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FKRP mutations cause congenital muscular dystrophy 1C and limb-girdle muscular dystrophy 2I in Asian patients

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Abstract

Mutation in the fukutin-related protein *(FKRP)* gene causes alpha-dystroglycanopathies, a group of autosomal recessive disorders associated with defective glycosylated alpha-dystroglycan (α-DG). The disease phenotype shows a broad spectrum, from the most severe congenital form involving brain and eye anomalies to milder limb-girdle form. *FKRP*-related alpha-dystroglycanopathies are common in European countries. However, a limited number of patients have been reported in Asian countries. Here, we presented the clinical, pathological, and genetic findings of nine patients with *FKRP* mutations identified at a single muscle repository center in Japan. Three and six patients were diagnosed with congenital muscular dystrophy type 1C and limb-girdle muscular dystrophy 2I, respectively. None of our Asian patients showed the most severe form of alpha-dystroglycanopathy. While all patients showed a reduction in glycosylated α-DG levels, to variable degrees, these levels did not correlate to clinical severity. Fifteen distinct pathogenic mutations were identified in our cohort, including five novel mutations. Unlike in the populations belonging to European countries, no common mutation was found in our cohort.

Keywords: fukutin-related protein, alpha-dystroglycanopathies, congenital muscular dystrophy type 1C, limb-girdle muscular dystrophy 2I

1. Introduction

Fukutin-related protein (*FKRP*) gene, located in chromosome 19q11.3, is ubiquitously expressed in human tissues with the highest levels detected in skeletal muscles and heart [1]. FKRP is a ribitol 5-phosphate transferase involved in the functional maturation of glycan chains on alpha-dystroglycan (α-DG) in the Golgi apparatus [2]. Glycosylated α-DG is a component of dystrophin-dystroglycan complex that maintains muscle stability by providing a physical linkage between the cytoskeleton and the extracellular matrix [3-5]; defect in α-DG glycosylation leads to the loss of normal interactions resulting in several forms of muscular diseases [1,6].

Alpha-dystroglycanopathy is an autosomal recessive disorder with muscular dystrophy associated with defective glycosylated α-DG. *FKRP* is considered a causative gene for alpha-dystroglycanopathy [1], and the clinical phenotype of alpha-dystroglycanopathy resulting from mutations in *FKRP* encompasses a broad spectrum. The most severe congenital form of *FKRP*-related alpha-dystroglycanopathy is the Walker–Warburg syndrome (WWS) or muscle-eye-brain disease (MEB), which includes brain and eye

anomalies [7,8]. Congenital muscular dystrophy type 1C (MDC1C) is a less severe form with or without mental retardation [9,10], while limb-girdle muscular dystrophy 2I (LGMD2I), also known as LGMDR9 according to reformed classification [11], is a milder form [12,13]. The variability in the severity of the *FKRP*-related disorders can be attributed to mutations in *FKRP*.

FKRP-related alpha-dystroglycanopathies are most frequently observed in the Caucasian population [14]. More than 200 different mutations have been listed in the Leiden database (www.dmd.nl). Among these, c.826C>A substitution in *FKRP* was reported to be frequent in the Northern European population and was associated with a milder phenotype of alpha-dystroglycanopathy [13,15,16]. In contrast to many reports of *FKRP* mutations in Europe, the number of reports from Asian countries is limited [17,18].

Here we report the clinical, pathological, and genetic findings of nine Asian patients of Japanese and Chinese origins with *FKRP* mutations.

2. Materials and methods

2.1. Patients

Patients tested at the National Center of Neurology and Psychiatry (NCNP), a major

referral center for muscle disease and the largest muscle repository in Japan, were enrolled in this study between January 1978 and May 2020. The patients who fulfilled the following inclusion criteria were enrolled: 1) absence or reduction of glycosylated α-DG by immunohistochemistry or western blotting analysis; 2) presence of pathogenic mutations in *FKRP*.

2.2. Histochemical and immunohistochemical analyses

A battery of routine histochemical stains and immunohistochemical analyses were performed for diagnostic purposes for the patients. The primary antibodies against glycosylated α-DG and β-DG used in this study were monoclonal anti-α-DG (VIA4-1, Merck KGaA, Darmstadt, Germany) and anti-β-DG (43DAG1/8D5, Leica Biosystems, Wetzlar, Germany), respectively. Immunohistochemistry was performed as described previously [19,20].

2.3. Immunoblotting and laminin overlay assay

Immunoblotting and laminin overlay assay were performed as described previously [17, 19,20]. Monoclonal anti-α-DG (VIA4-1, Merck KGaA), polyclonal goat anti-α-DG (GT20ADG, kindly provided by Prof. K. Campbell, University of Iowa), monoclonal

anti-laminin-α2 chain (5H2, Merck KGaA), and polyclonal anti-laminin-1 (Merck KGaA) were used for immunoblotting analysis.

2.4. Genomic DNA analysis

DNA was isolated from peripheral lymphocytes or muscle tissue specimens using standard techniques.

The nucleotide sequence of all exons and their flanking intronic regions in *FKRP* was determined by whole-exome sequencing, targeted gene panels [21], or Sanger sequencing using the primers reported previously [22]. Missense mutations were filtered by selecting those with an allele frequency <0.01 in the Human Genetics Variation Browser (HGVD; http://www.hgvd.genome.med.kyogo-u.ac.jp/), the Exon Aggregation Consortium (gnomAD: https://gnomad.broadinstitute.org/), and the NHLBI Exome Sequencing Project (ESP6500; http://evs.gs.washington.edu/EVS/). Mutation pathogenicity was determined according to the American College of Medical Genetics and Genomics (ACMG) guideline. In silico analysis of the pathogenicity prediction were performed by the following programs predicted pathogenicity: Mutation taster (http://www.mutationtaster.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and SIFT (https://sift.bii.a-star.edu.sg/).

Information on the mutations was obtained from the coding DNA reference sequence of *FKRP* (NM_024301.4) listed in the NCBI Reference Sequence (RefSeq) project.

2.5. Ethics

The ethics committee of the Graduate School of Medicine, Kobe University, and NCNP approved this study protocol (approval No. B190198, and A2019-079, respectively). Written informed consent for participation was obtained from either the patients or their parents. This study was conducted in compliance with the Declaration of Helsinki and the Ethical Guidelines for Human Genome and Gene Analysis Research.

3. Results

3.1. Patients with alpha-dystroglycanopathies caused by *FKRP* mutations

Nine patients positive for glycosylated α-DG and carrying *FKRP* mutation were enrolled in this study. None of the enrolled patients were related to each other. The clinical, pathological, biochemical, and genetic information of the patients is summarized in Table 1. Among these nine patients, three (Patients 1–3) and six (Patients 4–9) patients showed MDC1C and LGMD2I phenotypes, respectively.

3.2. Phenotypic features of the patients

The three patients with MDC1C were aged 6-14 years. Patient 1 was the third child of nonconsanguineous parents with no family history of neuromuscular disorders. He showed general hypotonia with facial muscle weakness, hip joint contracture at the age of 5 months, and severe Intellectual disability with polymicrogyria and white matter hyperintensity on brain magnetic resonance imaging (MRI). Ophthalmological examination revealed no abnormal finding on the lens, vitreous bodies, and retinas. Clinical summary of Patient 2 has been previously reported [23]. Patient 3 was the first child of nonconsanguineous healthy parents. From early infancy, she showed sucking difficulty and failure to thrive. By the age of 8 years, she was able to sit without support. Although abnormal brain MRI findings were observed from early infancy, she began to speak meaningful words by 11 months and framed two-word sentences by 18 months; she exhibited normal mental development until 13 years. Gastrostomy was performed at the age of 11 years due to dysphagia.

Six patients with LGMD2I were aged 3–33 years. One patient had Chinese ethnicity, whose parents were born in Mainland China, while the others were Japanese. Two patients were diagnosed with an unexpected elevation of serum creatine kinase level. The first symptom, either motor developmental delay, Gowers' sign, calf hypertrophy,

or difficulty with stair climbing, was observed between 14 months and 11 years. Although all acquired independent walk, three patients lost ambulation by early teenage owing to progressive muscular weakness. Four patients showed a reduction in forced vital capacity and one required non-invasive positive pressure ventilation (NPPV) from the age of 21 years [24]. Three patients showed cardiomyopathy from 14 to 20 years and received pharmacological therapy, including angiotensin-converting enzyme inhibitor and beta-blocker. None of the patients showed an intellectual disability.

3.3. Reduced glycosylated α-DG in patients with *FKRP* mutation

Immunohistochemical analysis revealed that immunoreactivity to VIA4-1, the monoclonal antibody for glycosylated α-DG, was absent or reduced in patients with *FKRP* mutation (Figure 1). Western blotting using VIA4-1 and GT20ADG was performed in all patients. Western blotting results of Patient 2 and 8 have already been reported [23,24]. The signals detected by VIA4-1 were decreased in all patients. GT20ADG binds to the core protein of α-DG. When α-DG was heavily glycosylated, the molecular mass in skeletal muscle corresponds to the 156 kDa band on the blot. Patients 6 and 9 had 156 kDa and 90 kDa bands with GT20ADG, suggesting a selective loss of immunoreactivity for glycosylated α-DG. The others found to lack a

well-glycosylated form of α-DG but showed a lower sized band (~70 to 140 kDa). Laminin overlay assay revealed that the binding between glycosylated α-DG and laminin was decreased in all patients.

3.4. Mutations in *FKRP*

A total of 15 distinct pathogenic mutations were identified in nine patients (Table 2). c.169G>A was the most detected mutation and was found in three alleles of two patients. Other identified mutations were individually unique.

Among the 15 mutations, five were novel and not found in the 1000 Genomes (https://www.internationalgenome.org/), Genome Aggregation (https://gnomad.broadinstitute.org/), or the NHLBI Exome Sequencing Project (https://esp.gs.washington.edu/drupal/) databases. c.778G>T were novel nonsense mutations causing premature stop codon. c.68_69delAT and c.540_570dup are frame-shift mutations that also induce premature translation termination. *In silico* analyses for novel missense mutations including c.157G>A and c.877A>G are shown in Table 2. At least one analysis predicted the pathogenicity of these mutations.

The remaining 10 mutations, c.169G>A, c.266C>G, c.501_502delinsCC, c.545A>G, c.692G>A, c.808C>T, c.878C>T, c.946C>T, c.1027G>T, c.1170_1171delCG, have been

reported previously. Compound heterozygosity for c.266C>G/c.1170_1171delCGand c.169G>A/c.692G>A identified in Patient 2 and 8, respectively was described in a previous stud [23,24]. Heterozygous c.808C>T missense mutation, identified in Patient 1 with MDC1C, was reported to be associated with LGMD2I [25]. c.1170_1171delCG was found in patients with both MDC1C and LGMD2I in the heterozygous state [23,26]. c.501_502delinsCC, a frame-shift mutation, was found in MDC1C patients [27]. c.169G>A [10], c.545A>G [28], and c.946C>T [29] are missense mutations, while c.1027G>T [30] is a nonsense mutation found in patients with LGMD2I in compound heterozygous form.

4. Discussion

This is the first of a series of case studies on patients with *FKRP* mutations in Japan, summarizing their clinical, immunohistochemical, protein-immunoblotting, and genetic features. We reported nine patients with alpha-dystroglycanopathies with varying severity, carrying *FKRP* mutations. We observed a variable reduction in glycosylated α-DG expression in the skeletal muscle of these patients. Further, 15 distinct pathogenic mutations, including five novel mutations, were identified in these patients. While common mutations have been reported in patients belonging to other ethnicities [10,

17,18], no common mutation was found in this cohort.

We identified two phenotypes of alpha-dystroglycanopathies, severe MDC1C and milder LGMD2I in our cohort; most severe WWS or MEB phenotypes were not detected. Although Patient 1 with MDC1C showed the most severe muscular weakness in our cohort, respiratory insufficiency requiring mechanical ventilation, profound mental retardation, and brain imaging anomalies that appeared to be compatible with WWS or MEB, he had no eye involvement, such as strabismus, anterior chamber abnormal, and retinal defect that are usually observed with severe phenotypes [7,31]. The varying severity of the patients with MDC1C in our cohort was similar to the phenotype of *FKRP*-related congenital muscular dystrophy [9,10,32]. Patients with LGMD2I have mainly been reported from non-Asian counties [16,33-36]. Recently, a few cases of LGMD2I have been reported in Asian countries [17,18,26,37]. In the present study, patients with LGMD2I showed highly variable presentation with different levels of motor, respiratory, and cardiac functions. Their disease onset and progression ranged from childhood-onset and Duchenne-like progression to adult-onset and slower progression with decades of walking ability. The incidence of cardiomyopathy was detected in 50% of our LGMD2I patients, consistent with 23%–83% incidence of cardiac problems reported in patients of other ethnicities [17,38,39]. The incidence of

respiratory dysfunction observed to different degrees of respiratory impairment in four out of six patients (66%) was higher than that reported previously (18%–44%) [33,38,39]. Intellectual disability that was not observed in our cohort is reportedly an uncommon complication in LGMD2I. However, a small number of patients with LGMD2I were reported to have cognitive impairment [15,34,36]. These results indicated that patients with LGMD2I in Japan exhibit varying severity of motor, cardiac, respiratory, and cognitive functions consistently with the disease phenotype of patients from other countries.

Several *FKRP* mutations are reportedly common to distinct populations. c.826C>A is the most common mutation detected in individuals from the United States and European countries [15,39,40]. Similarly, c.545A>G, c.948delC, c.1100C>T, c.1364C>A, c.1387A>G were also reported to be common mutations in Chinese [18], Taiwanese [17], South African Afrikaner [35], Tunisian [32], and Mexican [34] populations, respectively. In our cohort, Patient 4, born to Chinese parents, carried a common mutation seen in Chinese patients with LGMD2I. However, no common mutation was observed in all the Japanese patients in our cohort. c.169G>A, the most frequent mutation in our Asian population, was also reported in patients from the United States [28]. Nallamilli et al. categorized this mutation as a variant of uncertain significance

because it was the only mutation identified in a heterozygous state [28]. Based on ACMG guidelines [41], c.169G>A was considered to be likely pathogenic [24]. c.501_502delinsCC was first identified by Yoshioka et al. [27] in a heterozygous state in the Japanese patients with MDC1C, and the present study is the second to report this mutation in a homozygous state in Japanese patients. As this mutation has been found only in Japanese patients, it could be a founder mutation in the Japanese population. Further study is needed to confirm this in a larger cohort.

We identified five novel mutations in this study. Based on ACMG guidelines [41], we interpreted two protein-truncating mutations, c.778G>T and c.540_570dup, as pathogenic; two missense mutations, c.877A>G and c.68_69delAT, as likely pathogenic; and missense mutation, c.157G>A, as uncertain significance (Table 2). A comparison of FKRP protein sequences among different species (http://www.ncbi.nlm.nih.gov/homologene/) revealed that p.Y23, p.C191, and p.E260 are highly conserved in mammals, while p.V53 and p.293 are conserved in primates but not in rodents. As c.157G>A was interpreted as a significant uncertain mutation in the present study, further investigations, including identifying this mutation in other alpha-dystroglycanopathy patients and well-established in vitro or in vivo functional studies, are needed to determine the pathogenicity of this mutation.

Although in the present study, the patients showed a variable reduction in α-DG level in the skeletal muscles, detected by immunohistochemical analysis, the relationship between α-DG glycosylation and clinical severity was unclear. Two out of the three patients with MDC1C showed a complete absence of glycosylated α-DG. Even in the patient with a severe form of MDC1C, markedly reduced expression of glycosylated α-DG was observed. In patients with the milder LGMD2I, the degree of glycosylated α-DG expression was wide-ranging, from reduction to complete absence; Patient 8, who lost ambulation at the age of 12 years, showed reduced glycosylated α-DG at 28 years of age, while Patient 7, with no glycosylated α-DG expression at the age of 25 years, was able to walk. Similar findings were reported by Jimenez-Mallebrera et al. [42] and Alhamidi et al. [43], where the level of glycosylated α-DG (determined by immunohistochemistry) and clinical severity were not correlated in patients with MDC1C and LGMD2I. Several hypotheses, except for the level of glycosylated α-DG, have tried to explain the differences in clinical severity observed in *FKRP*-related disease. The effect of a mutation on protein function [44], deficiency in oligomerization [45], loss of Golgi localization [46], and endoplasmic reticulum stress triggered by accumulation of mutated FKRP [47] were proposed; however, none alone seem to explain the clinical variability thoroughly.

In summary, we found that *FKRP* mutations caused MDC1C and LGMD2I in our study cohort, but not the most severe forms WWS or MEB, indicating that the incidence of the most severe forms of alpha-dystroglycanopathy is less frequent in the Asian population. Five novel *FKRP* mutations were identified in our Asian cohort. We found no common *FKRP* mutations in Japanese patients. Therefore, comprehensive bioinformatics analyses, including targeted gene sequencing and whole-exome sequencing, are necessary to diagnose and evaluate a suspected case of alpha-dystroglycanopathy.

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Declaration of interest

All authors have no conflict of interest

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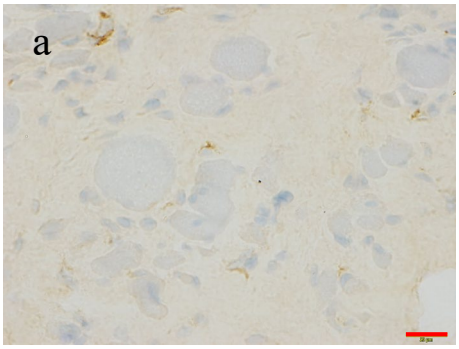
Figure legends

Figure 1. Immunohistochemical staining of skeletal muscles of (a–g) Patients 1-9 and control (j) with antibodies directed toward the glycosylated alpha-dystroglycan. Scale bars (yellow and red) indicate 50 μm and 20 μm, respectively.

Table legends

Table 1. Summary of the clinical features of patients with *FKRP* mutations. CK, creatine kinase; WB, western blot; IHC, immunohistochemistry; LOA, laminin overlay assay; ND, no data; VC, vital capacity; FEV, forced expiratory volume; NPPV, non-invasive positive pressure ventilation; M, male; F, female

Table 2. *FKRP* mutations were identified in this study and the results of *in silico* analysis for missense mutations. ACMG, American College of Medical Genetics and Genomics; MDC1C, Congenital muscular dystrophy type 1C; LGMD2I, limb-girdle muscular dystrophy 2I. Empty space indicates that no analysis was performed.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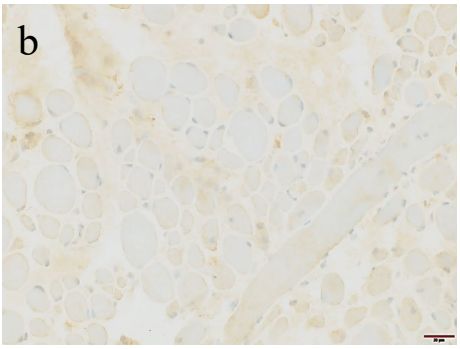
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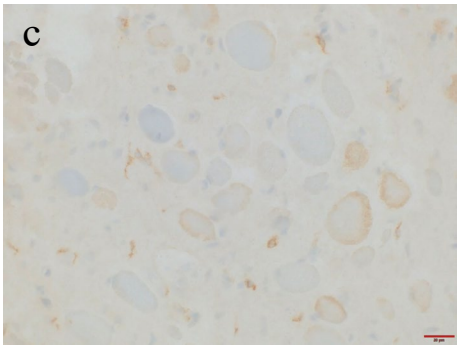
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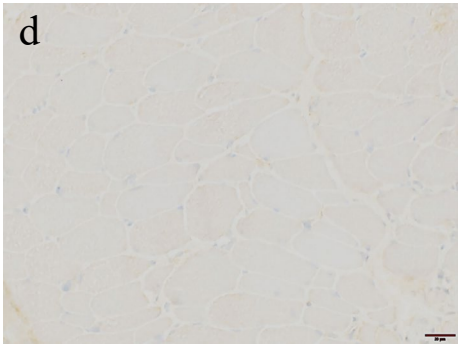
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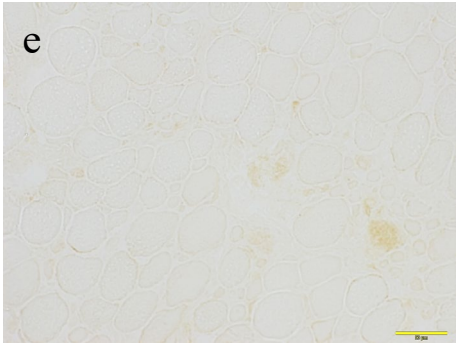
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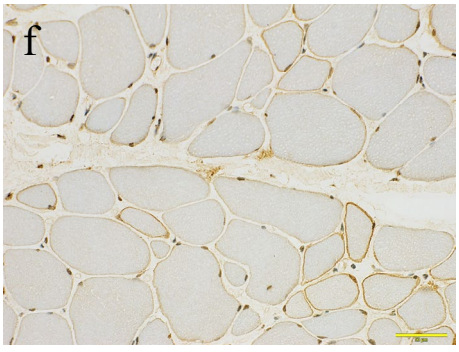
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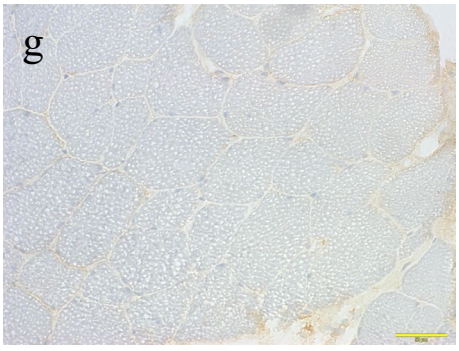
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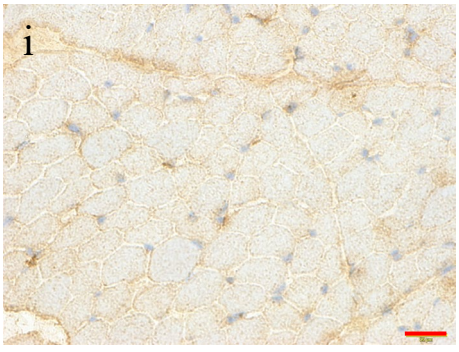
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