

Fukutin Gene Mutations Cause Dilated Cardiomyopathy with Minimal Muscle Weakness

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Objective: The fukutin gene (*FKTN*) is the causative gene for Fukuyama-type congenital muscular dystrophy, characterized by rather homogeneous clinical features of severe muscle wasting and hypotonia from early infancy with mental retardation. In contrast with the severe dystrophic involvement of skeletal muscle, cardiac insufficiency is quite rare. Fukuyama-type congenital muscular dystrophy is one of the disorders associated with glycosylation defects of α -dystroglycan, an indispensable molecule for intra-extra cell membrane linkage.

Methods: Protein and functional analyses of α -dystroglycan and mutation screening of *FKTN* and other associated genes were performed.

Results: Surprisingly, we identified six patients in four families showing dilated cardiomyopathy with no or minimal limb girdle muscle involvement and normal intelligence, associated with a compound heterozygous *FKTN* mutation. One patient died by rapid progressive dilated cardiomyopathy at 12 years old, and the other patient received cardiac implantation at 18 years old. Skeletal muscles from the patients showed minimal dystrophic features but have altered glycosylation of α -dystroglycan and reduced laminin binding ability. One cardiac muscle that underwent biopsy showed altered glycosylation of α -dystroglycan similar to that observed in a Fukuyama-type congenital muscular dystrophy patient.

Interpretation: *FKTN* mutations could cause much wider spectrum of clinical features than previously perceived, including familial dilated cardiomyopathy and mildest limb girdle muscular dystrophy.

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A group of disorders due to altered glycosylation of α -dystroglycan (α -DG), namely, α -dystroglycanopathy (α -DGP), is clinically characterized by a combination of muscular dystrophy, structural brain anomaly, and ocular involvement.^{1–7} Fukuyama-type congenital muscular dystrophy (FCMD) is the most common form of α -DGP in Japan, and the patients carry the founder mutation of 3kb retrotransposon insertion in the fukutin gene (*FKTN*), homozygously or heterozygously.^{7–9} Clinically, FCMD is characterized by severe congenital muscular dystrophy associated with mental retardation due to brain malformation.^{10–14} Most patients can speak only less than 20 meaningful words with no sentence formation. The peak motor function is seen from the age of 2 to 8 years, and their maximal motor ability

is usually unassisted sitting or sliding on the buttocks. A number of the patients never acquire head control. Few patients can obtain independent ambulation, but would soon lose this ability.^{14–16} The prognosis is poor with their mean life span of less than 20 years. The FCMD patients with a compound heterozygous mutation of 3kb insertion and missense mutation often show more severe clinical features compared with the patients with a homozygous 3kb insertion mutation.⁹ Reportedly, there has been only two non-Japanese patients harboring null mutation in *FKTN* gene in both alleles.^{17,18} Both were Turkish boys whose clinical features were quite severe resembling Walker–Warburg syndrome, which include generalized hypotonia, hy-

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Table. Clinical Summary of the Patients

Characteristics	Patient No.					
	1 ^a	2 ^a	3 ^a	4 ^a	5	6
Age (yr)/Sex	30/M	33/F	22/F	12/M	54/F	30/F
Age at onset of DCM (yr)	17	20	11	12	46	30
LVEF ^b	14%	40%	25%	14%	31%	32%
LVDD (mm)	69	59	64	75	78	63
LVDs (mm)	59	46	57	72	64	52
Age at biopsy (yr)	18	Not done	8	12 (autopsy)	46	30
Serum CK (IU/L)	400–3,000	1,500–1,800	2,000–4,000	High CK	1,200–2,000	1,139
Motor ability	Ambulant	Ambulant	Ambulant	Ambulant	Ambulant	Ambulant
Calf hypertrophy	Positive	Negative	Positive	Negative	Positive	Positive
Gowers' sign	Positive since age 24 yr	Negative	Negative	Negative	Positive since age 41 yr	Positive since 26 age yr
Intelligence	Normal	Normal	Normal	Normal	Normal	Normal
Eye abnormality	None	None	None	None	None	None
<i>FKTN</i> mutation	3kb insertion p.Q358P	3kb insertion p.Q358P	3kb insertion p.R179T	3kb insertion p.R179T	3kb insertion p.R179T	3kb insertion p.R179T

^aPatients 1 and 2 are siblings; Patients 3 and 4 are siblings.

^bLeft ventricular ejection fraction (LVEF): normal > 60%.

DCM = dilated cardiomyopathy; LVDD = left ventricular end-diastolic dimension; LVDs = left ventricular end-systolic dimension; CK = creatine kinase.

drocephaly, bilateral ocular abnormalities, and cataracts. They died during early infancy.

Here we report on six Japanese patients in four unrelated families with a compound heterozygous *FKTN* mutation presenting with dilated cardiomyopathy. All six patients show no muscle weakness until adulthood without any mental retardation. Our findings expand the phenotypic spectrum of *FKTN* mutations from severe congenital muscular dystrophy to dilated cardiomyopathy with mildest limb girdle muscular dystrophy.

Patients and Methods

Clinical Materials

All clinical materials used in this study were acquired with informed consent. The muscle samples were taken for diagnostic purpose and flash-frozen in isopentane chilled with liquid nitrogen.

Immunohistochemistry, Immunoblotting, and Laminin Overlay Assay

The detailed techniques of immunohistochemistry, immunoblotting, and laminin overlay assay have been described previously.^{7,19} The following antibodies were used for immunohistochemical and immunoblotting analyses: monoclonal anti- α -DG (VIA4-1; Upstate Biotechnology, Lake Placid, NY), polyclonal anti- α -DG (GT20ADG),²⁰ monoclonal anti-laminin α 2 chain (5H2; Chemicon, Temecula, CA), polyclonal anti-laminin-1 (Sigma, St. Louis, MO), and monoclonal anti- β -DG (43DAG1/8D5; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom).

Mutation Analyses of *FKTN*, *FKRP*, *POMGnT1*, *POMT1*, *POMT2*, and *LARGE*

DNA was isolated from peripheral lymphocytes or muscles using a standard technique. To detect the 3kb retrotransposon insertion in *FKTN*, we performed the genomic polymerase chain reaction using two primer sets, as described previously²¹; one is designed to amplify a 400bp product containing a part of retrotransposon insertion, and the other is designed to amplify a normal 269bp fragment. All exons and their flanking intronic regions of *FKTN* were sequenced directly in patients without homozygous retrotransposon insertion using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems, Foster City, CA).

Mutation analysis of *fukutin-related protein (FKRP)*, *protein O-mannose β 1,2-N-acetylglucosaminyltransferase 1 (POMGnT1)*, *protein O-mannosyltransferase 1 and 2 (POMT1 and POMT2)*, and *LARGE* was also performed by directly sequencing all exons and their flanking introns. Sequences of all the primers used in this study are available on request.

Results

Clinical Findings

Detailed clinical features and mutation in *FKTN* of six patients from four families are described in the Table.

Patient 1 is a 30-year-old man. He was a table tennis player with good health until 17 years of age. At this time, he started experiencing dyspnea and was diagnosed with idiopathic dilated cardiomyopathy in congestive heart failure. Echocardiography demonstrated dilated hypokinetic left ventricle with an ejection fraction of 14% (see the Table). Although he did not have

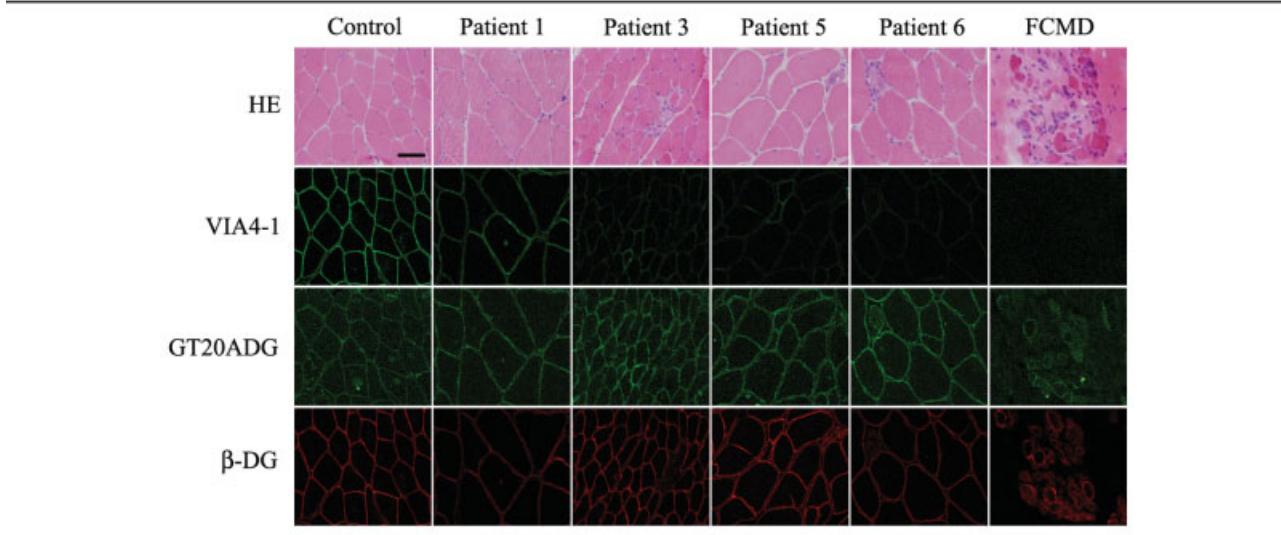


Fig 1. Hematoxylin and eosin staining (HE) and immunohistochemical analysis. On HE, mild variation in fiber size and a few regenerating fibers are seen in all four patients (Patient 1, 3, 5, and 6). Muscle from Fukuyama-type congenital muscular dystrophy (FCMD) shows severe dystrophic changes with dense deposition of fibrous and adipose tissues. Immunohistochemical analysis using antibodies VIA4-1, which recognize heavily glycosylated form of α -dystroglycan (α -DG), showed reduced sarcolemmal staining in all four patients and barely detectable in FCMD compared with control muscle. Well-preserved immunoreactivities of GT20ADG for core peptide of α -DG and β -DG are seen in all muscle specimens. Bar = 50 μ m.

any muscle weakness, elevation of serum creatine kinase (CK) levels ranging from 400 to 800IU/L (reference range, 51–197IU/L) led to muscle biopsy, showing mild variation in fiber size and internal nuclei in some fibers (Fig 1). His cardiac insufficiency progressed rapidly, necessitating heart transplantation, which he finally received at 18 years of age. After that, he had an uneventful clinical course until 24 years old when he noticed slowly progressive proximal muscle weakness of lower extremities. At 30 years of age, he shows calf hypertrophy, Gowers' sign, and mild waddling gait. Serum CK levels are elevated, ranging from 2,500 to 3,000IU/L. Muscle computed tomography shows atrophy in bilateral hamstring, gluteus, biceps brachii, and paravertebral muscles.

Patient 2 is a 33-year-old older sister of Patient 1. She enjoyed tennis during her college years. At the age of 20, cardiomegaly was suggested by chest radiograph on health checkup. She was followed up with no medication administered. She had no clinical symptoms until 27 years of age, when she developed rapidly progressive cardiac failure during pregnancy. She underwent induced abortion. Thereafter, her cardiac functions recovered and she has remained asymptomatic even at present.

Patient 3 is a 22-year-old woman. She was born term after an uneventful pregnancy from nonconsanguineous healthy Japanese parents. Her developmental milestones were at par with age. Elevation of serum CK level was incidentally found when she had a bout of flu at 8 years old. Calf hypertrophy was likewise

noticed. She subsequently underwent muscle biopsy showing a few necrotic and regenerating fibers in addition to fiber size variation (see Fig 1). At age 11, she was diagnosed to have cardiomyopathy, but she was still able to join volleyball team in her junior high school. She completed her university studies and currently is employed as an office worker. She can run, climb stairs, and does not have any limitation in her daily activities. She has no muscle weakness including facial muscles, but has calf hypertrophy. She does not have scoliosis and joint contractures. Serum CK levels are elevated from 2,000 to 4,000IU/L. Electrocardiogram shows right bundle branch block. Echocardiography shows left ventricular enlargement with decreased ejection fraction, suggestive of dilated cardiomyopathy. Despite detailed ophthalmological examinations, no abnormalities were found. Muscle computed tomography suggests mild fatty infiltration in hamstrings, gastrocnemius, and gluteus maximum muscles. Brain magnetic resonance imaging is normal except for one cerebellar cyst and probable focal pachygryia in the left occipital lobe.

Patient 4, older brother of Patient 3, was healthy and did not have either muscle weakness or calf hypertrophy. He was a swimmer during his early childhood. At 12 years old, he developed progressive dyspnea and was subsequently diagnosed to have cardiomyopathy. Despite intensive treatments, he died of cardiac failure after 1 month. Autopsy showed severe dilated cardiomyopathy with lymphocytic infiltration and fibrosis.

Patient 5 is a 54-year-old housewife. She was born

after an uneventful pregnancy from nonconsanguineous healthy Japanese parents. She had normal developmental milestones. At 40 years old, premature beats were observed on electrocardiogram. She also noticed difficulty in squatting. When she was 46 years old, she was admitted to a hospital because of dyspnea and was diagnosed to have dilated cardiomyopathy by echocardiography and endomyocardial biopsy. Mild proximal dominant muscle weakness without facial muscle involvement was also observed. Deep-tendon reflexes were normal. Serum CK level was elevated to 825IU/L. Muscle biopsy showed mild dystrophic changes with variation in fiber size, fibers with internal nuclei, and a few regenerating fibers (see Fig 1).

Patient 6 is a 30-year-old woman. She was a slow runner with calf hypertrophy since childhood, but no apparent muscle weakness was experienced. At 26 years old, she had an uneventful pregnancy. Postpartum, she noticed difficulties in walking while holding her baby. At 29 years old, she became unable to walk for long distances. At 30 years old, she was diagnosed with dilated cardiomyopathy from the results of echocardiography and an endomyocardial biopsy, which showed mild fibrosis with lymphocytic infiltration. She also developed proximal dominant muscle weakness and waddling gait. Serum CK level was increased to 1,139IU/L. Muscle biopsy showed mild dystrophic changes with a few necrotic fibers and mild fiber size variation (see Fig 1).

Immunohistochemical, Immunoblotting Analyses, and Laminin Overlay Assay

Immunohistochemical analysis was performed using biopsied skeletal muscles. Immunoreaction of α -DG (VIA4-1), which recognizes heavily glycosylated form of α -DG, demonstrated reduction of sarcolemmal staining from all four patients, whereas the antibody for the core peptide of α -DG (GT20ADG) and β -DG showed well-preserved membrane staining (see Fig 1). Muscle specimens from FCMD patients showed barely detectable immunoreaction for α -DG (VIA4-1), as described previously (see Fig 1).¹ No difference was seen between muscles from FCMD patients with a homozygous 3kb insertion and more severe patients with a compound heterozygous mutation of missense and 3kb insertion in *FKTN*. On immunoblotting analysis using VIA4-1, skeletal muscles from the four patients showed a fainter and smaller sized band than control muscle, whereas a muscle sample from FCMD showed no detectable band, as reported previously (Fig 2A).¹ By using GT20ADG, muscles from patients showed fainter broad bands, whose molecular masses were bigger than the approximately 90kDa band observed in FCMD muscles having either homozygous or compound heterozygous mutation. A 43kDa immunore-

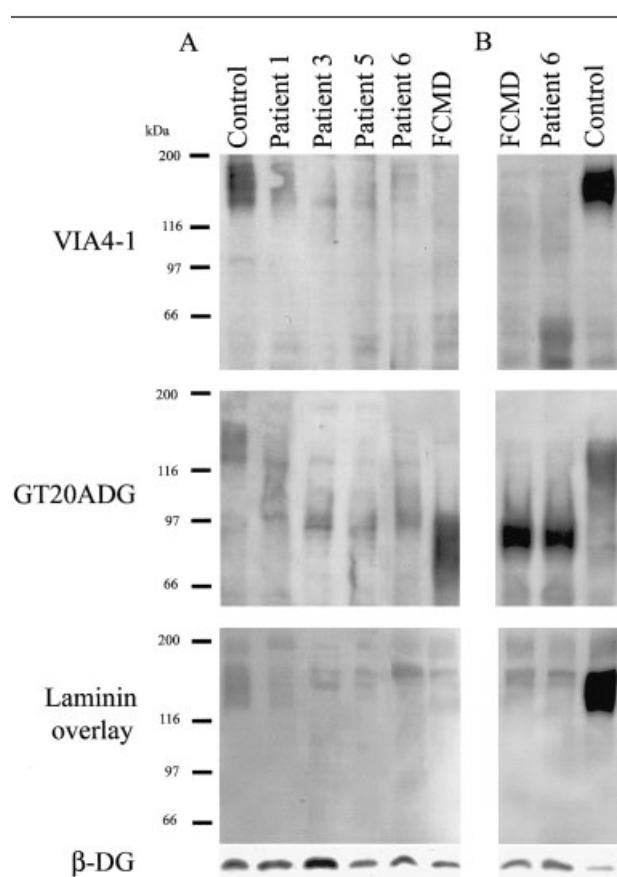


Fig 2. Immunoblotting analysis. (A) Immunoblotting analysis using antibodies of VIA4-1, GT20ADG, and β -dystroglycan (β -DG) and laminin overlay assay are performed using skeletal muscle from control, four patients (Patients 1, 3, 5, and 6), and Fukuyama-type congenital muscular dystrophy (FCMD). Both antibodies for α -DG (VIA4-1 and GT20ADG) recognize a broad band about 156kDa in size in control muscle. In the four patients' muscles, α -DG bands are reduced in size and amount compared with control. No detectable band is seen in FCMD muscle by VIA4-1; a band at approximately 90kDa was detected by GT20ADG. Laminin overlay assay shows greatly reduced but positive binding ability in the four patients, whereas it is not detectable in FCMD muscle. (B) Immunoblotting analysis of the biopsied cardiac muscle from Patient 6, autopsied control, and FCMD. Both Patient 6 and FCMD show similar reduced sized (approximately 90 kDa) band when compared with control.

active band for β -DG showed no difference between samples. Laminin overlay assay showed reduced but positive binding ability of α -DG to laminin in all four patients.

Immunoblotting analysis of biopsied cardiac muscle from Patient 6 showed no detectable band for α -DG by VIA4-1, whereas an approximately 90kDa band was observed by GT20ADG, which is similar to that observed in an autopsied FCMD heart carrying a homozygous 3kb insertion in *FKTN*. Laminin overlay as-

say showed no detectable band in both Patient 6 and FCMD (see Fig 2B).

Mutation Analysis

All patients had a compound heterozygous mutation of 3kb retrotransposal insertion and missense mutation in *FKTN* (see the Table). Patients 1 and 2 had the same compound heterozygous mutation of 3kb insertion and c.1073A>C (p.Q358P). Their healthy mother had a 3kb insertion, and the healthy father carries p.Q358P mutation, in heterozygous mode. Patients 3, 4, 5, and 6 had the same compound heterozygous mutation of 3kb insertion and c.536G>C (p.R179T). The father of Patients 3 and 4 had a 3kb insertion, and the mother had a p.R179T mutation, both in heterozygous mode. These two missense mutations were not detected in 100 chromosomes from Japanese healthy control individuals. The amino acids of both mutated positions are highly conserved among various species including chimpanzee, cow, mouse, rat, chicken, and fish (data not shown).

No mutation was found in the other responsible genes for α -DGP including *FKRP*, *POMGnT1*, *POMT1*, *POMT2*, and *LARGE* in these patients.

Discussion

FCMD is one of the most severe congenital muscular dystrophies with central nervous system involvement. Severe mental retardation and epilepsy are characteristic clinical features of FCMD, with brain showing polymicrogyria/pachygryia caused by altered neuronal migration. Muscle weakness is severe, and the patients show floppiness from infancy. Most patients cannot achieve independent ambulation and became bedridden by the second decade of life. Marked dystrophic changes of skeletal muscle with dense deposition of fibrous and adipose tissues are characteristic since early infancy. In contrast with the severely affected skeletal muscle, clinical manifestations of cardiac impairment are quite rare. Only a few patients were reported to have cardiac insufficiency, although fibrosis of myocardium is frequently seen in the autopsy studies.²²

The clinical features of the patients reported here are completely different from those seen in FCMD. All six patients show quite normal intelligence with no epileptic episodes. Muscle weakness is extremely mild, if any, and appears only in adulthood. Muscle computed tomography/magnetic resonance imaging showed mild signal changes in posterior thigh and hip muscles. These findings are also dissimilar from FCMD patients, because calf muscles are more severely affected than thigh muscles in FCMD patients.¹⁶ Pathological findings in the biopsied skeletal muscles showed only minimal dystrophic changes, which, again, is quite different from FCMD.

α -DG is a highly glycosylated surface membrane

protein, and its molecular mass in normal skeletal muscle is about 156kDa. In FCMD muscles, regardless the type of mutation or clinical severity, altered glycosylation of α -DG reduces its molecular mass to approximately 90kDa, resulting in a lack of laminin binding ability.^{1,20} Biopsied muscles from the patients described here, however, showed larger sized α -DG than FCMD muscles with reduction of laminin binding ability. These findings are similar to those observed in muscles from mild limb girdle muscular dystrophy type 2I (LGMD2I) patients caused by mutations in *FKRP*.² Minimal muscle involvement, if any, with partly glycosylated α -DG suggests that the mutated fukutin may preserve part of its function in skeletal muscle.

Cardiac involvement is the most remarkable finding in our patients. All the patients showed dilated cardiomyopathy, and two of them had life-threatening, rapidly progressive cardiac insufficiency. Cardiac involvement is rarely described in patients with α -DGP except for some patients with LGMD2I.^{23–25} In these patients, cardiac symptoms are not always correlated with severity of skeletal muscle weakness, and one family showed dilated cardiomyopathy without any muscular dystrophy.²⁶ It is unclear why the severity of affected organs is different, but one possible explanation is that the patients with no or minimal muscle weakness can run and perform exercise as normal individuals. Vigorous physical activities could induce stress and accelerate degeneration of cardiac muscles, resulting in progressive cardiomyopathy. The difference of glycosylation of α -DG between cardiac and skeletal muscles is also notable, although detailed composition of the sugar moiety in each tissue is still unknown. In contrast with the skeletal muscle, the molecular mass of α -DG in the biopsied cardiac muscle from Patient 6 was similar to that from FCMD patient. The mutant fukutin caused by missense mutations in *FKTN* identified in this series may have a different effect on glycosylation of α -DG between skeletal and cardiac muscles. To date, the molecular mechanism of cardiomyopathy associated with *FKTN* mutation is still unclear. Further extensive analyses are needed to clarify the roles of fukutin in the glycosylation process of α -DG in the different organs.

Our data indicate that the clinical spectrum caused by *FKTN* mutations is much wider than previously perceived, from the most severe form of congenital muscular dystrophy with central nervous system involvement to dilated cardiomyopathy with mildest limb girdle muscular dystrophy. Careful follow-up of cardiac function is necessary for the patients with α -DGP. More importantly, patients with cardiomyopathy should also be examined for skeletal muscle involvement, including determination of serum CK levels. Several responsible genes for dilated cardiomyopathy have been identified; however, mutations are successfully identified in only a fraction of the patients.

It is important to consider the mutation in *FKTN* for diagnosis of familial dilated cardiomyopathy, although it is rarely found outside of Japan.

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