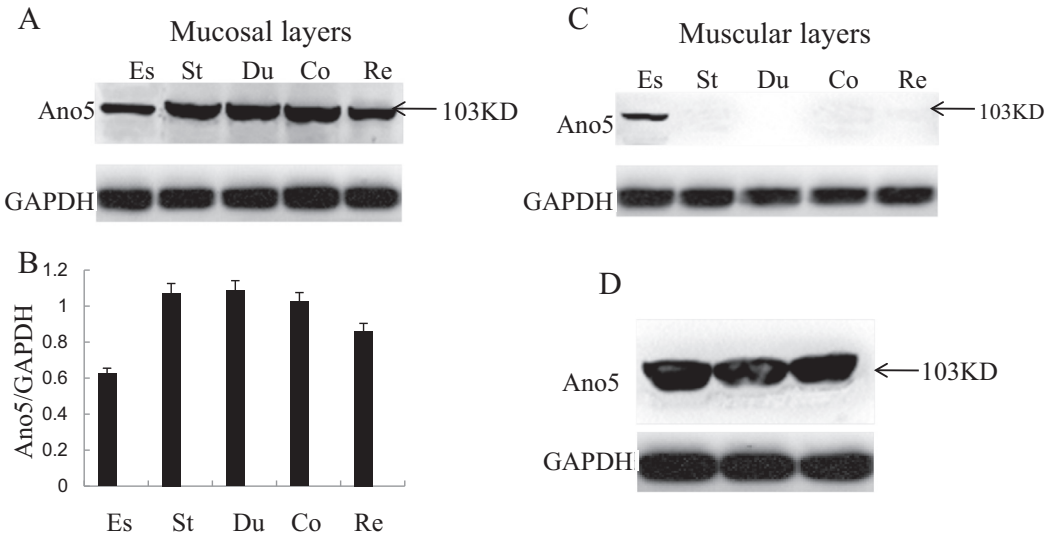


**Fig. 2.** Ano5 protein expression in the mouse GI tract. (A) Mucosal layer. (B) Summary histogram showing the Ano5 protein expression levels in the mucosal layer. (C) Muscular layer. (D) The mouse quadriceps muscle was used as a positive control. GAPDH was used as a loading control. M, marker; Es, esophagus; St, stomach; Du, duodenum; Co, colon; Re, rectum.

clear distribution of Ano5 immunoreactivity in the sarcolemmal membrane (Fig. 3A). Similarly, Ano5 immunoreactivity was also detected in the muscular layer in the upper esophagus, which consists of skeletal muscle (Fig. 3B). No Ano5 immunoreactivity was detected in the muscular layer in the stomach, the duodenum, the colon or the rectum. Furthermore, substantial amounts of Ano5 immunoreactivity were distributed in the epithelium and the mucosal glands of in the mouse GI tract (Fig. 4), including the esophagus (Fig. 4A), the stomach (Fig. 4B), the duodenum (Fig. 4C), the colon (Fig. 4D) and the rectum (Fig. 4E).

## 4. Discussion

In the present study, for the first time, we demonstrate Ano5 expression in the GI tract. Ano5 is distributed in the mucosal layer in all regions of the GI tract, but in the muscular layer, only the upper esophagus displays Ano5 expression. No Ano5 expression was detected in the muscular layer of the stomach, the duodenum, the colon or the rectum.

Ano5, also referred to as TMEM16E, is an anoctamin family member. Dominant mutations in the Ano5 gene are associated

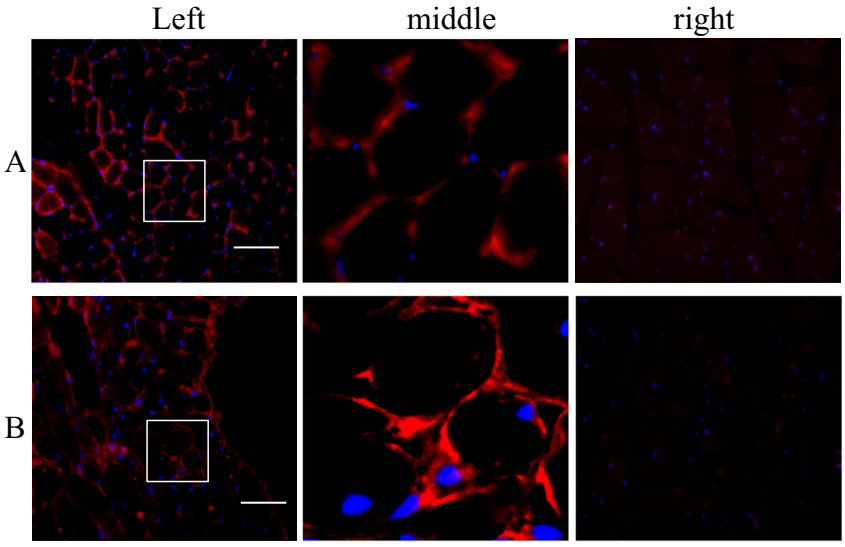


**Fig. 3.** The distribution of Ano5 immunoreactivity in the quadriceps femoris muscle and the muscular layer in the upper esophagus of mice. (A) Ano5 immunoreactivity (red) in the quadriceps muscle. The middle panel displays a higher-magnification image of the skeletal muscle shown in the left panel. The right panel displays the negative control. (B) Ano5 immunoreactivity (red) in the muscular layer in the upper esophagus. The middle panel displays a higher-magnification image of the esophageal skeletal muscle shown in the left panel, and the right panel displays the negative control. The nuclei are shown in blue (DAPI staining). *Bars*, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

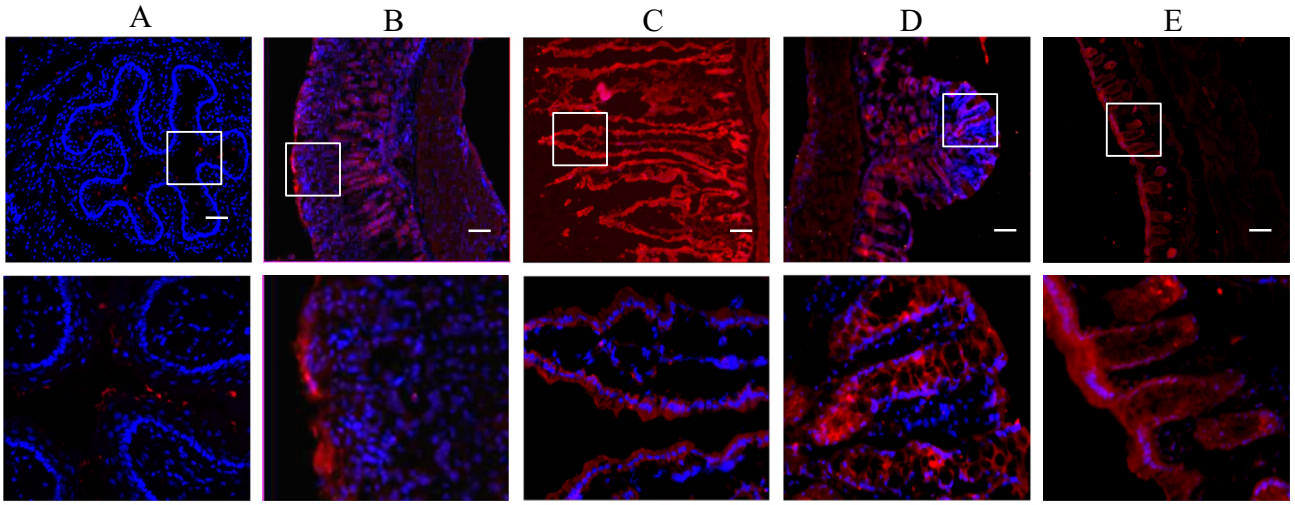


**Fig. 4.** The distribution of Ano5 immunoreactivity in the mouse GI mucosa. (A) Upper esophagus. (B) Stomach. (C) Duodenum. (D) Colon. (E) Rectum. Ano5 immunoreactivity is shown in red. The nuclei are shown in blue (DAPI staining). The lower panel displays a higher-magnification image of the upper panel. *Bar*, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with the skeletal disorder gnathodiaphyseal dysplasia (GDD) [18]. Recessive mutations in Ano5 can cause LGMD, which is associated with atrophy of the asymmetric quadriceps femoris and biceps brachii muscles [19]. Ano5-induced muscular dystrophy is considered as one of the most common forms of muscular dystrophy [20,21], and these patients commonly exhibit dysphagia [16]. In the present study, based on an examination of the muscular layer in different regions of the mouse GI tract, we detected Ano5 expression in the muscle of the upper esophagus but not in the other GI tract regions. Because the muscle tissue in the upper esophagus is skeletal muscle, Ano5 expression in this tissue provides strong morphologic evidence for the cause of dysphagia in LGMD2 and MMD patients.

Anoctamin family proteins are endogenous CaCCs that produce $Ca^{2+}$-activated $Cl^-$ currents [5]. Ano5 itself functions as a $Cl^-$ channel, but its activation requires a much higher $Ca^{2+}$ concentration than that for other Ano members [7,15]. The intestinal epithelium is a columnar, non-ciliated epithelium that is responsible for secretion and absorption. Ano1 is expressed in the large intestine [4,22], and epidermal growth factor up-regulates Ano1 expression in colonic epithelial cells [14]. As an epithelial anoctamin, the highest level of Ano5 expression was reported in the epithelial tissue of the thyroid gland [23]. The amino acid sequence of Ano1 displays 59% similarity to that of Ano5 [15]. The present study demonstrates the distribution of Ano5 expression in the entire mucosal layer from the esophagus to the rectum. Ano5 might act as a CaCC to modulate epithelial $Cl^-$ transport the GI tract, but further investigation must be performed. Many physical and chemical factors and diseases can impair the GI epithelium. Ano5 might also be involved in the membrane repair process in the vulnerable GI epithelia.

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