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ORIGINAL ARTICLE

Deep-intronic variant of *fukutin* is the most prevalent point mutation of Fukuyama congenital muscular dystrophy in Japan

Kazuhiro Kobayashi[1,5], Reiko Kato[1,5], Eri Kondo-Iida[2], Mariko Taniguchi-Ikeda[1,3], Makiko Osawa[4], Kayoko Saito[2] and Tatsushi Toda[1]

Fukuyama congenital muscular dystrophy (FCMD), which is caused by mutations in the *fukutin* gene, is the second most common form of childhood muscular dystrophy in Japan. The founder haplotype is the most prevalent in the chromosomes of Japanese FCMD patients, and corresponds to an SVA retrotransposal insertion in the 3′-untranslated region of *fukutin*. Although other mutations have been reported, the mutation corresponding to the second most prevalent haplotype in Japanese FCMD patients remained unknown. Recently a deep-intronic point mutation c.647+2084G>T was identified in Korean patients with congenital muscular dystrophy. Here, we performed mutational analysis of 10 patients with the second most prevalent haplotype and found that all of them were compound-heterozygous for the SVA insertion and this c.647+2084G>T mutation. The *fukutin* mRNA of these patients contained a pseudoexon between exon 5 and exon 6, which was consistent with the previous Korean study. As expected, the mutated fukutin protein was smaller than the normal protein, reflecting the truncation of fukutin due to a premature stop codon. Immunostaining analysis showed a decrease in the signal for the glycosylated form of α-dystroglycan. These findings indicated that this mutation is the second most prevalent loss-of-function mutation in Japanese FCMD patients.
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INTRODUCTION

Abnormal glycosylation of α-dystroglycan (α-DG) is known to cause some types of muscular dystrophy and lissencephaly, which are collectively called the α-dystroglycanopathies.[1] Several causative genes of the α-dystroglycanopathies have been identified to date. Fukuyama congenital muscular dystrophy (FCMD) is a member of this disease group and is one of the most common autosomal recessive disorders in Japan.[2] Patients with FCMD typically present with generalized hypotonia and weakness in infancy, followed by marked muscle atrophy, joint contractures and psychomotor developmental delay in childhood. Upright ambulation, even with support, is attained only rarely. The brain malformations are micropolygyria, pachygyria and agyria. Intellectual, cognitive and communicative functions are moderately delayed. Ophthalmological findings such as peripheral abnormalities of the retina or abnormal eye movements are often observed. The clinical course is inexorably progressive, with an average age at death of 16 years. We identified *fukutin* as the causative gene for FCMD through positional cloning,[3] found that the clinical manifestations vary greatly among patients depending on the type of mutation in the *fukutin* gene,[4] and demonstrated the possibility of splicing modulation therapy by antisense oligonucleotides as a clinical treatment for FCMD.[5] A founder haplotype on chromosome 9q31 is seen in most FCMD patients (138-192-147-183, in terms of sizes of the PCR products of the markers D9S2105-D9S2170-D9S2171-D9S2107),[6] and corresponds to an SVA retrotransposal insertion in the 3′-untranslated region of *fukutin*,[3] which causes abnormal splicing.[5] Several other mutations in FCMD patients have been found and linked to their own haplotypes. However, the mutation corresponding to the second most prevalent FCMD haplotype (139-201-155-183), which was found in nine out of the 107 patients heterozygous for the founder haplotype, has not been identified to date.[4]

In a recent report, mutations in *fukutin* were found to be responsible for the majority of Korean cases of congenital muscular dystrophy (CMD) with defective glycosylation of α-DG, and the two major types of *fukutin* mutations were the SVA insertion and a novel deep-intronic mutation (c.647+2084G>T).[7] The latter point mutation occurred in intron 5, creating a new strong splicing donor site, and hence caused additional splicing to form a 64-bp pseudoexon between exon 5 and exon 6, and resulted in a frameshift and a

[1]Division of Molecular Brain Science/Neurology, Kobe University Graduate School of Medicine, Kobe, Japan; [2]Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan; [3]Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan and [4]Department of Pediatrics, School of Medicine, Tokyo Women's Medical University, Tokyo, Japan
[5]These authors contributed equally to this work.
Correspondence: Professor T Toda, Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-chou, Chuo-ku, Kobe 650-0017, Japan.
E-mail: toda@med.kobe-u.ac.jp
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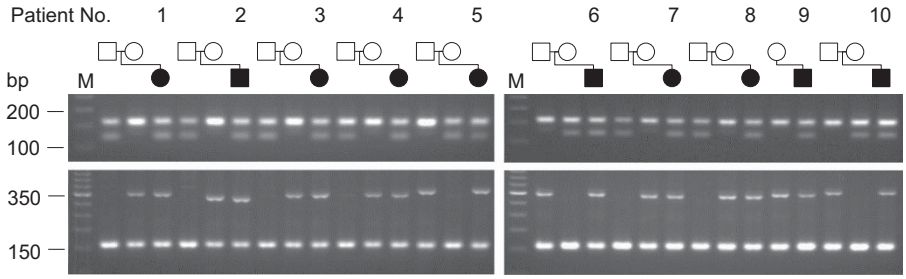

Figure 1 Mutation status of the DNA of patients carrying the second most common haplotype and the founder haplotype heterozygously. Upper panels show the results of PCR-RFLP analysis to detect the c.647+2084G>T mutation. The lower bands (120 bp) demonstrate the existence of the mutation. Lower panels show the results of PCR analysis to detect the SVA insertion mutation. The upper bands (375 bp) demonstrate the existence of the insertion. M: 50-bp ladder DNA marker; 1–10: patient numbers.

premature stop codon. Considering the close ethnic interactions between Japan and Korea throughout history, we suspected that the second most prevalent haplotype in Japanese FCMD patients might be associated with this mutation identified in Korean patients, as in the case of the SVA insertion mutation, which is also shared between these two populations. We hence conducted a mutation analysis in 10 Japanese FCMD patients with the second most prevalent haplotype, to test this hypothesis.

MATERIALS AND METHODS

Nine patients with the second most common haplotype (139-201-155-183 for D9S2105-D9S2170-D9S2171-D9S2107) that were studied previously[4] were analyzed in the present study. Another patient with this haplotype whose muscle biopsy specimen was available was also included. Cultured lymphoblastoid cell lines derived from three of the former nine patients were also available. All 10 patients have been genotyped and found to be heterozygous for this haplotype and the founder haplotype 138-192-147-183, which is linked to the SVA retrotransposal insertion.[4] Parents of the patients who were available and willing to participate, one patient carrying the SVA retrotransposal mutation homozygously, two normal controls, and a person suspected of having a connective tissue disease were also included as controls. Informed consent was obtained from all participants. This study has been approved by the Human Ethics Review Committees of Kobe University Graduate School of Medicine and Tokyo Women's Medical University.

Genomic DNA was extracted from peripheral blood. PCR was carried out with primers FKTNINT5f1 (CATGTGCAAAAATTTATCTTTGGCTATCTC) and FUKUTINT5r1 (GGTCATTTTGAAAATATGGCTTGGTTCAG) flanking the c.647+2084G>T mutation, using Ex Taq polymerase (Takara Bio, Shiga, Japan). RNA from lymphoblastoid cell lines was extracted using RNeasy plus kit (Qiagen, Venlo, The Netherlands), and RNA from skeletal musle was extracted using TRIzol Reagent (Invitrogen, Carlsbad, California, US). RT-PCR with the primer pair FCMDex4F (CATGCGATCCACTTGGTAGTC) and FCMDex6-7 (GGTACTGCTGAAAGAATGCTCG) was carried out with SuperScript III Reverse Transcriptase (Invitrogen) and Ex Taq polymerase. PCR products were purified and direct-sequenced. PCR-restriction fragment length polymorphism (RFLP) analysis to detect the c.647+2084G>T mutation was performed as follows: a mismatched primer pair was designed so that the PCR product consistently contains an *Rsa* I restriction enzyme recognition site (GTAC) in the 5′ part as a positive control for *Rsa* I digestion, and also in the 3′ part only when the template DNA carries the c.647+2084G>T mutation, namely, primers FKTN5f3M (GGATTAAAAACATTCTTGAAGTTATACTT GGAGTActAAGTTTC) and FKTN5rM (CTCACTGGAAGTTACTAAGAAGG AGTTTTTAATGTGAAAgT) (the mismatched sequences are written in lowercase letters). Genomic DNA was amplified with the mismatched primer pair using AmpliTaq Gold master mix (Applied Biosystems, Foster City, CA, USA), and the PCR product, with a length of 195 bp, was then treated with *Rsa* I. The product was digested into two fragments of 32 and 163 bp, and if the c.647+2084G>T mutation is present, the 163-bp fragment is additionally digested to 120-bp and 43-bp fragments. Thus, we can conclude that a sample carries the c.647+2084G>T mutation if the 120-bp fragment is observed. The SVA retrotransposal insertion and the endogenous fukutin protein were detected as described previously.[5,8] Immunostaining of muscle biopsy cryosections was performed using antibodies against α-DG (IIH6: Millipore, Billerica, MA, USA; AP-074G-C, reported previously.[9] Briefly, AP-074G-C was raised in Goat against recombinant α-DG lacking a signal sequence and the mucin domain that was produced in *Escherichia coli*, and affinity-purified using recombinant α-DG secreted from HEK293.), β-dystroglycan (β-DG) (8D5: Novocastra, Newcastle upon Tyne, UK), dystrophin (ab15277: Abcam, Cambridge, MA, USA), and laminin-α2 (4H8-2: Abcam).

RESULTS

We first attempted direct sequencing of the PCR products from genomic DNA of three (patient no. 7, 8 and 9) of the 10 patients with the haplotype 139-201-155-183 for D9S2105-(FCMD)-D9S2170-D9S2171-D9S2107 around the *fukutin* gene, to check if these patients carried the deep-intronic point mutation c.647+2084G>T in *fukutin* that was previously found in Korean CMD patients.[7] We found that all three patients carried this point mutation in heterozygous form (data not shown). We then constructed a PCR-RFLP system using a mismatched primer pair to detect c.647+2084G>T and applied this method to the 10 patients with the haplotype 139-201-155-183 and their parents, together with analysis of the SVA retrotransposal insertion (Figure 1). The results showed that all 10 patients have the c.647+2084G>T mutation and clearly demonstrated the parental origins of the c.647+2084G>T mutation and the SVA insertion in each patient. Considering their haplotypes that had been analyzed previously, we concluded that the haplotype 139-201-155-183 is linked to the c.647+2084G>T mutation in intron 5 of the *fukutin* gene.[4]

Next, we performed RT-PCR and direct sequencing using RNA from the cultured lymphoblastoid cell lines of patients no. 7, 8 and 9. We found that *fukutin* RNA of all three patients carried the 64-bp pseudoexon insertion between exon 5 and exon 6 that is caused by abnormal splicing, which is consistent with the previous report (Figure 2).[7] We also confirmed that RNA from skeletal muscle of patient no. 10 also contains this pseudoexon.

The pseudoexon insertion is thought to cause a frameshift and a premature stop codon. To confirm that this mutation results in the synthesis of a shorter fukutin protein (presumably ~26 kDa, compared with ~55 kDa for normal fukutin and ~62 kDa for mutant fukutin from the SVA insertion allele), we performed immunoprecipitation and western blotting to detect the endogenously expressed fukutin protein in cultured lymphoblastoid cell lines derived from patients no. 7, 8 and 9 (Figure 3). A larger-sized band derived from the heterozygous SVA insertion allele was observed in all patients as described previously,[5] and, consistent with our prediction, another smaller-sized weak band (~26 kDa) was also observed exclusively in the three patients carrying the pseudoexon insertion. The expression

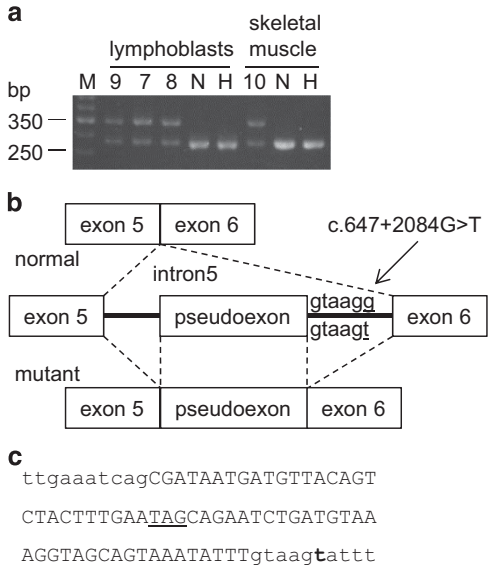

Figure 2 RNA status of the patients carrying the c.647+2084G>T mutation. (**a**) Upper bands (347 bp) show the RT-PCR products containing a pseudoexon. (**b**) Schematic representation of the abnormal splicing caused by this mutation, which results in the pseudoexon. (**c**) The nucleotide sequences of the pseudoexon and the flanking intron parts. The capital letters represent the pseudoexon sequence and the small letters represent the flanking intron sequence. A premature stop codon is underlined. The c.647+2084G>T mutation is shown as a bold letter. M: 50-bp ladder DNA marker; 7–10: patient numbers; N: normal control; H: a patient carrying the SVA insertion homozygously.

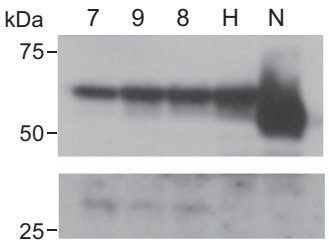

Figure 3 Fukutin protein status of patients carrying the c.647+2084G>T mutation. The upper panel shows the normal fukutin protein (~55 kDa) and the mutant fukutin protein (~62 kDa) derived from the SVA insertion allele. The lower panel shows the truncated fukutin protein that is possibly derived from the c.647+2084G>T mutation allele, detected by long exposure. H: a patient carrying the SVA insertion homozygously; N: normal control; 7–9: patient numbers.

level of the truncated fukutin seems to be very low probably due to the nonsense-mediated mRNA decay mechanism.

We also performed immunostaining to assess the effects of the c.647+2084G>T mutation on the integrity of the dystrophin-glycoprotein complex in the skeletal muscle from one patient (no. 10). Components of the dystrophin-glycoprotein complex, including dystrophin, β-DG, α-DG and laminin-α2 were analyzed (Figure 4). All of these proteins showed normal distributions and expression; however, the glycosylated form of α-DG was not detected, indicating that c.647+2084G>T causes the abnormal glycosylation of α-DG. The α-DG core protein seemed to be slightly reduced in muscle of this patient, although the staining of the α-DG core protein seemed to be normal in previous report.[10] The mechanism behind this discrepancy has yet to be clarified, and it might be due to the variability in the strength of staining depending on patient's background or something. Hematoxylin and eosin staining showed dystrophic changes, including fiber size variability and fibrosis (data not shown).

DISCUSSION

In our previous study, 80 out of 107 Japanese FCMD patients were homozygous for the founder haplotype (138-192-147-183), which is linked to a 3-kb SVA retrotransposal insertion mutation in the *fukutin* gene.[4] Twenty-five of the remaining 27 patients were compound-heterozygous for the founder haplotype and another haplotype. Nine of the 25 patients carried the second most common haplotype 139-201-155-183; however, a specific mutation linked to this haplotype has not yet been found. In this study, we successfully showed that c.647+2084G>T in intron 5 of the *fukutin* gene is linked to this haplotype. Seven of the 25 patients had the third most common haplotype 130-201-157-183, which corresponds to the c.139C>T nonsense mutation in exon 3 of the *fukutin* gene. Therefore, these three mutations account for approximately 90% (96/107) of the mutations of Japanese FCMD patients. The PCR-RFLP method to detect the c.647+2084G>T mutation mentioned in this study will be clinically useful for genetic testing of FCMD patients, together with the PCR method to detect the SVA insertion and the PCR-RFLP method to detect the c.139C>T nonsense mutation.[3] We previously demonstrated that splicing modulation therapy by antisense oligonucleotides is a possible radical treatment for FCMD.[5] Patients who are compound-heterozygous for the SVA insertion and c.647+2084G>T are also expected to benefit from this potential therapy.

Patients carrying the c.647+2084G>T mutation clinically present with severe phenotypes, such as no head control or sitting with support, compared with patients carrying the SVA insertion homozygously.[4] This might be explained by the sites of abnormal splicing. Both the c.647+2084G>T mutation and SVA insertion cause abnormal splicing. The normal *fukutin* gene consists of 10 exons. The c.647+2084G>T mutation results in a pseudoexon between exon 5 and exon 6 and produces a half-truncated fukutin protein that does not contain the DXD motif, which is a putative catalytic site of the fukutin protein (p.Arg216Serfs*10). On the other hand, a splicing acceptor site located within the SVA insertion in exon 10 activates a rare alternative splicing donor site in exon 10, causes abnormal splicing involving the authentic stop codon, and produces a truncated fukutin protein lacking a small part of the C-terminus but containing the DXD motif (p.Gly423_Tyr461delins129).[5] Recently, we found that fukutin is a ribitol-phosphate transferase that is essential for laminin-binding glycan synthesis on α-DG.[11] Fukutin transfers ribitol-phosphate from CDP-ribitol that is produced by ISPD, one of the gene products responsible for the α-dystroglycanopathies, to the C3 position of *N*-acetylglucosamine at the top of the phosphorylated *O*-mannose glycan called CoreM3 on α-DG. Fukutin-related protein, FKRP, another α-dystroglycanopathy gene product, sequentially transfers ribitol-phosphate from CDP-ribitol to the C1 position of ribitol-phosphate which is transferred by fukutin. Thus, the former mutant fukutin protein produced by the c.647+2084G>T mutation may be dysfunctional, whereas the latter by the SVA insertion may retain some enzyme activity. The difference in the small amount of normal fukutin protein that is produced from the very small amount of normally spliced products may be another possible reason.

FCMD was once thought to be endemic only in Japan, but many non-Japanese patients have been found to have mutations in the *fukutin* gene. Moreover, the SVA insertion, which is the Japanese

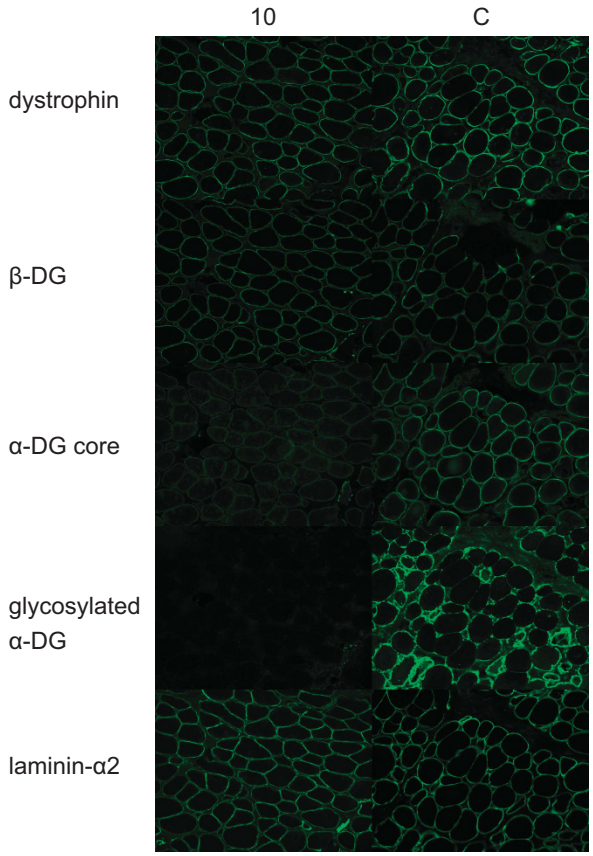

Figure 4 Muscle tissue status of the patient carrying the c.647+2084G>T mutation. Each panel shows immunostaining of the skeletal muscle. C: a patient with suspected connective tissue disease, used as a control; 10: patient number.

founder mutation, has been found in several Korean and Chinese CMD patients.[12,13] Thus, it was not beyond our expectation that the c.647+2084G>T mutation is shared between Japanese and Korean patients, considering their ethnic interactions throughout history. When and where this mutation appeared and how it spread among the modern Japanese and Korean population might be of great interest from the view of ethnology. As for the SVA insertion, its age was calculated to be ~100 generations in our previous study, indicating that it emerged in the Yayoi period when immigrants from Asian continents settled in the Japanese islands.[14] For such discussion regarding the c.647+2084G>T mutation, haplotype analysis of Korean patients will offer a large amount of useful information. If Korean patients with the c.647+2084G>T mutation have the same haplotype (i.e., 139-201-155-183), these patients probably originate from a common ancestor with Japanese patients carrying this mutation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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