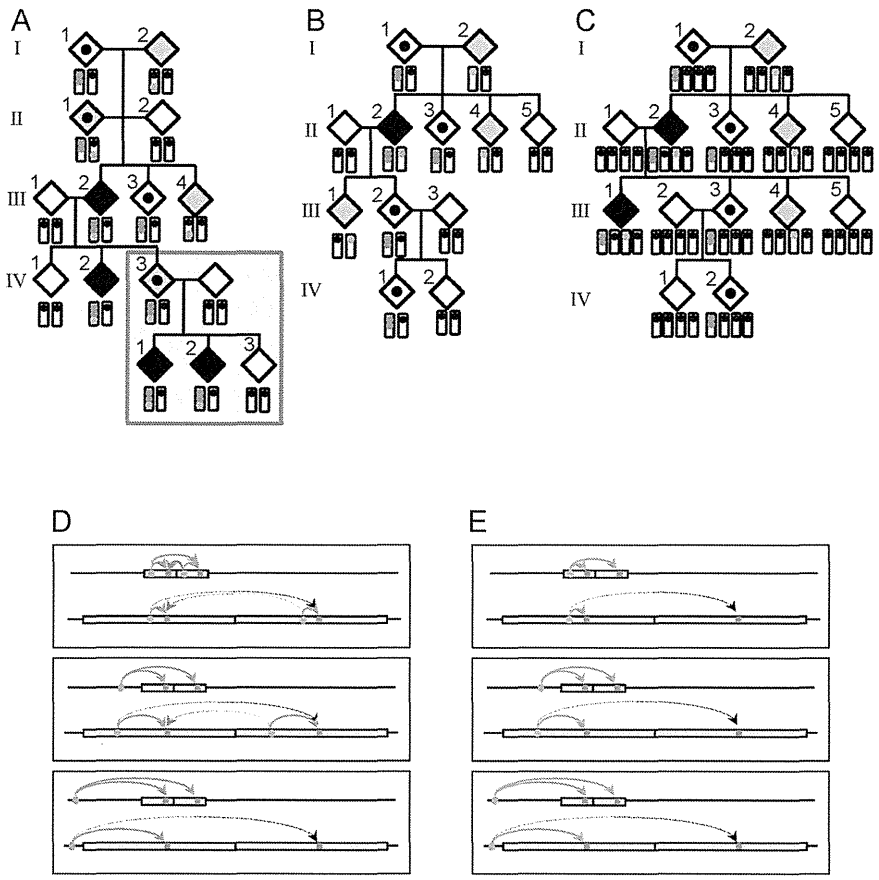

Figure 3 Models for a modifier(s) and effects of the duplication size. In models **A–C**, the yellow bars show chromosome 17, and the light green bars indicate other chromosomes. The two red dots represent the duplication at 17p13.3, and the blue dots indicate a putative modifier(s). Black painted diamonds represent limb malformation positive patients, dot-associated and gray painted diamonds indicate clinically normal carriers with the duplications and the modifier(s) respectively, and white painted diamonds denote clinically normal subjects without both the duplications and the modifier(s). **A**. This model assumes that co-existence of the duplication and a *cis*-acting modifier(s) causes limb malformation. If co-existence of the duplication and the *cis*-acting modifier(s) is associated with incomplete penetrance, this can explain all the transmission patterns observed to date, including the patient-to-carrier transmission and the presence of ≥ 2 affected children. **B**. This model postulates that the presence of a *cis*-acting modifier(s) on the normal chromosome 17 leads to limb malformation by enhancing the expression of the single *BHLHA9*, together with duplicated *BHLHA9* on the homologous chromosome. **C**. This model postulates that co-existence of the duplication at 17p13.3 and a modifier(s) on other chromosome causes limb malformation. In models **D–E**, the red bars represent *BHLHA9*, the blue circles indicate a physiological cis-regulatory element for *BHLHA9*, and the green circles indicate a non-physiological modifier(s) for *BHLHA9*. **D**. The physiological cis-regulatory element may be duplicated or non-duplicated, depending on its position relative to the size of the duplications. *BHLHA9* expression can be higher in small duplications than large duplications. **E**. The non-physiological modifier(s) can be transferred to various positions of the duplication positive chromosome 17, depending on the recombination places (see Model A). *BHLHA9* expression can be higher in small duplications than large duplications irrespective of the position of the modifier(s).

small duplications were ascertained primarily due to developmental retardation and limb malformation, respectively [8]. It is likely that a physiological cis-regulatory element for *BHLHA9* (e.g., an enhancer) can frequently but not invariably work on both of the duplicated *BHLHA9* when the duplication size is small but is usually incapable of working on duplicated *BHLHA9* when the duplication size is large, probably because of the difference in the chromatin structure (see Model D in Figure 3). Similar findings have also been reported in other genes. For example, small (~150 kb) and relatively small (600–800 kb) duplications involving a putative testis-specific enhancer(s) for *SOX9* have caused 46,XX testicular and ovotesticular disorders of sex development respectively, whereas large duplications (~2 Mb) involving the enhancer(s) have permitted normal ovarian development in 46,XX individuals [30].

Thus, a plausible explanation may be that a range of limb malformations emerge when the effects of *BHLHA9* overdosage exceed the threshold for the development of SHFM, SHFLD, or GWC, depending on the conditions of

other genetic and environmental factors including the size of duplications/triplications as an important but not definitive factor. One may argue that this notion is inconsistent with the apparent anticipation phenomenon that is suggested by the rare patient-to-carrier transmission and the frequent carrier-to-patient transmission of the duplications/triplications, because no specific factor(s) exaggerating the development of limb malformations is postulated in the next generation. However, the skewed transmission pattern would primarily be ascribed to ascertainment bias rather than anticipation [31]. Indeed, while clinically normal parents of disease positive children would frequently be examined for the underlying genetic factor(s) of the children, clinically normal children born to disease positive parents would not usually be studied for such factor(s), as exemplified in this study. Similarly, the frequent patient-to-patient transmission of the duplications/triplications would also be ascribed to ascertainment bias, because molecular studies would preferentially be performed in such families. Nevertheless, the apparently autosomal dominant inheritance pattern of limb malformations in several families may still suggest the relevance of a non-physiological *cis*-acting modifier(s) (see Models A and E in Figure 3). It is possible that such a modifier(s), once transferred onto the duplication/triplication positive chromosome 17, is usually co-transmitted with the duplications/triplications, leading to a specific condition in which the effects of *BHLHA9* overdosage frequently but not invariably exceed the threshold for the development of limb malformations in offsprings with the duplications/triplications.

Remarks

Several matters should be pointed out in the present study. First, in contrast to diverse duplication sizes in non-Japanese populations [5-9], the size of the genomic segment subjected to duplications/triplications was identical in this study. Since families 1–27 were derived from various places of Japan, there is no selection bias in terms of a geographic distribution. Rather, since the small duplications/triplications identified in this study were not associated with developmental retardation, it is likely that they spread throughout Japan primarily via carriers with normal fitness and were found via patients with limb malformations. Obviously, this notion does not exclude the possible presence of other types of duplications/triplications at 17p13.3 in Japan. Second, except for the duplications/triplications at 17p13.3, we could reveal a homozygous *WNT10B* mutation (SHFM6) only in a single SHFM family and chromosome 10q24 duplications (SHFM3) only in three SHFM families. Thus, underlying factors are still unknown in the remaining 20 families, although tiny deletions and/or duplications affecting the known SHFM loci might have been overlooked because of the low resolution of the array. In addition, although all the probands had a normal karyotype, there might be cryptic translocations and/or inversions involving the known SHFM loci. Third, no deletion of *BHLHA9* was identified in the 51 probands and in the 200 control subjects. This argues against the relevance of *BHLHA9* haploinsufficiency to limb malformations, and coincides with the Japanese founder duplication being produced by a replication-mediated mechanism rather than an interchromatid/interchromosomal (but not an intrachromatid) NAHR that can lead to both deletions and duplications as a mirror image [17]. Furthermore, it remains to be determined (i) whether gain-of-function mutations (and possibly loss-of-function mutations as well) of *BHLHA9* are identified in patients with limb malformations, (ii) whether duplications/triplications involving *BHLHA9* underlie limb malformations other than SHFM, SHFLD, and GWC, and (iii) whether *BHLHA9*-containing duplications/triplications are also the most frequent underlying factors for limb malformations in non-Japanese populations.

Conclusions

The results imply that (i) duplications/triplications involving *BHLHA9* at chromosome 17p13.3 constitute a strong susceptibility factor for the development of a range of limb malformations including SHFM, SHFLD, and GWC; (ii) the Japanese founder duplication was generated by a replication-based mechanism and spread with subsequent triplication and haplotype modification through recombination-based mechanisms; and (iii) clinical variability appears to be due to multiple factors including the size of duplications/triplications. Thus, the present study provides useful information on the development of limb malformations.

Additional files

Additional file 1: Table S1. Primers utilized in this study.

Additional file 2: Figure S1. Real-time PCR analysis.

Additional file 3: Figure S2. *D17S1174* analysis in 200 Japanese control subjects, showing discontinuous distribution of the CA repeat numbers, as observed in the Japanese families with limb malformations.

Additional file 4: Table S2. *In silico* analysis for specific structures around the breakpoint-flanking regions and control regions.

Additional file 5: Table S3. Phenotypes in patients/subjects with increased copy number of *BHLHA9*.

Additional file 6: Figure S3. Genomic region encompassing *BHLHA9* examined in this study.

Additional file 7: Table S4. Polymorphism analysis of rs3951819 (A/G SNP) in *BHLHA9*.

Additional file 8: Table S5. Polymorphism analysis of rs34201045 (4 bp insertion) in *TP63*.

Additional file 9: Figure S4. Genomic basis of the Japanese founder copy number gain.

Abbreviations

AER: Apical ectodermal ridge; CEP17: Centromere of chromosome 17; CGH: Comparative genomic hybridization; Dup: Duplication; FoSTeS: Fork stalling and template switching; GWC: Gollop-Wolfgang complex; L: Lower; LBD: Long bone defect; MMBIR: Microhomology-mediated break-induced replication; NAHR: Non-allelic homologous recombination; N.E.: Not examined; NHEJ: Non-homologous end-joining; qPCR: Quantitative real-time PCR; SF: Split foo17)t; SH: Split hand; SHFLD: SHFM with long bone deficiency; SHFM: Split-hand/foot malformation; Trip: Triplication; U: Upper.

Competing interests

The authors have nothing to declare.

Authors' contributions

Molecular analysis using human samples was performed by EN, HK, FK. RY, SN, SW, KY, TT, SS, MF, and TT, ST, and SY; clinical assessment and blood sampling by RK, HT, SM, TK, TH, MK, AS, KS, HO, NH, HN, EH, TN, HY, GN, and TO; design of this study and interpretations of the data by HA, SI, and TO; and paper writing by TO. All authors read and approved the final manuscript.

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ANNALS of Clinical and Translational Neurology
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BRIEF COMMUNICATION

ABCG2 variant has opposing effects on onset ages of Parkinson's disease and gout

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Abstract

Uric acid (urate) has been suggested to play a protective role in Parkinson's disease onset through its antioxidant activity. Dysfunction of ABCG2, a high-capacity urate exporter, is a major cause for early-onset gout based on hyperuricemia. In this study, the effects of a dysfunctional *ABCG2* variant (Q141K, rs2231142) were analyzed on the ages at onset of gout patients ($N = 507$) and Parkinson's disease patients ($N = 1015$). The Q141K variant hastened the gout onset ($P = 0.0027$), but significantly associated with later Parkinson's disease onset ($P = 0.025$). Our findings will be helpful for development of more effective prevention of Parkinson's disease.

Introduction

Parkinson's disease (PD) is a multifactorial disease characterized by selective cell death of dopaminergic neurons. Oxidative stress is well known to be one of the major causes of PD development.[1] On the other hand, uric acid (UA), which has an antioxidant effect on the central nervous system (CNS), may play a protective role in onset and development of PD.[2,3] Gout, a consequence of hyperuricemia, is also associated with a lower risk of PD.[4] Previously, common dysfunctional variants of ATP-binding cassette transporter, sub-family G, member 2 (*ABCG2*, also known as *BCRP*), a urate transporter gene,[5,6] have been revealed to be a major cause of early-onset gout.[7] The common variant (Q141K, rs2231142) of *ABCG2* is proven to be a dysfunctional variant by in vitro functional studies.[5,6]

This study aimed to evaluate whether the Q141K variant of *ABCG2* could delay the age at onset (AAO) of PD in a relatively large population of Japanese patients.

Patients and Methods

Study participants

This study was approved by the institutional ethical committees, and all procedures involved in this study were performed in accordance with the Declaration of Helsinki. Informed consent in writing was obtained from each subject participating in this study. A total of 1015 PD patients (464 male and 548 female) and 507 gout male patients was collected and then genetically analyzed. PD patients were collected in Juntendo University (Tokyo, Japan) and Kobe University (Kobe, Japan). Diagnosis of PD was made by board-certified neurologists of the Japanese Society of Neurology, based on the presence of at least two cardinal features of PD with no secondary cause, no levodopa unresponsiveness, or no early signs of more extensive nervous system involvement.[8] Clinically defined gout cases were collected in the Kyoto Industrial Health Association (Kyoto, Japan).

Genetic analysis

Genomic DNA was extracted from whole peripheral blood cells.[9] For PD patients, genotyping of Q141K (rs2231142) in *ABCG2* gene was performed by direct sequencing using the following primers: forward, 5′-ATGGAGTTAACTGTCATTTGC-3′, and reverse, 5′-CACGTTCATATTATGTAACAAGCC-3′. DNA sequencing analysis was performed with a 3130xl Genetic Analyzer[10] (Life Technologies Corporation, Carlsbad, CA). The genotyping data of PD patients collected in Kobe University were obtained from the result of previous GWAS[11] using the Illumina Infinium HumanHap550 array (Illumina, Inc., San Diego, CA). For gout patients, genotyping of Q141K in *ABCG2* gene was performed by TaqMan assay (Life Technologies Corporation) with a LightCycler 480 (Roche Diagnostics, Mannheim, Germany).[12,13]

Statistical analysis

In the statistical analysis, SPSS v.17.0J (IBM Japan Inc., Tokyo, Japan) was used for all calculations. Regression analysis was used for the association analysis.

Results

The results of genotyping of gout and PD patients are shown in Table 1. Figure 1 shows the AAO of gout and PD participants of each genotype of *ABCG2* Q141K. The AAO (mean ± standard error) of gout were 40.4 ± 1.1 years old, 42.0 ± 0.7 years old, and 45.0 ± 1.1 years old for patients with Q141K homozygous (A/A), heterozygous (C/A) mutation, and without Q141K mutation (C/C), respectively. On the other hand, the AAO of PD were 58.5 ± 1.1 years old, 58.2 ± 0.5 years old, and 56.6 ± 0.5 years for patients with Q141K homozygous, heterozygous mutation, and without mutation, respectively. The AAO of gout with homozygous mutation was 4.6 years younger than those without Q141K mutation, while the AAO of PD with homozygous mutation was 1.6 years older than those without Q141K mutation.

The Q141K mutation of *ABCG2* hastened the onset of gout significantly ($P = 0.0027$; see Fig. 1A); on the contrary, this variant significantly delayed the PD onset ($P = 0.025$; see Fig. 1B).

Discussion

This study revealed for the first time that a common dysfunctional variant of *ABCG2* (Q141K, rs2231142) has surprisingly differential effects on two common diseases, significantly delaying the AAO of PD, while hastening that of gout. *ABCG2* encodes ATP-dependent transporter for urate excretion both in gut[14,15] and kidney.[16] Molecular functional studies revealed that ABCG2 dysfunction elevates serum UA levels.[5,6] As UA is the strong antioxidant, ABCG2 dysfunction might have a neuroprotective effect. In fact, our study showed that the dysfunctional variant of this UA-related gene, *ABCG2*, could have a protective effect against PD, which is wholly consistent with the previous studies suggesting that the higher levels of serum UA are negatively correlated with the risk of PD[17] and its rate of progression.[18]

So far, only a few genetic analyses have been performed about the association between PD onset and UA-related genes.[19,20] However, there is no report demonstrating that

Table 1. Genotype of *ABCG2* variant Q141K (rs2231142) for gout and PD patients.

	N (%)				
Q141K (rs2231142[1])	C/C	C/A	A/A	Total	MAF
Gout cases	131 (25.8)	257 (50.7)	119 (23.5)	507 (100.0)	0.49
PD cases	509 (50.1)	425 (41.9)	81 (8.0)	1015 (100.0)	0.29

PD, Parkinson's disease; MAF, minor allele frequency.
[1]For alleles of rs2231142 (C for cytosine; A for adenine), allele A is the minor allele.

a single variant of *ABCG2* could significantly affect the AAO of PD.

Together with the antioxidant effect of UA, our results strongly support the hypothesis that UA should reduce the risk of PD as an antioxidant, because oxidative stress is involved in the pathogenesis of PD. In addition to its expression in gut and kidney, ABCG2 highly expresses in the blood brain barrier (BBB).[21] Therefore, we propose a physiological model that ABCG2 exports urate from the brain side to the blood side at BBB (see Fig. 2). Since ABCG2 dysfunction decreases urate excretion via gut[14,15] and kidney,[16] which results in serum UA elevation,[5,6,14,16] it therefore has a pathogenic effect on earlier onset of gout. Elevated serum UA also should result in elevated UA levels in CNS. In addition, ABCG2 dysfunction could decrease urate excretion via BBB that enhances the

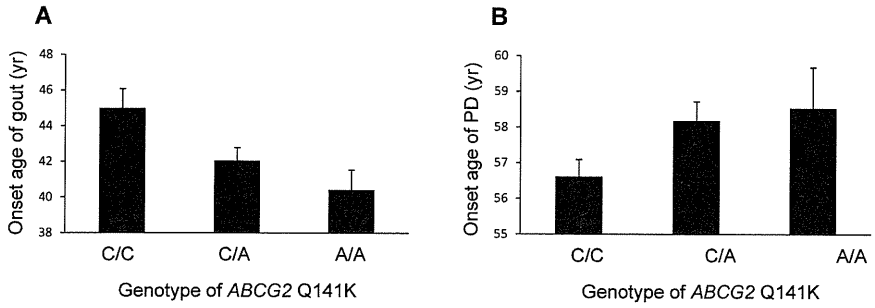

Figure 1. *ABCG2* dysfunctional variant (Q141K) and the age at onset (AAO) of gout/PD. The AAO of gout was significantly hastened as the number of minor alleles of Q141K increased ($P = 0.0027$); on the contrary, the AAO of PD was significantly delayed as the number of minor alleles of Q141K increased ($P = 0.025$). The AAO of gout with homozygous mutation (A/A) was 4.6 years younger than those without Q141K mutation (C/C). And the AAO of PD with homozygous mutation was 1.6 years older than those without Q141K mutation. Each bar represents the mean with standard error.

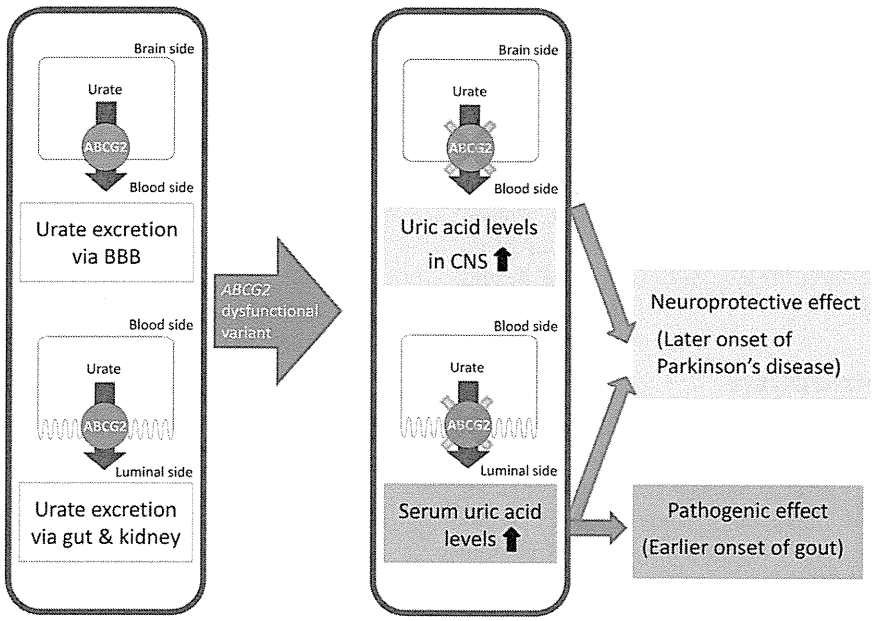

Figure 2. Contrary effects of ABCG2 dysfunction on PD and gout. ABCG2 is expressed in gut, kidney, and blood brain barrier (BBB) and exports urate. ABCG2 dysfunction in gut and kidney elevates the serum uric acid (UA) levels and subsequently causes gout. In this proposed model, ABCG2 dysfunction in BBB plays an important role on increasing UA levels in central nervous system (CNS), together with increased serum UA by ABCG2 dysfunction in gut and kidney.

elevation of UA levels in CNS as shown in our proposed model (see Fig. 2). In this model, ABCG2 dysfunction coordinately increases UA levels in CNS by the combined two differential mechanisms shown in Figure 2, although other UA-related gene variants have not been reported to have such differential mechanisms to elevate UA levels in CNS. Thus, the dysfunction of ABCG2 both in gut/kidney and BBB could cooperatively contribute to the elevated UA levels in CNS. These proposed differential mechanisms are consistent with our present result, which showed the differential effects on AAO of two common diseases, gout and PD. By these two differential mechanisms, therefore, ABCG2 dysfunction could have a significant neuroprotective effect for later onset of PD through increased UA, the strong antioxidant (see Fig. 2). That is why ABCG2 dysfunction could have significant effects on PD and be important in PD pathogenesis. Furthermore, the regulation of UA levels in serum and CNS could be applicable for prevention and therapy of PD.[22]

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Author Contribution

H. M., W. S., T. C., Y. K., A. N., S. S., M. S., T. T., and N. S. performed genetic analyses. H. T., W. S., H. O., M. F., K. N., T. S., K. Kaida., K. Kamakura., T. T., and N. H. performed clinical evaluations and medical record reviews. H. M. and T. C. wrote the paper. All authors contributed to data interpretation and manuscript preparation.

Conflicts of Interest

None declared.

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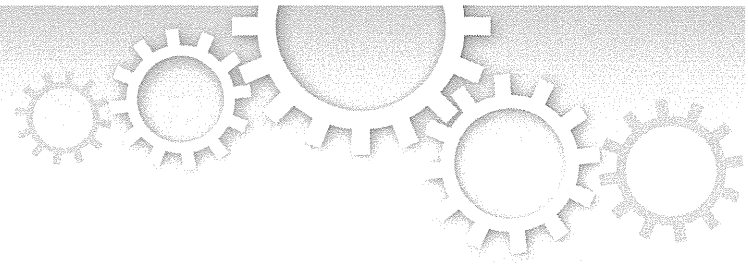

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Fukutin is prerequisite to ameliorate muscular dystrophic phenotype by myofiber-selective LARGE expression

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α-Dystroglycanopathy (α-DGP) is a group of muscular dystrophy characterized by abnormal glycosylation of α-dystroglycan (α-DG), including Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease, Walker-Warburg syndrome, and congenital muscular dystrophy type 1D (MDC1D), etc. LARGE, the causative gene for MDC1D, encodes a glycosyltransferase to form [-3Xyl-α1,3GlcAβ1-] polymer in the terminal end of the post-phosphoryl moiety, which is essential for α-DG function. It has been proposed that LARGE possesses the great potential to rescue glycosylation defects in α-DGPs regardless of causative genes. However, the in vivo therapeutic benefit of using LARGE activity is controversial. To explore the conditions needed for successful LARGE gene therapy, here we used Large-deficient and fukutin-deficient mouse models for MDC1D and FCMD, respectively. Myofibre-selective LARGE expression via systemic adeno-associated viral gene transfer ameliorated dystrophic pathology of Large-deficient mice even when intervention occurred after disease manifestation. However, the same strategy failed to ameliorate the dystrophic phenotype of fukutin-conditional knockout mice. Furthermore, forced expression of Large in fukutin-deficient embryonic stem cells also failed to recover α-DG glycosylation, however coexpression with fukutin strongly enhanced α-DG glycosylation. Together, our data demonstrated that fukutin is required for LARGE-dependent rescue of α-DG glycosylation, and thus suggesting new directions for LARGE-utilizing therapy targeted to myofibres.

α-Dystroglycanopathy (α-DGP) is a genetically and clinically heterogeneous group of muscular dystrophy[1,2] for which more than 15 causative genes have been identified[3–21]: *POMT1, POMT2, POMGnT1, fukutin, FKRP, LARGE, ISPD, GTDC2* (*POMGnT2*), *DAG1, TMEM5, B3GALNT2, SGK196* (*POMK*), *B3GNT1* (*B4GAT1*), *GMPPB, DOLK, DPM1, DPM2 and DPM3*. Regardless of the causative gene, α-DGP is characterized by abnormal glycosylation of α-DG, indicating that the disease is associated with defects in the glycosylation pathway for α-DG. α-DG is a cell surface receptor for matrix and synaptic proteins such as laminins, agrin, perlecan, neurexin, and pikachurin[22,23]. A unique *O*-mannosyl glycosylation is required for the ligand-binding activity of α-DG, and abnormal glycosylation leads to reduced ligand-binding activity[24,25]. α-DG also interacts with a transmembrane β-DG, which in turn binds to intracellular dystrophin[23]. Thus, proper glycosylation of α-DG is necessary for the connection between the basement membrane and cytoskeleton. Disruption of this linkage is thought to cause myofibre membrane weakness, leading to disease-predisposing muscle cell necrosis[26]. Although myofibres can regenerate after necrosis, it has been shown that muscle regeneration activity is impaired in α-DGP[27]. Thus, α-DG glycosylation is important for maintenance of skeletal muscle viability and defects in this process underlie the pathogenesis of α-DGP.

α-DGP includes Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), Walker-Warburg syndrome (WWS), and several types of congenital muscular dystrophies (MDCs) and limb-girdle muscular dystrophies (LGMDs)[1,2]. The clinical spectrum of α-DGP is wide; the most severe cases exhibit congenital muscular dystrophy with structural abnormalities in the brain and eyes, whereas the mildest form presents as adult-onset LGMD with no central nervous system involvement[28–30]. In addition, there is no clear genotype-phenotype correlation. Thus, it has been proposed that α-DGPs can be classified into three broad phenotypic groups, MDDG (muscular dystrophy dystroglycanopathy) type A, B and C[29]: MDC with brain/eye abnormalities (A), MDC with milder brain structural abnormalities (B), and LGMD (C). FCMD is the first

identified α-DGP[31] and the second most common childhood muscular dystrophy in Japan[32,33]. The causative gene for FCMD is *fukutin*[6]. *LARGE* was identified as the gene responsible for MDC1D[8]. Fukutin and LARGE are involved in a novel phosphodiester-linked modification, namely, post-phosphoryl modification, of *O*-mannose on α-DG[24,34]. Although the exact function of fukutin is unknown, LARGE was recently shown to be a glycosyltransferase that catalyses the formation of a repeating [-3Xyl-α1,3GlcAβ1-] polymer, which is modified on the distal end of the post-phosphoryl moiety[35]. These repeating units likely serve as the ligand-binding domain of α-DG[36]. Interestingly, overexpression of LARGE causes hyperglycosylation of α-DG with increased ligand-binding activity not only in wild-type and *Large*-deficient muscle cells, but also in cells from WWS, MEB, FCMD patients and mouse models[37]. This finding inspired a novel therapeutic strategy based on the unique activity of LARGE—modulation of LARGE activity can be a versatile treatment for α-DGP, regardless of the causative gene.

After this breakthrough finding, several reports showed that overexpression of LARGE in mice induced hyperglycosylation of α-DG in skeletal muscle of α-DGP mouse models such as *POMGnT1*- and *FKRP*-deficient[38,39]. However, LARGE overexpression in cells lacking GTDC2 expression or POMT1 activity did not induce hyperglycosylation of α-DG[9,40]. Moreover, some studies have shown the beneficial effects of LARGE overexpression in *POMGnT1*- or *FKRP*-mutant mice[38,39], but others showed a deterioration in *FKRP*- or *fukutin*-mutant mice crossed with LARGE-overexpressing transgenic mice[41,42]. Thus, it remains unclear whether LARGE could be a target molecule for α-DGP treatment. We hypothesized that the conditions for LARGE expression such as way of gene delivery, timing of intervention, and target cells may affect α-DG glycosylation and therapeutic consequences. Here, we examined therapeutic benefits of myofibre-selective *Large* gene expression after disease manifestation in *Large*- or *fukutin*-deficient α-DGP mouse models. Our data also showed that fukutin is a prerequisite for LARGE-dependent rescue of α-DG glycosylation.

Results

Myofibre-selective expression of *Large* after disease onset restores α-DG glycosylation and ameliorates dystrophic pathology of *Large^{myd}* mice. We performed systemic *Large* gene delivery after disease manifestation and myofibre-selective *Large* gene expression in α-DGP mouse models. For this purpose, we constructed recombinant adeno-associated virus (AAV) 9 vectors containing the *Large* cDNA under the myofibre-selective muscle creatine kinase (MCK) promoter (AAV9-MCK-*Large*). We first examined the therapeutic benefits of muscle-selective LARGE expression in *Large*-deficient *Large^{myd}* mice. New-born *Large^{myd}* mice (1 week old) showed no signs of muscle pathology (Fig. 1a), but at 4 weeks of age, the *Large^{myd}* skeletal muscles showed signs of muscular dystrophy such as necrotic and regenerating fibres (Fig. 1a). After 4 months, *Large^{myd}* mice showed severe dystrophic pathology in the hind-limb muscles (Fig. 1a). The dystrophic changes include the presence of myofibres with loss of polygonal contour, high population of regenerating fibres with centrally located nuclei, and infiltrations of macrophages and connective tissues. Therefore, we administered intravenous AAV9-MCK-*Large* via the tail vein to 5-week-old *Large^{myd}* mice exhibiting dystrophic symptoms, and then analysed α-DG glycosylation status and therapeutic effects after 5 months. Glycosylation status was evaluated by assessing the reactivity of the monoclonal IIH6 antibody, which recognizes properly glycosylated α-DG[25].

Western blot analysis confirmed LARGE was overexpressed in AAV-treated *Large^{myd}* mice; consequently, the reactivity of IIH6 antibody exceeded even the baseline levels observed in untreated heterozygous animals (Fig. 1b). Immunofluorescence analysis also confirmed increased IIH6-reactivity in the treated *Large^{myd}* skeletal muscles (Fig. 1c). Haematoxylin and eosin (H&E) staining of skeletal muscles indicated decreases in the number of necrotic fibres and recovery of the polygonal contour of myofibres in AAV-treated *Large^{myd}* versus untreated *Large^{myd}* mice (Fig. 1d). The number of muscle fibres with centrally located nuclei as well as infiltration of connective tissues and macrophages were significantly reduced in comparison to the findings obtained for untreated *Large^{myd}* mice (Fig. 2a–c). After the AAV-injection, we tracked changes in grip strength, body weight, and serum creatine kinase (CK). Our results showed significant improvements of these parameters even 4 weeks after the injection (Fig. 2d–f). These results demonstrated that myofibre-selective LARGE expression in *Large^{myd}* mice via systemic administration ameliorates the dystrophic pathology even if the initial intervention occurs after onset.

***Large* gene therapy failed to restore glycosylation and ameliorate muscle pathology of *fukutin*-deficient α-DGP models.** LARGE overexpression increases glycosylation and ligand-binding activity of α-DG in *fukutin*-deficient cells from FCMD patients[37]. We examined whether the muscular dystrophic phenotype of *fukutin*-deficient mice can be improved by LARGE overexpression in vivo. We used muscle precursor cell (MPC)-selective *fukutin*-deficient conditional knock-out (cKO) mice as a *fukutin*-deficient model (Myf5-*fukutin*-cKO mice)[27]. Myf5-*fukutin*-cKO mice showed loss of IIH6-positive glycosylation of α-DG in the skeletal muscles at birth[27]. The dystrophic pathology begins around 4 weeks of age and becomes severe at 12 weeks[27]. We administered intravenous AAV9-MCK-*Large* into 4-week-old Myf5-*fukutin*-cKO mice via the tail vein, and then analysed the glycosylation status of α-DG and therapeutic efficacy after 2 months. Interestingly, although we observed expression of LARGE protein in the AAV-treated Myf5-*fukutin*-cKO skeletal muscles, IIH6-positive α-DG was hardly produced in AAV-treated Myf5-*fukutin* cKO mice (Fig. 3a). Immunofluorescence staining also confirmed failure to restore IIH6-positive glycosylation of α-DG by AAV-treatment in Myf5-*fukutin*-cKO mice (Fig. 3b). H&E staining of skeletal muscles and quantitative muscle pathology showed no significant improvement with AAV treatment (Fig. 3c and Fig. S1a–c). In addition, we found no evidence to support improvements in grip strength, body weight, and serum CK activity after AAV treatment (Fig. S1d–f). These data indicate that the failure to restore α-DG glycosylation in Myf5-*fukutin*-cKO mice is associated with failure of LARGE therapeutic efficacy.

The amount of LARGE protein expressed in the AAV-treated Myf5-*fukutin*-cKO mice was comparable to that in AAV-treated *Large^{myd}* (Fig. 3a). α-DG glycosylation was recovered in *Large^{myd}* skeletal muscle after AAV9-MCK-*Large* treatment, suggesting something other than protein expression levels is responsible for the failure of glycosylation recovery in Myf5-*fukutin*-cKO mice. We hypothesized that complete loss of fukutin caused failure to build the part of post-phosphoryl moiety, which may be required for LARGE-dependent glycosylation; therefore, even excess LARGE protein could not form the [-3Xyl-α1,3GlcAβ1-] polymer on α-DG. To test this hypothesis, we expressed LARGE in *fukutin*-null embryonic stem (ES) cells. Transfection of the *fukutin* cDNA restored IIH6 reactivity in *fukutin*-null ES cells, but transfection of the *Large* cDNA failed to restore α-DG glycosylation, although LARGE expression in wild-type ES cells produced strong IIH6-reactivity (Fig. 4). When the *fukutin* and *Large* cDNAs were co-transfected into *fukutin*-null ES cells, we observed increases in IIH6-reactivity in comparison to *fukutin* singly transfected cells although expression levels of both fukutin and LARGE were much lower than they were in each single transfection (Fig. 4, lanes 6–8). These data show that fukutin-dependent modification is a prerequisite for LARGE-dependent formation of [-3Xyl-α1,3GlcAβ1-] repeating units.

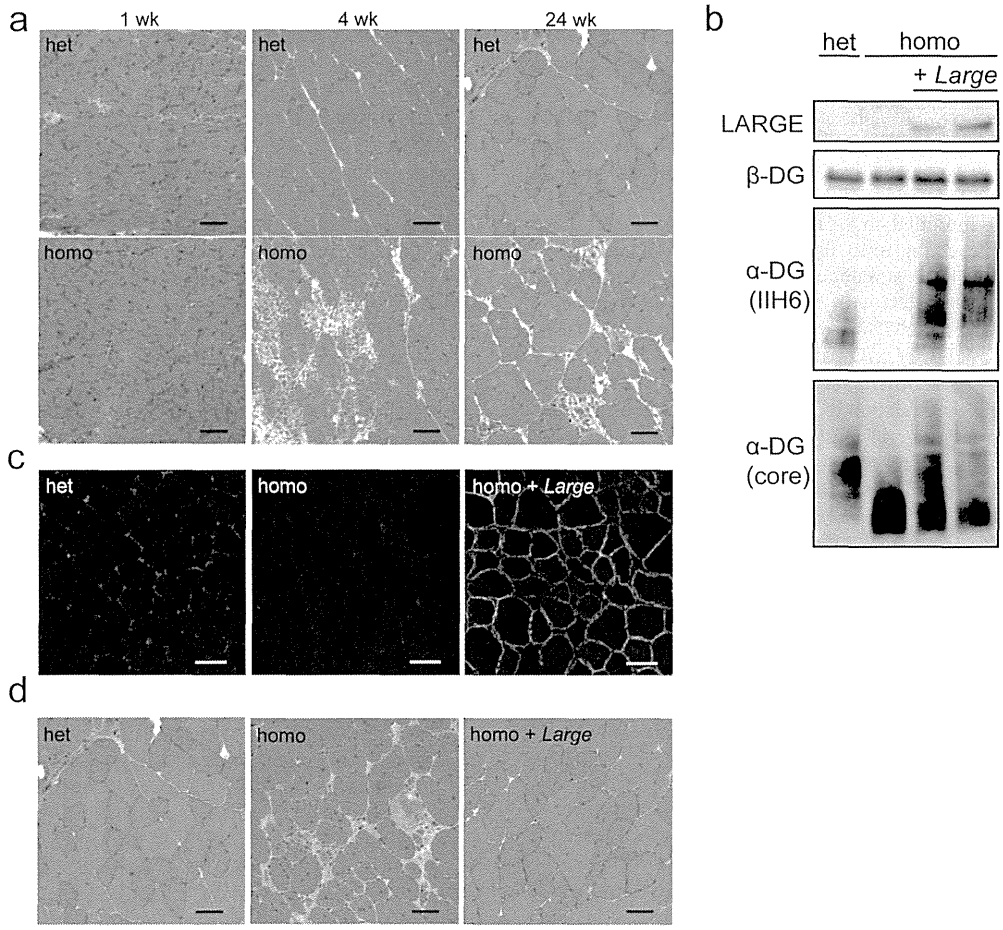

Figure 1 | Systemic gene transfer of Large into Large^{myd} mice after onset. (a) Histopathological analysis (H&E staining) of the skeletal muscle of *Large^{myd}* (homo) and heterozygous (het) mice at 1, 4, and 24 weeks of age. Bar = 50 μm. (b–d) AAV9-MCK-*Large* was administered to 5-week-old *Large^{myd}* mice via the tail vein; after 5 months, the skeletal muscles were harvested and analysed for α-DG glycosylation (b, c) and histology (d). LARGE was detected by western blotting of total lysates (b). β-DG was used as a loading control. DG proteins enriched with WGA-beads were analysed by western blotting to assess α-DG glycosylation (b). The full-length blots with α-DG (IIH6), α-DG (core), LARGE, and β-DG are presented in Supplementary Figure S2a–d, respectively. Immunofluorescence analysis with IIH6 antibody confirmed the increase in α-DG glycosylation (c). H&E staining of the tibialis anterior muscle indicated amelioration of the muscular dystrophic phenotype after treatment with AAV9-MCK-*Large* (d). Het, *Large^{myd}* heterozygous controls; homo, untreated *Large^{myd}* homozygous mice; and homo + *Large*, *Large^{myd}* homozygous mice with AAV9-MCK-*Large* treatment. Bar = 50 μm.

Discussion

It has been widely recognized that LARGE possesses the great potential to rescue glycosylation defects of α-DG regardless of causative genes for α-DGP, however, therapeutic benefits of using LARGE activity is controversial. To assess the feasibility of LARGE-utilizing therapy, in this study we explored the conditions needed for successful *Large* gene therapy. We demonstrated for the first time that AAV-mediated *Large* gene expression targeted to myofibres is therapeutically beneficial in a *Large*-deficient α-DGP model even when the intervention is performed after disease manifestation. On the other hand, the same strategy failed to restore α-DG glycosylation in *fukutin*-deficient skeletal muscles and ES cells, suggesting that fukutin is required for LARGE-utilizing therapeutic strategy.

Several reports have shown that AAV-mediated LARGE expression, which was driven by the chicken β-actin (CB) promoter, restored glycosylation of α-DG in *Large^{myd}* mice[38,39]. However, expression of exogenous genes by non-selective promoter such as the CB promoter in various non-muscle cells may be detrimental. For example, because glycosylation levels of α-DG and the expression levels of LARGE change during muscle differentiation and regeneration[36], excess glycosylation may disturb cellular homeostasis and tissue regeneration. In fact, LARGE overexpression in C2C12 myoblasts impairs differentiation[42]. Furthermore, there may be situations or cells in which glycosylation is physiologically unnecessary even in wild-type tissues[34]. Although AAV-mediated *Large* gene transfer using the CB promoter rescued the muscular dystrophic phenotype in *Large^{myd}* mice[38,39], this study was designed to introduce viral vectors into new-born pups; thus, it is likely that α-DG restores proper glycosylation before disease manifestation, preventing disease-causing myofibre necrosis. However, in humans, clinical and genetic diagnoses of α-DGP are made after disease manifestation, making gene delivery in new-borns unfeasible. We demonstrated that myofibre-selective expression of LARGE ameliorated the dystrophic phenotype of *Large^{myd}* mice, and the intervention even after disease manifestation was effective by using systemic AAV-mediated gene delivery. The effect continues at least for 5 months after the intervention, the longest period observed in gene therapy studies of α-DGP mouse models. Myofibre-selective rescues of fukutin or LARGE expression by AAV gene transfer or crossing with transgenic mice improved the dystrophic phenotypes of Myf5-*fukutin*-cKO or

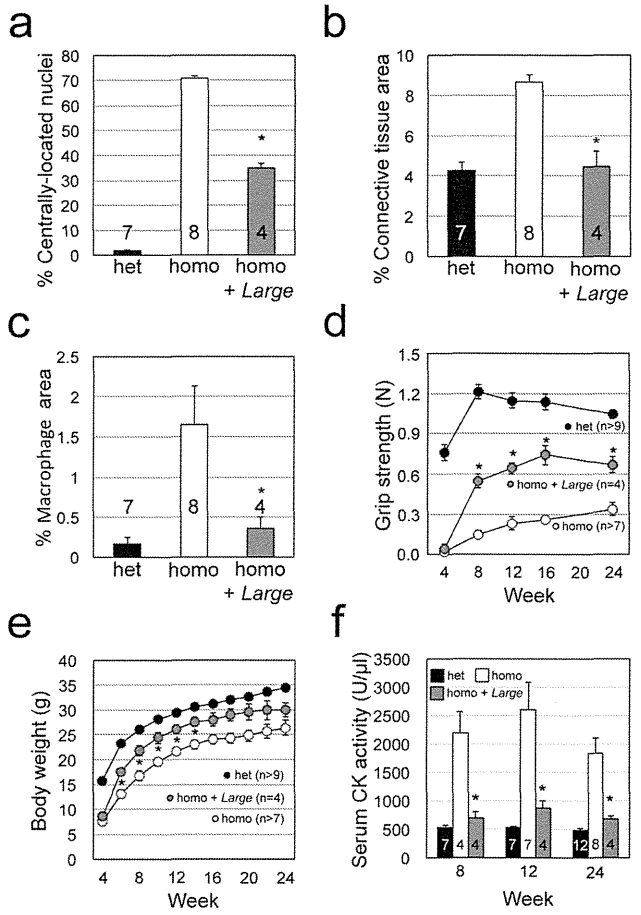

Figure 2 | Quantitative analysis of the therapeutic effects of AAV9-MCK-Large treatment in Large^{myd} mice. Amelioration of dystrophic histology after AAV9-MCK-*Large* treatment was evaluated by quantifying muscle fibres with centrally located nuclei (a; $P = 0.007$), measuring infiltration of connective tissue by collagen I-immunofluorescence staining (b; $P = 0.007$) and infiltration of macrophages by F4/80-immunofluorescence staining (c; $P = 0.011$). Therapeutic efficacy over time was evaluated by grip strength (d; $P = 0.007$, 0.006, 0.008, and 0.014 for 8, 12, 16, and 24 weeks), body weight (e; $P = 0.019$, 0.019, 0.024, 0.017, and 0.032 for 6, 8, 10, 12, and 14 weeks), and serum CK activity (f; $P = 0.021$, 0.008, and 0.011 for 8, 12, and 24 weeks). Data shown are mean ± s.e.m. for each group (*n* is indicated in the graph). $*P \leq 0.05$ vs. non-treated *Large^{myd}* homozygous mice (Mann–Whitney U test). Het, *Large^{myd}* heterozygous controls; homo, untreated *Large^{myd}* homozygous mice; and homo + *Large*, *Large^{myd}* homozygous mice with AAV9-MCK-*Large* treatment.

Large^{myd} mice[27,43], supporting the validity of myofibre-selective gene rescue for treatment of α-DGP. We propose that myofibre-targeting rescue/reinforcement of glycosylation is an effective treatment for α-DGP.

We also examined the potential of LARGE as a therapeutic target for *fukutin*-deficient α-DGP; however, our data showed that LARGE expression failed to restore α-DG glycosylation in *fukutin*-null myofibres and ES cells. LARGE synthesises [-3Xyl-α1,3GlcAβ1-] repeating units in the terminal end of the post-phosphoryl moiety, which is modified on phospho-mannose in the Core M3 structure, GalNAc-GlcNAc-Man(P)-O[24,35,44]. The activities of POMT and GTDC2 are required for Core M3 synthesis and fukutin mediates formation of the post-phosphoryl modification[24,34,44]. In cells lacking POMT activity or GTDC2 expression, LARGE overexpression failed to induce hyperglycosylation or restore glycosylation of α-DG[9,40]. Thus, even when overexpressed, LARGE requires the Core M3 structure and fukutin-dependent modification in order to form the [-3Xyl-α1,3GlcAβ1-] repeating units. However, several reports have shown LARGE-dependent glycosylation recovery in genetically distinct α-DGP models[38,39], which seems contradictory but can be explained by the presence of residual glycosylation of α-DG. Presumably, α-DG is hypoglycosylated in such cells, but there might be a small amount of normally glycosylated α-DG species produced by residual activity of the mutant gene products. In fact, the *fukutin*-knock-in and *POMGnT1*-KO mice showed slight reactivity against IIH6 antibody, indicating the presence of normally glycosylated α-DG[45,46]. In such cases, LARGE can reinforce [-3Xyl-α1,3GlcAβ1-] repeats on the small amount of residual Core M3 structure. Importantly, many mild cases of FCMD patients show the presence of a small fraction of normally glycosylated α-DG[30]. Thus, the concept of LARGE modulation therapy remains attractive for a wide range of mild cases of α-DGP such as MDDG type C.

An important issue when considering therapeutic applications is that α-DGP is accompanied by central nervous system abnormalities including severe defects in structural development and mental retardation[28,29]. Although the AAV9 vector delivers genes to central nervous tissues[47], there are many obstacles to treatment for developing central nervous tissues. For example, the structural abnormalities in the central nervous system occur during fetal developing stage[48,49], and such abnormalities are thought to be irreversible after birth. Moreover, fetal therapy also contains many concerns such as ethical, legal, technical, and clinical safety issues. However, improvement of muscle functions via muscle-targeting strategies will improve patients' activities of daily living and caregivers' burdens, and may have an impact on patient mental development. We propose that gene therapy is an effective approach to α-DGP even after disease progression, and that LARGE therapy may be applied to mild cases of α-DGP. Our study will provide a new direction for therapeutic approached to α-DGP.

Methods

Animals. *Large*-deficient *Large^{myd}* mice were from Jackson Laboratories. Generation of muscle precursor cell (MPC)-selective *fukutin* conditional knock-out (cKO) mice (Myf5-*fukutin*-cKO mice) was described previously[27]. All animal procedures were approved by the Animal Care and Use Committee of Kobe University Graduate School of Medicine (P120202-R2) in accordance with the guidelines of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and the Japan Society for the Promotion of Science (JSPS). The animals were housed in cages (2–4 mice per cage) with wood-chip bedding in an environmentally controlled room (25°C, 12 h light-dark cycle) and provided food and water *ad libitum* at the animal facility of Kobe University Graduate School of Medicine. Well-trained and skilled researchers and experimental technicians, who have knowledge of methods to prevent unnecessary and excessive pain, handled the animals and performed the experiments. Euthanisation was done by cervical dislocation. At sacrifice, the muscles were harvested and snap-frozen in liquid nitrogen (for biochemistry) or in liquid-nitrogen-cooled isopentane (for immunofluorescence and histology). The number and ages of the animals used in each experiment are indicated in the Figure legends and graphs.

Adeno-associated viral gene transfer and evaluation of therapeutic efficacy. To generate a *Large*-encoding AAV9 vector, the complete open reading frame of the mouse *Large* gene was cloned into pAAV-IRES-hrGFP[50]. The MCK promoter was subcloned from AAV-MCKLacZ[51]. The recombinant *Large*-encoding AAV9 vector (AAV9-MCK-*Large*) was produced as described[50]. The AAV9-MCK-*Large* viral vectors (5×10^{11} vector genome) were injected into *Large^{myd}* (n = 4) and Myf5-*fukutin* cKO (n = 6) mice via tail vein at 4–5 weeks of age. Before the injection, body weight, grip strength, and serum CK activity were measured. These clinical parameters (body weight, grip strength, and serum CK activity) were continuously measured until the AAV-treated mice were sacrificed for histological evaluation (the time points of the clinical tests were shown in the figures). The H&E and immunofluorescence images shown in the figures are representative of the AAV-treated and non-treated mice.

Quantitative evaluation of muscle pathology was performed by assessing the number of myofibres with centrally located nuclei at least 1,000 fibres. Macrophage and connective tissue infiltration was quantified by analysing the immunofluorescence signals of F4/80-positive and collagen I-positive areas with Image J software. Serum CK activity was measured with the CPK kit (WAKO). Grip strength was measured for 10 consecutive trials for each mouse using a strength meter (Ohara Ika

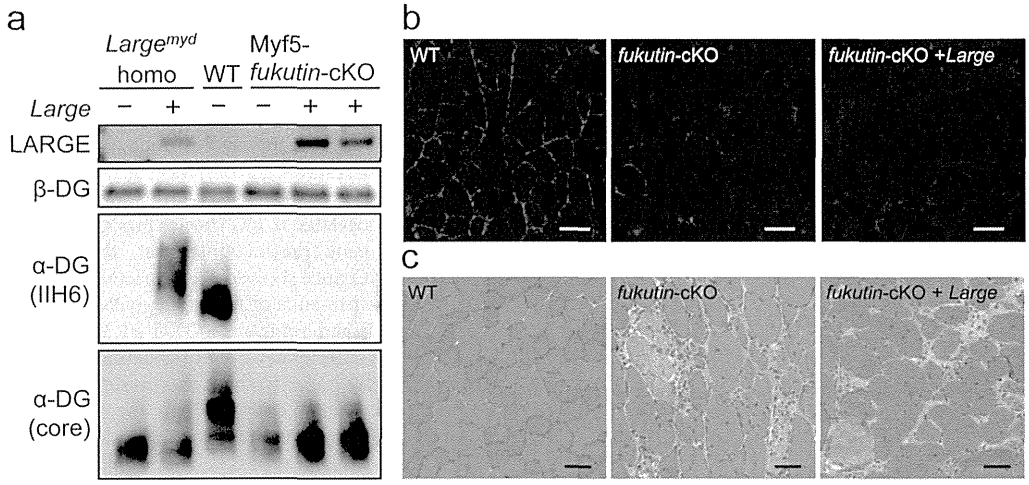

Figure 3 | Systemic gene transfer of Large into Myf5-fukutin cKO mice after onset. AAV9-MCK-*Large* was administered to 4-week-old Myf5-*fukutin* cKO mice via tail vein injection; after 2 months, the skeletal muscles were harvested and analysed for α-DG glycosylation (a, b) and histology (c). Although LARGE was expressed (a), the levels of α-DG glycosylation were unchanged in AAV-treated Myf5-*fukutin*-cKO mice (a, b). H&E staining for the tibialis anterior muscle did not show improvement of the muscular dystrophic phenotype of Myf5-*fukutin*-cKO mice (c). WT, litter control mice (*fukutin*$^{lox/lox}$ without cre-transgene); *fukutin*-cKO, untreated Myf5-*fukutin*-cKO mice; and *fukutin* cKO + *Large*, Myf5-*fukutin*-cKO mice with AAV9-MCK-*Large* treatment. Bar = 50 μm. The full-length blots with α-DG (IIH6), α-DG (core), LARGE, and β-DG are presented in Supplementary Figure S2e-h, respectively.

Sangyo Co. Ltd., Tokyo); 20% of the top and bottom values were excluded to obtain the mean. Statistical analysis was performed to determine means and s.e.m.; *P*-values <0.05 were considered significant (Mann–Whitney U test).

Antibodies. Antibodies for western blotting and immunofluorescence were as follows: mouse monoclonal antibody 8D5 against β-DG (Novocastra); mouse monoclonal antibody IIH6 against α-DG (Millipore); rat monoclonal antibody against mouse F4/80 (BioLegend); rabbit polyclonal antibody against collagen I (AbD Serotec); mouse monoclonal antibody against FLAG tag (Sigma); and rabbit polyclonal antibody against c-Myc tag (Santa Cruz). Rabbit polyclonal antibody against LARGE was raised using recombinant human LARGE protein expressed in *E. coli*. Antisera were purified by Melon gel IgG purification kit (Pierce). Rat monoclonal antibody against the α-DG core protein (3D7) was generated from a α-DG-Fc fusion protein[52].

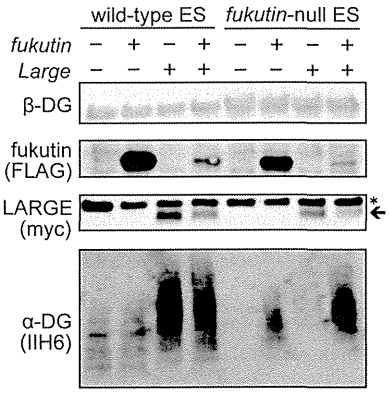

Figure 4 | α-DG glycosylation in fukutin-null ES cells after fukutin or LARGE expression. Fukutin (FLAG-tagged) and/or LARGE (myc-tagged) were expressed in wild-type or *fukutin*-null mouse ES cells and α-DG glycosylation status was analysed by western blotting. Exogenous LARGE expression produced highly glycosylated α-DG in wild-type but not *fukutin*-null ES cells, indicated by IIH6 staining. Co-transfection of *fukutin* and *Large* yielded LARGE-dependent glycosylation of α-DG in *fukutin*-null ES cells. Arrow and asterisk indicate LARGE protein and non-specific signals, respectively. The full-length blots with α-DG (IIH6), LARGE (myc), fukutin (FLAG), and β-DG are presented in Supplementary Figure S2i-l, respectively.

Protein preparation and western blotting. DG was enriched from solubilized skeletal muscle as described[27]. Briefly, skeletal muscles (TA ~30 mg, calf ~100 mg) were solubilised in Tris-buffered saline containing 1% Triton X-100 and protease inhibitors (Nacalai). The solubilised fraction was incubated with wheat germ agglutinin (WGA)-agarose beads (Vector Laboratories) at 4°C for 16 h; DG was eluted with SDS-PAGE loading buffer. To detect LARGE protein expression, total lysates were analysed by western blotting. Proteins were separated in 4–15% linear gradient SDS gels, then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Blots were probed with antibodies and developed with horseradish peroxidase (HRP)-enhanced chemiluminescence reagent (Supersignal West Pico, Pierce; or ECL Prime, GE Healthcare).

Histology and immunofluorescence analysis. For H&E staining, cryosections (7 μm) were stained for 2 min in haematoxylin, 1 min in eosin, and dehydrated with ethanol and xylene. IIH6-immunofluorescence analysis was performed after treating the with cold ethanol/acetic acid (1 : 1) for 1 min, blocking with 5% goat serum in MOM Mouse Ig Blocking Reagent (Vector Laboratories) at room temperature for 1 h, and incubation with primary antibodies diluted in MOM Diluent (Vector Laboratories) overnight at 4°C. For F4/80- and collagen I-immunofluorescence, sections were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature for 1 h, and then with primary antibodies diluted in 1% BSA overnight at 4°C. The slides were washed with PBS and incubated with Alexa Fluor 488-conjugated or Alexa Fluor 555-conjugated secondary antibodies (Molecular Probes) at room temperature for 30 min. Permount (Fisher Scientific) and TISSU MOUNT (Shiraimatsu Kikai) were used for H&E staining and immunofluorescence, respectively. Sections were observed by fluorescence microscopy (Leica DMR, Leica Microsystems).

ES cell culture. ES cells were cultured in DMEM with 20% heat-inactivated foetal bovine serum, 100 μM 2-mercaptoethanol, 1 mM non-essential amino acids (Gibco), 2 mM L-glutamine (Nacalai), and 10^3 U/mL leukaemia inhibitory factor (Millipore). Targeted disruptions of the *fukutin* gene in ES cells have been described previously[53]. Transfection was performed with Lipofectamine LTX reagents (Invitrogen) according to manufacturer protocols. After 48 h transfection, cells were lysed with Tris-buffered saline containing 1% Triton X-100 and protease inhibitors (Nacalai). DG proteins were enriched with WGA-beads and analysed by western blotting.

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Author contributions

Y.O., M.K., T.O., K.K., S.T. and T.T. conceived and designed the research. Y.O., M.K. and C.I. performed experiments and analysed the data. C.Y., K.K., T.C. and T.O. constructed the AAV vectors. Y.O., M.K. and T.T. wrote the paper. All authors reviewed the manuscript.

Additional information

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筋ジストロフィー治療の新しい展開

戸田　達史

要　旨

最も多いDuchenne型筋ジストロフィー(Duchenne muscular dystrophy：DMD)原因遺伝子ジストロフィンのクローニングを契機として，これまでに様々な筋ジストロフィーの原因遺伝子が同定されている．Disease-modifying therapyの開発が期待されており，現在臨床治験段階にあるエクソン・スキップ治療とリードスルー治療を中心に，将来臨床応用が期待されている分子標的治療の開発の現状について紹介する．福山型筋ジストロフィーは筋と脳を侵す日本に多い常染色体性劣性遺伝性疾患である．我々は原因遺伝子を同定，原因蛋白質をフクチンと命名した．患者ではフクチン遺伝子の3'非翻訳領域にSVA型レトロトランスポゾンの挿入を認めるが，本症がスプライシング異常症であることを見出し，これを制御するアンチセンス核酸を用い，患者細胞およびモデルマウスでの治療に成功した．この治療法は全ての患者に適応となる初の根治療法となる可能性があり，臨床応用を目指す．

〔日内会誌　103：2820～2828，2014〕

Key words　Duchenne型筋ジストロフィー，エクソンスキッピング，アンチセンス治療，リードスルー治療，福山型筋ジストロフィー

はじめに

筋ジストロフィーは，骨格筋線維の変性・壊死と不完全再生のサイクルを繰り返しながら間質の線維化・脂肪化が進行する遺伝性疾患群である．臨床的には進行性の骨格筋萎縮と筋力低下を呈しADLが低下するだけでなく，呼吸筋不全や心不全といった重篤な合併症を併発することもある難病である．新聞などを通し，または一般人の感覚で筋ジストロフィーというと，1個の疾患のように感じられるかもしれない．しかし，表に見るように筋ジストロフィーは遺伝形式も症状も遺伝子も異なる40種以上からなる疾患群であり，うちDuchenne型，肢帯型，顔面肩甲上腕型，先天型(福山型)，筋強直型，遠位型などが代表的である．

最も多いDuchenne型筋ジストロフィー(Duchenne muscular dystrophy：DMD)の原因遺伝子ジストロフィンのクローニングを契機として，これまでに様々な筋ジストロフィーの責任遺伝子が同定されているが，現在も治療の中心は対症療法である．リハビリテーション，心不全に対する薬物療法，呼吸筋麻痺に対する呼吸管理により，生命予後は約10年延長したといわれる．唯一エビデンスのある薬物療法は副腎

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The Cutting-edge of Medicine ; Recent development of therapy for muscular dystrophy.

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表

疾患名	遺伝形式	遺伝子座	シンボル名	遺伝子産物
Duchenne型	XR（x劣性）	Xp21.2	DMD	ジストロフィン
Becker型	XR	Xp21.2	DMD	ジストロフィン
Emery-Dreifuss型	XR	Xq28	EMD	エメリン
	AD	1q11-23	EDMD-AD	ラミンA/C
肢帯型（1型）	AD（常染色体優性）	5q31	LGMD1A	ミオチリン
	AD	1q11-21	LGMD1B	ラミンA/C
	AD	3p25	LGMD1C	カベオリン3
肢帯型（2型）	AR（常染色体劣性）	15q15.1-21.1	LGMD2A	カルパイン3
	AR	2p13	LGMD2B	ジスフェルリン
	AR	13q12	LGMD2C	γサルコグリカン
	AR	17q12-21	LGMD2D	αサルコグリカン
	AR	4q12	LGMD2E	βサルコグリカン
	AR	5q33-34	LGMD2F	δサルコグリカン
	AR	17q11-12	LGMD2G	テレソニン
	AR	9q31-34.1	LGMD2H	TRIM32
	AR	19q13.3	LGMD2I	FKRP
顔面肩甲上腕型	AD	4q35	FSHD	?
先天性（メロシン欠損型）	AR	6q2	MDC1A	ラミニンα2
先天性（福山型）	AR	9q31	FCMD	フクチン
先天性（muscle-eye-brain病）	AR	1p33-34	MEB	POMGnT1
先天性（Walker-Warburg症候群）	AR	9q34	WWS	POMT1
先天性（インテグリン欠損型）	AR	12q	?	インテグリンα7
先天性（rigid-spine型）	AR	1p3	RSMD1	SEPN1
先天性（その他）	AR	19q13.3	MDC1C	FKRP
筋強直性	AD	19q13	DM	ミオトニンキナーゼ
	AD	3q21	DM2	ZNF9
遠位型（三好型）	AR	2p12-14	MM	ジスフェルリン
遠位型（rimmed-vacuole型）	AR	9p1-q1	DMRV	GNE
遠位型（家族性封入体型）	AR	9p1-q1	HIBM	GNE
眼咽頭型	AD	14q11.2-13	OPMD	ポリA結合蛋白2
表皮水疱症型	AR	8q24-qter	MD-EBS	プレクチン
デスミン関連型	AD/AR	2q35	DES	デスミン

皮質ステロイド薬の内服であるが，治療効果は限られており，より有効なdisease-modifying therapyの開発が期待されている．ここでは最近のトピックスとして，現在臨床治験段階にあるエクソン・スキップ治療とリードスルー治療を中心に，将来臨床応用が期待されている分子標的治療の開発の現状について紹介するとともに，筆者らが主導する日本に特異的に多い福山型筋ジストロフィーが治療可能かもしれないことを解説する．

1．筋ジストロフィーの臨床治験段階にある治療

1）エクソン・スキップ治療

DMDの責任遺伝子であるジストロフィン遺伝子はX染色体短腕上Xp21.1に存在する巨大な遺伝子であり，79個のエクソンからなる．ジストロフィン異常症の遺伝子異常はエクソン単位の欠失が70～80%，ナンセンス変異などの微小変

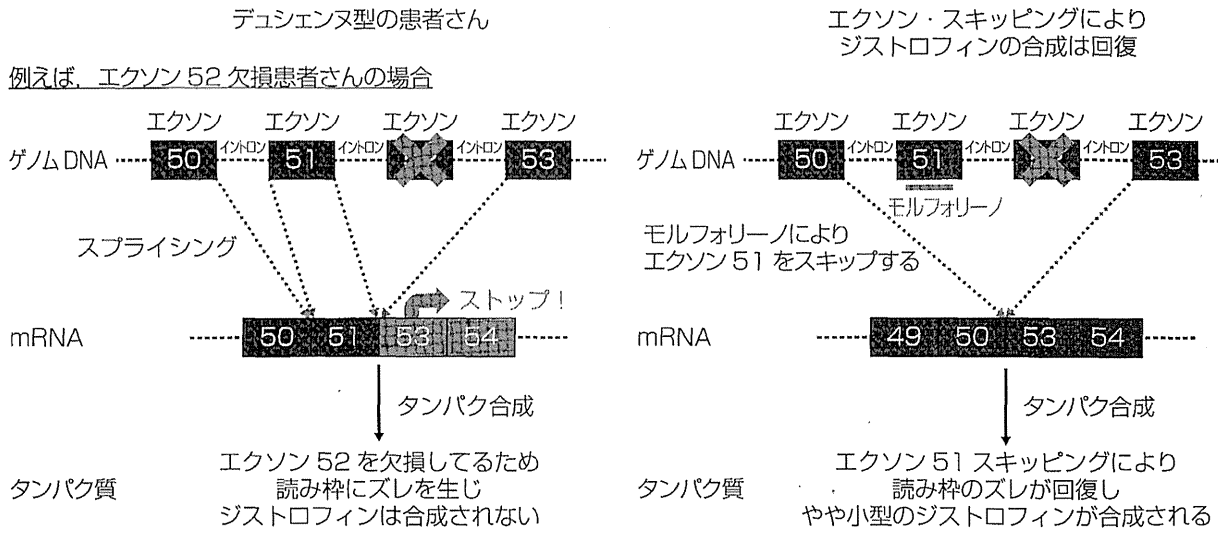

図1．アンチセンス核酸によるエクソンスキッピング
筋ジス犬が走り出す．Duchenne患者治験中．

異が20%とされている．同じジストロフィン遺伝子の欠失であるにも関わらず，DMDとBecker型筋ジストロフィー(Becker type muscular dystrophy：BMD)で重症度に違いがあるのは，フレームシフト則により説明される．すなわち，欠失エクソンの総塩基数が3の倍数であれば翻訳の際の読み枠は維持され(in-frame欠失)，機能を有する少し短くなったジストロフィン蛋白が合成されるためBMDとなるのに対して，3の倍数でなければ読み枠にずれが生じ(out-of-frame欠失)，未熟終始コドンの出現によりジストロフィン蛋白が合成されないため重症のDMDとなる．このフレームシフト則に基づいて，アンチセンス核酸化合物により欠失エクソンに隣接するエクソンをスプライシングの過程でスキップさせ，mRNAレベルでの欠失を3の倍数にして読み枠を修正し，DMDを軽症化しようとするのが，エクソン・スキップ治療である(図1)．

現在，スキッピングの標的とされているのはエクソン51で，欠失によるDMD患者の約10%が治療対象となる．エクソン・スキップを誘導するアンチセンス核酸化合物としては，低毒性でヌクレアーゼ耐性の安定な人工核酸である2'-O-methyl oligonucleotide (2OMeAS) とphosphorodiamidate morpholino oligomer (PMO) が用いられている．Prosensa Therapeutics社が開発しているPRO051 (drisapersen®) は前者の，またSarepta社のAVI-4658 (eteplirsen®) は後者の化合物である．2011年これら2剤について欧米で第2相の臨床治験の結果が報告された[1,2]．両者ともエクソン51スキップの誘導とジストロフィン蛋白発現の回復が確認され，安全性にも問題がなかった．PRO051はグラキソ社が第3相国際共同治験を行い，2013年10月残念ながら患者の6分間歩行に有意な改善が認められなかったため，現在グループ化してサブ解析中である．

また，2012年9月Sarepta社はeteplirsenの治験において，6分間歩行テストの改善データを公表した．50 mg/kg投与群の4名のDMD患児では投与後48週の時点で歩行距離が21 m延長したのに対して，プラセボで開始し24週目から実薬を投与された遅延開始群では36週の時点で歩行距離は−78 mまで減少した．48週時点では−68 mまで回復したが，早期開始群との間に89 mという有意差がついた．症例数は少ないが，この成績はエクソン・スキップ治療に期待を抱かせる．

また，日本ではエクソン53スキップの医師主導治験が2013年開始された．

2）リードスルー治療

点変異により終始コドンが生じると，蛋白質が合成されず欠損症状を呈する．こうしたナンセンス変異はDMD患者の約15％を占めるとされているが，その場合の治療戦略としてリードスルー治療が考えられる．すなわち，薬物を用いて翻訳過程で終始コドンを読み飛ばす（リードスルー）ことにより，蛋白発現を回復させようという試みである．アミノグリコシド系抗生物質は，真核細胞においてもmRNAの翻訳忠実度を低下させ，時として終始コドンを読み越えさせることが知られていた．DMDのモデル動物である*mdx*マウスはジストロフィン遺伝子のナンセンス変異を持つが，Sweeneyらは*mdx*マウスにゲンタマイシンを投与して，ジストロフィン蛋白発現の回復と筋力の増大に成功した[3]．しかし，アミノグリコシド系抗菌薬には聴覚毒性と腎毒性があるため，高用量を長期にわたり遺伝性疾患の患者に投与することは困難である．そこで安全でリードスルー活性の高い化合物の開発が行われている．PTC Therapeutics社が開発したリードスルー薬PTC124（ataluren®）[4]は米国でDMD患者を対象とした治験が実施されたが，残念ながら患者の臨床症状に有意な改善が認められなかったため，現在は開発が延期されている．

我が国でも，低分子化合物のスクリーニングにより独自のリードスルー薬の開発が行われている．さらに，アミノグリコシド系抗生物質であるネガマイシンを基本骨格として，抗菌活性とリードスルー活性を分離した誘導体の開発も進行中である．一方で，すでにMRSA治療薬として認可されているアルベカシンが比較的高いリードスルー活性を有することがわかり，DMDに対して日本で医師主導臨床治験が進行中である．

3）ミオスタチン阻害による筋量増大と筋再生の促進

ミオスタチンは骨格筋特異的に発現するTGF-βファミリー分子である．以前からヨーロッパでは筋肉量が2～3倍に増大した肉牛（Belgian blue, Piedmontese）や羊（Texel）などの家畜が知られていたが，こうした家畜のミオスタチン遺伝子を調べてみるといろいろな変異が発見された．さらに，2005年にはミオスタチン遺伝子変異を持つ男児も報告された．この男児は新生児の頃から筋肉がよく発達していて，5歳にして3 kgのダンベルを水平挙上できたという．このようにミオスタチンは骨格筋量を抑制的に調節していることが明らかになってきた．

*mdx*マウスにミオスタチン中和抗体を投与したところ，ジストロフィー病理変化と筋力が改善したことから[5]，ミオスタチン阻害療法は新たな筋ジストロフィー治療法として注目されるようになった．Weyth社はヒト化抗体MYO-029を開発し，Becker型筋ジストロフィー，肢帯型筋ジストロフィー，顔面肩甲上腕型筋ジストロフィー患者を対象として第2相治験を実施した．安全性は確認されたが，エンドポイントである徒手筋力テストや筋MRI検査で有意な改善が得られなかったことから，開発が断念された．

一方，bimagrumabはアクチビンのタイプII受容体に働く抗体医薬であり，ミオスタチンやアクチビンなどリガンドの結合を阻害し，これら因子からのシグナルを遮断することにより筋発達を刺激する[6]．ノバルティス社が共同開発しており，封入体筋炎の他，COPD，癌悪液質，サルコペニアの治療薬としても開発を進めている．

4）縁取り空胞を伴う遠位型ミオパチー（distal myopathy with rimmed vacuoles：DMRV）のシアル酸補充療法

DMRVは常染色体劣性遺伝疾患で原因遺伝子はGNE（UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase，シアル酸の合成を

触媒する酵素の1つ)で,最近はGNEミオパチーともよばれる.通常10歳代後半から30歳代後半に発症する.遠位筋,特に前脛骨筋が好んで侵され,大腿四頭筋は侵されにくい.徐々に筋萎縮が進行し,発症から平均12年で歩行不能となる.日本には300～400人程度の患者が存在すると予想される.モデルマウス研究により,シアル酸補充療法の有効性が明らかになっており[7],この研究成果を受けて,シアル酸補充療法は,日本では第1相,米国では第2相試験が行われている.

2. 福山型筋ジストロフィーの分子標的治療

1) ジストログリカノパチーの新たなメカニズム

福山型先天性筋ジストロフィー(Fukuyama congenital muscular dystrophy:FCMD)は1960年福山らにより発見された常染色体劣性遺伝疾患である.我が国の小児期筋ジストロフィーではDuchenne型の次に多く,日本人の約90人に1人が保因者とされる.日本に1,000～2,000人ほどの患者が存在すると推定され,日本人特有の疾患とされていたが,近年海外からの報告が相次いでいる.本症は重度の筋ジストロフィー病変とともに,多小脳回を基本とする高度の脳奇形(小多脳回)が共存し,さらに最近は近視,白内障,視神経低形成,網膜剝離などの眼症状も注目されている.すなわち,本症は遺伝子異常により骨格筋-眼-脳を中心に侵す一系統疾患である.

FCMDの疾患責任遺伝子であるフクチン遺伝子(*fukutin*, 9q31)は1998年に筆者らにより同定された.ほとんどのFCMD患者は,フクチン遺伝子の末端側の,蛋白質をコードしない3'非翻訳領域に「動く遺伝子」である約3千塩基長(3 kb)のSVA(Sine-VNTR-*Alu*)型レトロトランスポゾンの挿入型変異を認める[8].この変異は約100世代前,日本人祖先の1人に生じたとされるため,日本人の90人に1人が保因者で約3万出生に1人発症すると考えられている[8].

フクチン遺伝子が同定された後に,FCMDでは筋膜上の糖蛋白であるαジストログリカンの糖鎖に対する抗体の反応性が低いことが報告された.そして,糖転移酵素POMGnT1, POMT1/2がそれぞれ,FCMDの類縁疾患であるmuscle-eye-brain病, Walker Warburg症候群の原因遺伝子であることが次々と明らかにされた.これら患者の骨格筋では細胞膜と基底膜を繋ぐ糖蛋白α-ジストログリカンのO-マンノース型糖鎖修飾Siaa2-3Galb1-4GlcNAcb1-2Manに欠損があり,この糖鎖を介する細胞膜-基底膜間の結合が破綻するために重度の筋ジストロフィーが発症すると考えられるようになり,これらの疾患をαジストログリカノパチーと総称し新しい疾患概念ができた(図2A)[9].フクチン蛋白はゴルジ体に局在し,既知の糖転移酵素とのアミノ酸配列相同性よりαジストログリカン(αDG)の糖鎖修飾に関与する糖転移酵素ではないかと考えられているが,その機能を含め未知な点が多い.

ところで,ラミニン結合に関わる糖鎖として,Siaa2-3Galb1-4GlcNAcb1-2Manに加え,最近,O-マンノシル糖鎖にはリン酸基を介した側鎖構造があり,リン酸基より先の修飾もラミニン結合に必要であることが報告された(図2B)[10].この構造の合成にはLARGEが関与することが示されているが,興味深いことに,FCMD患者由来の細胞でもホスホジエステル結合を介した構造が欠如している.詳細な構造同定に加え,この修飾におけるフクチンの役割の解明が急がれる.また我々は,FKRP(fukutin-related protein)モデルマウスでもこのホスホジエステル結合を介した構造が欠如していること,さらには正常の肺や精巣組織でもαジストログリカンのリン酸基を介した側鎖構造が欠如しており,ラミニン結