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Neuromuscular Disorders 29 (2019) 628–633

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Clinical spectrum and gene mutations in a Chinese cohort with anoctaminopathy

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Received 4 April 2019; received in revised form 4 June 2019; accepted 5 June 2019

Abstract

Recessive mutations in anoctamin-5 (*ANO5*) are causative for limb-girdle muscular dystrophy (LGMD) 2L and non-dysferlin Miyoshi-like distal myopathy (MMD3). ANO5 mutations are highly prevalent in European countries; however it is not common in patients of Asian origin, and there is no data regarding the Chinese population. We retrospectively reviewed the clinical manifestations and gene mutations of Chinese patients with anoctaminopathy. A total of five ANO5 mutations including four novel mutations and one reported mutation were found in four patients from three families. No hotspot mutation was found. Three patients presented with presymptomatic hyperCKemia and one patient had limb muscle weakness. Muscle imaging of lower limbs showed preferential adductor magnus and medial gastrocnemius involvement. No hotspot mutation has been identified in Chinese patients to date.

Keywords: Anoctaminopathy; Limb girdle muscular dystrophy; Presymptomatic hyperCKemia; Mutation; Muscle MRI.

1. Introduction

Recessive mutations in anoctamin-5 (*ANO5*) are responsible for limb girdle muscular dystrophy (LGMD) 2L, the most prevalent forms of muscular dystrophy in Europe [1], as well as the non-dysferlin Miyoshi-like distal myopathy (MMD3) [2]. The clinical manifestations of anoctaminopathy include presymptomatic hyperCKemia, myalgia, muscle stiffness [3], recurrent rhabdomyolysis and limb girdle muscle weakness. Suspected cardiomyopathy was also reported [4]. Muscle involvement in patients with anoctaminopathy was usually asymmetric and selective at early stage with preferential involvement of quadriceps, hamstrings and calves [5,6]. To date, more than 100 disease associated mutations have been reported in *ANO5* gene (https://databases.lovd.nl/shared/variants/ANO5). Two recurrent mutations were particularly noted: c.191dupA in exon 5 was the most common one as the founder mutation of Northern European origin [6], while c.2272C > *T* in exon 20 was the most common mutation in the Finnish population [7]. However, no clear genotype-phenotype correlation was revealed.

Only a few cases of anoctaminopathy have been reported in the Asian population and there is no published data regarding the Chinese population [8–11]. Here we present the clinical presentations in four patients with confirmed anoctaminopathy and one patient with suspected anoctaminopathy, and

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https://doi.org/10.1016/j.nmd.2019.06.005
0960-8966/

reviewed muscle pathology and muscle magnetic resonance imaging (MRI) in these cases.

2. Materials and methods

2.1. Patients ascertainment

Patients with a clinical diagnosis of LGMD in Huashan neuromuscular biosample database from 2005–2018 were reviewed. Most of the patients came from East Mainland China and were of Han origin. Inclusion criteria of suspected LGMD were as follows: (1) progressive limb girdle muscle weakness; (2) elevated serum creatine kinase(CK) level (>170 u/L); (3) electromyogram (EMG) study: myopathic changes. Duchenne muscular dystrophy (DMD), facioscapulohumeral dystrophy type 1 and type 2 (FSHD1 and FSHD2), myotonic dystrophy type 1 and type 2 (DM1 and DM2), oculopharyngeal muscular dystrophy (OPMD), inflammatory myopathies and congenital myopathies were excluded according to clinical presentation and genetic tests when needed. Patients from dominant-inherited pedigrees were also ruled out. Together with the LGMD cohort, patients with presymptomatic hyperCKemia admitted to the outpatient clinic were also included for further screening for potential *ANO5* gene mutations by targeted next-generation sequencing (NGS).

2.2. Genetic analysis

Genomic DNA was extracted from peripheral blood using the High Pure Polymerase chain reaction (PCR) Template Preparation Kit (Roche, Basel, CH) according to the manufacturer's instructions. The DNA fragments were enriched by performing solution-based hybridization capture, followed by sequencing using an Illumina Miseq platform (Illumina, San Diego, CA, USA) with the 2×300 bp paired-end read module. The hybridization capture procedure was performed using the SureSelect Library Prep Kit (Agilent, Santa Clara, CA, USA). The DNA samples were screened for targeted NGS of 245 genes related to neuromuscular diseases (Suppl.Tab.1). All mutations identified by NGS were subsequently confirmed by Sanger sequencing.

2.3. Clinical data collection

Medical records of the 4 patients with genetically confirmed *ANO5* mutations and 1 case with suspected anoctaminopathy were reviewed and evaluated. Physical examination, electrocardiogram (ECG), echocardiogram, electromyography (EMG) and serum CK levels were collected when available. Detailed family history, motor milestones and personal past medical history were also inquired during out-patient follow-up or through phone call follow-up.

2.4. Muscle imaging

Muscle MRI of the lower limbs was performed in three patients (P1, P3 and P4). T1 weighted image (T1WI) and short time inversion recovery (STIR) sequences were employed on a 3-T MR scanner (Ingenia; Philips Medical Systems, The Netherlands). Each study was done using a 15-channel head coil, 12-channel posterior coils, and two 16-channel anterior Torso coils. The transverse sections were analyzed according to abnormal muscle bulk and signal intensities.

2.5. Histology and immunohistochemistry

Open muscle biopsies were obtained from the biceps of three cases (P2, P4 and P5). Histochemical and immunohistochemical studies including H&E, Gomori modified trichrome (GMT), periodic acid-Schiff (PAS) and congo red were performed. The following primary antibodies were used for immunohistochemistry with standard procedures: dystrophin (DYS1: Rod domain; DYS2: C-terminus; DYS3: N-terminus, Novocastra, Newcastle upon Tyne, UK), dysferlin (NCL-Hamlet, Novocastra), Sarcoglycans (NCL-g-SARC, NCL-a-SARC, NCL-b-SARC, Novocastra).

3. Results

3.1. Genetic mutations of ANO5

Among the 301 LGMD cases and 15 cases with presymptomatic hyperCKemia, most common subtypes are LGMD2B (172/301, 57.1%) and LGMD2A (50/301, 16.6%). Out of a total of 316 individuals, 66 LGMD cases and 6 cases with presymptomatic hyperCKemia were genetically undiagnosed, we identified compound heterozygous or homozygous ANO5 gene variants in 5 individuals (P1-5).

Four patients were considered as confirmed anoctaminopathy (P1-4, Table 1). Five mutations identified in Patient 1–4 distributed from the middle region to the C-terminal of *ANO5* gene (Fig. 1), including 4 novel mutations and one reported mutation. Among these novel mutations, c.1103C>*T* was predicted to be probably damaging with a score of 1 by polyphen-2 and the corresponding amino acid was highly conserved among different species. Another novel homozygous mutation c.2498T>*G* identified in P4 from a consanguineous family had a score of 0.995 by polyphen-2. The mutation c.1969C>*T* was a novel nonsense mutation leading to premature termination. Compound heterozygous mutations including a novel frameshift mutation c.2596_2597del and a reported missense mutation c.1640G>*A* were identified in Patient 2 and Patient 3 from the same family. A novel homozygous c.2423A>*T* variant was identified in Patient 5 born in a consanguineous family (Fig. 1). The variant is predicted to be possibly damaging by polyphen-2 with a score of 0.880

Table 1
Clinical manifestations and mutations of the patients with confirmed anoctaminopathy.

Confirmed anoctaminopathy	P1	P2	P3	P4
Gender	M	M	M	F
Onset age(yrs)	–	–	–	25
Diagnostic age (yrs)	22	60	59	34
Phenotype	presymptomatic hyperCKemia	presymptomatic hyperCKemia	presymptomatic hyperCKemia	LGMD
Weakness	–	–	–	+
Cardiac problems	–	+	+	–
CK (U/L)	2400	2076	Elevated (Specific data not available)	3049
Muscle MRI (age at MRI(yrs))	GM(24)	NA	MAGN(59)	VM/BF/GM/ MAGN(34)
Pathology	NA	Mild	NA	Mild
Mutations	c.1969C>*T* p.Q657X	c.2596_2597del p.K866fs	c.2596_2597del p.K866fs	c.2498T>*G* p.M833R
	c.1103C > *T* p.T368M	c.1640G > *A* p.R547Q	c.1640G>*A* p.R547Q	c.2498T>*G* p.M833R

NA: not available; M male; F female; GM: Gastrocnemius medial; MAGN: adductor magnus; VM: vastus medialis; BF: biceps femoris.

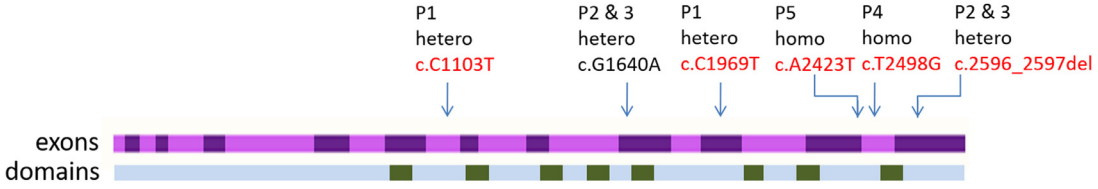

Fig. 1. Mutations of the 5 patients and their locations on *ANO5* gene. Dark and light purple indicate exons; green indicates transmembrane helical domains, and blue indicates cytoplasmic or extracellular topological domains. The red text indicates novel mutations.

and the corresponding amino acid was highly conserved. In view of the unspecific clinical presentation of patient P5 and the possibility of this individual having pathogenic variants in genes not investigated by our NGS panel, in absence of strong pathogenic evidence for the ANO5 variant found we classified this patient as with suspected anoctaminopathy.

3.2. Clinical presentation, serum CK and muscle MRI

The clinical presentations of these 4 confirmed cases include presymptomatic hyperCKemia in 3 cases(P1-P3) and limb girdle muscle weakness in 1 patient (P4). P3 is the younger brother of P2. One patient (P4) presented limb girdle muscle weakness, accounting for 0.33% (1/301) of the whole LGMD population. The clinical, pathological, and molecular data of the four patients with confirmed anoctaminopathy were summarized in Table 1.

For presymptomatic hyperCkemia cases like P1, P2 and P3, there was no complaint of weakness, myalgia or muscle cramps. Muscle strength was normal in all three patients and only mild calf hypertrophy was demonstrated in P2. P4 experienced progressive limb weakness for 9 years. Physical examination showed decreased muscle strength graded 4-/5 on Medical Research Council (MRC) scale of the hip extension, knee extension and flexion, as well as muscle atrophy of the quadriceps bilaterally (Fig. 2). No consanguinity was reported in the patients' families. Serum CK levels were elevated in all four patients.

For patient 2, elevated serum CK was noticed during the pre-operative tests for percutaneous coronary intervention (PCI). He reported chest distress and palpitation for 10 years due to stenocardia. Ventricular premature beats (571 beats), ventricular trigemini (49 times), atrial premature beats (5823 beats) and supraventricular tachycardia (4 times) were recorded during the Holter examination. Interventricular septum thickness, enlargement of the left atrium and moderate diastolic dysfunction of left ventricle were observed on echocardiogram. The patient had a clinical history of hypertension for 10 years and diabetes for 12 years with good control of blood pressure and glucose. Patient 3, the younger brother of Patient 2, had a medical history of atrial septal defect.

Muscle MRI revealed fatty infiltration in medial gastrocnemius (Fig. 3A-B), adductor magnus and adductor longus (Fig. 3C-D) for the presymptomatic cases like P1 and P3. For P4 with LGMD phenotype, fatty infiltration of vastus lateralis, vastus intermedius, adductor magnus and biceps femoris in thigh, and medial gastrocnemius in calf (Fig. 3E-F) was observed.

We also identified one case with suspected anoctaminopathy (P5). This was a 62-year-old male born to consanguineous parents, who experienced exercise intolerance with post-exercise muscle pain since childhood. Elevated serum CK was demonstrated in 2015 and it fluctuated from 1577 to 3049 U/L. He had difficulty in keeping supine at night from the age of 35. Physical examination revealed slight

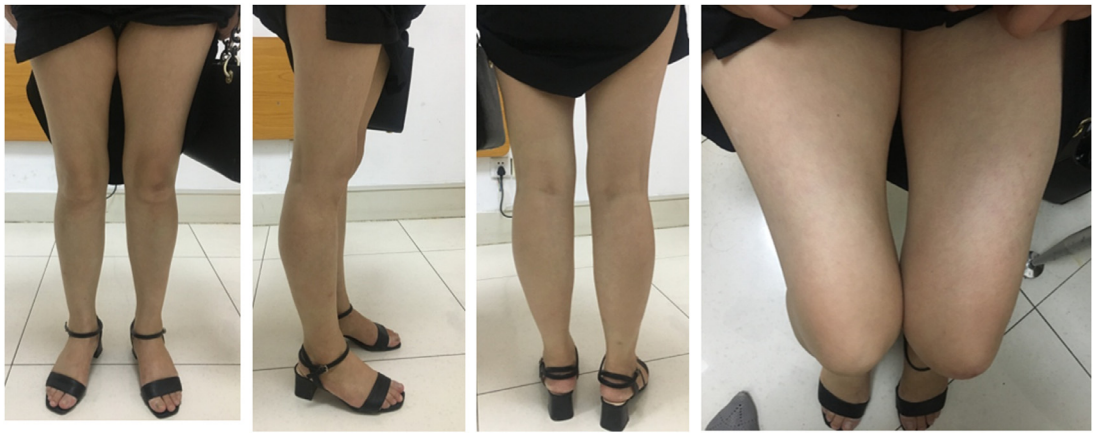

Fig. 2. Clinical aspects of patient 4 showed asymmetric atrophy of quadriceps with the right side being more prominent and atrophy of medial head of gastrocnemius.

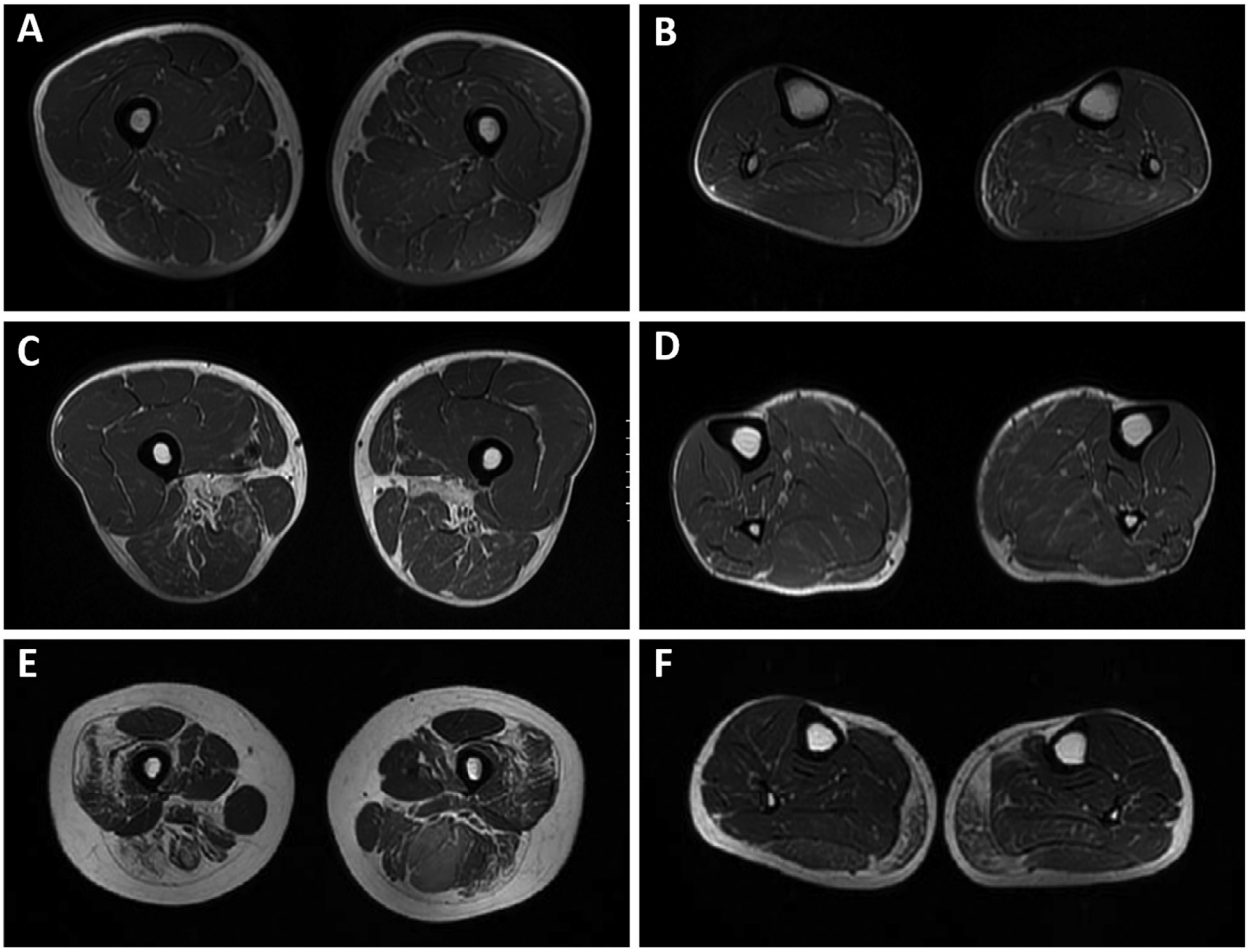

Fig. 3. Muscle MRI in lower limbs revealed mild fatty infiltration in medial gastrocnemius in Patient 1 (A: thigh, B: Calf), and adductor magnus and adductor longus involvement in Patient 3 (Fig. 3C-D). Fatty infiltration of vastus lateralis, vastus intermedius, adductor magnus and biceps femoris in thigh, and medial gastrocnemius in the calf was revealed in Patient 4 (Fig. 3E-F).

muscle atrophy in his left leg. Muscle strength was normal. EMG showed myogenic changes. ECG showed first-degree atrioventricular block and Echocardiogram showed diastolic dysfunction in left ventricle and enlargement of the left atrium. No other explanation for diastolic cardiomyopathy was revealed throughout his medical history.

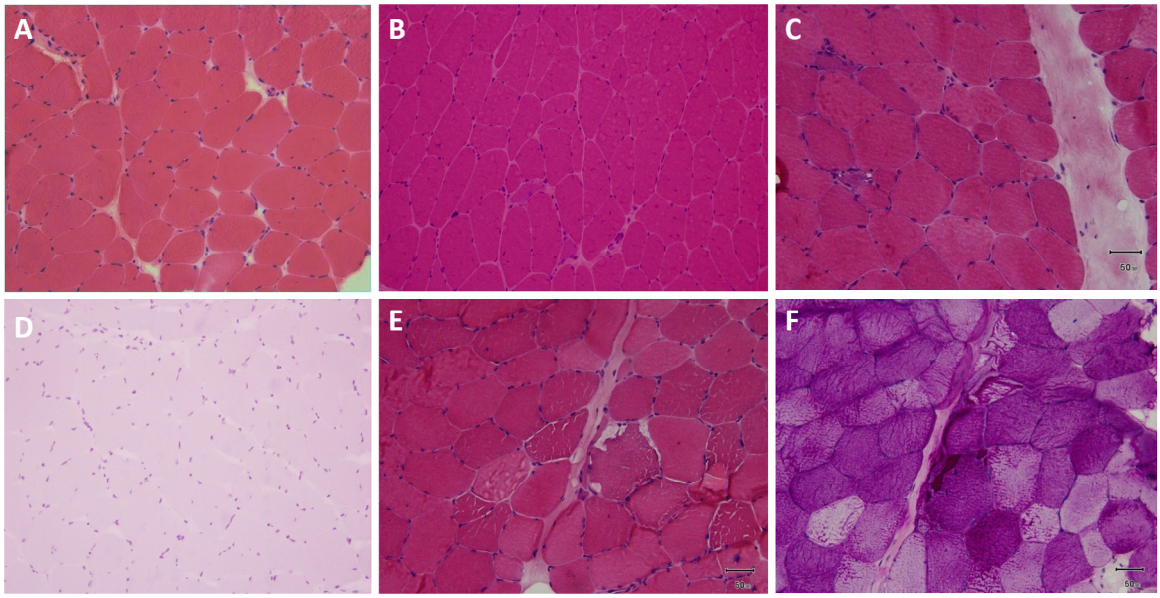

Fig. 4. Muscle biopsy of Patient 2,4 and 5. HE staining (×200) of patient 2(A), patient 4(B) and patient 5(C) showed mild myopathic changes. Congo red staining did not reveal any abnormal changes of patient 2 (×100) (D). HE staining (×200) (E) and PAS staining (×200) (F) of patient 5 showed rimmed vacuoles and submembranous vacuoles with positive PAS staining in a few muscle fibers.

3.3. Muscle pathology

Muscle biopsy was performed in P2, P4 and P5. Myopathic changes with mild muscle fiber size variation, scattered necrotic fibers, regenerating fibers and slightly increased internal nuclei were observed in muscle biopsies from all three patients. Congo red staining did not reveal any significant findings in muscle specimens in P2 and P4 (Fig. 4). Muscle biopsy of P5 revealed mild changes with rimmed vacuoles and submembranous vacuoles with positive PAS staining in a few muscle fibers. Immunostaining for dystrophin, sarcoglycan and dysferlin in these 3 patients were all normal.

4. Discussion

We report for the first time a Chinese cohort with anoctaminopathy. Four patients present with presymptomatic hyperCKemia and 1 case with LGMD2L. In our single neuromuscular disorder diagnostic center, LGMD2B and LGMD2A are the most prevalent forms accounting for 73.8% in overall LGMD cases. For the remaining 66 LGMD patients, we diagnosed 4 patients with Sarcoglycanopathies and 5 patients with LGMD2T, and one case for LGMD2I/2 J/2 M/2Q/2T, relatively. Current study identified 1 case with confirmed anoctaminopathy (P4), accounting for only 0.33% (1/301) of the whole LGMD cohort. The prevalence of *ANO5* muscular dystrophy varied in different regions. It was reported to be 10.5% in a Dutch population [12] and 11% in a Denmark population [13], most of whom presented LGMD or MMD3. In contrast, the prevalence of *ANO5* muscular dystrophy in an Italian cohort was 2.6% [5]. In other reports, a large proportion of patients with *ANO5* mutations presented with presymptomatic hyperCKemia or isolated cardiac manifestation without muscle weakness [7]. It is possible that the diagnosis of anoctaminopathy is biased towards more severe phenotypes and that a proportion of milder phenotypes remain undiagnosed.

Apart from the typical LGMD and MMD3 phenotypes, one of our genetically confirmed patients (P2) presented cardiac involvement. Cardiac involvement was reported to be part of the phenotypic spectrum in some cases, The higher prevalence compared to healthy age-matched controls and the occurrence of cardiac abnormalities in young patients of 30–43 years old made it reasonable that anoctaminopathy patients had increased risk of cardiac involvement [3,4,13,14]. It is also known that *ANO5* is highly expressed in cardiac muscle [15]. However, Cardiomyopathy has not been convincingly confirmed in anoctaminopathy. In our study, Patient 2 had long-term hypertension and diabetes type 2, probably responsible for his cardiac arrhythmia.

ANO5 genotypes are diversified among different ethnic groups. The mutation c.191dupA was reported to be the founder mutation of Northern European origin and had a very high prevalence in Dutch and Danish population. The frequency was much lower in non-north European populations such as Italy or United States [4,5]. It is reported to be cause a more severe phenotype [16]. In Asian populations, different mutations were reported: c.352delG and c.1025G > *A* in a Jordanian patient, c.1181–1811_c.1898+2287del resulting in an out-of-frame *ANO5* transcript in an Arabic patient and c.2394dup and c.1648C > *T* in two Japanese patients. In our study, the mutation c.1640G > *A* identified in Patient 2 and 3, was previously found in the British population [12]. Up to date, no recurrent mutation has been identified in Asian populations [8–11]. In our cohort, Patient 4 with

limb-girdle muscle weakness had a homozygous mutation c.2498T > *G* which was adjacent to the transmembrane domain (Fig. 3). All the other mutations from presymptomatic patients(P1-P3) were located inside the topological domains. Whether mutations near the transmembrane domain is more detrimental to *ANO5* expression and functioning, leading to severer phenotypes needs further investigation.

With regard to the muscle MRI of our patients with anoctaminopathy, fatty infiltration was confined to certain muscles at the early stage. In line with previous reports, adductor magnus was preferentially involved in Patient 3, while medial gastrocnemius was selectively involved in Patient 1 and Patient 3. At the more advanced stage as in Patient 4, fatty infiltration was more widespread on the thigh level, while remained selective on the calf level in medial gastrocnemius. This is in accordance with a 24-year follow-up study by Mahjneh et al. [17]. Highly selective adductor magnus fatty infiltration to a very late stage of the disease was also reported [7,10,11]. The most distinguishable imaging clue between anoctaminopathy and dysferlinopathy was selective involvement of adductor magnus [18]. Thus, the preferential involvement of adductor magnus and medial gastrocnemius might provide some diagnostic clues for anoctaminopathy.

In the current study, we concluded that LGMD2L was relatively rare in Asian LGMD population and there is no recurrent mutation so far identified in *ANO5* gene. In our cohort of Chinese patients with anoctaminopathy, presymptomatic hyperCKemia was more frequent than limb-girdle weakness. However, the number of confirmed cases is too small for a final conclusion of the phenotypic spectrum in Asian populations.

Acknowledgement

We would like to express our gratitude to all the participants and their family members involved in this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2019.06.005.

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