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Developmental Expression of Calcium Activated Chloride Ion Channels Anoctamin 5 in Mouse Skeletal Muscle

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Abstract

Anoctamin 5 (Ano5), also named TMEM16E, belongs to the Anoctamin gene family. The mutations in the Ano5 gene cause Limb-girdle muscular dystrophy (LGMD) 2L type and Miyoshi muscular dystrophy (MMD3). Both patients showed sarcolemmal lesions. The studies showed that TMEM16E mRNA expressed in the somites during embryogenesis, particularly in the myotomal cells, and also in the muscle myotome-derived progenitor cells. However, no report has been done to examine Ano5 expression during mouse skeletal muscle development. In the present study, we investigated the distribution and quantification of Ano5 in the skeletal muscles of mice during their development, with the methods of immunofluorescence, Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses. The results indicated that Ano5 mRNA and protein are expressed in skeletal muscle of the mouse from 1 day to 6 months, but with development and aging, the expression of Ano5 reduced gradually. Taken together, our results demonstrate that Ano5 expression level decreased throughout development and aging, and this may explain why the muscular dystrophy syndrome of Ano5 mutant patients only starts during the later stage of their lives.

Keywords: Ano5; Skeletal muscle; Development; Expression

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

Introduction

Anoctamin5 (Ano5) also named Gnathodiaphyseal dysplasia 1 (GDD1) or TMEM16E, which belongs to a novel Anoctamin gene family [1]. Since 2010 Ano5 deficiency was described firstly as a cause of muscular dystrophy [2]. Many articles have reported myopathy was related with Ano5 mutations, especially non-dysferlin Miyoshi muscular dystrophy (MMD3) and proximal myopathy limb-girdle muscular dystrophy 2L(LGMD2L) [3-7]. The studies showed that Ano5 mRNA is firstly expressed in the somites during embryogenesis, particularly in the myotomal cells, and later on in the muscle myotome-derived progenitor cells. In addition, Ano5 expression in adult mouse cardiac muscle, bone tissues [9] and skeletal muscle tissue has also been detected [10]. The study about the exact function of Ano5 is rare.

Although Tsutsumi et al. [10] have examined that GDD1 expression was upregulated during the course of myogenic differentiation in C2C12 cells. It has been reported that patients with MMD3 or LGMD2L caused by Ano5 mutations began to have unpleasant muscle sensations when they were in their 20s, then symptoms worsened and lasted for many years [8]. So we want to ask, in different development stages, is there a change of Ano5 expression in skeletal muscles? Does Ano5 play a role in development of skeletal muscle *in vivo*? We conducted a series of experiments aimed at detecting the Ano5 expression pattern during mouse skeletal muscle tissues development stages, and suggesting a possible role of Ano5 in skeletal muscle development.

Materials and Methods

Animals

All male C57BL/6J mice were purchased from the Laboratory Animal Services Center of Capital Medical University. In this study, all of the experiments were performed in accordance with the guidelines established by the National Institutes of Health (NIH, USA) and were approved by the Animal Care and Use Committee of the Capital Medical University, Beijing, China. All efforts in this study were made to minimize the animal suffering, and the minimal number of animals necessary to produce reliable scientific data.

Immunofluorescence

Six-micron-thickness cryostat sections were cut from frozen mice leg muscles of 1-day-old mice, 1-month-old mice and 6-months-old mice. The sections were washed for 5 minutes in Phosphate-buffered saline (PBS) for three times, then were immersed in citrate buffer (0.01 M, pH 6.0) to heat in a microwave oven for 15 min for antigen retrieval. After washing for three times with PBS, The sections were treated with blocking solution for 1 hour at room temperature. Incubations with an anti-Ano5 (sc-169628, Santa Cruz, USA) polyclonal antibody at 4°C overnight, the sections were stained with a secondary (conjugated with 594) donkey anti-goat antibody (A11058, Invitrogen, USA) for 2 hours at room temperature. Nuclei were stained with DAPI for 5 min. Immunofluorescence analysis was performed using a Nikon 80i fluorescence microscope equipped with Leica DFC300 FX.

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RNA isolation and RT-PCR

Total RNA was isolated from the mice leg skeletal muscles using the TRIzol Reagent. The primers sequences were ordered specifically as follows: Ano5: forward 5'-CGTGGAGGATTTGAAGAAAGAT-3', reverse 5'-TGTTGAGGATGGAAAGAAAGTG-3', products 379bp. RNA was reversed by GoScript™ Reverse Transcriptase (A5000, Promega, USA) Each reaction consisted of 12.5μl of GoTaq® Green Master Mix (M7122, Promega, USA), 2.5μl primer of each terminal, 5μl of cDNA. After 5 minutes at 95°C for activation of Taq polymerase, the cDNA was amplified by 40 seconds at 95°C, 40 seconds at 54°C, and 1 minute at 72°C, for 30 cycles, ending with 5 minutes at 72°C and at 4°C conserved (MyCycler, Bio-Rad, USA). In order to verify accuracy of the amplification, PCR products were further analyzed on 1.5% agarose gels stained by ethidium bromide. Pictures were collected with UV transilluminator. Data were analyzed with Light Cycler 480 software (Roche Applied Science). The target expressions were normalized using GAPDH expression as reference.

Western blot

Tissues were isolated from the mice skeletal muscles of 1-day-old mice, 1-month-old mice and 6-months-old mice, and then were homogenized in RIPA buffer (P0013C, Beyotime, China) for protein extraction. All of the proteins were separated on an 8% polyacrylamide gel (PAGE) and were transferred to Nitrocellulose (NC) membranes (Millipore). The membranes were incubated with 10% bovine serum albumin in TBST (20 mM Tris–Cl, pH, 7.5, 0.15 M NaCl, 2.7 mM KCl, 0.05% Tween-20) for 1h at room temperature and were probed using a rabbit polyclonal anti-Ano5 antibody (or39268, Biorbyt, UK) diluted 1:500 overnight at 4°C. The secondary antibody, HRP-conjugated goat anti-rabbit (sc-2004, Santa Cruz, USA) antibody was used at 1:5000 dilutions for 2h at room temperature. After using Super ECL (P1020, APPLYGEN, China) for chemiluminescence, the protein bands were visualized using a Bio-Rad ChemiDoc Imaging System (Bio-Rad, USA). The Ano5 protein levels were normalized using GAPDH expression as reference.

Statistical analyses

All the results were presented as the means ± Standard deviation (SD) from at least three independent experiments. The statistical analyses were conducted using Student's paired t-test (GraphPad Prism software 4.0 package, GraphPad Software, La Jolla, CA, USA). *P* values less than 0.05 indicated a significant difference.

Results

Ano5 immunoreactivity in the mouse skeletal muscle

In our previous study [11] we found Ano5 was expressed in the mucosal layer of Gastrointestinal (GI) tract and skeletal muscle of the mice. To confirm Ano5 protein is expressed in skeletal muscle of different age, we examined endogenous Ano5 expression in skeletal muscle at different age from mouse legs. We performed immunofluorescence with Ano5 antibody, and found at the first day of birth, Ano5 localized mostly in cytosol (Figure 1A). At the age of one month, Ano5 showed clear sarcolemma membrane expression (Figure 1B). At the age of 6 months, Ano5 still expressed on sarcolemma membrane, but with a reduced pattern, when compare with one month old (Figure1C).

Ano5 mRNA and protein expression in the mouse skeletal muscles

To confirm Ano5 mRNA expression in different developmental stage of skeletal muscle in mice, we used quadriceps femoris muscle at different age of the mice and performed RT-PCR to examine Ano5 mRNA expression. The skeletal muscle of all the different parts that we have examined showed clearly Ano5 mRNA expression. However, the expression of Ano5 showed significant decrease through development. Ano5 in skeletal muscle of 1-day-old pups was highest, and decreased significantly at 1-month-old pups, and at 6-months-old muscles Ano5 expression level was only 1/3 as compared to 1-day-old pups (Figure 2).

To test whether a similar protein expression in different developmental stage of the mouse skeletal muscles, we performed Western blot to examine Ano5 protein expression in different age of skeletal muscle tissues. From 1-day-old mice to 6-month-old mice, the expression of Ano5 mRNA declined gradually (Figure 3), which was consistent with mRNA expression.

Discussion

In this present study, for the first time, we demonstrate that Ano5 is expressed in skeletal muscle in different developing stage with peak expression at 1 day after birth and gradually decreased *in vivo*. Our data demonstrate that Ano5 may play a role in skeletal muscle development, and this may explain the late onset and worsening of Ano5 mutant patients' muscular dystrophy.

The Anoctamin family, also referred to as TMEM16 family has 10 members, and each possess eight transmembrane domains, with intracellular N- and C-terminal tails [1,12]. Recently, several members of the Anoctamin family have been identified as CaCCs in several tissues and cell lines [13-19], including Ano5 can confer chloride conductance in cell culture [18]. Dominant mutations in the Ano5 gene were associated with the skeletal disorder GDD [20]. Recessive mutations in Ano5 were identified as the cause of an autosomal recessive form of LGMD associated with asymmetric quadriceps femoris and biceps brachii atrophy [21], and MDD distal weakness of calf muscles. Although the studies showed Ano5 was expressed in growth-plate chondrocytes and osteoblasts at sites of active bone

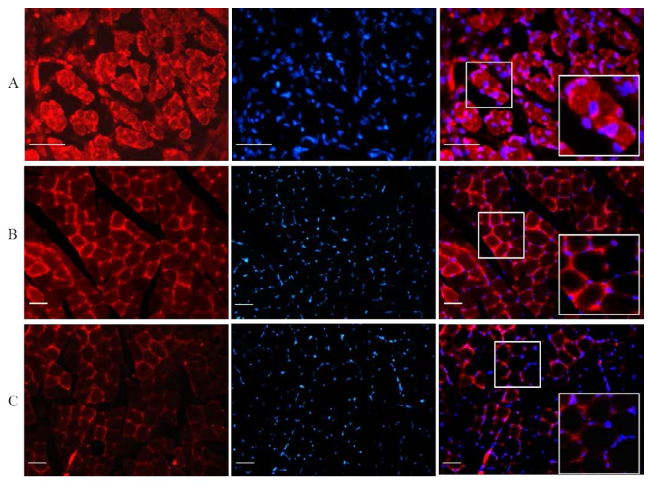

Figure: 1 Ano5 immunoreactivity (red) in the quadriceps muscle of the mice at 1 day(A), 1 month(B) and 6 months(C) of age. The nuclei are shown in blue (DAPI staining). Bars, 100μm.

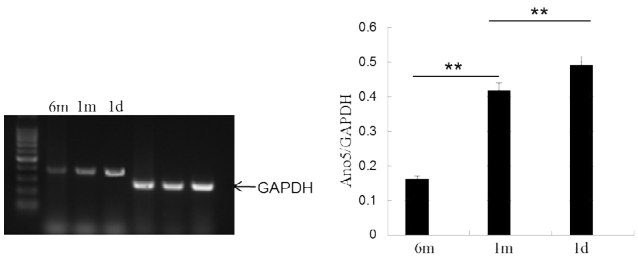

Figure 2: Relative expression of Ano5 mRNA (normalized to GAPDH) examined by RT-PCR in the quadriceps muscles of mice at 6 months, 1 month and 1 day of age. ***P<0.01*

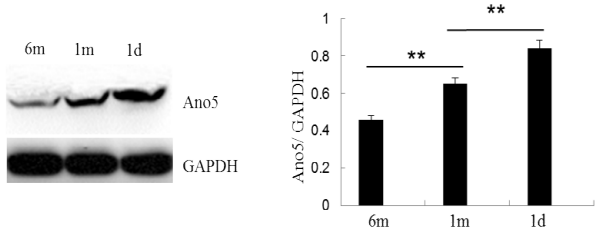

Figure 3: Relative expression of Ano5 protein (normalized to GAPDH) examined by Western blot in the quadriceps muscles of mice at 6 months, 1 month and 1 day of age. ***P<0.01*

turnover, indicating an important role in bone formation. Ano5 was also expressed in somites and in developing skeletal muscle. But whether Ano5 expression pattern changes in the different stages of skeletal muscle *in vivo*, and if this change influences skeletal muscle development remain to be determined.

Tons of work has been done to characterize Anoctamin family. Ano1, as CaCCs could stimulate or inhibit the cell proliferation [22,23]. If Ano1 was inhibited, it could inhibit CaCCs currently and reduce proliferation of interstitial cells of Cajal and a pancreatic cancer cell line CFPAC-1 [24]. Ano6 was a special CaCC, which regulated myoblast proliferation likely through the ERK/AKT signaling pathway [25]. Taken together, it showed a possibility that TMEM16 proteins may regulate cell proliferation and development. In our results, we found that the expression of Ano5 mRNA and protein were significantly decreased through skeletal muscle development. Ano5 in skeletal muscle of 1-day-old pups was the highest, and decreased significantly at 1-month-old pups and only 1/3 left in 6-month-old muscles as compared to 1-day-old pups. Therefore, it is possible that Ano5 may play a role in cell proliferation or differentiation in skeletal muscle. The ability of proliferation and differentiation decreased in skeletal muscle through development and aging. When Ano5 mutated, skeletal muscle could not be repaired effectively. The symptom appeared, such as muscle atrophy. This is an explanation why patients with myopathy caused by Ano5 mutation have symptoms in adult. However, the exact mechanism remains to be determined. Future investigations are required to fully understand the molecular and cellular functions of Ano5 in muscle tissue.

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