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Journal of the Neurological Sciences 219 (2004) 89–93

Journal of the Neurological Sciences

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# FSHD-like patients without 4q35 deletion

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Received 27 June 2003; received in revised form 10 October 2003; accepted 19 December 2003

**Abstract**

Facioscapulohumeral muscular dystrophy (FSHD) is characterized by progressive weakness and wasting of facial, shoulder-girdle and upper arm muscles. Despite of the characteristic clinical features, the diagnosis of FSHD is sometimes difficult because clinical symptoms are extremely variable including facial sparing type, limb-girdle type, and distal myopathy type. Most of the FSHD patients have a deletion in the subtelomeric region of chromosome 4q35 (FSHMD1A), however the linkage analysis in some families suggested genetic heterogeneity. In the present study, we identified 40 patients without a deletion in the 4q35 region (non-4q35del) among 200 Japanese patients who were clinically suspected to have FHSD. All non-4q35del patients had shoulder-girdle weakness and 75% also had facial weakness. Eight patients showed clinical features that were indistinguishable from FSHD, but two of them had Becker muscular dystrophy. FSHD is clinically, and most likely genetically, as well, variable. Other forms of muscular dystrophy can also mimic FSHD.



*Keywords:* Facioscapulohumeral muscular dystrophy (FSHD); Southern blotting; Chromosome 4q35; *Eco*RI fragment; Deletion; Becker muscular dystrophy

## 1. Introduction

Autosomal dominant facioscapulohumeral muscular dystrophy (FSHD) is the third most common form of muscular dystrophy. The gene locus has been mapped to the subtelomeric region of chromosome 4q35 and most patients show a deletion of this region (FSHMD1A; MIM 158900), although the responsible gene for FSHD has not yet been identified. Southern blotting analysis using probe p13E-11 reveals a short *Eco*RI fragment in the 4q35 region, which contains the 3.3-kb *Kpn*I repeats (D4Z4) [1–8]. The homologous tandem repeats of 3.3-kb *Kpn*I units are also present on chromosome 10q26 and crosshybridized by p13E-11. However, a *Bln*I restriction enzyme site within each repeat from 10q26 allows differentiation of the two loci without haplotype analysis [9].

Clinical features of FSHD are characterized by progressive weakness and wasting of facial, shoulder-girdle and upper arm muscles. Most patients subsequently show involvement of peroneal and pelvic girdle muscles as well, and eventually 20% of the patients are wheelchair bound by the age of 40 years [10,11]. Nevertheless, clinical diagnosis of FSHD is sometimes difficult, because the phenotypic expression, even within the same family, is extremely variable, ranging from severe disability to being almost asymptomatic [12]. Facial muscles are spared in some patients, and others may show limb-girdle or distal dominant muscle involvement.

During genetic analysis of the FSHD patients, we found a certain number of patients who did not have a short *Eco*RI fragment on chromosome 4q35 although they had a clinical diagnosis of FHSD. In the present study, we focused on these patients and compared their clinicopathological find-

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0022-510X/$ - see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.jns.2003.12.010

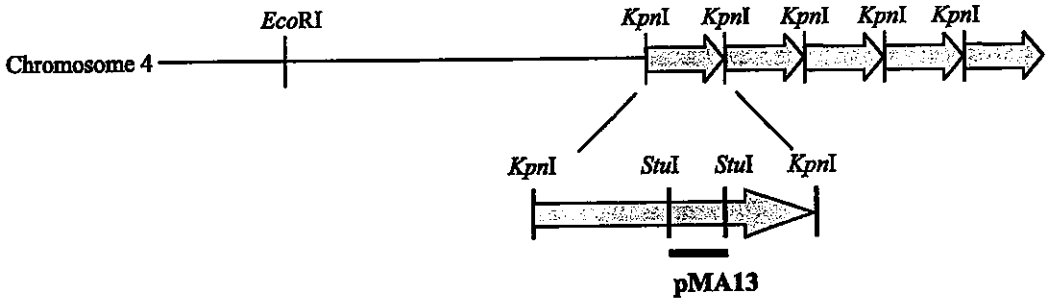


Fig. 1. The location of the probe pMA13. pMA13 is a 1.3kb *Stu*I fragment within each repeated *Kpn*I unit (D4Z4) on chromosome 4q35.

ings with those of patients with genetically confirmed FSHMD1A.

## 2. Materials and methods

### 2.1. Clinical materials

We studied 200 unrelated Japanese patients clinically suspected to have FSHD on examination by at least one neurologist. Since the clinical features of FSHD is quite variable, we labeled the patients as 'typical-FSHD-type' in the present study when all of the following four clinical criteria were satisfied: (1) weakness initially in facial or shoulder-girdle muscles, (2) progressive weakness or wasting of facial and shoulder-girdle muscles, (3) sparing of extra-ocular, masticatory, pharyngeal, lingual and cardiac muscles, (4) absence of severe or diffuse joint contractures.

Ninety-six unrelated healthy individuals of age over 60 years were also examined as control, since these older individuals had low risk to become symptomatic FSHD patients.

### 2.2. Genetic analysis

We obtained informed consent from all the patients and controls. Genomic DNA was isolated from peripheral blood lymphocytes using standard methods. To distinguish between the *Eco*RI fragments from 4q35 (*Bln*I-resistant) and from 10q26 (*Bln*I-sensitive), the DNA was digested with *Eco*RI and *Eco*RI/*Bln*I for conventional analysis and *Eco*RI/*Hin*dIII and *Eco*RI/*Bln*I for pulsed field gel electrophoresis (PFGE). After separation by both conventional gel electrophoresis and PFGE, we performed Southern blot analysis using two probes of p13E-11 and pMA13. The probe pMA13 is a 1.3 kb *Stu*I fragment within each repeated *Kpn*I unit (Fig. 1), which can identify patients with a deletion in the region of probe p13E-11, and also detect the exact size of the *Eco*RI fragment in patients with hybrid repeats of 4q35 and 10q26.

We also performed multiplex PCR analysis for the dystrophin gene, as previously described [13,14].

### 2.3. Histopathological analysis of muscle biopsies

Muscle biopsies from nine patients with 'non-4q35del' were frozen in liquid nitrogen-cooled isopentane. Serial frozen sections were stained with a battery of histochemical methods and immunostained with the following monoclonal antibodies: anti-dystrophin (NCL-DYS1, DYS2 and DYS3, Novocastra Laboratories, Newcastle upon Tyne, UK), anti-α-sarcoglycan (LNC-a-SARC, Novocastra Laboratories), anti-α-dystroglycan (VIA4-1, Upstate Biotechnology, Lake Placid, NY, USA), anti-β-dystroglycan (43 DAG1/8D5, Novocastra Laboratories), anti-laminin α2 chain (5 H2, Chemicon International, CA, USA), anti-caveolin-3 (BD Transduction Laboratories, KY, USA) and anti-dysferlin (NCL-Hamlet2, Novocastra Laboratories).

## 3. Results

We analyzed 96 healthy individuals over 60 years old by Southern blot analysis using the probe p13E-11, as control.

Table 1
Comparison of clinical features of FSHMD1A and non-4q35del patients

| | Weakness | | | | Regression of symptoms[a] | Sever joint contracture[a] | Initial symptom | | | | High CK[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Facial | Shoulder-girdle | limb-girdle | e, m, p, t[a] | | | Facial | Shoulder-girdle | Facial and shoulder-girdle | Limb-girdle[a] | |
| FSHMD1A | 96.0 | 100 | 86.0 | 12.5 | 0 | 0 | 35.6 | 49.3 | 9.4 | 5.6 | 5.0 |
| Non-4q35del | 75.0 | 100 | 90.0 | 35.0 | 2.5 | 0 | 12.5 | 47.5 | 0 | 22.5 | 1.3 |

e, extra-ocular; m, masticatory; p, pharyngeal; t, tongue.
[a] Exclusion criteria of FSHD.
[b] Serum CK level over 1000 IU\log.

Table 2
Clinical features of eight non-4q35del patients showing typical-FSHD-type

| Patient no. | SP/F | Age/sex (year) | Onset (year) | Initial symptom | Muscle weakness | | | Asymmetry | Progression | CK (IU/l) | *Eco*RI (kb) | Other clinical features |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | f | s | l | | | | | |
| 1 | SP | 24/M | 8 | s | + | + | + | + | + | ND | 38 | arrhythmia |
| 2 | SP | 24/M | 10 | f | + | + | + | + | + | 665 | 40 | |
| 3 | F | 20/M | 7 | s | + | + | + | + | + | 738 | 48 | RD |
| 4 | SP | 13/M | 11 | s | + | + | + | – | + | 69 | >50 | HL |
| 5 | F | 25/M | 15 | s | + | + | ND | + | + | 956 | >50 | |
| 6 | SP | 63/F | 40 | s | + | + | + | + | + | 455 | >50 | HL, RD |
| 7[a] | SP | 35/M | 5 | s | + | + | + | – | + | 1266 | 36 | |
| 8[a] | F | 29/M | 7 | f | + | + | + | + | + | ND | >50 | |

SP/F, sporadic/familial; M, male; F, female.
f, facial muscle (orbicularis oculi, peri-oral muscles); s, shoulder-girdle muscle; l, limb-girdle muscle.
*Eco*RI, *Eco*RI fragment size from 4q35; ND, not described.
HL, hearing loss; RD, respiratory disturbance.
[a] BMD.

We found five individuals who had a *Eco*RI digested fragment from 4q35 less than 40 kb; one had a 35 kb, one had a 36 kb, and three individuals had a 38 kb fragment. From this result, we designated our patients who had a short *Bln*I-resistant *Eco*RI fragment less than 35 kb on chromosome 4q35 as 'FSHMD1A', and the remaining were as 'non-4q35del', in the present study.

Among 200 patients clinically suspected to have FSHD, 160 patients had a short *Eco*RI fragment (<35 kb) on chromosome 4q35 and FSHMD1A was the diagnosis. The fragment size varied from 10 to 27 kb. The clinical features of the 160 patients are summarized in Table 1. In these patients, 126 (79%) showed all the typical-FSHD-type features, while 34 patients showed only some of the four criteria of typical-FSHD. Interestingly, 29 patients (18%) had symptoms usually considered to be exclusion criteria of FSHD [15]: extra-ocular (one patient), masticatory (four patients), pharyngeal (seven patients), lingual (13 patients) and onset in limb-girdle muscles (nine patients), which indicated that these exclusion criteria are not always reliable for ruling out the diagnosis of FSHD. No FSHMD1A patient showed regression of symptoms, severe joint contractures or cardiomyopathy.

By both of conventional and pulsed-field gel electrophoresis, 40 among 200 unrelated patients (20%) did not have a short *Eco*RI fragment from 4q35 of less than 35 kb, although they were clinically suspected to have FSHD (non-4q35del). The other probe pMA13 detected the same size fragments in all patients. The mean age at onset of the 40 non-4q35del patients was 19.2 years. Eight patients had family members with similar clinical symptoms. All patients showed shoulder-girdle muscle weakness. Ten patients (25%) did not have facial weakness (Table 1). Four patients had hearing loss, but none of the patients had retinal vasculopathy. Asymptomatic arrhythmia (five patients), mild respiratory disorders (11 patients), swallowing disturbances (four patients) were also found. The creatine kinase (CK) levels varied from 8 to 11,118 (normal ≦ 152 IU/l). There were myopathic changes in 94% and myopathic changes with scattered small angular fibers in 6% of the muscle biopsies. Nine muscle specimens were examined immunohistochemically, and showed no abnormality for all

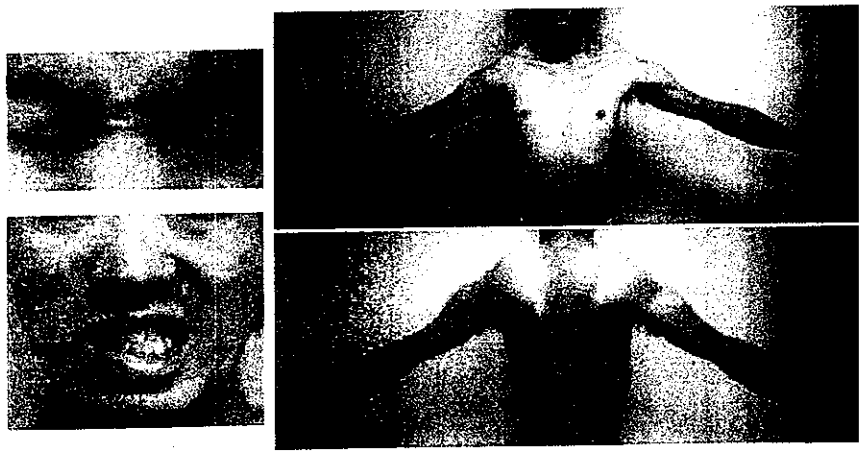

Fig. 2. A 24-year-old patient with typical-FSHD-type clinical features without a short *Eco*RI fragment (Pt.2 in Table 2). He first noted an asymmetrical smile and difficulties in lifting his arm with scapular winging at 10 years of age. Five years later, limb-girdle muscles were also involved.

the antibodies we used, except for one which showed a deficiency of caveolin-3. Electromyography (EMG) showed myopathic changes in 81% and mixed changes in 11%.

Eight patients in the non-4q35del group showed clinical features seen in the typical-FSHD patients, even though they had no short *Eco*RI fragment (Table 2). The age of onset was under 20 years except for one patient (Pt. 6) who became symptomatic at age 40 years. In all eight patients, facial muscles were involved and weakness of the shoulder and upper limb muscles was progressive (Fig. 2). The initial symptom in two patients was facial muscle involvement, while the other six patients first noted shoulder-girdle muscle weakness. Serum CK levels were mildly elevated in all except one patient (Pt. 4) whose level was within the normal range. To our great surprise, two of eight patients were found to have BMD by multiplex PCR analysis for the dystrophin gene (Fig. 3).

The remaining 32 patients without a short *Eco*RI fragment did not satisfy all of the four clinical criteria of typical FSHD. All had weakness of the shoulder-girdle muscles, but facial muscle involvement was present in 65%. There also were involvement of extraocular (nine patients), masticatory (four patients), lingual (three patients) and pharyngeal (three patients) muscles. By protein and gene analyses or electrophysiological analysis, we found that one patient had Emery-Dreifuss muscular dystrophy (AD-EDMD), another had limb-girdle muscular dystrophy type 1C (LGMD1C) and the third myasthenia gravis (MG).

Although shoulder-girdle muscles were involved in all 200 patients examined, facial muscle weakness was less common in non-4q35del group (75%) compared with FSHMD1A (96%). Although patients in both FSHMD1A and non-4q35del groups had exclusion criteria symptoms, they were more common in patients with non-4q35del (18 patients; 45%) than in FSHMD1A (29 patients; 18%). There was no marked difference in the histopathological and electrophysiological findings, except for one FSHMD1A patient who showed no abnormality on these examinations.

## 4. Discussion

Typical clinical symptoms of FSHD are characterized by a unique pattern of muscle involvement, which usually progresses in a descending manner, including weakness and atrophy of facial muscles, followed by shoulder-girdle, the scapula fixators, and the upper arm muscles. The clinical diagnosis, however, is sometimes difficult because of the extremely variable clinical features from the early onset severe form to clinically asymptomatic individuals. Several unusual clinical presentations have been reported including facial sparing type, limb-girdle type, and distal myopathy in genetically confirmed cases of FSHMD1A [16,17]. In the present study, 3.7% of genetically confirmed FSHMD1A patients showed facial sparing, and 18% presented with symptoms of the so-called exclusion criteria of FSHD [15], confirming the clinical variability of FSHMD1A.

On the other hand, 8 of 200 (4%) patients showed clinically typical-FSHD-type, although they had no deletion in the FSHD gene region. Interestingly, two of them turned out to have BMD by genetic analysis. The initial symptom was weakness of facial or shoulder-girdle muscles, but not limb-girdle muscles. Calf hypertrophy and cardiac involvement were not seen. One patient showed elevation of serum CK level (1266 IU/l). It should be noted that BMD patients can show clinical features indistinguishable from FSHD.

The remaining six clinically typical-FSHD patients without a short *Eco*RI fragment showed no abnormality by immunohistochemical and genetic analyses. It has been reported that approximately 5% of FSHD families fail to

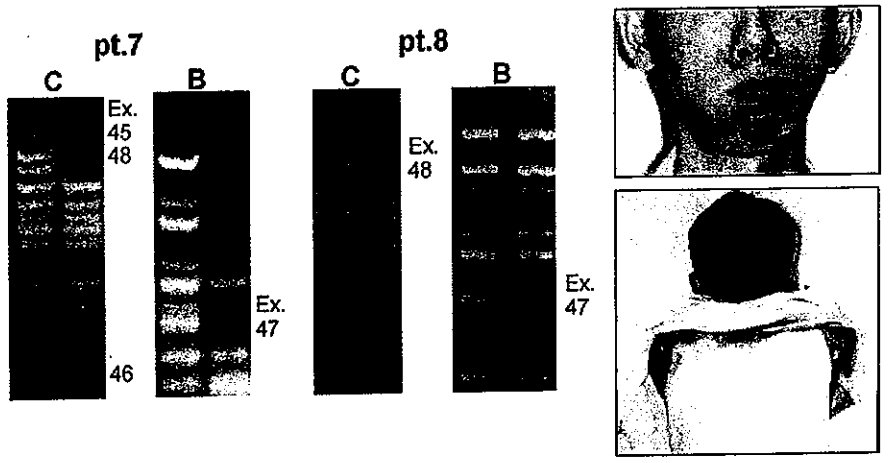


Fig. 3. Multiplex PCR analysis for the dystrophin gene of two typical-FSHD-type patients without a short *Eco*RI fragment. Pt.7 had a deletion from exons 45 to 48, and Pt.8 showed a deletion of exons 47 and 48. Asymmetrical facial muscle involvement and also atrophy of shoulder-girdle muscles were observed in Pt. 8. Two primer sets by Chamberlain (C) and Beggs (B) were used [13,14].

exhibit linkage to 4q35 [18]. These results suggest the genetic heterogeneity of this disease.

Since the responsible gene for FSHD has not been identified, genetic diagnosis is based on Southern blot analysis. The size of the *Eco*RI fragment is a critical determinant of clinical severity. Patients with the smallest size (10–11 kb) showed severe and wide varieties of clinical features [19–21]. On the other hand, several families were recently reported to have typical clinical features of FSHD with a *Eco*RI fragments greater than 35 kb [22,23]. These findings may expand the range of the *Eco*RI fragment size diagnostic of FSHD, but recent report showed the difficulty to define the molecular diagnostic cut-off point between FSHMD1A patients and the control population [24]. In the present study, we found five healthy individuals with a short *Eco*RI fragment of less than 40 kb. On the other hand, five non-4q35del patients had *Eco*RI fragment between 35 and 40 kb, and three of them showed typical-FSHD phenotypes, including BMD patient with 36 kb (Pt.1, 2 and 7 in Table 2). However, we did not label them as having FSHMD1A, since we could not perform linkage analysis because of the small size of the families. The role of the shortened D4Z4 repeats should be clarified to understand the pathomechanism of this complicated disease.

## Acknowledgements

We thank Dr. S.M. Sumi, University of Washington, for his helpful discussion and advice. This work was supported by the Research Grants-in-Aid for Nervous and Mental Disorders, Research on Psychiatric and Neurological Diseases from the Ministry of Health, Labour and Welfare, and Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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J. Biochem. 135, 709–712 (2004)
DOI: 10.1093/jb/mvh086

# Subcellular Localization of Fukutin and Fukutin-Related Protein in Muscle Cells

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Received March 29, 2004; accepted April 13, 2004

**Fukuyama-type congenital muscular dystrophy and congenital muscular dystrophy 1C are congenital muscular dystrophies that commonly display reduced levels of glycosylation of α-dystroglycan in skeletal muscle. The genes responsible for these disorders are *fukutin* and *fukutin-related protein* (*FKRP*), respectively. Both gene products are thought to be glycosyltransferases, but their functions have not been established. In this study, we determined their subcellular localizations in cultured skeletal myocytes. FKRP localizes in rough endoplasmic reticulum, while fukutin localizes in the *cis*-Golgi compartment. FKRP was also localized in rough endoplasmic reticulum in skeletal muscle biopsy sample. Our data suggest that fukutin and FKRP may be involved at different steps in *O*-mannosylglycan synthesis of α-dystroglycan, and FKRP is most likely involved in the initial step in this synthesis.**

**Key words: *cis*-Golgi, fukutin, fukutin-related protein, rough, endoplasmic reticulum, skeletal muscle.**

Abbreviations: DG, dystroglycan; ER, endoplasmic reticulum; FCMD, Fukuyama-type congenital muscular dystrophy; FKRP, fukutin-related protein; MDC1C, congenital muscular dystrophy 1C; POMT1, protein *O*-mannosyltransferase 1.

Congenital muscular dystrophy comprises a genetically heterogeneous group of disorders. Fukuyama-type congenital muscular dystrophy (FCMD), the most common congenital muscular dystrophy in Japan, is characterized by muscle weakness and hypotonia from early infancy, and is associated with mental retardation and a brain anomaly called type II lissencephaly. The causative gene was cloned in 1998 and named *fukutin*, and a 3-kb retrotransposal insertion in the 3′-untranslated region of *fukutin* is found in most patients with FCMD (*1*). Fukutin-related protein (FKRP) was characterized as a homolog of fukutin in 2001 (*2*), and mutations in *FKRP* were also shown to cause muscular dystrophies (congenital muscular dystrophy 1C (MDC1C) and limb-girdle muscular dystrophy 2I) (*2, 3*).

Skeletal muscle biopsy samples from FCMD and MDC1C patients show a marked reduction in α-dystroglycan (α-DG) by immunostaining with an antibody against the glycosylated epitope (*2, 5*), whereas α-DG could still be visualized even in FCMD skeletal muscle with an antibody that recognizes the peptide epitope (*6*). The reduction in α-DG glycosylation in skeletal muscle leads to a loss in its ability to bind to extracellular ligands such as laminin, agrin and neurexin (*6*). Thus, the abnormality in posttranslational glycosylation of α-DG plays a crucial role in the pathogenesis of FCMD and MDC1C. The functions of fukutin and FKRP have not been clarified. However, they are supposed to act as glycosyltransferases, because they both are type-II transmembranous proteins and possess a DXD motif in the C-terminal side that is often found in glycosyltransferases (*4*). These facts strongly indicate that these two proteins may participate in the glycosylation of α-DG.

Protein glycosylation is a highly organized orderly process. Newly synthesized naked proteins are sequentially modified *en route* by glycosyltransferases during transport from the rough endoplasmic reticulum (ER) to the *trans*-Golgi network (*7*). Therefore, the expression and localization of a glycosyltransferase must be precisely regulated to synthesize the specific glycostructure in each tissue and cell. Determining the localization of putative glycosyltransferases will help to predict their functions. In this study, we determined the precise localization of fukutin and FKRP in muscle cells.

## MATERIALS AND METHODS

*Antibodies*—Anti-FKRP and anti-fukutin antibodies were raised in rabbits against the recombinant fragments comprising the carboxyl-terminal 112 and 121 amino acids, respectively. Expression vectors for the FKRP and fukutin fragments with a glutathione-*S*-transferase tag at the N-terminus were constructed by inserting the cDNA fragments into pGEX-4T (Amarsham). Both antibodies were affinity-purified on antigen-immobilized activated thiol-Sepharose 4B (Amersham Pharmacia Biotec). Other antibodies used in this study are anti-emerin (Novocastra Laboratories), anti-BiP, anti-calnexin, anti-

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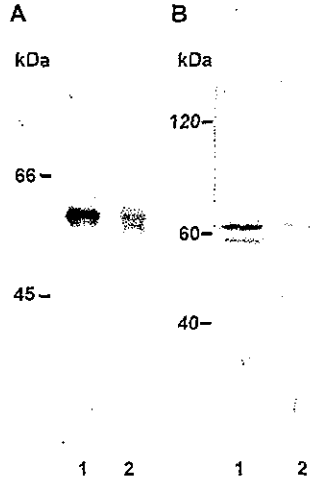


Fig. 1. **Characterization of newly raised polyclonal antibodies by Western blotting.** A: The anti-FKRP antibody detected a band at approximately 60 kDa in COS cell lysates with transiently expressed myc-FKRP (lane 1) and in human skeletal muscle cells (lane 2). B: Western blotting of a C2C12 cell lysate transfected with myc-fukutin. Both anti-fukutin (lane 1) and anti-myc (lane 2) antibodies detected doublet bands at approximately 60 kDa.

GM130 (BD Biosciences Pharmingen), and anti-myc 9E10 (Oncogene Science). Primary antibodies were used at the following dilutions: anti-FKRP (1:200), anti-fukutin (1:100), anti-myc (1:100), anti-BiP (1:100), anti-calnexin (1:100), anti-GM130 (1:500) and anti-emerin (1:500). Alexa 488-labeled anti-rabbit IgG and Alexa 568-labeled anti-mouse IgG (Molecular probes) were used as secondary antibodies.

*cDNA Transfection and Immunocytochemistry*—The open reading frames of FKRP and fukutin were amplified by PCR and subcloned into the expression vector CMV-myc (BD Clontech). These expression vectors encoding myc-epitope tagged FKRP and fukutin were transfected into the C2C12 cell line using Lipofectamine as described in the manufacturer's instructions (Invitrogen Carlsbad). The C2C12 cells and human skeletal muscle cells were induced to differentiate as described in a previous report (*8*). The immunostaining of myocytes was performed as described earlier (*9*). Laser confocal fluorescence images were obtained using an Olympus FLUOVIEW confocal microscope (Olympus).

*Western Blotting and Immunohistostaining of Human Skeletal Muscles*—Western blotting and immunohistostaining of human skeletal muscle were performed as described previously (*5*, *9*). The immunoreactive bands on Western blot were visualized using an ECL kit (Amersham Pharmacia Biotec). The immunostained sections were observed under a Zeiss Axiophot2 microscope (Carl Zeiss)

*Muscle Biopsy Sample*—Control muscle was obtained from surgical discards from a 24-yr-old male patient. Informed consent was obtained using the form approved by the Ethical Review Board at NCNP.

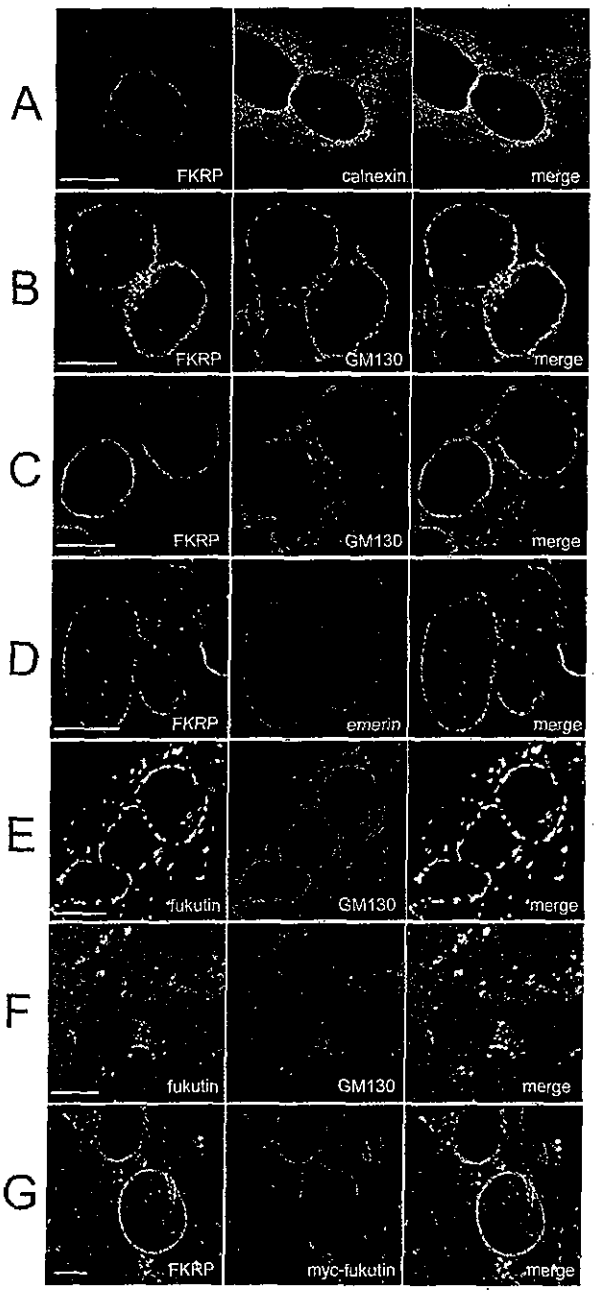


Fig. 2. **Subcellular localization of FKRP and fukutin.** A: FKRP and calnexin staining of human skeletal myotubes. FKRP (red) was continuously stained around nuclei with the innermost part of calnexin staining (green). B, C: FKRP and GM130 staining in mouse C2C12 myotubes. FKRP (green) was stained in the inner aspect of GM130 localization (red). Treatment with brefeldin A induced the dissociation of the Golgi apparatus, but FKRP remained localized around nuclei (C). D: FKRP and emerin staining in human skeletal myotubes. FKRP (green) was stained on the outer aspect of emerin staining (red). E, F: Immunostaining of transfected fukutin and GM130 in C2C12 myotubes. The merged image shows the co-localization of transfected fukutin and GM130. Treatment with brefeldin A dispersed both stainings into the cytosol (F). G: Spatial relationship between FKRP and fukutin. Endogenous FKRP (green) and transfected fukutin (red) in C2C12 myotubes showed different localizations. Bars denote 10 μm.

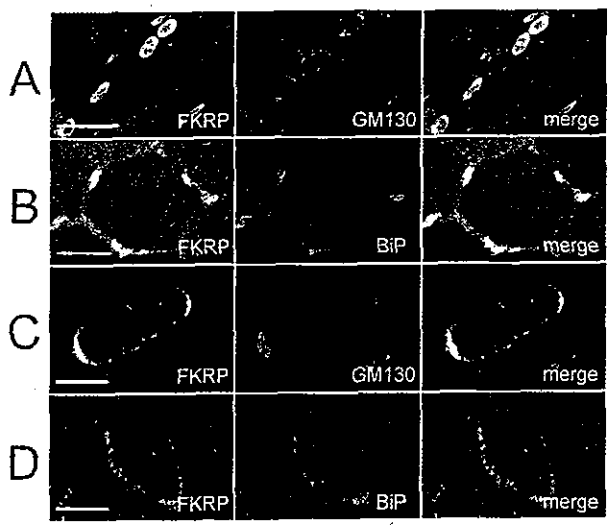


Fig. 3. **Immunohistochemical staining of FKRP in skeletal muscle.** The anti-FKRP antibody stained continuously around nuclei (A–D, green), while the anti-GM130 antibody stained nuclear poles and granular concentrates in cytosol (A, C, red). On higher magnifications, FKRP and GM130 are seen to overlap partially at both nuclear poles (C). BiP staining merges with FKRP in muscles (D). Bars denote 50 μm (A, B) and 5 μm (C, D).

## RESULTS

*Characterization of Newly Raised Antibodies against FKRP and Fukutin*—Polyclonal antibodies against FKRP and fukutin were raised using recombinant fragments comprising the carboxyl-terminal 112 and 121 amino acids, respectively; the fragments share no homology to each other. Western blot analysis of a homogenate from cultured human skeletal muscle cells using the anti-FKRP antibody detected a band migrating at approximately 60 kDa (lane 2 in Fig. 1A), which matches the size of transiently expressed myc-FKRP in COS cells (lane 1).

The anti-fukutin antibody was not adequately sensitive to detect endogenous fukutin in skeletal muscle or in cultured muscle cell homogenates. In C2C12 muscle cells, in which myc-fukutin was transiently expressed, the anti-fukutin antibody detected two adjacent bands migrating at 60 kDa (lane 1 in Fig. 1B). The anti-myc antibody also detected two same sized bands (lane 2), suggesting that some posttranslational modification, such as glycosylation, generates the two bands. The specificity of each antibody was also examined by cross Western blot experiments in which the anti-FKRP antibody was allowed to react with the recombinant fukutin protein expressed in C2C12 cells, and the anti-fukutin antibody to react with the recombinant FKRP expressed in COS cells. No band was detected in either blotting, demonstrating the specificity of each antibody.

*Subcellular Localization of FKRP and Fukutin in Differentiated Myocytes*—To determine the subcellular localization of FKRP and fukutin in differentiated myocytes, human myocytes or C2C12 cells were immunostained with the two antibodies (Fig. 2). FKRP staining was detected surrounding the nuclei only in myotubes, but not in myoblasts. We compared this localization with those of organelle marker proteins against calnexin, a chaperone protein in rough ER (*10*). Calnexin localizes around the nuclei and merges with FKRP staining (Fig. 2A), although calnexin is also diffusely distributed in the cytosol. Double staining with antibodies for GM130, a matrix protein in the *cis*-Golgi compartment (*11*) or emerin, an inner nuclear membranous protein, showed that FKRP co-localizes with neither of them, the inner aspect of GM130 and outer aspect of emerin (Fig. 2B, D). After treating of the cells with brefeldin A, which induces the dissociation of the Golgi apparatus (*12*), GM130 staining was dispersed in the cytoplasm while FKRP staining was unaffected (Fig. 2C). These results suggest that FKRP is not localized in the Golgi or inner nuclear membrane but in the rough ER.

The staining of transfected C2C12 cells with antibodies against fukutin and its introduced tag showed the same distribution in the perinuclear area in a discontinuous dot-like pattern under confocal microscopy (data not shown). When transfected C2C12 cells were stained for fukutin and GM130, the two stains precisely overlapped (Fig. 2E), and treatment with brefeldin A dispersed both proteins (Fig. 2F). These phenomena indicate that fukutin is localized in the *cis*-Golgi in differentiated myocytes.

We compared the localization of FKRP and fukutin in C2C12 cells in which myc-fukutin was transiently expressed. FKRP and myc-fukutin displayed different localizations in myocytes (Fig. 2G).

*Localization of FKRP in human skeletal muscle*—In skeletal muscle biopsy specimens, FKRP was detected continuously around the nuclei of skeletal muscle fibers (Fig. 3A–D, green). After absorption of the FKRP antibody with recombinant FKRP protein, no staining was observed (data not shown). We compared this localization with those of organelle marker proteins against BiP, a chaperone protein in rough ER (*10*), and GM130. BiP was localized around the nuclei and merged with FKRP staining (Fig. 3B, D). GM130 was present at the poles of the nuclei and, in addition, a few concentrated granules were present within the cytosol and did not overlap with FKRP (Fig. 3A, C). These findings suggest that FKRP is localized in rough ER *in vivo*. Interestingly, in some nuclear poles, GM130 and FKRP staining was found to be partly merged (Fig. 3C). It is important to note that ER and the Golgi apparatus form an essentially continuous structure in the intracellular transport system, and that this is a possible explanation for the partial co-localization of FKRP and GM130.

## DISCUSSION

The localization of FKRP is distinctly different from that of fukutin. Recently, Esapa et al. reported the subcellular localization of FKRP and fukutin in the medial-Golgi apparatus using rat kidney fibroblasts (*13*). Our data do not support the localization of FKRP in the Golgi apparatus, although immunohistochemical staining of skeletal muscle biopsy sample showed the partial incorporation of FKRP into other Golgi resident proteins. We assume that this difference is a result of the different cell types used in these two studies because FKRP colocalized with fukutin in CHO cells in which FKRP and myc-fukutin were transiently expressed (data not shown). In our experiments using C2C12 cells, endogenous FKRP was seen

only in differentiated myotubes, and the localization was different from those of GM130 and fukutin. Thus, we believe during muscle cell development, FKRP is expressed and functions in the rough ER after myotube formation. In addition, preliminary observations by immunoelectron microscopy demonstrated FKRP localization on the outer nuclear membrane or in transporting vesicles between the nuclear membrane and the Golgi apparatus, close to the distribution of ribosomes (unpublished data). It is unlikely that the localization of extrinsic fukutin is an artefact due to overexpression of this protein, because the extrinsic fukutin localizes only in the *cis*-Golgi apparatus in all transfected cells despite of its variable expression level.

Newly synthesized α-DG in the rough ER is thought to be modified by a series of glycosyltransferases, finally forming unique sugar chains consisting of *O*-mannosyl tetrasaccharide (Siaα2–3Galβ1–4GlcNAcβ1–2Man-*O*-protein) (*14*, *15*). The first *O*-mannosylation step is catalyzed by protein *O*-mannosyltransferase 1 (POMT1) (*16*), and this step may occur in rough ER. In fact, in yeast, several O-mannosyltransferases and their donor substrate, dolichyl-*P*-mannose, are indeed localized in rough ER (*17*). A mutation in *POMT1* causes Walker-Warburg syndrome, the most severe form of congenital muscular dystrophy with brain involvement, and it is also known that skeletal muscle biopsy samples from patients with Walker-Warburg syndrome show markedly reduced levels of α-DG (*16*, *18*). Our observations predict that FKRP localizes in rough ER, suggesting that FKRP may play a role in the first *O*-mannosylation step of α-DG with POMT1. Further analyses of the relationship between POMT1 and FKRP may clarify the role of FKRP in the *O*-mannosylation of α-DG.

In this study, we have clarified the precise subcellular localization of fukutin and FKRP in myocytes. The localization of both proteins suggests that they are involved in the glycosylation of α-DG, but in different manners. The biological characterization of fukutin and FKRP as glycosyltransferases and structural analyses of the α-DG sugar chains in patients are necessary to gain a complete understanding of the glycosylation process of α-DG, and, hence, the possible elucidation of the pathomechanism of FCMD and MDC1C.

This work was partly supported by the AYAKA Foundation.

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European Journal of Neurology 2004, 11: 657–661

# Dysferlin mutation analysis in a group of Italian patients with limb-girdle muscular dystrophy and Miyoshi myopathy

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**Keywords:**
dysferlin, limb-girdle muscular dystrophy (LGMD), Miyoshi myopathy (MM), mutation, polymorphism

Received 28 March 2003
Accepted 24 October 2003

Mutations in the dysferlin gene (*DYSF*) on chromosome 2p13 cause distinct phenotypes of muscular dystrophy: limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy (MM), and distal anterior compartment myopathy, which are known by the term 'dysferlinopathy'. We performed mutation analyses of *DYSF* in 14 Italian patients from 10 unrelated families with a deficiency of dysferlin protein below 20% of the value in normal controls by immunoblotting analysis. We identified 11 different mutations, including eight missense and three deletion mutations. Nine of them were novel mutations. We also identified a unique 6-bp insertion polymorphism within the coding region of *DYSF* in 15% of Italian population, which was not observed in East Asian populations. The correlation between clinical phenotype and the gene mutations was unclear, which suggested the role of additional genetic and epigenetic factors in modifying clinical symptoms.

## Introduction

Miyoshi myopathy (MM) is an early-adult onset, autosomal recessive form of distal muscular dystrophy, characterized by predominant involvement in the calf muscles and highly elevated serum creatine kinase (CK) levels (Miyoshi *et al.*, 1967, 1986). MM is caused by mutations in the dysferlin gene (*DYSF*) on chromosome 2p13 (Liu *et al.*, 1998a,b). Mutations in the same gene were also identified in patients with limb-girdle muscular dystrophy type 2B (LGMD2B) (Bashir *et al.*, 1998; Liu *et al.*, 1998a), and distal anterior compartment myopathy (Illa *et al.*, 2001). These diseases caused by mutations in *DYSF* are known by the term 'dysferlinopathy'. Dysferlin is a 237-kDa protein expressed predominantly in skeletal muscle which localizes to the plasma membrane of muscle fibers (Anderson *et al.*, 1999; Matsuda *et al.*, 1999). It is still unknown why mutations of the same gene cause either proximal LGMD or distal myopathies.

In the present study, we performed mutation analysis of *DYSF* in a group of families with a dysferlin protein deficiency, and found nine novel mutations and one unique insertion polymorphism in the coding region.

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## Methods

All clinical materials were obtained for diagnostic purposes with informed consent. We examined 14 Italian patients from 10 unrelated families who were clinically suspected of LGMD or MM, and showed a deficiency of dysferlin protein below 20% of the control value by immunoblotting analysis (Matsuda *et al.*, 1999; Fanin *et al.*, 2001). Genomic DNA and total RNA were isolated either from peripheral blood lymphocytes or biopsied skeletal muscles using a standard technique. Control genomic DNA samples were also obtained from 60 Italian, 50 Japanese, 50 Korean and 50 Chinese unrelated individuals.

For the haplotype analysis, we used three microsatellite markers: D2S292, D2S291, and D2S286 spanning *DYSF* on chromosome 2p, as described previously (Bejaoui *et al.*, 1995).

Specific primer sets for each 55 exon of *DYSF* were described previously (Liu *et al.*, 1998a). Polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP) was performed at three different temperatures: 5, 10 and 15°C, using a GenePhor DNA Separation System and a GeneGel Excel 12.5/24 Kit (Amersham Biosciences Co., Tokyo, Japan). To detect the deletion mutation from exons 25 to 29, reverse transcriptase-PCR (RT-PCR) was performed using following primer sets; Dysf6F: 5′-GGTGACATCCATGAGA CAAC-3′ (nt 2546–2565) and Dysf6R: 5′-GGACA-CACGAACCAATCTCC-3′ (nt 3155–3174), Dysf7F: 5′-GGAGATTGGTTCGTGTGTCC-3′ (nt 3155–3174)

and Dysf7R: 5′-CGATCTCGTAGAAGATGAGCG-3′ (nt 3979–3998), and Dysf6F and Dysf7R.

Direct sequencing of the PCR products was performed using a Long Read Tower (Amersham Biosciences). For the screening of control individuals, PCR-SSCP or PCR-restriction fragment length polymorphism (PCR-RFLP) was performed. For PCR-RFLP, we used *Hin*PI for exon 29, *Taq*I for exon 37, *Mae*III for exon 42, and *Ava*II for exon 44.

## Results

The clinical summary of 14 patients from 10 unrelated families is shown in the Table 1. Different clinical phenotypes were observed within the same family in F2 and F6. From the results of PCR-SSCP and direct sequencing analyses, we identified 11 different mutations that included eight missense and three deletion mutations (Table 1). Nine of them were novel mutations. A total of 120 chromosomes from Italian control individuals were screened for all mutations by either PCR-SSCP or PCR-RFLP, and no control individual showed the same substitution or deletion that the affected patients had.

Patient 9 showed a possible homozygous large deletion of the gene which was the same one previously reported by Anderson *et al.* (2000) as a 'probable deletion'. Haplotype analysis using three microsatellite markers, D2S292, D2S291, and D2S286 on chromosome 2p, revealed that only the affected patients with the typical MM phenotype were homozygous in this region, while other unaffected family members were heterozygous (Fig. 1a). PCR amplification using genomic DNA showed no amplified products from exons 25 to 29, although the other exons were clearly amplified at the expected sizes (Fig. 1b). RT-PCR and direct sequencing analyses revealed two truncated transcripts with different sizes corresponding to the deletions from exons 25 to 29 and from exons 25 to 30 (Fig. 1c). Both of these altered transcripts were in-frame and produced truncated dysferlin proteins; however, immunoblotting analysis showed no detectable band in this patient (data not shown).

During the mutation screening, we found nine kinds of polymorphisms. They were 766C > T (P131P), 1315C > T (H314H), 2200T > C (D609D), 2446G > A (Q691Q), 2956T > C (S861S), 3438G > A (R1022Q), 3853G > A (Q1160Q), 4381C > A (I1336I), and one 6-bp insertion. The 3438G > A substitution resulted in an amino acid change in R1022Q, but this was observed in 21% of Italian control individuals. The 6-bp insertion of AGGCGG was located within the coding region (exon 30) of *DYSF*, causing a two amino acid (AE) insertion at amino acid number 1062 (Fig. 2a, b). Nine of 60 Italian control individuals (15%) had this 6-bp insertion heterozygously. This insertion was also observed in two families with dysferlinopathy. Patient 3 had this insertion heterozygously, and her healthy mother had it homozygously. All three patients in F6 also had this 6-bp insertion homozygously, together with a homozygous 3247C > T missense mutation. We searched for this insertion in 50 Japanese, 50 Korean and 50 Chinese control individuals, but none had it (Fig. 2a).

Although all 55 exons of *DYSF* were examined by the PCR-direct sequencing method, we could identify only one allelic mutation in four families (Table 1). No

**Table 1** The clinical summary and mutations of the patients

| Family no. | Patient no./sex | Age at onset (years) | Clinical phenotype | Exon | Mutation | Inheritance pattern |
|---|---|---|---|---|---|---|
| 1 | 1a/M | 20 | MM | 20 | **2234G > A (G618R)** | Heterozygous |
| | 1b/F | 25 | | 45 | **5358C > G (T1662R)** | |
| 2 | 2a/M | 33 | LGMD | 32 | **3817–8TG > AA (1148X)** | Homozygous |
| | 2b/F | 11 | MM | | | |
| 3 | 3/F | 12 | MM | 38 | **4454T > C (C1361R)** | Heterozygous |
| | | | | 44 | **5245delG(1633X)** | |
| 4 | 4/F* | 23 | LGMD | 42 | **4887A > G (Y1505C)** | Heterozygous |
| 5 | 5/F* | 26 | MM | 37 | **4376G > A (E1335K)** | Heterozygous |
| 6 | 6a/M | 60 | High CK | 27 | **3247C > T (R958W)** | Homozygous |
| | 6b/M | 20 | MM/LGMD | | | |
| | 6c/M | 18 | MM | | | |
| 7 | 7/M* | 17 | LGMD | 37 | **4376G > A (E1335K)** | Heterozygous |
| 8 | 8/M | 17 | MM | 29 | **3483C > T (R1041C)** | Homozygous |
| 9 | 9/M | 19 | MM | 25–29 | 2885–3547del (Y838-R1058 del) | Homozygous |
| 10 | 10/M* | 17 | LGMD | 23 | 2573–7del (750X) | Heterozygous |

M, male; F, female; MM, Miyoshi myopathy; LGMD, limb-girdle muscular dystrophy; CK, creatine kinase.

Bold text, novel mutation.

*families where only one allelic mutation was identified.

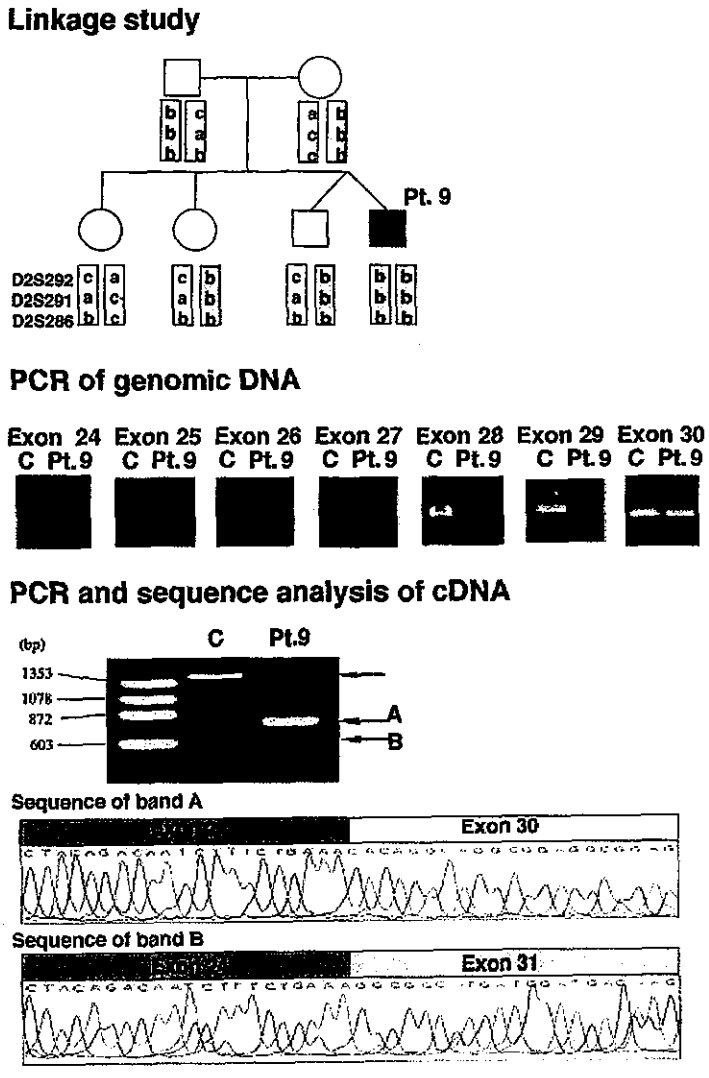


Figure 1 Large deletion of *DYSF* in a family (F9). (a) Haplotype analysis. Only one patient (patient 9) showed homozygous in three markers for chromosome 2p. (b) PCR of genomic DNA. In this patient, no amplified product was observed from exons 25 to 29. PCR products of the other exons were clearly amplified with the expected size. (c) RT-PCR. Two types of PCR products (A and B) were amplified using cDNA from biopsied muscle. Sequence analysis of these bands revealed deletions from exons 25 to 29 (band A) and from 25 to 30 (band B).

truncated transcript was amplified by RT-PCR in these four heterozygous families, either.

## Discussion

Dysferlin is a FER-1 member protein and contains six putative C2 domains (Liu *et al.*, 1998a; Britton *et al.*, 2000), which can bind to phospholipids, inositol polyphosphates, $Ca^{2+}$ and intracellular proteins (Nalefski and Falke, 1996; Rizo and Sudhof, 1998). The function of dysferlin is not yet known. However, it localizes to the plasma membrane of skeletal muscle and might have a role in membrane fusion (Anderson *et al.*, 1999; Matsuda *et al.*, 1999). Recently, Bansal *et al.* (2003) reported the possible involvement of dysferflin in the membrane-repair machinery in skeletal muscle. Mutations in *DYSF* cause different clinical phenotypes of muscular dystrophy and the distribution of affected muscles is different between patients, even in the same family. Some patients show onset in proximal muscles, while others show initial selective atrophy and weakness of calf muscles and are diagnosed as MM. However, muscle weakness and atrophy are progressive, and subsequently both proximal and distal muscles are involved in most of the patients.

In the present study, we performed mutation analysis on 14 Italian patients from 10 unrelated families who were clinically suspected of having LGMD or MM and showed a deficiency in the dysferlin protein by immunoblotting analysis. As *DYSF* on chromosome 2p13 contains 55 exons and encodes a 6911-bp mRNA (AF075575), the mutation screening is complex and time consuming. Furthermore, many polymorphisms have been reported. PCR-SSCP analysis is thought to

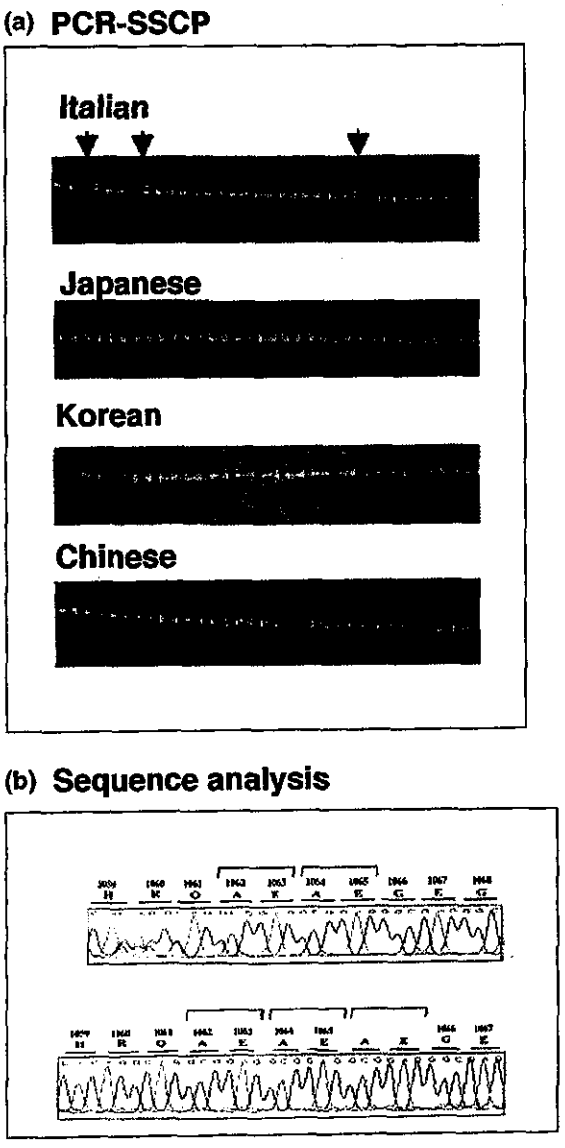


**Figure 2** Six basepair insertion in exon 30. (a) Fifteen per cent of Italian control individuals had a heterozygous 6-bp insertion polymorphism in exon 30 (arrows). This polymorphism was absent in East Asian populations of Japanese, Korean and Chinese origin. (b) Sequence analysis of the 6-bp insertion. This insertion caused two additional amino acids AE following AEAE.

be a powerful method for *DYSF* mutation analysis. In fact, all the mutations identified in this study showed abnormal shifted patterns by PCR-SSCP.

We identified 11 possible mutations in 10 families. All missense mutations were observed only in the affected patients, but not in the control individuals. Distribution of the mutations in *DYSF* varied along the molecule, and no hot spot was observed. The 4376G > A missense mutation was observed in two unrelated patients (patients 5 and 7), and the 5-bp frame-shift deletion mutation observed in patient 10 was the same as previously reported in an Italian MM patient (Aoki *et al.*, 2001). These mutations may be relatively common in Italian patients. One nonsense mutation of 5358C > G observed in one family (F1) was within the fifth putative C2 domain. This patient lacked the last C2 domain, but the clinical severity was intermediate. The large deletion we found in one family (F9) contained two dysferlin domains, whose functions are not yet known. This deletion mutation produced two different truncated transcripts, the larger one being a major product corresponding to the deletion from exons 25 to 29, while the shorter one from exons 25 to 30. PCR of genomic DNA failed to amplify exons between 25 and 29, but not 30, and sequence analysis showed no abnormality of the exon–intron boundary of exons 24 and 30. Haplotype analysis showed that the patient was homozygous in the *DYSF* gene region. These results suggest that the patient has a homozygous deletion, including exons from 25 to 29, and two transcriptional variants, although detailed genomic sequence of the breakpoints and Southern blotting analysis were not performed. Both the truncated transcripts were in-frame, although immunoblotting analysis showed no detectable band. Further, dysferlin protein was either greatly reduced or absent in the muscles of patients with missense mutations. The truncated or altered protein produced by mutations in *DYSF* may degrade rapidly and was therefore barely detectable in biopsied muscles.

In one family (F6), the father and two sons had the same homozygous mutation, although there was no consanguinity reported in this intriguing family. Haplotype analysis also showed a homozygous pattern in all three patients. We could not perform genetic analysis on the mother, but she could be a mutation carrier as the father and the mother in this family were from the same small village. Interestingly, the two siblings had a very active myopathy, while the father with the identical mutation showed no apparent clinical symptoms up to the age of 58 years, except for an elevation of serum CK levels, and had only mild myopathic changes at muscle biopsy. In addition, the younger brother (patient 6c) showed predominant distal weakness and the elder brother (patient 6b) revealed both proximal and distal muscle involvement. Different clinical features were also observed in F2, in which the brother showed proximal dominant muscle involvement, while the sister showed distal myopathy. Although it is unclear as to why clinical features were different among the same family with the same mutation, similar findings have often been observed (Matsumura *et al.*, 1999; Weiler *et al.*, 1999; Illarioshkin *et al.*, 2000; Nakagawa *et al.*,

2001). Additional genetic and epigenetic factor(s) may play a role in modifying clinical symptoms.

In the four heterozygous patients in our study (patients 4, 5, 7, 10), only one allelic mutation was identified, although all 55 exons were sequenced directly. No truncated transcript was observed by RT-PCR, either. These patients may have a mutation in the promoter region or a regulatory region of an intron of *DYSF*.

The 6-bp insertion in exon 30 was frequently observed in Italian controls heterozygously. Two families with dysferlinopathy also had this insertion, and additional possible mutations were identified in these families that were not observed in 60 control individuals. Furthermore, an unaffected mother from F3 had this insertion homozygously, while an affected patient had it in one allele. Hence, the 6-bp insertion is considered to be a polymorphism. This 6-bp insertion occurred after two repeats of the same 6-bp AGGCGG, and the amino acid sequence was changed from AEAE to AEAEAE. It is interesting to speculate on the functional difference, if any, of the dysferlin molecule with this insertion. This 6-bp insertion was observed only in an Italian population, but not in East Asian populations of Japanese, Korean and Chinese origin. It is important for the mutation analysis to choose control samples from individuals with a similar genetic background.

## Acknowledgements

We dedicate this article to the memory of Dr Kiichi Arahata (National Institute of Neuroscience, NCNP), who passed away while this project was underway. This work was supported by Grants-in-Aid for Research on Psychiatric and Neurological Diseases and Mental Health from the Ministry of Health and Welfare, Ichiro Kanehara Memorial Foundation, Japan, Grants of Telethon Italy (GTF02009) and MURST (no. 2001068328).

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PERGAMON

Neuromuscular Disorders 14 (2004) 810–814

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# Two novel *CAV3* gene mutations in Japanese families

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Received 21 April 2004; received in revised form 16 August 2004; accepted 27 August 2004

**Abstract**

Caveolin-3 deficiency is a rare, autosomal dominant, muscle disorder caused by caveolin-3 gene (*CAV3*) mutations and consists of four clinical phenotypes: limb-girdle muscular dystrophy type 1C (LGMD-1C), rippling muscle disease, distal myopathy, and familial hyperCKemia. So far, only 13 mutations have been reported. We here report two novel heterozygous mutations, 96C>G (N32K) and 128T>A (V43E), in the *CAV3* gene in two unrelated Japanese families with LGMD-1C. Both probands presented with elevated serum CK level with calf muscle hypertrophy in their childhood but without apparent muscle weakness. However, their mothers showed mild limb-girdle weakness in addition to high CK level. Caveolin-3 was deficient and caveolae were lacking in muscles from both patients. Our data confirm that caveolin-3 deficiency causes LGMD-1C and expand the variability in *CAV3* gene mutations.


*Keywords:* CAV3; Caveolin-3; Caveolinopathy; LGMD-1C

## 1. Introduction

Caveolin is an integral membrane protein and is the principal component of caveolae membranes in vivo. Caveolae are vesicular invaginations of the plasma membrane and play a role in vesicular trafficking events and in signal transduction processes. The caveolin gene family consists of caveolin-1, -2, and -3. Caveolin-3 is muscle-specific and is found in both cardiac and skeletal muscles [1].

The first mutation in the human caveolin-3 gene (*CAV3*) was identified in an autosomal dominant limb-girdle muscular dystrophy type 1C (LGMD-1C) [2]. This mutation was an in-frame 9-bp deletion but all the subsequently reported mutations were missense mutations. Muscles from these patients show caveolin-3 deficiency at the protein level. Since monomers of caveolin-3 oligomerize to form the molecular scaffolding of caveolae, it is thought that heterozygous mutations in the *CAV3* gene would have a dominant negative effect in oligomerization and thus in caveolae formation [3].

So far, 13 different heterozygous mutations in the *CAV3* gene have been associated with four different muscle disorders: LGMD-1C, rippling muscle disease (RMD), familial hyperCKemia, and distal myopathy (Table 1) [2,4–17]. Nevertheless, their genotype–phenotype correlations have only been poorly determined because of the rarity of these diseases.

Here, we describe two novel mutations in the *CAV3* gene in two unrelated Japanese families. We also reviewed all the *CAV3* gene mutations reported previously.

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doi:10.1016/j.nmd.2004.08.008

Table 1
Summary of the *CAV3* gene mutations

| Mutation | Exon | Position | Nucleotide change | Phenotypes | Reference (ethinic background) |
|---|---|---|---|---|---|
| R26Q | 1 | N-terminal | 77G>A | LGMD-1C | [4] (French) |
| | | | | RMD | [5] (German), [6] (Japanese) |
| | | | | Distal myopathy | [7] (Japanese) |
| | | | | HyperCKemia | [8] (Italian) |
| D27E | 1 | N-terminal | 81C>A | LGMD-1C | [9] (German) |
| | | | | RMD | [9] (German) |
| P28T | 1 | N-terminal | 82C>A | RMD | [10] (Bergian) |
| P28L | 1 | N-terminal | 83C>T | HyperCKemia | [11] (Italian) |
| N32K | 1 | N-terminal | 96C>G | LGMD-1C | Present report (Japanese) |
| V43E | 2 | N-terminal | 128T>A | LGMD-1C | Present report (Japanese) |
| A45T | 2 | N-terminal | 133G>A | LGMD-1C | [12] (German) |
| | | | | RMD | [13] (Norwegian, German) |
| A45V | 2 | N-terminal | 134C>T | RMD | [13] (German) |
| V57M | 2 | Caveolin-scaffolding domain | 169G>A | HyperCKemia | [14] (Spanish) |
| T63P | 2 | Caveolin-scaffolding domain | 187A>C | LGMD-1C | [15] (Japanese) |
| TFT63-65del | 2 | Caveolin-scaffolding domain | 186-194del | LGMD-1C | [2] (Italian) |
| L86P | 2 | Membrane-spanning domain | 215T>C | RMD | [16] (Colombian) |
| A92T | 2 | Membrane-spanning domain | 232G>A | RMD | [16] (Italian) |
| F97del | 2 | Membrane-spanning domain | 328-330del | LGMD-1C | [17] (Italian) |
| | | | | RMD | [17] (Italian) |
| | | | | HyperCKemia | [17] (Italian) |
| P104L | 2 | Membrane-spanning domain | 311C>T | LGMD-1C | [2] (Italian), [13] (German) |

## 2. Patients, materials and methods

### 2.1. Patients

Patient 1 was a 6-year-old Japanese boy. His psychomotor development was normal. However, he started occasionally complaining of fatigability and muscle pain at age 3. On examination, his calf muscles were hypertrophic but otherwise normal. He had neither muscle weakness nor rippling. Serum CK was elevated (3430 IU/l (normal range <200 IU/l)). He had no brother. His 33-years-old mother had noted easy fatigability after his birth. Her calf muscles were hypertrophied. She had mild weakness of MRC 4 level predominantly in the proximal muscles of all four extremities. She was ambulatory but could not run. She showed Gowers' sign. Her serum CK level was elevated to 1700 IU/l. Both did not show either muscle rippling, percussion-induced rapid muscle contractions (PIRCs), or muscle mounding on percussion.

Patient 2 was a 3-year-old Japanese boy unrelated to patient 1. His psychomotor development was normal. Elevated serum CK level (1578 IU/l) was found by chance at age 3. On examination, he had bilateral calf muscle hypertrophy but no muscle weakness. His brother (6-year-old) showed no neurological abnormality and had normal serum CK level. His mother (30-year-old) showed mild weakness in limb-girdle muscles and bilateral calf muscle hypertrophy on examination but did not have any difficulty for her daily activities. His maternal grandfather (age 62) had proximal dominant muscle weakness at MRC 4 level and muscle wasting in limb-girdle muscles. He showed Gowers' sign but was ambulatory. Both had elevated CK levels and often experienced pain in the calf muscles after exercise. Any member of this family did not show muscle rippling, PIRCs, or muscle mounding on percussion.

### 2.2. CAV3 gene analyses

We sequenced the entire coding region and the exon/intron junctions of the *CAV3* gene. Genomic DNA was extracted from peripheral blood lymphocytes of both patients and affected mothers. We amplified each exon and flanking sequences of the *CAV3* gene by polymerase chain reaction and directly sequenced the amplified fragments using an ABI 3100 Sequencer (PE Applied Biosystems, Foster City, CA) as described previously [7]. We searched for the identified mutations in control DNA from 100 Japanese individuals.

### 2.3. Muscle biopsy: histochemistry and immunohistochemistry

Muscle biopsies were performed from the biceps brachii muscle in both patients. One portion of each muscle biopsy specimen was frozen in liquid nitrogen-cooled isopentane for histochemistry and the other portion was divided in two for electron microscopy. Transverse serial frozen sections of 10 μm thickness were stained with hematoxylin and eosin (H&E), modified Gomori trichrome and a battery of histochemical methods.

We performed indirect immunofluorescence staining on 6 μm serial cryosections of muscle. These sections were incubated at 37 °C for 2 h with the primary mouse monoclonal IgG antibodies against caveolin-3

(BD Transduction laboratories, Lexington, KY), dysferlin, the C-terminal of dystrophin, α-, β-, γ-, δ-sarcoglycan, α-, β-dystroglycan and laminin α2 (Novocastra, Newcastle Upon Tyne, UK). They were subsequently incubated at room temperature for 1 h with a secondary antibody fluorescein isothiocyanate (FITC)-labeled goat F(ab')2 anti-mouse IgG (Leinco Technology, St Louis, MO). These sections were examined by fluorescence microscopy. Control specimens were obtained from 10 individuals with morphologically normal muscle.

### 2.4. Electron microscopy

For electron microscopy, one piece of each biopsy was fixed in buffered 2% isotonic glutaraldehyde at pH 7.4, post-fixed in osmium tetroxide and were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead nitrate, and examined with an H-7000 electron microscope (Hitachi, Tokyo, Japan). For lanthanum staining, the remaining samples werefixed in 2% glutaraldehyde-2% paraformaldehyde in 0.1 mol/l cacodylate buffer, pH 7.2 for 30 min. The samples were then minced into 1×2 mm pieces, washed three times in buffer, and rinsed overnight at 4 °C in 0.5 mol/l cacodylate buffer. The specimens were postfixed at room temperature by vibratory agitation for 2 h in a medium containing 1% osmium tetroxide in 0.2 M s-collidine buffer at pH 7.2 and 2% lanthanum nitrate. They were dehydrated, washed in 100% propylene oxide and embedded in epoxy resin.

## 3. Results

### 3.1. Gene analyses

Patient 1 and his affected mother had a heterozygous C to G substitution on at nucleotide position 96 that is predicted to change neutral amino acid, asparagine, at codon 32 to basic amino acid, lysine (96C>G (N32K)) (Fig. 1). Patient 2 and his affected mother carried a heterozygous T to A substitution at nucleotide position 128 predicted to cause an amino acid change from neutral valine to acidic glutamic acid (128T>A (V43E)). Both mutations were absent in 100 control Japanese individuals.

### 3.2. Histochemistry and immunohistochemistry

On H&E, both patients showed scattered necrotic and regenerating fibers in addition to mild to moderate variation in fiber size. There were scattered type 2C fibers and mild endomysial fibrosis. By immunohistochemistry, dystrophin, the four sarcoglycans, the two dystroglycans and laminin α2 were normally expressed in the sarcolemma in both patient. However, immunoreactivity of caveolin-3 was almost completely absent and that of dysferlin was markedly reduced compared to a normal control (Fig. 2A–F).

### 3.3. Electron microscopy

On electron microscopy (Fig. 2G–H), caveolae were identified by their characteristic flask or oval shape and location at or near the plasma membrane of muscle and endothelial cells in a normal control. Plasma membrane was highlighted by lanthanum staining. In contrast, in any of the tested muscle from the patients, we did not find caveolae over the surface of the muscle fibers although the non-muscle caveolae were present in the endothelial cells.

## 4. Discussion

We have described two novel missense mutations, 96C>G (N32K) and 128T>A (V43E), in the *CAV3* gene in two unrelated Japanese patients with caveolin-3 deficiency. These mutations are likely to be the cause of caveolin-3 deficiency because they are the only nucleotide changes in the *CAV3* gene open reading frame and because both mutations were absent in 100 control individuals.

Both affected mothers showed typical LGMD-1C while the patients had clinically atypical LGMD-1C with no

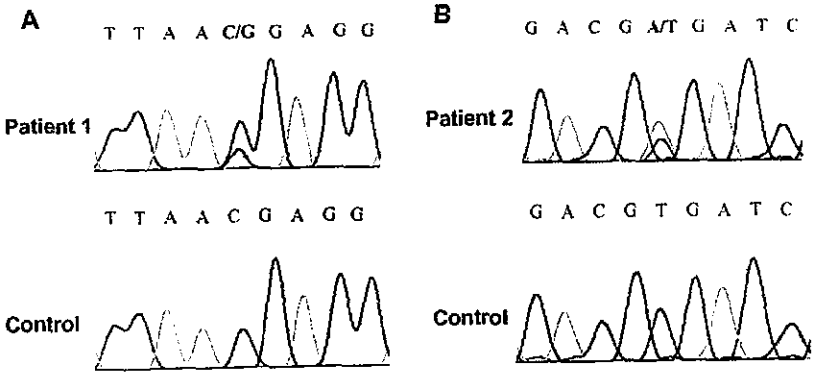


Fig. 1. Direct sequence analysis of the *CAV3* gene. (A) The heterozygous C to G mutation at nucleotide position 96 in patient 1. This change is absent in normal controls. (B) The heterozygous T to A mutation at nucleotide position 128 in patient 2. This change is also absent in normal controls.

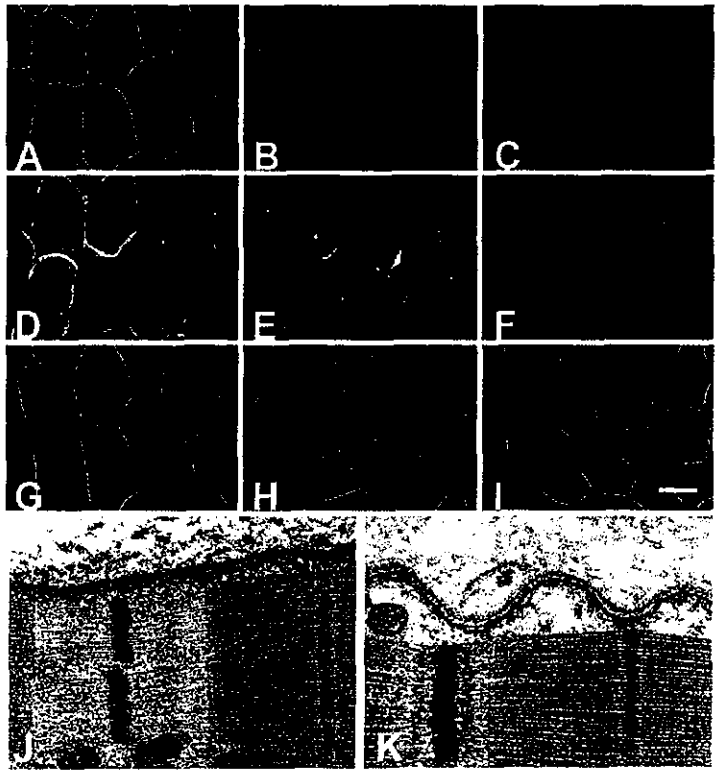


Fig. 2. Immunohistochemistry against caveolin-3 (A–C), dysferlin (D–F) and dystrophin (G–I). Transverse sections of skeletal muscle biopsies from a normal control (A, D, G), patient 1 (B, E, H) and patient 2 (C, F, I) with caveolin-3 deficiency. Normal expression of dystrophin but reduced expression of caveolin-3 and dysferlin at the sarcolemma in both patients, as compared with a control. Electron microscopy. In normal control muscle (J), caveolae are identified by their characteristic flask or oval shape and location at or near the plasma membrane which is accentuated by lanthanum staining. In contrast, in patient 1 with caveolin-3 deficiency (K), there is loss of caveolae in muscle fibers. (A–I) *Bar* 40 μm. (J and K) Original magnification, ×10,000.

apparent weakness. Nevertheless, muscle pathology showed dystrophic changes, with marked caveolin-3 deficiency and probable secondary dysferlin deficiency, on light microscopy and loss of caveolae on electron microscopy. In addition, inheritance pattern was compatible with an autosomal dominant trait and both patients had calf hypertrophy. Therefore, both patients were therefore diagnosed as having probable LGMD-1C rather than hyperCKemia, although it was reported that the same mutation may lead to a different phenotypes even within the same family [9].

The secondary dysferlin deficiency in our patients supports the previously proposed hypothesis that dysferlin may play a role in the signaling functions of caveolae by interacting with caveolin-3 [15]. The lack of caveolae in the sarcolemma is most likely due to severe impairment of caveolae formation in the subsarcolemma of muscle fibers, as previously described [18].

Caveolinopathy is a rare muscular disorder [1]; and LGMD-1C is a rare subtype of LGMD as compared to other LGMDs, especially LGMD-2A and LGMD-2B. The *CAV3* gene mutations have been associated with four different muscle diseases; LGMD-1C, RMD, hyperCKemia, and distal myopathy. So far, world-wide, only 13 different *CAV3* gene mutations have been reported as we summarized in Table 1 [2,4–17]. Besides, the G55S, C71W, and R125H were reported in limb-girdle muscular dystrophy patients. However, these mutations are now thought to be polymorphisms rather than pathogenic mutations, because they affected neither the expression nor the localization of the caveolin-3 protein [19]. The R26Q is the most common mutation in the *CAV3* gene and this mutation can cause any phenotype of caveolin-3 deficiency [20]. The mutations associated with LGMD-1C phenotype seem to be scattered throughout the open reading frame.

So far, five Japanese patients with caveolinopathy have been reported including the two LGMD-1C patients in the present study, carrying 96C>G (N32K) and 128T>A (V43E), and two other patients previously reported by our group, one with a peculiar distal myopathy, who had a 77G>A (R26Q) mutation [7] and the other with LGMD-1C who carried a 187A>C (T63P) mutation [15]. The other Japanese patient had RMD phenotype and had a 77G>A (R26Q) mutation [6]. Among these mutations, N32K, V43E, T63P were found only in Japan and all were only associated with LGMD-1C.

However, it is probably too early to define the genotype–phenotype correlation and we will have to await

accumulation of additional patients with genetically-determined caveolinopathy since the number of patients reported so far is too small to characterize this disease. Alternatively, the mutation site may not be the only determinant factor for the different clinical manifestations since all four phenotypes are associated with caveolin-3 deficiency at the protein level.

## Acknowledgements

The authors thank Dr Shuzo M. Sumi for his reviewing the manuscript. We also thank Ms Fumie Uematsu and Ms Chiharu Yoshioka for their technical assistance. This work is, in part, supported by the Research Grant (14B-4) for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare.

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