Limb-girdle muscular dystrophy type 2I is not rare in Taiwan

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Abstract

Alpha-dystroglycanopathy is caused by the glycosylation defects of α-dystroglycan (α-DG). The clinical spectrum ranges from severe congenital muscular dystrophy (CMD) to later-onset limb girdle muscular dystrophy (LGMD). Among all α-dystroglycanopathies, LGMD type 2I caused by *FKRP* mutations is most commonly seen in Europe but appears to be rare in Asia. We screened uncategorized 40 LGMD and 10 CMD patients by immunohistochemistry for α-DG and found 7 with reduced α-DG immunostaining. Immunoblotting with laminin overlay assay confirmed the impaired glycosylation of α-DG. Among them, five LGMD patients harbored *FKRP* mutations leading to the diagnosis of LGMD2I. One common mutation, c.948delC, was identified and cardiomyopathy was found to be very common in our cohort. Muscle images showed severe involvement of gluteal muscles and posterior compartment at both thigh and calf levels, which is helpful for the differential diagnosis. Due to the higher frequency of LGMD2I with cardiomyopathy in our series, the early introduction of mutation analysis of *FKRP* in undiagnosed Taiwanese LGMD patients is highly recommended.
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1. Introduction

Alpha-dystroglycanopathy is a group of muscular dystrophies caused by altered glycosylation of α-dystroglycan (α-DG), which is one of the components of dystrophin–glycoprotein complex [1,2]. The clinical phenotypes form a broad spectrum, ranging from severe congenital muscular dystrophy (CMD) with or without ocular and central nervous system involvement to later-onset limb girdle muscular dystrophy (LGMD) [3–5].

A number of genes have been reported to cause α-dystroglycanopathy, including *POMT1*, *POMT2*, *POMGnT1*, *FKTN*, *FKRP*, and *LARGE* that are known to be involved in glycosylation of α-DG, and *DAG1*, which encodes DG itself [6–11]. Recently, the number of genes associated with α-dystroglycanopathy has been increasing to include *ISPD*, *TMEM5*, *GTDC2*, *B3GNT1*, *DOLK*, *DPM2* and *DPM3* [12–19]. Patients with all

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kinds of α-dystroglycanopathy are inherited with autosomal recessive trait.

Among those causative genes for α-dystroglycanopathy, *FKRP* mutations are the most frequently seen in the Caucasian population, causing LGMD2I and congenital muscular dystrophy type 1C (MDC1C). In the Asian population, on the other hand, the most common α-dystroglycanopathy is Fukuyama congenital muscular dystrophy and LGMD2M caused by the mutations in *FKTN* [20–24]. This phenomenon may be caused by the founder effect of c.826C>A substitution in *FKRP* and the ancestral insertion of a SINE-VNTR-Alu (SVA) retrotransposon in *FKTN* in different geographic areas [21,25]. Recently, an increasing number of patients having *FKTN* mutations were identified outside Asia but so far few Asian patients with LGMD2I caused by *FKRP* mutations have been reported [26–29].

In this study, we found that LGMD2I is common in the Taiwanese patients with α-dystroglycanopathy due to a common mutation, c.948delC (p.Cys317Alafs∗111), which may cause more severe phenotype and cardiomyopathy.

2. Materials and methods

2.1. Patients

Forty patients clinically and pathologically diagnosed as LGMD and 10 patients with CMD who received muscle biopsy in Kaohsiung Medical University Hospital from January, 2008 to December, 2011 were enrolled. LGMD was defined as progressive proximal-dominant muscle weakness with characteristic dystrophic changes in muscle pathology. CMD was recognized as infantile floppiness with dystrophic muscle. Patients with deficiencies of dystrophin, sarcoglycans, dysferlin, merosin or collagen VI were excluded by immunohistochemistry beforehand. All merosin deficiency patients were confirmed to have *LAMA2* mutations [30]. This study was approved by the institutional review board of the Kaohsiung Medical University Hospital.

2.2. Histochemistry

Biopsied muscle specimens were frozen in isopentane cooled in liquid nitrogen. A serial frozen section was stained by a battery of histochemical methods including hematoxylin and eosin (H&E), modified Gomori-trichrome (mGt) and NADH-tetrazolium reductase (NADH-TR).

2.3. Immunohistochemistry

Frozen sections of 6 μm thickness were used for immunohistochemistry according to the standard protocols with Vantana Benchmark automated stainer. Primary antibodies used in this study were monoclonal anti-α-DG (VIA4-1; Upstate Biotechnology, Lake Placid, NY, USA) and anti-β-DG (43DAG1/8D5; Novocastra Laboratories, Newcastle upon Tyne, UK) antibodies.

2.4. Immunoblotting and laminin overlay assay

The detailed techniques of immunoblotting, and laminin overlay assay have been described previously [31]. The following antibodies were used for immunoblotting analysis: monoclonal anti-α-DG (VIA4-1) and polyclonal anti-α-DG (GT20ADG, kindly provided by Prof. K. Campbell, Iowa Univ.), polyclonal anti-laminin-1 (Sigma, St. Louis, MO, USA), and monoclonal anti-β-DG (43DAG1/8D5).

2.5. Mutation analyses of α-DGP associated genes

Genomic DNA was extracted from leukocytes in peripheral blood lymphocytes according to standard protocols. All exons and their flanking intronic regions of *FKRP* (NM_024301.4), *FKTN* (NM_001079802.1), *POMGnT1* (NM_001243766.1), *POMT1* (NM_007171.3), *POMT2* (NM_013382.5), and *LARGE* (NM_004737.4) were amplified and sequenced using an automated 3100 DNA sequencer (Applied Biosystems, Foster, CA, USA). Primer sequences are available upon request. DNA samples from 100 Taiwanese individuals without apparent neuromuscular disorders were analyzed as controls.

3. Results

3.1. Patients with α-DGP caused by FKRP mutations

Seven of 50 patients with unclassified LGMD and CMD had a reduced α-DG immunoreaction using VIA4-1antibody, which recognizes glycosylated forms of α-DG on muscles, and they were thus considered to have α-dystroglycanopathy (Fig. 1). Among these seven patients, six had LGMD phenotype and one was CMD. Mutation screening revealed that five LGMD patients from four families harbored *FKRP* mutations (Fig. 2). No mutation in *FKTN*, *POMGnT1*, *POMT1*, *POMT2* and *LARGE* was identified in these seven patients. The clinical, pathological and biochemical information of all five patients with *FKRP* mutations are summarized in Table 1 together with that of a previously reported Taiwanese LGMD2I patient who was the first reported case in East-Asia (Patient 6) [26]. The c.948delC (p.Cys317Alafs∗111) mutation was found heterozygously in four newly diagnosed patients (Patients 2, 3, 4 and 5) as well as in Patient 6. Patients 2, 3, and 4 carried a c.545A>G (p.TyrY182Cys) mutation, which was previously reported in two Brazilian patients, and a c.823C>T (p.Arg275Cys) mutation was identified in Patients 5 and 6. The compound heterozygous mutations for Patients 2, 5, and 6 were also found to lie on different parental alleles. Patient 1 bears two different novel
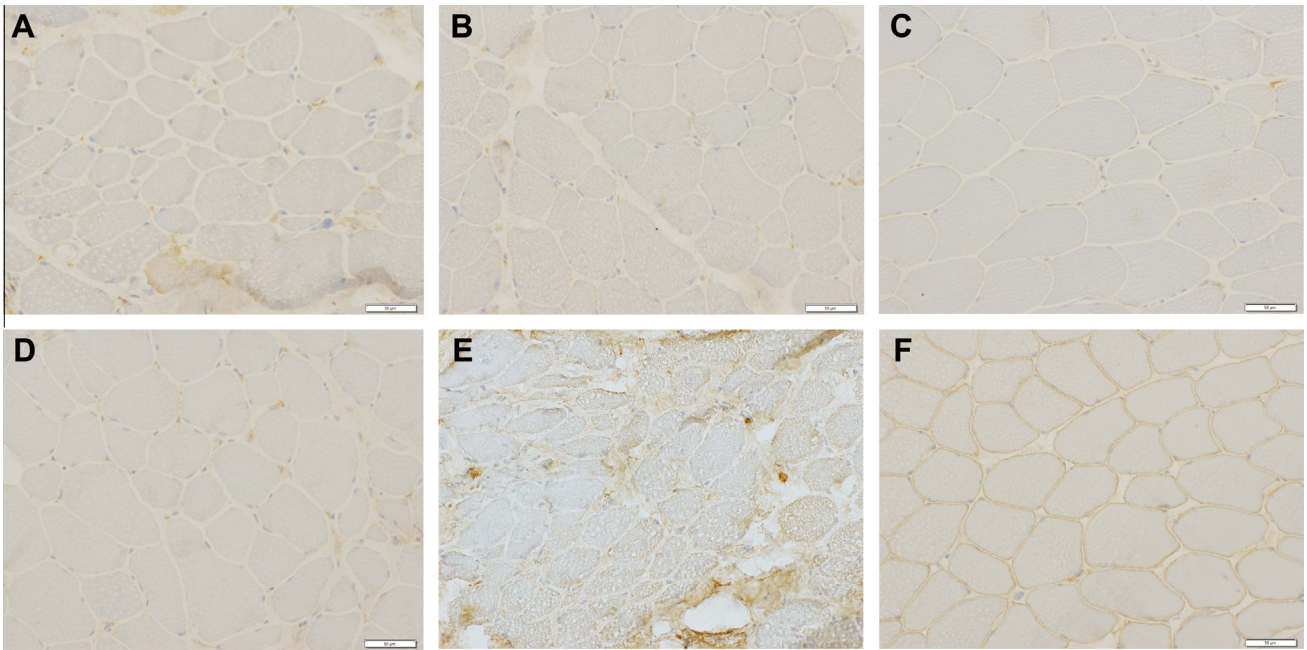
Fig. 1. Immunohistochemistry for α-DG (VIA4-I) in Patients 1, 2, 4, 5, and 6 (A–E). All patients' muscle samples showed markedly reduced staining, as compared with controls (F). Bar: 50 μm.
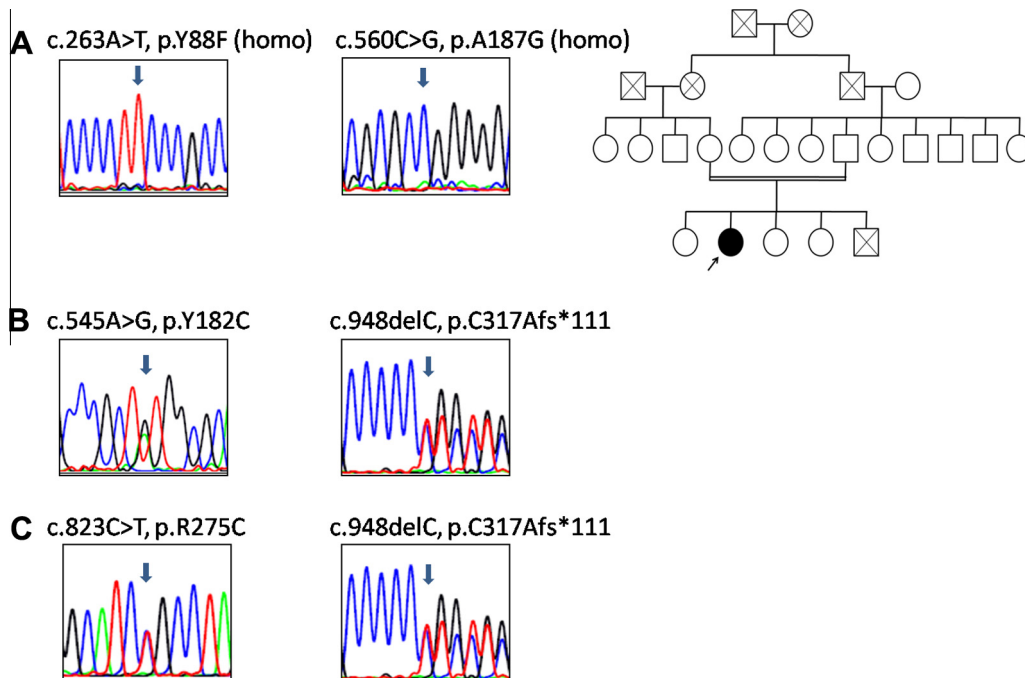

Fig. 2. Sequence analysis of *FKRP* revealed homozygous c.263A>T and c.560C>G mutations in Patient 1 (A), compound heterozygous c.545A>G and c.948delC mutations in Patients 2, 3, and 4 (B), and compound heterozygous c.823C>T and c.948delC mutations in Patients 5 and 6 (C). The pedigree of Patient 1 is also shown; the youngest brother of Patient 1 died of unknown causes at 7 months of age (A).

homozygous mutations, c.263A>T (p.Try88Phe) and c.560C>G (p.Ala187Gly), neither of which were identified in the human genome mutation database (HGMD) and 100 healthy individuals. The consanguineous healthy parents of Patient 1 carried these two missense mutations, heterozygously.

3.2. Reduced glycosylation of α-DG in LGMD2I patients

We further confirmed the altered glycosylation of α-DG in our LGMD2I patients (P2, 4, 5 and 6) using immunoblotting analysis and laminin overlay assay. On immunoblotting analysis using VIA4-1 antibody, skeletal

Table 1
Summary of clinical, pathological, biochemical and molecular analyses for the patients with α-dystroglycanopathy.

	P1	P2	P3[a]	P4[a]	P5	P6[b]
Sex/age (years)	F/35	M/16	M/31	M/30	M/10	F/23
Age of onset (years)	2	5	10	17	2	2
Calf hypertrophy	Y	Y	Y	Y	Y	Y
Cardiomyopathy (age of diagnosis, years)	Y (28) DCM	Y (14) DCM	Y (31) DCM	Y (30) DCM	N	Y (17) DCM
Loss of ambulatory ability (age, years)	N 6-min walk: 76 m	Y 6-min walk: 343 m	Y (29)	N 6-min walk: not done	N 6-min walk: 210 m	Y (12)
Cognition	Normal	Normal	Normal	Normal	Normal	Normal Brain MRI: negative finding
Other anomalies	Over-active bladder	N	N	N	N	Scoliosis with op
CK (IU/L)	1000–1500 max: unknown	4000–8000 max:>10,000	1500–2000 max: unknown	1500–2000 max: unknown	6000–9000	200–500 max: >10,000
Lung function	FVC: 32% FEV1: 33% PCF: 2.12 L/s	FVC: 45% FEV1: 53% PCF: 6.91 L/s	FVC: 43% FEV1: 36% PCF: 3.46 L/s	FVC: 62% FEV1: 73% PCF: 3.7 L/s	FVC: 64% FEV1: 76% PCF: 4.64 L/s	FVC: 10% FEV1: 12% PCF: 0.42 L/s BiPAP use at night
FKRP mutations	c.263A>T homo (F & M, hetero) c.560C>G homo (F & M, hetero)	c.545A>G (F, hetero) c.948delC (M, hetero)	c.545A>G c.948delC	c.545A>G c.948delC	c.823C>T (M, hetero) c.948delC (F, hetero)	c.823C>T (F, hetero) c.948delC (M, hetero)

Y: yes; N: nil; DCM: dilated cardiomyopathy; min: minute; m: meter; op: operation; max: maximum; F: Father; M: Mother.
[a] Siblings.
[b] Previously reported (Reference [21]).

muscles from all four patients showed fainter and smaller sized bands than the control (Fig. 3A). With GT20ADG antibody for the core region of α-DG, all skeletal muscles from these patients showed fainter broadbands with smaller molecular mass than that detected in the control (Fig. 3B). Laminin overlay assay displayed greatly reduced binding ability of α-DG to laminin in all patients (Fig. 3C).

3.3. Clinical findings of LGMD2I patients (Table 1)

The mean age of all 6 LGMD2I patients at examination was 24.2 ± 9.7 years, and the mean disease duration was 17.8 ± 9.1 years. The disease onset was variable, ranging from early childhood to late teens (2–17 years; 6.3 ± 6.1). All patients had calf hypertrophy and proximal dominant muscle weakness, starting from lower extremities and
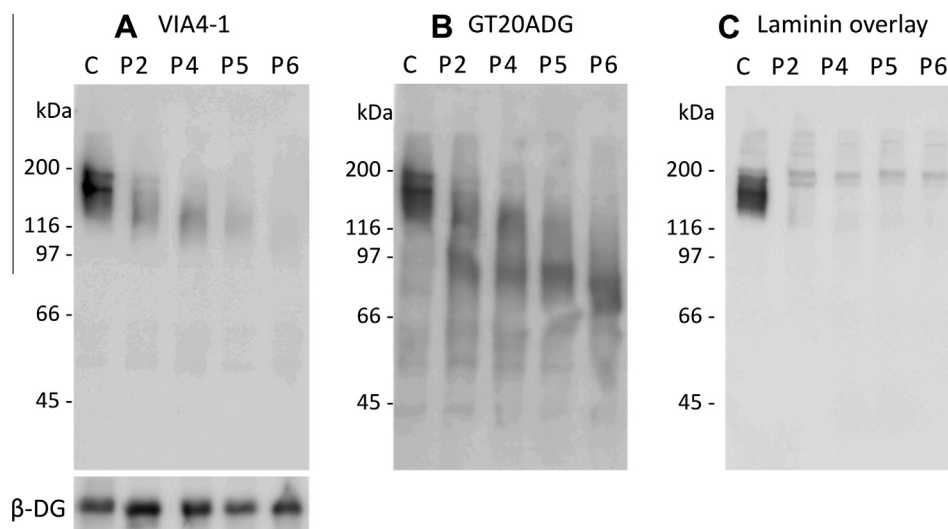

Fig. 3. Immunoblotting analysis. All 4 patients (P2, P4, P5 and P6) examined showed fainter and smaller sized bands than controls using α-DG (VIA4-1) (A). With the antibody of GT20ADG, fainter broadbands with smaller molecular weights were detected (B). Laminin overlay assay displayed greatly reduced binding ability of α-DG to laminin in all patients (C).

then extending to shoulder girdle and arms. Patient 2 became wheelchair-bound at the age of 29 years while Patient 6 lost her ambulatory ability at 14 years of age. Dilated cardiomyopathy (DCM) was seen in five of six patients (83.3%) and they are currently under medication. DCM was diagnosed with echocardiogram in Patients 2, 3, and 4 at their first visit to our hospital, so that the exact onset age of cardiac involvement was unclear. All patients had impaired pulmonary function with different degrees of severity but only Patient 6 required ventilator assistance (1 in 6; 16.7%). All patients had normal cognitive functions and the brain MRI of Patient 6 showed no notable abnormal changes. As for other abnormalities, only Patient 6 received an operation for scoliosis at 13 years of age. Serum creatine kinase levels were usually up to 10,000 IU/L at disease onset and then declined to hundreds at a later stage.

3.4. Muscle CT of LGMD2I patients

On muscle CT, all assessed patients (Patients 1–5) showed similar patterns of muscle involvement (Fig. 4) Lower extremities were more severely affected than upper extremities. Gluteus maximus was the most affected muscle (Fig. 4A), followed by posterior compartment of thigh muscles, among which biceps femoris and then adductors showed marked hypodensity (Fig. 4B). In the anterior compartment of thigh, vastus muscles and rectus femoris were equally involved. At the calf level, posterior compartment muscles, especially gastrocnemius and soleus, were also more affected than anterior part (Fig. 4C). As for upper extremities, involvement of shoulder girdle muscles including subscapularis, infraspinatus and supraspinatus was more prominent than trapezium and deltoid muscles (Fig. 4D).

4. Discussion

Wide variability in clinical picture has been reported in LGMD2I, of which the clinical features can be Duchenne muscular dystrophy-like, late-onset LGMD phenotypic and even asymptomatic [32,33]. In European countries, homozygosity of the most common missense mutation of c.826C>A (p.Leu276Ile) has been reported to confer a relatively milder phenotype than patients with compound heterozygous mutations [34]. A homozygous mutation of c.545A>G identified in the Brazilian patients has previously been reported to cause mild clinical phenotypes and disease course [32]. In our series, Patients 2–4 harbor the same compound heterozygous mutations of c.545A>G and c.948delC while Patients 5 and 6 both carry the same c.823C>T and c.948delC mutations. The patients carrying c.823C>T and c.948delC seem to show more severe clinical features than the patients having c.545A>G and c.948delC in terms of the age at onset, disease course, motor deterioration and complications. Because only a limited number of patients were included, however, additional patients with each mutation are required to clarify the phenotype and genotype correlation more clearly.
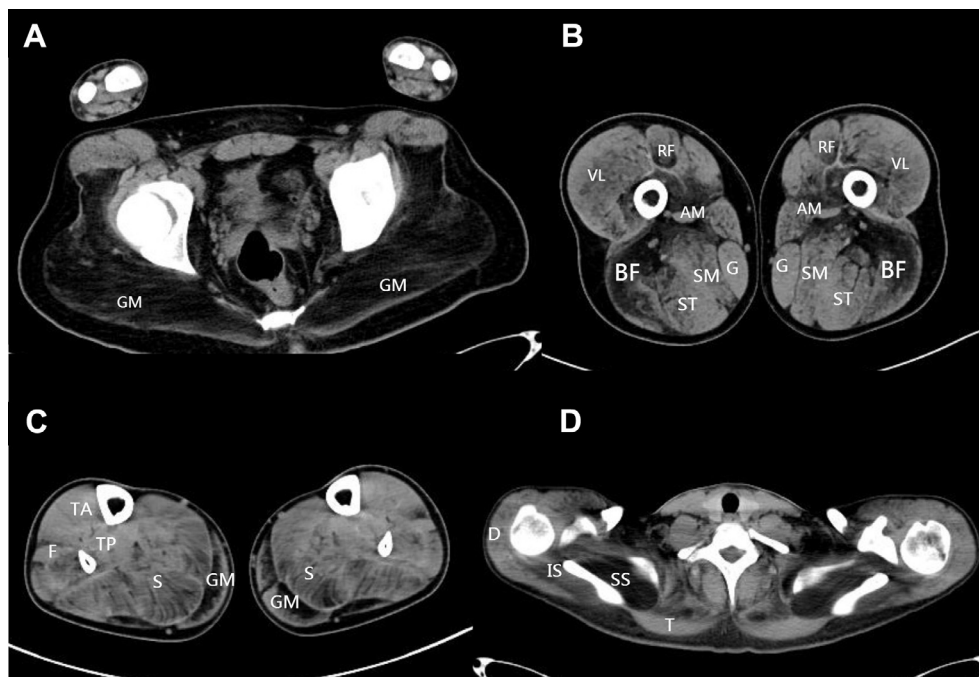

Fig. 4. Muscle CT on Patient 2. Gluteus maximus muscles were severely affected (A), followed by biceps femoris and adductors (B). At the calf level, gastrocnemius and soleus muscles were severely involved (C). In the upper extremities, involvement of subscapularis, infraspinatus and supraspinatus were more severe than trapezium and deltoid (D). (D: deltoid; IS: infraspinatus; SC: subscapularis; BF: biceps femoris; ST: semitendinosus; SM: semimembranous; S: soleus; F: fibularis; G: gastrocnemius; GM: gluteus maximus; RF: rectus femoris; VL: vastus lateralis; AM: adductor magus; G: gracilis).

Noteworthily, c.948delC in *FKRP* is a common mutation in Taiwanese LGMD2I patients. The mutation could cause frame shift and premature termination in translation (p.Cys317Alafs∗111). We further screened 300 controls without neuromuscular diseases to determine the carrier frequency of c.948delC but none carried this mutation. This result suggests that the prevalence of the homozygosity of c.948delC is at least lower than 1 in 360,000, which may be too low to identify a homozygous patient. On the other hand, this result may also indicate that the homozygosity of this frame shift mutation is too severe to survive, since none of the homozygous null mutations in *FKRP* has been reported to date and *FKRP* knockout mice also showed embryonic lethality [35].

Interestingly, two different homozygous mutations, c.263A>T (p.Tyr88Phe) and c.560C>G (p.Ala187Gly), were found in Patient 1, but not in 100 controls. Her parents were consanguineous (cousins) and both harbored these two mutations heterozygously. Compared the amino acid sequences of the FKRP protein among different species, p.Tyr88 is highly conserved in mammals while p.Ala187 is preserved among primates and some mammals, but not in rodents. Furthermore, predictions of functional effects of these two variants using software showed that p.Tyr88Phe change is probably damaging but p.Ala187Gly is benign in terms of functional impact (http://genetics.bwh.harvard.edu/pph2/index.shtml). Accordingly, c.263A>T (p.Tyr88Phe) is more likely to be pathogenic in Patient 1 although further functional studies are still necessary.

In our cohort, cardiomyopathy accounted for 83% of our patients, whereas about 10–55% of European LGMD2I patients were reported to have cardiac problems [36]. As for respiratory function, only one of our patients (Patient 6) was ventilator-dependent at night although the other five developed variable degrees of respiratory impairment. However, the proportion of respiratory aid requirement was slightly lower than other reports [20, 36–38], probably because the assessment age and disease duration of our patients were also lower. Similar to previously reported LGMD2I patients, none of our patients had overt mental retardation.

So far few papers have focused specifically on the muscle imaging of LGMD2I patients [37,39]. Based on previous related literature, gluteal muscles and posterior compartment of thigh muscles were more affected than anterior compartment in LGMD2I. In our report, similar muscle involvement was seen on CT images in which gluteal maximus was the most severely affected, followed by adductors and biceps femoris. Some of these changes may overlap with those seen in other common LGMD, especially LGMD2A [39], such as the early involvement of gluteal muscles and predominant involvement of posterior compartment. However, selective involvement of medial gastrocnemius and soleus and relative sparing of vastus lateralis are characteristic for LGMD2A [12,21,40], which suggests that muscle images are still helpful for a differential diagnosis. In addition, different clinical phenotypes including commonly-seen calf hypertrophy and cardiac involvement in LGMD2I and the presence of characteristic lobulated fibers on muscle pathology of LGMD2A are also important to make the differentiation. In our series, all patients showed calf hypertrophy and 83% had cardiac problems; lobulated fibers were not observed in skeletal muscle from any patient and molecular analysis of *CAPN3* revealed no mutation.

LGMD2I is one of the most prevalent LGMD in Europe but is very rare in Asia. Only one from Taiwan (P6), two from China and another Asian patient from North America have been reported on thus far [26–28]. Also in Japan, only one LGMD2I patient was identified by the National Center of Neurology and Psychiatry, which has the largest muscle repository in Japan. Therefore, our report discloses that LGMD2I is not rare at least in Taiwan. Considering that the glycosylation defect may be too mild to be detected by immunohistochemical screening, there must be more LGMD2I patients who are as yet undiagnosed in Taiwan. Larger scale mutation analysis for uncategorized LGMD patients may be necessary for an early diagnosis of LGMD2I to be made. One common mutation, c.948delC, in the Taiwanese population may be associated with higher frequency and early development of cardiomyopathy although a larger number of patients is required to make this conclusive. However, it is still suggested that clinicians should closely monitor the cardiac function of LGMD2I patients harboring this mutation from late childhood or their early teens.

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