Matrix Metalloproteinase Activity that Disrupts the Dystroglycan Complex: Its Role in the Molecular Pathogenesis of Muscular Dystrophies

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Abstract

The dystroglycan (DG) complex, composed of two subunits αDG and βDG, interacts with the sarcoglycan complex to form the dystrophin-glycoprotein complex. αDG is a cell surface peripheral membrane protein which binds to the components of the extracellular matrix, while βDG is a type I integral membrane protein which anchors αDG to the cell membrane via the N-terminal extracellular domain. Although defects of the DG gene have not been identified as the primary causes of hereditary diseases in humans, secondary but significant abnormalities of the DG complex have been revealed in a number of muscular dystrophies. In this article, we characterize the matrix metalloproteinase (MMP) activity that disrupts the DG complex by cleaving the extracellular domain of βDG and discuss if this MMP plays a role in the molecular pathogenesis of muscular dystrophies. We also address the therapeutic potential of the drugs that inhibit this MMP activity to decelerate muscle degeneration in these diseases.

Key Words: Dystroglycan; Matrix metalloproteinase; Extracellular matrix; Cardiomyopathic hamster; Sarcoglycanopathy.

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I. Introduction: Molecular organization of the dystroglycan (DG) complex

The DG complex is a cell membrane-spanning complex composed of two subunits αDG and βDG [13]. In muscle, the DG complex interacts with the sarcoglycan complex to form the dystrophin-glycoprotein complex. αDG is a cell surface peripheral membrane protein which binds to the components of the basement membrane, laminin, agrin and perlecan, while βDG is a type I integral membrane protein which anchors αDG to the cell membrane via the N-terminus of the extracellular domain and binds to the cytoskeletal protein dystrophin via the C-terminal cytoplasmic domain [8, 13, 20, 27, 29]. Thus, the DG complex provides a tight link between the extracellular matrix (ECM) and intracellular cytoskeleton via the cell membrane. At present, the role of the DG complex in the assembly and maintenance of the basement membrane remains controversial [7, 11, 15, 30].

These recent findings indicate that the DG complex needs to be disrupted efficiently when tissue remodeling takes place in various conditions and suggest that a specific device may exist for this purpose [31]. As such a device, we have recently identified a matrix metalloproteinase (MMP) activity that cleaves the extracellular domain of βDG [31]. In this paper, we characterize this MMP activity and discuss its role in the molecular pathogenesis of muscular dystrophies. We also address the therapeutic potential of MMP inhibitors to decelerate muscle degeneration in these diseases.

II. Characterization of the MMP activity that disrupts the DG complex

In skeletal muscle, βDG is detected as a single 43-kDa band (βDG_{full}) by immunoblot analysis using the monoclonal antibody 43DAG/8D5 against the C-terminal cytoplasmic tail of βDG [1, 31] (fig. 1). 43DAG/8D5 also detects a 30-kDa fragment of βDG (βDG_{30}) in several non-muscle tissues [31]. We clarified the tissue distribution of βDG_{30} by immunoblot analysis of various bovine tissues using 43DAG/8D5. βDG_{30} was detected in peripheral nerve, smooth muscle, lung and kidney, whereas it was obscure or undetectable in cardiac muscle, skeletal muscle, cerebrum and cerebellum [31].

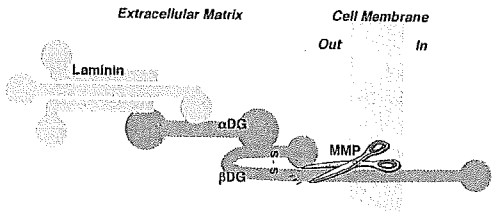

Fig. 1. Schematic model of the MMP activity (DG-MMP) that disrupts the DG complex by cleaving the extracellular domain of βDG.

To compare the biochemical properties of βDG_{30} with βDG_{full}, we performed extraction analysis of the crude membranes of bovine peripheral nerve and rat RT4 schwannoma cells, which express both βDG_{full} and βDG_{30} [31]. Although βDG_{full} and βDG_{30} were both extracted by 2% Triton X-100, they were not extracted by pH 11 or by 10 mM EDTA [31]. Because βDG is a type I integral membrane protein having a single transmembrane domain, these results indicate that βDG_{30} retains this transmembrane domain. Also because βDG_{30} is recognized by 43DAG/8D5 directed against the C-terminus of the cytoplasmic domain of βDG, these results indicate that the predicted cleavage site exists in the extracellular domain of βDG and βDG_{30} is its C-terminal fragment.

To test the hypothesis that MMP may be responsible for the processing of βDG_{full} into βDG_{30}, we cultured RT4 cells in the presence or absence of N-biphenyl-sulfonyl-phenylalanine hydroxamic acid (BPHA), which is a highly specific hydroxamate MMP inhibitor [19], harvested the living cells and performed immunoblot analysis using 43DAG/8D5. βDG_{30} decreased with increasing concentrations of BPHA [31]. We also tested the effects of 1,10-phenanthroline, which is a transition metal ion chelator and well established as a MMP inhibitor. Because RT4 cells did not grow well in the presence of 1,10-phenanthroline, we instead incubated the total homogenates of harvested RT4 cells in the presence of 1,10-phenanthroline at 37°C for varying time periods. 1,10-Phenanthroline inhibited the proteolysis of βDG in a concentration-dependent manner (Fig. 2). These results indicate that βDG_{30} is the processing fragment by MMP and that this MMP is active for the living RT4 cells. We tentatively refer to this MMP activity as DG-MMP in this article.

We next tested the effects of another newly developed hydroxamate derivative, (2R)-3-(1H-Indol-3-yl)-2-[4-(2-phenyl)-2H-tetrazol-5-yl] benzenesulfonylamino] propionoc acid (MMI-166), which has a narrower range of inhibitory activity than BPHA [18]. When RT4 cells were cultured in the presence of BPHA or MMI-166, BPHA, but not MMI-166, inhibited the proteolysis of βDG in a concentration-dependent manner (Fig. 3). In addition, BPHA, but not MMI-166, inhibited the

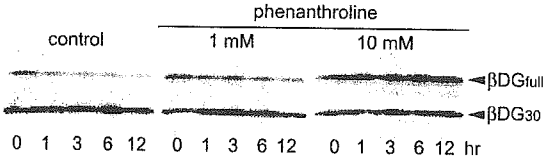

Fig. 2. 1,10-Phenanthroline inhibits the processing of βDG by DG-MMP. After 3 days of culture, RT4 cells were harvested, homogenized and then incubated in the presence or absence of 1,10-phenanthroline at 37°C for various time periods. The homogenates were analyzed by immunoblotting using 43DAG/8D5. 1,10-Phenanthroline inhibited the proteolysis of βDG in a concentration-dependent manner.

proteolysis of βDG when added to the total homogenates of harvested RT4 cells (not shown). Based on the inhibitory profiles of these reagents [18, 19], we presume that DG-MMP is distinct from MMP-1, MMP-2/MMP-14 or MMP-9. To clarify if DG-MMP is present in RT4 cells themselves or secreted into the culture medium, RT4 cells were harvested, homogenized and then incubated at 37°C for various times. βDG_{full} decreased and βDG_{30} increased with time, indicating that DG-MMP was present in RT4 cells themselves, not secreted into the culture medium [31]. We are currently investigating the possibility that DG-MMP might be a membrane-type MMP.

To know if the processing of βDG by DG-MMP affects the integrity of the DG complex, we performed sucrose density gradient sedimentation analysis of RT4 cell membranes. βDG_{full}, but not βDG_{30}, co-sedimented with αDG (Fig. 4). We isolated the DG complex from the RT4 cell membranes by laminin affinity chromatography. βDG_{full} co-isolated with αDG, which bound to laminin-Sepharose directly as a laminin-binding protein [31]. However, βDG_{30} did not co-isolate with αDG [31]. We isolated the DG complex from the RT4 cell membranes by wheat germ agglutinin (WGA) affinity chromatography. αDG and βDG_{full} were completely absorbed by WGA-Sepharose and recovered in the eluates [31]. However, βDG_{30} was not absorbed and undetectable in the eluates [31]. All together, these results indicate that βDG_{full}, but not βDG_{30}, is complexed with αDG and thus that the MMP cleavage of βDG into βDG_{30} disintegrates the DG complex. This is consistent with the report that the αDG-binding site exists in the N-terminus of the extracellular domain of βDG [27], since βDG_{30} is the C-terminal fragment of the cleavage. Because αDG and βDG are responsible for the binding to the ECM and cell membrane respectively, DG-MMP disrupts the link between the ECM and cell membrane via the DG complex.

III. Implications of DG-MMP in the pathogenesis of diseases

1. Cancer invasion/metastasis and infectious diseases

The aforementioned findings will have important implications in an array of pathological phenomena. For instance, it has been shown recently that certain carcinoma cell lines express β-DG_{30} abundantly [17]. Taken together with our results, carcinoma cells are presumed to employ DG-MMP to disrupt the dystroglycan complex. This will enable carcinoma cells to metastasize and invade other tissues. Interestingly in this respect, BPHA has been developed as a drug to inhibit cancer spread and metastasis [18, 19].

Processing of βDG by DG-MMP may also play a role in the molecular pathogenesis of viral and bacterial infections. It has been shown recently that pathogens such as arena viruses (several strains of lymphocytic choriomeningitis virus and Lassa fever virus) and *Mycobacterium leprae* bind to the cell surface αDG as an initial step of host cell infection [5, 23, 26]. Therefore, DG-MMP might be a natural defense mechanism against these pathogens, in analogy to matrilysin (MMP-7), which has been shown to play a defensive role against microorganisms in mucosal epithelial cells [16].

2. Muscular dystrophies

Another situation where DG-MMP is implicated is the molecular pathogenesis of hereditary neuromuscular diseases. Over the last 10 years, primary genetic defects have been identified in a number of these diseases. However, the precise molecular pathways by which the primary defects lead to muscle cell degeneration eventually in these diseases have not necessarily been clarified. Studies to elucidate the biological functions and dysfunctions of the proteins which work in close concert with the causative proteins *in vivo* will be useful in this context. As such, a research on DG processing by DG-MMP could provide us precious clues concerning the molecular pathogenesis of muscular dystrophies caused by the primary defects of the components of the dystrophin-glycoprotein complex and its related proteins, because abnormalities of the DG complex are well known in theses diseases.

For instance, defective glycosylation of αDG has been demonstrated in several forms of congenital muscular dystrophies, including Fukuyama-type congenital muscular dystrophy, MDC1C/LGMD2I, muscle-eye-brain disease, Walker-Warburg syndrome and $Large^{myd}$ mice, which are the model animals of congenital muscular dystrophy [2, 3, 9, 10, 14, 21, 32]. These diseases are all caused by the primary defects of the genes encoding glycosyltransferases, which are presumed to disturb glycosylation of αDG. Because glycosylation of αDG is crucial for the binding of laminin [6, 20], its defect is expected to perturb this binding and result in the disruption of the ECM-cell membrane linkage via the DG complex. This scenario is supported by the finding that the antibody against the carbohydrate residues of αDG involved in the binding of laminin induced a dystrophic phenotype in cultured muscle cells [4].

Another intriguing example is sarcoglycanopathy. Having the mutation of the δ-sarcoglycan gene, a genomic deletion including the first exon, cardiomyopathic hamsters are the model animals of sarcoglycanopathy LGMD2F [22, 25]. It is noteworthy that αDG has been shown to be dissociated from βDG and not recovered in the membrane fraction in the muscle of these animals [24, 28]. It has also been shown that αDG is reconstituted into the DG complex when dystrophic changes are corrected by the adenovirus transfer of the δ-sarcoglycan gene [12]. Furthermore, similar observations have been reported in other types of sarcoglycanopathies and their model animals. Based on these observations, we are currently analyzing the activity of DG-MMP in cardiomyopathic hamsters. Preliminary results indicate that DG-MMP is activated in the muscle of these animals (manuscript in preparation). We presume that activation of DG-MMP causes the disruption of the link between the ECM and

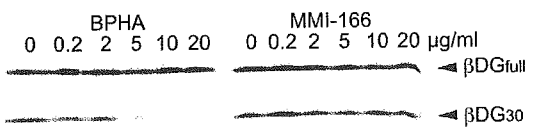

Fig. 3. BPHA, but not MMI-166, inhibits the processing of βDG by DG-MMP. Various concentrations of BPHA or MMI-166 were added to the culture medium of RT4 cells. After 3 days of culture, cells were harvested, homogenized and analyzed by immunoblotting using 43DAG/8D5. While BPHA inhibited the proteolysis of βDG in a concentration-dependent manner, MMI-166 did not.

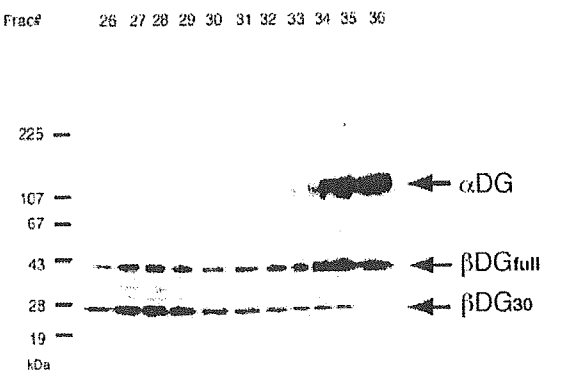

Fig. 4. βDG_{full}, but not βDG_{30}, co-sediments with αDG. Sucrose density gradient sedimentation of RT4 cell membranes was performed and analyzed by immunoblotting using 43DAG/8D5. βDG_{full}, but not βDG_{30}, co-sedimented with αDG.

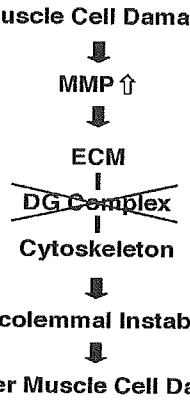

Fig. 5. Hypothetical scheme of the role of DG-MMP in the molecular pathogenesis of muscular dystrophies.
DG-MMP is hypothesized to be activated by the initial muscle cell damage in certain muscular dystrophies. This results in the disruption of the ECM-cell membrane linkage via the DG complex, which, in turn, destabilizes the sarcolemma and further augments muscle cell damage in a vicious cycle.

cell membrane via the DG complex in the muscle of cardiomyopathic hamsters and this may play an important role in the pathogenesis of muscle degeneration.

IV. Therapeutic implications of DG-MMP: do inhibitors of DG-MMP decelerate muscle degeneration in muscular dystrophies?

If DG-MMP turns out to be activated in certain muscular dystrophies, this will have significant implications for the molecular pathogenesis of muscle degeneration in these diseases, because the resulting disruption of the ECM-cell membrane linkage via the DG complex is expected to further augment muscle cell damage in a vicious cycle (Fig. 5). Moreover, it will raise the intriguing possibility of a novel pharmacological therapy for these diseases. The MMP inhibitors effective against DG-MMP have been developed as anti-cancer reagents and demonstrated to be not only effective but also safe without serious side effects when administered orally [19]. We are currently planning the oral administration of DG-MMP inhibitors to cardiomyopathic hamsters to see their effects on the progression of muscle degeneration.

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Worldwide distribution and broader clinical spectrum of muscle–eye–brain disease

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Muscle–eye–brain disease (MEB), an autosomal recessive disorder prevalent in Finland, is characterized by congenital muscular dystrophy, brain malformation and ocular abnormalities. Since the MEB phenotype overlaps substantially with those of Fukuyama-type congenital muscular dystrophy (FCMD) and Walker–Warburg syndrome (WWS), these three diseases are thought to result from a similar pathomechanism. Recently, we showed that MEB is caused by mutations in the protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (*POMGnT1*) gene. We describe here the identification of seven novel disease-causing mutations in six of not only non-Finnish Caucasian but also Japanese and Korean patients with suspected MEB, severe FCMD or WWS. Including six previously reported mutations, the 13 disease-causing mutations we have found thus far are dispersed throughout the entire *POMGnT1* gene. We also observed a slight correlation between the location of the mutation and clinical severity in the brain: patients with mutations near the 5′ terminus of the *POMGnT1* coding region show relatively severe brain symptoms such as hydrocephalus, while patients with mutations near the 3′ terminus have milder phenotypes. Our results indicate that MEB may exist in population groups outside of Finland, with a worldwide distribution beyond our expectations, and that the clinical spectrum of MEB is broader than recognized previously. These findings emphasize the importance of considering MEB and searching for *POMGnT1* mutations in WWS or other congenital muscular dystrophy patients worldwide.

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INTRODUCTION

Muscle–eye–brain disease (MEB: MIM 253280) is an autosomal recessive disorder characterized by congenital muscular dystrophy (CMD), ocular abnormalities and brain malformation (type II lissencephaly) (1). Patients with MEB show congenital muscular dystrophy, severe congenital myopia, congenital glaucoma, pallor of the optic discs, retinal hypoplasia, mental retardation, hydrocephalus, abnormal electroencephalograms and myoclonic jerks. From birth, infants with MEB are floppy with generalized muscle weakness, including facial and neck muscles. Muscle biopsies show dystrophic changes, and brain MRIs reveal pachygyria-type cortical neuronal migration disorder, flat brainstem and cerebellar hypoplasia. Walker–Warburg syndrome (WWS: MIM 236670) is another extreme of CMD, which shows the most severe brain malformation, characterized by type II lissencephaly and eye involvement. WWS is usually lethal within the first year of life (2). Fukuyama-type congenital muscular dystrophy (FCMD: MIM 253800) is a recessively inherited CMD with type II lissencephaly that occurs exclusively in Japan (3). In some cases, the clinical resemblance makes it difficult to differentiate between MEB, FCMD and WWS. These three diseases are thought to be caused by a similar pathomechanism.

Molecular genetic studies have been helpful in defining subgroups of CMD. The genes responsible for both MEB and FCMD have been identified and characterized. Through linkage analysis, the gene responsible for MEB was localized to chromosome 1p32–34 (4), and we recently showed that MEB is caused by loss of function mutations in the gene encoding protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) (5). *O*-mannosylation is a rare type of glycosylation in mammals, occurring in a limited number of brain, nerve and skeletal muscle glycoproteins (6). Sialyl *O*-mannosyl glycan is known to be a laminin-binding ligand of α-dystroglycan (7), and POMGnT1 catalyzes the transfer of *N*-acetylglucosamine to *O*-mannose of glycoproteins.

FCMD is caused by mutations in the *fukutin* gene on chromosome 9q31, which we positionally cloned previously (8–11). The function of fukutin is not yet clear; however, sequence analysis predicts it to be an enzyme that modifies cell-surface glycoproteins or glycolipids (12). Immunoreactivity to the glycans of α-dystroglycan has been undetectable in skeletal muscle from both MEB and FCMD patients (13–15), and the core α-dystroglycan protein shows an electrophoretic mobility shift (15). These findings have suggested a common pathomechanism for MEB and FCMD, in which defects in *O*-mannosylation compromise laminin binding. Identification of the genes responsible for MEB and FCMD now enables the definition of these complicated diseases at the molecular level, since their symptoms are often similar and complicated. In particular genetic analysis of FCMD is being performed frequently and has been highly informative (16).

WWS has been observed in many population groups with a worldwide distribution. In contrast, both MEB and FCMD show striking founder effects. MEB was first described in Finland, where it is most prevalent, owing to a strong founder effect followed by genetic drift (17). Consequently, most MEB patients have come from a small, geographically isolated population in Finland, with few Caucasian exceptions. Most FCMD mutations can be traced to a single ancestral founder, who carried a 3 kb retrotransposal insertion in the 3′ non-coding region of the *fukutin* gene (11,18). Thus far, FCMD patients have been identified exclusively in Japan.

We describe here the identification of different MEB-causing mutations in Japanese and Korean patients as well as Caucasian patients initially diagnosed as FCMD, MEB, or WWS. Our results show that MEB is present in diverse population groups with a worldwide distribution and has a broader clinical spectrum than previously expected. Furthermore, we have shown a slight genotype–phenotype correlation in the brain among the patients.

RESULTS

Patients and mutation analysis

In a previous study, we showed that mutations in the *POMGnT1* gene are the primary genetic defect in MEB. Mutation analysis and characterization of the gene product has demonstrated that MEB is inherited in a loss-of-function manner (5). In this study, we extended our analysis to screen the entire coding region and exon/intron flanking sequences of the *POMGnT1* gene for mutations in 30 patients who were clinically diagnosed for WWS, severe FCMD, or MEB. To determine whether MEB patients exist in Asia, we included Japanese and Korean patients in this study.

Our analysis identified seven novel mutations and one recurrent mutation in six patients (Fig. 1, Table 1). None of these individuals harbored mutations in the *fukutin* gene. Combined with our previous results, we have now identified a total of 13 different mutations in the *POMGnT1* gene.

Patient EV carried a homozygous C281T transition in exon 3, which results in an Arg63Stop nonsense mutation (Fig. 1A). EV is a 12-year-old Italian female who was hospitalized at one year of age for a ventriculo-peritoneal shunt operation for hydrocephalus. She is unable to speak or walk (Table 2).

Patient HS is a 12-year-old Japanese male. He is a compound heterozygote who carried a 1 bp deletion at base 541 in exon 6 (frameshift and premature termination at codon 167) and a G761A transition in exon 8 (Glu223Lys) (Fig. 1B). Severe hydrocephalus was observed prenatally by an ultrasonograph, and an MR image at 6 years of age showed extreme ventricular dilatation and agenesis of the septum pellucidum (Fig. 3A). Of all the patients examined, HS showed one of the more severe phenotypes (Table 2).

SI, a 7-year-old female Japanese patient, was identified as a compound heterozygote with a G900A transition in exon 9 (Cys269Tyr) and a 1 bp insertion at base 1077 in exon 11 (frameshift and premature termination at codon 338; Fig. 1C). Dilated ventricles were observed prenatally by an ultrasonograph, and, at one year of age, hydrocephalus required a ventriculo-peritoneal shunt. SI also shows a more severe phenotype (Table 2).

Patient DC, an 8-year-old Belgian female, is compound heterozygous for a G761A transition in exon 8 (Glu223Lys) and a G-to-A substitution in intron 17, which alters the conserved GT splicing donor sequence to AT (Fig. 1D). In our

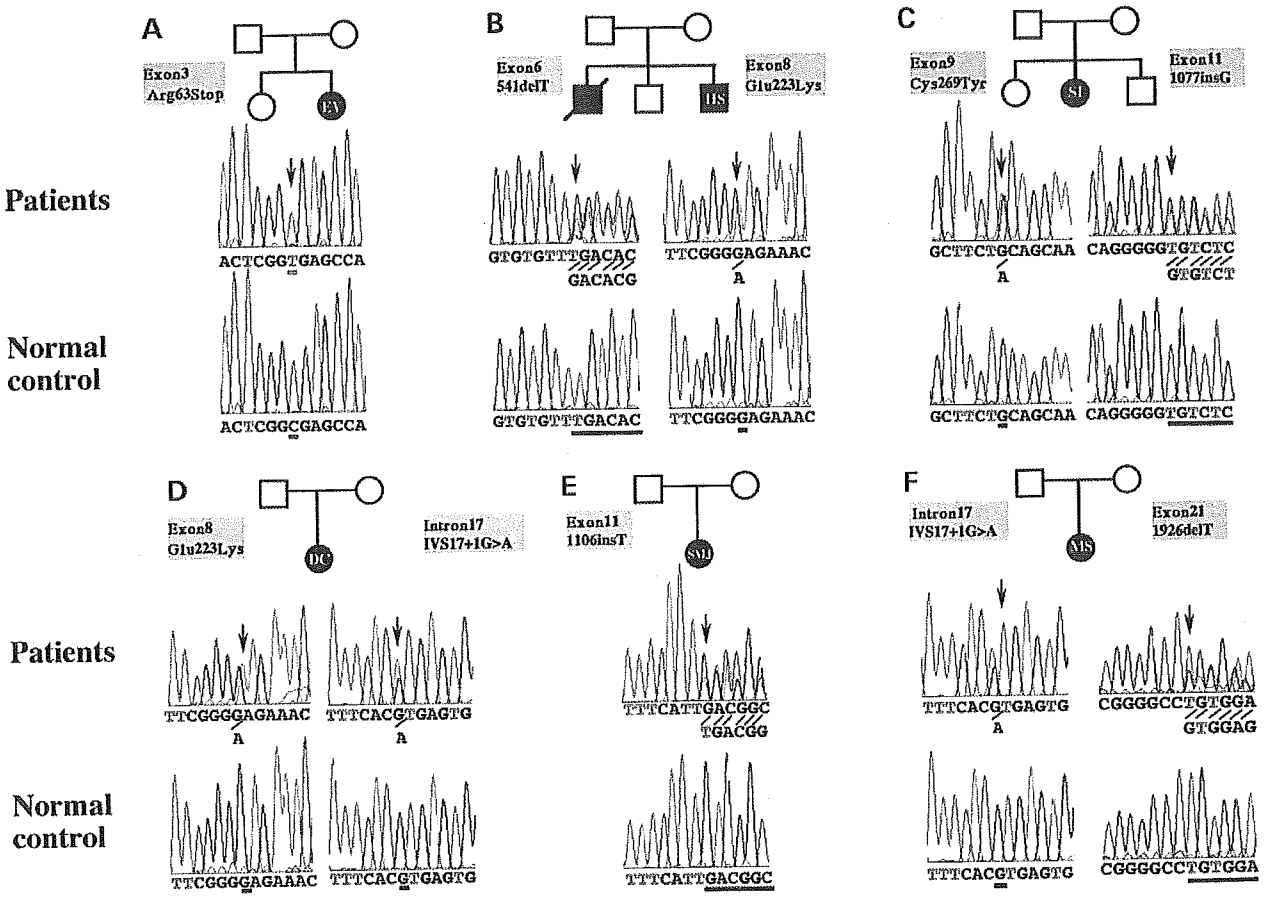

Figure 1. Novel point mutations in six patients with MEB. (**A**) Patient EV carried a homozygous C281T transition in exon 3, which results in an Arg63Stop nonsense mutation. (**B**) Patient HS is a compound heterozygote who carries a 1 bp deletion at base 541 in exon 6 (frameshift) and a G761A transition in exon 8 (Glu223Lys). (**C**) Patient SI is compound heterozygous for a G900A transition in exon 9 (Cys269Tyr) and a 1 bp insertion at base 1077 in exon 11 (frameshift). (**D**) Patient DC is compound heterozygous for a G761A transition in exon 8 (Glu223Lys) and a G-to-A substitution in intron 17, which causes abnormal splicing. (**E**) Patient SMJ was shown to be a putative compound heterozygote who carries a 1 bp insertion at base 1106 in exon 11 (frameshift). Thus far, no coding region mutation has been detected in the other allele. (**F**) Patient MS is compound heterozygous for a G-to-A substitution in intron 17 (abnormal splicing) and a 1 bp deletion at base 1926 in exon 21 (frameshift).

Table 1. Summary of novel mutations of the *POMGnT1* gene in MEB patients

Patients	Mutation	Location	Effect	Status
EV	281C>T	Exon3	Arg63Stop Nonsense	Homozygote
HS	541 del T	Exon6	Phe149 frameshift 167Stop	Compound heterozygote
	761G>A	Exon8	Glu223Lys Missense	
SI	900G>A	Exon9	Cys269Tyr Missense	Compound heterozygote
	1077 ins G	Exon11	Val328 frameshift 338Stop	
DC	761G>A	Exon8	Glu223Lys Missense	Compound heterozygote
	IVS17+1G>A[a]	Intron17	Glu514read-through 526Stop/Leu472-His513del	
SMJ	1106 ins T	Exon11	Asp338 frameshift 338Stop	Compound heterozygote
	?	Noncoding region?	?	
MS	IVS17+1G>A[a]	Intron17	Glu514read-through 526Stop/Leu472-His513del	Compound heterozygote
	1926 del T	Exon21	Leu611 frameshift 633Stop	

[a]Mutation was reported in the previous study (5).

Table 2. Clinical features of MEB patients

Patient	EV	HS	SI	DC	SMJ	KO[a]	YA[a]	SA[a]	MK[a]	CC[a]	MS	TLG[a]
Origin	Italy	Japan	Japan	Belgium	Korea	Turkey	Turkey	Turkey	Turkey	Turkey	USA/Japan	France
Age (years)	12	12	7	8	6	12	6	7	5	10	25	3
Clinical diagnosis	Atypical WWS	WWS or MEB	MEB or severe FCMD	FCMD or MEB	FCMD	MEB	MEB	MEB	MEB	MEB	A milder WWS	MEB
Mutation (location)	Arg63Stop (exon 3)	541delT (exon 6)	Cys269Tyr (exon 9)	Glu223Lys (exon 8)	1106insT (exon 11)	IVS17+ 1G>T (intron 17)	IVS17+ 1G>T (intron 17)	IVS17+ 1G>A (intron 17)	Ser535-Ser550 del (exon 19)	1813delC (exon 20)	IVS17+ 1G> (intron 17)	Pro493Arg (exon 17)
		Glu223Lys (exon 8)	1077insG (exon 11)	IVS17+ 1G>A (intron 17)	ni (noncoding?)						1926delT (exon 21)	1970delG (exon 21)
Brain												
Mental retardation	+++	+++	+++	+++	ni	+++	++	++	+++	+++	++	+++
Speech	No words	No words	No words	Single words	No words	No words	No words	ni	ni	No words	A single word	No words
IQ/DQ	IQ < 30	DQ < 10	DQ < 20	IQ−30	ni	ni	ni	ni	ni	ni	DQ−43	ni
Hydrocephalus	√	√	√	√	—	√	ni	—	—	—	—	ni
Brainstem hypoplasia	+++	+++	++	+++	ni	+++	++	++	+++	+	+	+++
Septum pellucidum agenesis	—	√	√	—	—	√	ni	—	—	—	—	ni
Corpus callosum hypoplasia	—	√	√	—	√	√	ni	√	√	√	—	ni
White matter lucency	—	—	++	++	—	++	++	+	++	++	++	+
Type II lissencephaly	++	++	++	++	—	++	ni	+++	+++	+	++	++
Cerebellar vermis hypoplasia	√	√	—	—	√	√	√	√	√	√	√	ni
Eyes												
Myopia	√	√	√	√	ni	√	ni	ni	—	√	√	√
Retinal dysplasia	√	√	√	—	ni	—	ni	ni	√	—	√	—
Anterior chamber malformation	—	—	—	—	ni	√	√	ni	—	√	—	—
Microphthalmia	—	—	√	—	ni	—	√	ni	—	—	—	—
High VEP	—	√	√	—	ni	ni	ni	—	ni	ni	√	—
Muscle												
Maximum motor function (age)	Sit with support (10 years)	Head control (8 years)	No head control (7 years)	Sit with support (2 years)	ni	No Head control (12 years)	Head control (4 years)	head control (3 years)	No head control (8 years)	Sit with support (5 years)	Sit with support (3 years)	No head control (3 years)
CK(U/l) (age)	700 (10 years)	2365 (6 years)	852 (6 years)	1844 (ni)	ni	42271 (2 years)	434 (10 months)	844 (4 years)	628 (4 years)	1212 (3 years)	1339 (12 years)	4778 (1 year)

[a]Patients whose mutations were reported in the previous study (5). +++, severe; ++, moderate; +, mild; √, observed; —, not observed; ni, no information was obtained in these patients.

previous study, we found that the intron 17 mutation caused both read-through of intronic sequences, resulting in introduction of a premature termination codon, and skipping of the upstream exon 17, resulting in the deletion of 42 amino acids (5). In DC, severe myopia was found upon opthalmological examination in the first months of life, although retinal dysplasia was not observed. Although she was able to sit with support and her speech was limited to three single words at 2 years of age, by 7 years of age she was severely hypotonic and mentally retarded. DC shows a relatively mild phenotype compared with the other patients examined (Table 2).

A 6-year-old female Korean patient, SMJ is a putative compound heterozygote who carried a 1 bp insertion at base 1106 in exon 11, causing a frameshift and premature termination at codon 338 (Fig. 1E). We were unable to detect a mutation in the other *POMGnT1* allele. It is possible that the second mutation may lie outside the coding sequence, perhaps in the promoter or a regulatory region of an intron.

Patient MS is the 25-year-old female child of a Japanese mother and an American father of Scandinavian origin (19). The *POMGnT1* allele inherited from her father harbors a G-to-A substitution in intron 17, which alters the GT splicing sequence. From her mother, MS inherited a 1 bp deletion at base 1926 in exon 21, which results in a frameshift and premature termination at codon 633 (Fig. 1F). MS was previously diagnosed with a milder form of WWS because her symptoms included relatively severe eye abnormalities and specific features such as severe hypoplasia of the cerebellar vermis and cataracts, which are common in WWS. However her mental retardation is relatively mild for MEB and she can indicate 'yes' or 'no' with gestures (Table 2).

In each case, mutations cosegregated within the pedigree (families HS, SI, SMJ, and MS). We screened at least 92 normal individuals for two missense changes (Cys269Tyr and Glu223Lys), excluding the possibility of polymorphism.

In addition, patient MK is one of the subjects examined in our previous report (5). MK is a 5-year-old Turkish male who does not show MEB-specific eye symptoms such as myopia. MK carried a homozygous G1743A transition in the final base of exon 19, which was previously reported as a missense mutation (Ser550Asn) (5). However, subsequent RT–PCR analysis of skeletal muscle from this patient has shown that this mutation causes skipping of exon 19, resulting in the deletion of 15 amino acids (data not shown).

Genotype–phenotype correlation

We found that patients with MEB showed a broad range of severity of symptoms. In addition, we found that these patients possessed mutations that were scattered throughout the *POMGnT1* gene. To assess whether there is a genotype–phenotype correlation, we investigated the clinical features of the patients with regard to brain, eye and muscle, relative to the distribution of mutations throughout the *POMGnT1* gene (Table 2, Fig. 2). This analysis revealed a wider clinical spectrum of MEB than recognized previously. Taking into account each patient's clinical features, correlations between the location of the mutation and clinical severity seemed difficult to assess. However, a slight correlation of clinical severity in the brain was observed. Patients with mutations near the 5′ terminus of the *POMGnT1* coding region showed relatively severe brain symptoms, while patients with mutations near the 3′ terminus had milder phenotypes. (Table 2). Hydrocephalus showed a particular correlation with mutations near the 5′ terminus (Table 2, Fig. 3). For example, patient HS, who carried a 1 bp deletion in exon 6 and a missense mutation in exon 8, near the 5′ terminus, was diagnosed as WWS or MEB and showed relatively severe phenotypes such as hydrocephalus (Table 2, Fig. 3A). On the other hand, patient CC carried a homozygous 1 bp deletion in exon 20, near the 3′ terminus of the *POMGnT1* coding region. CC had a relatively mild phenotype without hydrocephalus (Table 2, Fig. 3B). These analyses suggest that the location of a mutation influences the severity of the MEB phenotype.

In addition, we examined the skeletal muscle tissue from both patient SI who carried mutations near the 5′ terminus (Fig. 3C–E) and patient TLG who carried mutations near the 3′ terminus (Fig. 3F–H) and found normal immunoreactivity for β-dystroglycan and lamininα2 chain but greatly reduced staining for α-dystroglycan. No obvious differences could be observed in the skeletal muscle of the two patients.

DISCUSSION

The six *POMGnT1* mutations identified in the previous study were all simple point mutations (5), while most FCMD patients carry a quite rare insertion mutation in the *fukutin* gene and are found exclusively in Japan. These findings led us to hypothesize that MEB mutations might have a broader distribution outside Finland. A recent linkage study reported the occurrence of MEB in some Caucasians and classified MEB and WWS as distinct disorders (20). To test our hypothesis, we examined 30 patients from various countries, including Japan and Korea, who were diagnosed as WWS, severe FCMD or MEB. In addition to the six previously described mutations, we identified seven new mutations in this study. Therefore, MEB patients may exist with a broader distribution and more varied phenotypes than previously expected.

The 13 known mutations in the *POMGnT1* gene are dispersed throughout the entire coding region (Fig. 2), with no accumulation in any particular domain. The clinical features of MEB vary among patients, and evaluation of clinical severity in each individual patient is difficult. However, we observed a slight correlation between genotype and brain phenotype: patients with mutations in the vicinity of the 5′ terminus of the *POMGnT1* gene show relatively severe WWS-like symptoms. All of the patients with *POMGnT1* mutations are still alive; hence, lifespan may be one of the differences between MEB and typical WWS, in which almost patients die before one year of age.

The amino acid sequence of POMGnT1 is homologous to α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT-I), which is a Golgi-resident enzyme involved in the *N*-linked oligosaccharide biosynthetic pathway. While the crystal structure of GnT-I has been determined (21), the structure of POMGnT1 itself has not been analyzed in detail. Computer analysis predicts that the 660-amino-acid POMGnT1 protein is divided into four domains: a cytoplasmic tail (Met1-Arg37), a transmembrane domain (Phe38-Ile58), a stem domain (Leu59–Leu300), the catalytic domain consisting of the UDP-GlcNAc

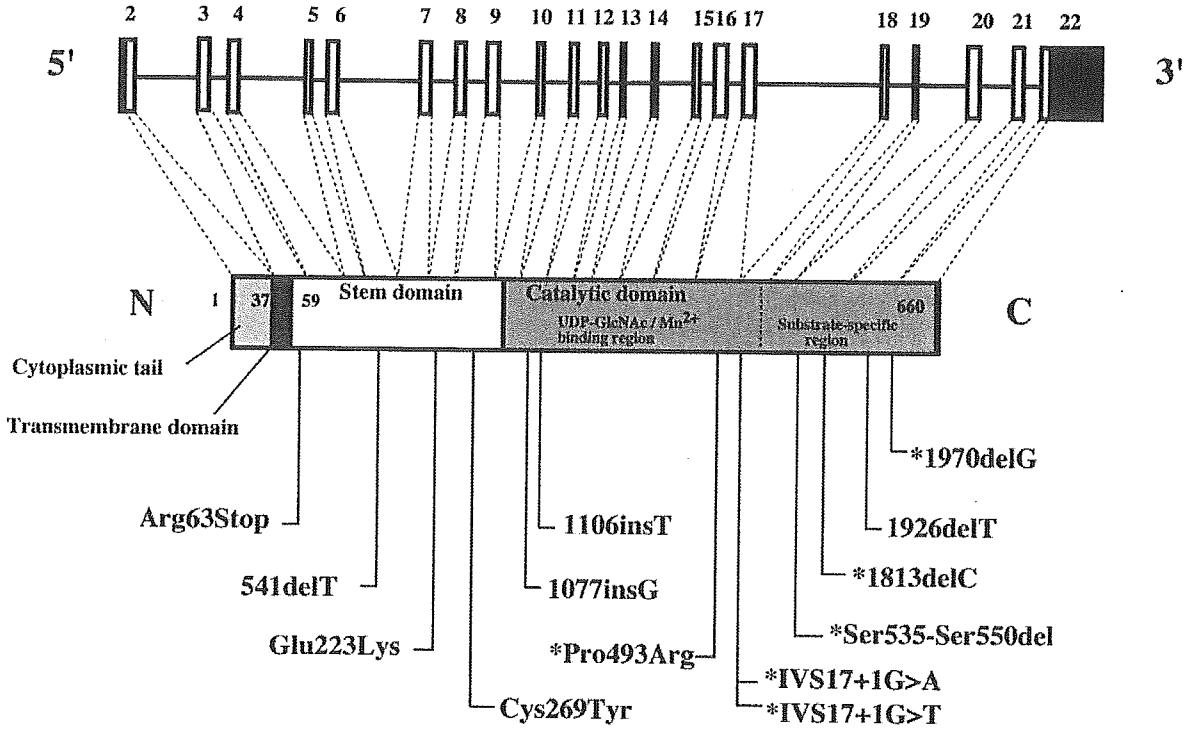

Figure 2. Schematic representation of the *POMGnT1* gene and the corresponding protein, showing the location of mutations found in MEB. Exons are represented by boxes, and introns are represented by lines. POMGnT1 protein is divided into four domains. Mutations detected in this study and in the previous study are shown below the protein. The asterisk represents mutations reported in the previous study (5).

and Mn^{2+} binding regions (around Asn301–Leu530), and the substrate-specific region (around Arg531-Thr660) (5). Nonsense or frameshift mutations near the 5′ terminus shorten the POMGnT1 protein significantly, probably resulting in loss of function. Missense mutations in the stem domain may diminish retention of POMGnT1 in the Golgi apparatus (22). Mutations in the 3′ region of the gene may retain some ability to transfer sugars, since the catalytic domain of the protein is preserved to some extent. Measurement of the enzymatic activity of mutant POMGnT1 proteins will be necessary to explain possible mechanisms for the genotype–phenotype correlation seen in this study.

MEB, FCMD and WWS are clinically similar, and the nosological classification of these disorders has been controversial. In MEB and FCMD patients, the lack of full *O*-mannosylation of α-dystroglycan significantly disrupts the interactions of α-dystroglycan with extracellular matrix ligands (15). This result suggests that post-translational disruption of dystroglycan–ligand interactions may be a common mechanism for muscular dystrophy with brain abnormalities. The structure of laminin-binding *O*-mannosyl glycan in dystroglycan is Siaα2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr (7), where POMGnT1 catalyzes the GlcNAcβ1-2Man linkage (5). Since the clinical presentations of MEB and WWS significantly overlap, and the most severe brain malformation and shortest life span are striking features of WWS, we postulated that the gene product responsible for WWS may be a glycosyltransferase that catalyzes the Man-Ser/Thr linkage in *O*-mannosyl glycans. Quite recently 20% of WWS patients have been found to have mutations in *POMT1*, a putative human counterpart of a yeast *O*-mannosyltransferase (23). In FCMD, compound heterozygotes for the FCMD founder mutation in the *fukutin* gene show severe phenotypes like WWS, and no patients have been identified with non-founder (point) mutations on both alleles, suggesting that such patients are embryonic lethal (16,24). Unlike FCMD, MEB patients with point mutations on both alleles can survive. We suppose that fukutin may perform a more essential role in early development than POMGnT1.

Further molecular genetic study will open new avenues for understanding the pathophysiological mechanisms underlying these complex disorders. It may be necessary and possible to re-classify muscular dystrophies based on genetic rather than clinical criteria. This study emphasizes the importance of considering MEB and searching for *POMGnT1* mutations in WWS or other CMD patients worldwide.

MATERIALS AND METHODS

Patients

We analyzed genomic DNA from 30 patients with CMD, brain malformation and ocular abnormalities. Information about the

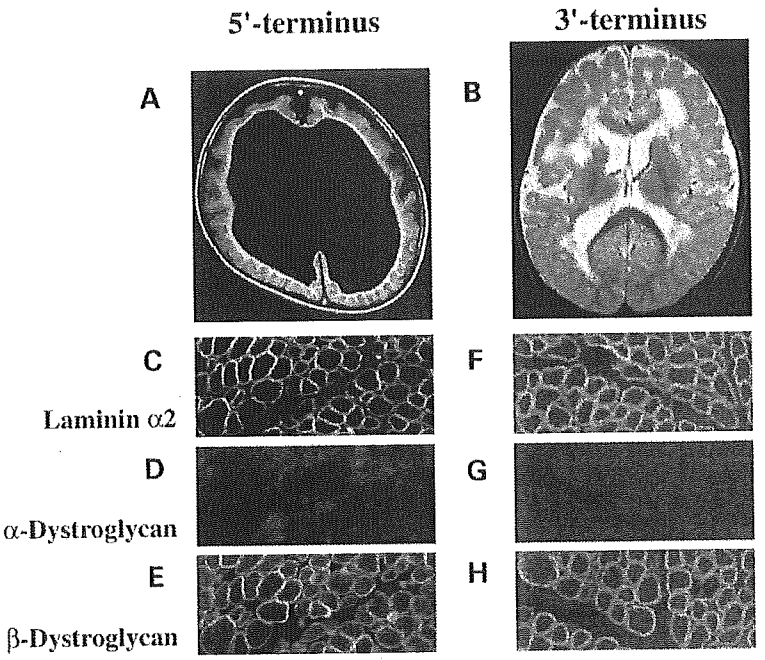

Figure 3. Comparison of cranial MR images and immunohistochemical analysis in MEB patients. (**A**) An axial T_1-weighted image of patient HS (at 6 years of age) shows extreme ventricular dilatation with a slightly smooth cortical surface. (**B**) An axial T_2-weighted image of patient CC (at 13 months of age) shows pachygyria, slightly enlarged ventricles, and white matter abnormality. The mutation in patient CC has been reported previously (5). Consecutive frozen sections of skeletal muscle from patient SI (**C–E**) and patient TLG (**F–H**) immunostained with anti-lamininα2 chain (C, F), α-dystroglycan (D, G), and β-dystroglycan antibodies (E, H).

six patients whose mutations were identified in this study (EV, HS, SI, DC, SMJ and MS) is briefly described in the Results section. All parents of these patients are not consanguineous. The six patients KO, YA, SA, MK, CC and TLG were described in the previous study (5). All phenotypes are summarized in Table 2.

Mutation analysis

Primers used for mutation analysis have been described previously (5). PCR products from patient genomic DNA were excised from gels, and direct sequencing was performed using Bigdye terminators (Applied Biosystems). Fragments were electrophoresed on an ABI Prism 3100 sequencer (Applied Biosystems).

Immunohistochemistry

Immunodetection was performed using a mouse monoclonal anti-α-dystroglycan antibody for patient SI (clone VIA4-1, Upstate Biotechnology), affinity-purified sheep antiserum directed against a 20-amino-acid C-terminal sequence of chick α-dystroglycan (25) for patient TLG, a monoclonal anti-β-dystroglycan (clone 8D5, Novocastra), a polyclonal anti-β-dystroglycan (26) and a monoclonal anti-laminin α2 chain antibody (clone 5H2, GibcoBRL, Chemicon). Skeletal muscle staining was performed as described previously (13).

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A New Mutation of the *fukutin* Gene in a Non-Japanese Patient

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Fukuyama-type congenital muscular dystrophy (FCMD), Walker–Warburg syndrome, and muscle-eye-brain disease are clinically similar autosomal recessive disorders characterized by congenital muscular dystrophy, cobblestone lissencephaly, and eye anomalies. FCMD is frequent in Japan, but no FCMD patient with confirmed *fukutin* gene mutations has been identified in a non-Japanese population. Here, we describe a Turkish CMD patient with severe brain and eye anomalies. Sequence analysis of the patient's DNA identified a homozygous 1bp insertion mutation in exon 5 of the *fukutin* gene. To our knowledge, this is the first case worldwide in which a *fukutin* mutation has been found outside the Japanese population. This report emphasizes the importance of considering *fukutin* mutations for diagnostic purposes outside of Japan.

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Fukuyama-type congenital muscular dystrophy (FCMD),[1] Walker–Warburg syndrome (WWS),[2] and muscle-eye-brain (MEB) disease[3] are clinically similar autosomal recessive disorders characterized by congenital muscular dystrophy, lissencephaly, and eye anomalies. FCMD patients survive beyond infancy, and ocular manifestations are rare and usually mild. Patients with WWS are severely affected from birth, and few live beyond infancy. In MEB, the cerebral and ocular anomalies are severe, but some patients reach adulthood. Although FCMD is frequent only in Japan, WWS has been found in many different nationalities, and MEB has been observed mainly in Finland.

We previously identified the gene responsible for FCMD, *fukutin*, on chromosome 9q31.[4–6] Most FCMD patients carry an ancestral founder mutation,[7] which arose from the integration of a 3kb retrotransposon element into the 3′ untranslated region of the *fukutin* gene.[6] A small fraction of FCMD patients are compound heterozygous, carrying one founder insertion and one point mutation. No FCMD patients have been identified with nonfounder (point) mutations on both alleles, suggesting that such patients are embryonic lethal and that fukutin is essential for normal embryonic development.[8] Targeted homozygous mutation of this gene in mice leads to lethality at embryonic day 6.5 to 7.5, before development of skeletal muscle, cardiac muscle, or mature neurons (H. Kurahashi, S. Takeda, C. Meno, M. Horie, M. Taniguchi, H. Otani, H. Hamada, T. Toda, unpublished data).

Until now, no CMD patient has been identified with confirmed *fukutin* gene mutations in a non-Japanese population. Here, we describe a Turkish patient with a severe phenotype carrying a homozygous point mutation in the *fukutin* gene.

Case Report and Results

A full-term male infant was referred to our hospital with macrocephaly, hypotonia, and dyspnea. His parents were first cousins, and their first son is unaffected. In Turkey, there are many origins (Cherkez, Gurcu, Laz, Kurdish, and Turkish) because of immigration. His family was of Turkish origin (Oguz Turks from Central Asia), not Kurdish or others. The mother had not seen a physician before the onset of labor. Ultrasonographic examination during the intrapartum period showed polyhydramnios, macrocephaly, and cephalopelvic disproportion. The infant was born by cesarean section and weighed 2,700gm (25th percentile); his height was 50cm (50th percentile), and head circumference was 47cm (>97th percentile). Physical examination showed respiratory difficulties, central cyanosis, generalized hypotonia, hydrocephaly, bilateral buphthalmus, and cataracts (Fig 1). The suction, Moro, and tendon reflexes were absent, and he had no eye movement.

Upon ocular examination, both eyes were proptotic. The left eye had buphthalmus, with a horizontal corneal diameter of 13mm. There was a central corneal ulcer due to exposure keratopathy on the left eye. Both eyes contained central polar cataracts. We noticed iris atrophy with visible iris vessels, and there were peripheral corneal adhesions, suggesting Rieger's anomaly. We removed the cataract on the right eye by a lensectomy, posterior capsulotomy, and anterior vitrectomy procedure. Ocular examination after cataract extraction showed that the optic disc and the retina were hypoplastic.

Computed tomography showed hydrocephalus and gener-

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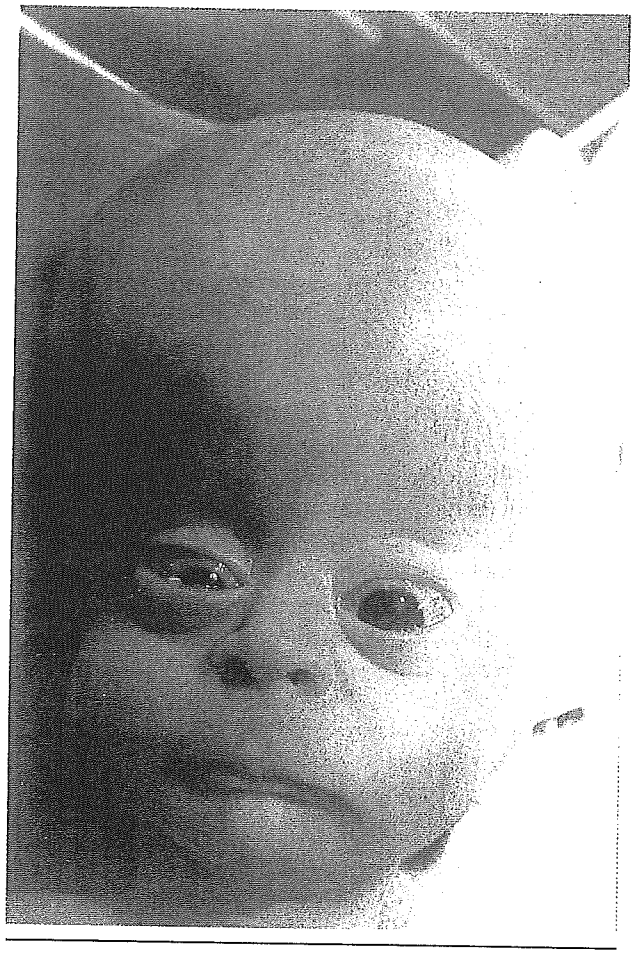


Fig 1. Patient has macrocephaly, cataract, and buphthalmus.

alized brain atrophy (Fig 2A). Muscle enzymes were elevated: serum total creatine kinase levels were 4,214U/ml (76–600U/ml is normal for that age), and myocardial-binding creatine kinase (CK-3) levels were 4,032U/ml (normal for age, 72–576U/ml). Other biochemical analyses were within normal limits. The infant was supported by mechanical ventilation for 10 days.

The patient died on the 10th day, and an autopsy was performed. Neuropathological examination showed severe malformations of the central nervous system. Principal anomalies included agyric hemispheres with polymicrogyria in several cortical segments and severe cortical disorganization in other segments (data not shown). The ventricles released 600ml of cerebrospinal fluid.

Congenital muscular dystrophy was also seen, with variation in fiber size, fibrosis, and fat replacement (see Fig 2B). Immunohistochemical analysis showed greatly reduced stain-

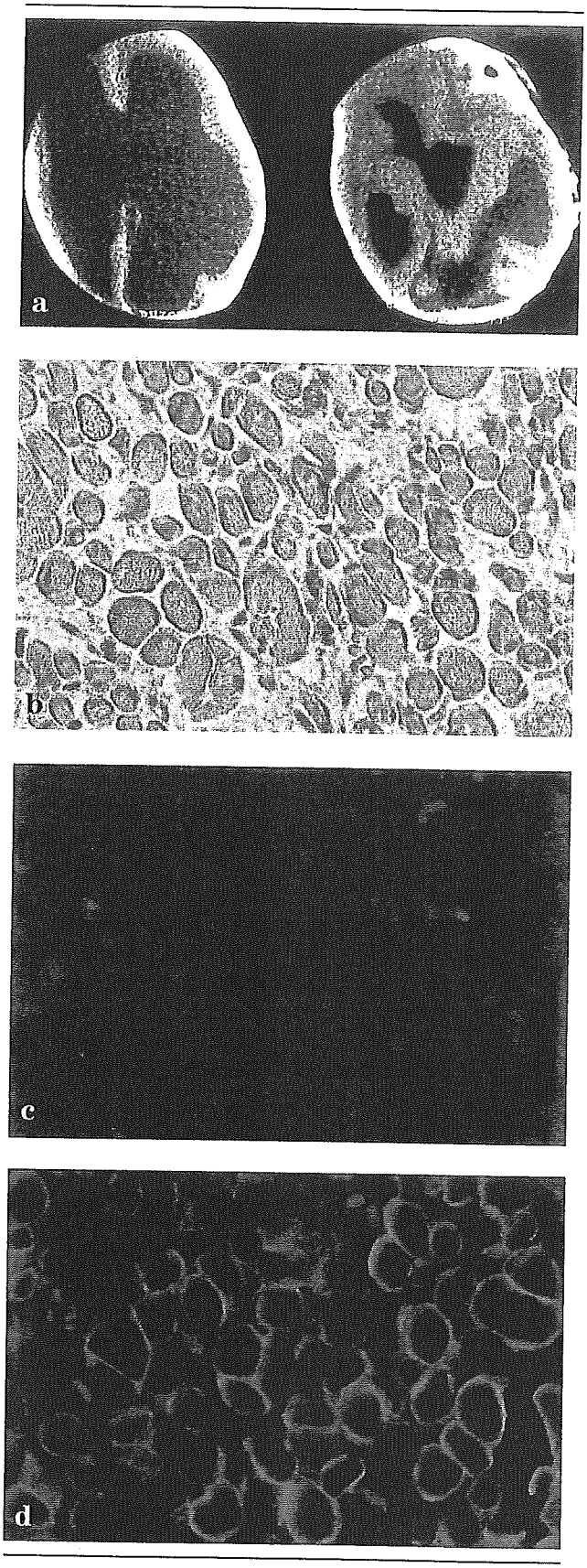


Fig 2. (a) Brain computed tomography shows hydrocephalus and cortical atrophy. (b) Hematoxylin and eosin staining of skeletal muscle shows dystrophic changes with variation in fiber size, fibrosis, and fat replacement. Consecutive sections of skeletal muscle immunostained with anti-α-dystroglycan (c) and β-dystroglycan (d) antibodies.

ing for α-dystroglycan (IIH6; Upstate Biotech, Lake Placid, NY; see Fig 2C) but normal immunoreactivity for β-dystroglycan (8D5; Novocastra, Newcastle upon Tyne, UK; see Fig 2D) in the skeletal muscle membrane.

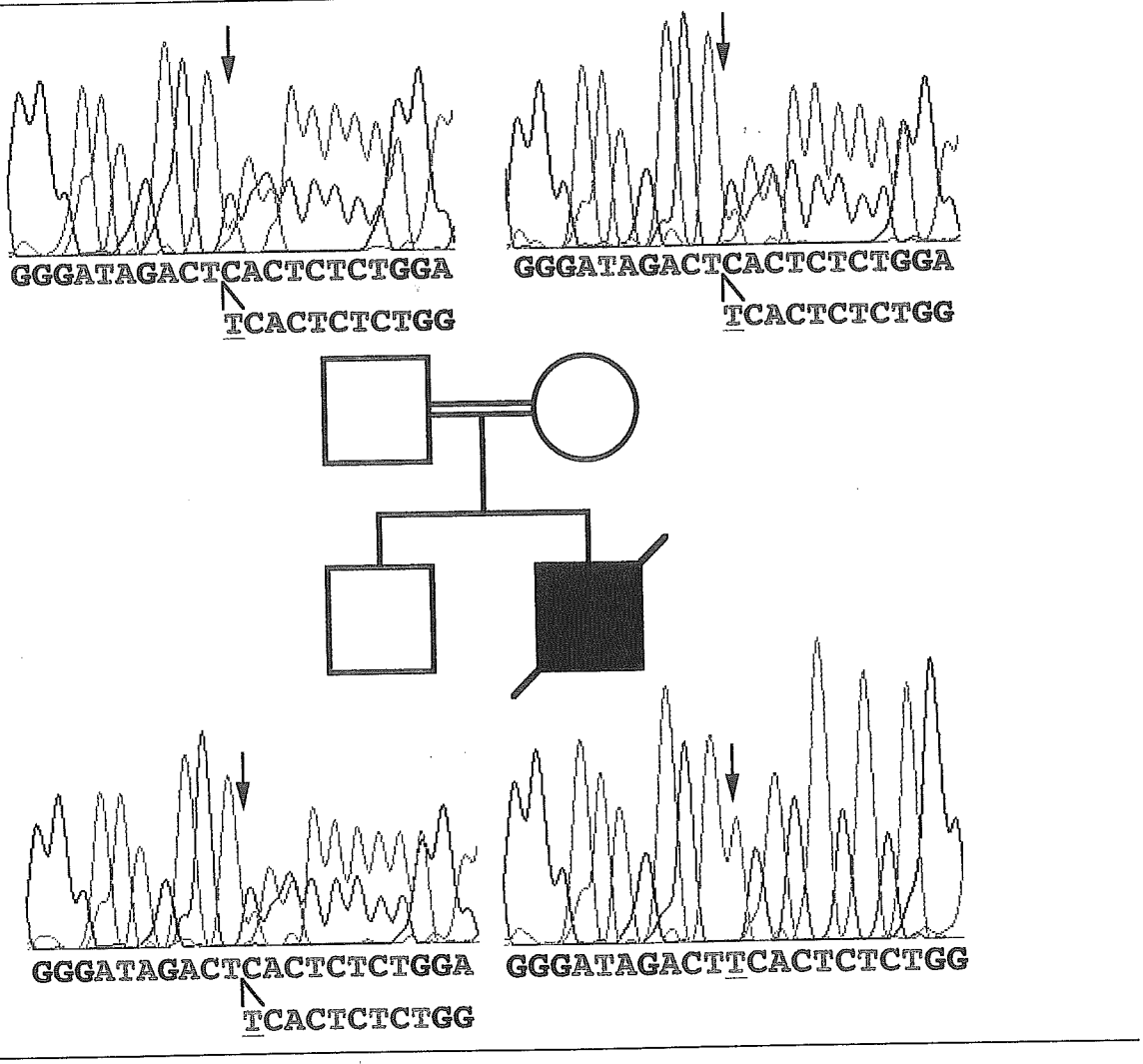
Genomic DNA was extracted from peripheral blood leukocytes of the patient, his brother, and the parents. After digestion of genomic DNA with *Pvu*II, Southern hybridization was performed using fEco8-1 as a probe.[6] As expected, the result indicated that the patient had no Japanese founder insertion. We then screened all exons and flanking introns of the *fukutin* gene in the patient by polymerase chain reaction direct sequencing. We detected a homozygous 1bp insertion mutation, nt504(insT), in exon 5 of the *fukutin* gene. This mutation causes a frameshift, resulting in a premature termination at codon 157. Both parents and the brother were heterozygous for this mutation (Fig 3).

Discussion

The syndrome of congenital muscular dystrophy with central nervous system dysplasia and ocular anomaly has been categorized into FCMD, WWS, and MEB. However, the relationship of these syndromes remains uncertain. The clinical manifestations of WWS are much more severe than those in FCMD. Patients with WWS die earlier and have severe retinal and cerebellar malformations, hydrocephalus, and occasionally occipital encephalocele.[2] Although these findings are uncommon in FCMD patients, several known cases have shown retinal detachment, hydrocephalus, and/or encephalocele.[9]

FCMD is relatively common only in the Japanese. It is the second most common form of childhood muscular dystrophy and one of the most common autosomal recessive disorders in Japan: the incidence of FCMD is 5.6 to 11.9 per 100,000 or nearly half that of Duchenne muscular dystrophy in the Japanese population.[1] In ethnic groups other than the Japanese population, FCMD appears almost nonexistent; only a few white families showing an "FCMD-like phenotype" have been reported, although their phenotypes are not

Fig 3. Point mutation in the fukutin *gene. Our patient was homozygous for a 1bp insertion at base 504 in exon 5 of the* fukutin *gene, which causes a frameshift and a premature termination at codon 157.*



necessarily typical, and mutational analyses of these cases have not yet been performed.[10–12] Two Taiwanese patients were diagnosed by Japanese expert clinicians with clinically typical FCMD, but they carried no *fukutin* mutations.[13]

The strikingly high prevalence of FCMD among the Japanese appears to result from the initial founder effect, whose expansion occurred in relative isolation. Most FCMD-bearing chromosomes in Japan are derived from a single ancestral founder who lived a few thousands years ago.[7] The majority of FCMD patients carry two founder insertions, and fewer are compound heterozygous. Kondo-Iida and colleagues[8] demonstrated a higher frequency of severe phenotypes, including WWS-like manifestations such as hydrocephalus and microphthalmia, among probands who were compound heterozygotes than among those who were homozygous for the founder insertion.

The observed lack of Japanese FCMD patients with two nonfounder mutations suggests that such cases might be embryonic lethal. This may explain why few FCMD cases are reported in non-Japanese populations[8] in which the founder mutation does not occur. Chromosomes carrying the founder insertion in the noncoding region may produce a lower level of mature fukutin than normal and generate a relatively mild FCMD phenotype. Nonfounder mutations, which include nonsense and frameshift mutations within the coding region, cause major structural changes in fukutin protein and thus are likely to produce more severe effects.[8]

The patient described here was affected with a very severe CMD phenotype that resembled WWS. According to the genetic classification, this case may be included in FCMD; however, the mutation is different from that present in most FCMD, and the clinical phenotype is also different. It may not be appropriate to classify this case at this time in existing categories. Although it is postulated that individuals carrying two point mutations will be embryonic lethal, this patient was born alive. Therefore, homozygous disruption within the first third of the fukutin protein (157/461 amino acids) generated a very severe but not embryonic lethal phenotype.

Currently, the function of fukutin remains unknown. However, sequence analysis predicts fukutin to be an enzyme that modifies cell surface glycoproteins or glycolipids.[14] This is supported by recent reports of selective deficiency of highly glycosylated α-dystroglycan in FCMD,[15] as well as defective glycosylation of α-dystroglycan in muscular dystrophies caused by genetic defects in the putative glycosyltransferases, fukutin-related protein,[16] and mouse like-acetylglucosaminyltransferase (large).[17] In addition, we have observed a selective deficiency of α-dystroglycan in MEB, which is caused by loss-of-function mutations in the gene encoding *O*-linked mannose β1, 2-*N*-acetylglucosaminyltransferase (POMGnT1).[18] Quite recently, 20% of WWS patients has been found to have mutations in POMT1, a putative human counterpart of yeast *O*-mannosyltransferase.[19] Moreover, Michele and colleagues showed, in MEB, FCMD, and myodystrophy mouse, that α-dystroglycan is expressed at the muscle membrane, but similar hypoglycosylation in the diseases directly abolishes binding activity of dystroglycan for the ligands laminin, neurexin, and agrin.[20] These findings suggest that defective glycosylation of α-dystroglycan due to genetic defects in glycosyltransferases may be the common denominator causing muscle cell degeneration in these diseases.

This report emphasizes the importance of considering *fukutin* mutations for diagnostic purposes outside of Japan. The possibility exists that non-Japanese patients carrying two *fukutin* point mutations may be misdiagnosed with WWS. As the phenotype observed here was severe, there may be a high probability of early death without a proper diagnosis. Examination of WWS patients and their parents for *fukutin* point mutations may improve the accuracy of diagnosis as well as genetic counseling.

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Fukutin is required for maintenance of muscle integrity, cortical histiogenesis and normal eye development

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Fukuyama-type congenital muscular dystrophy (FCMD), one of the most common autosomal-recessive disorders in Japan, is characterized by congenital muscular dystrophy associated with brain malformation due to a defect during neuronal migration. Through positional cloning, we previously identified the gene for FCMD, which encodes the fukutin protein. Here we report that chimeric mice generated using embryonic stem cells targeted for both *fukutin* alleles develop severe muscular dystrophy, with the selective deficiency of α-dystroglycan and its laminin-binding activity. In addition, these mice showed laminar disorganization of the cortical structures in the brain with impaired laminin assembly, focal interhemispheric fusion, and hippocampal and cerebellar dysgenesis. Further, chimeric mice showed anomaly of the lens, loss of laminar structure in the retina, and retinal detachment. These results indicate that fukutin is necessary for the maintenance of muscle integrity, cortical histiogenesis, and normal ocular development and suggest the functional linkage between fukutin and α-dystroglycan.

INTRODUCTION

Since the discovery of the Duchenne muscular dystrophy (DMD) gene product dystrophin (1), many studies have focused on understanding the pathophysiology of muscular dystrophies and on developing therapeutic approaches. Structural defects in the dystrophin–glycoprotein complex (DGC) can result in a loss of linkage between laminin-2 (merosin) in the extracellular matrix and actin in the subsarcolemmal cytoskeleton, and this can lead to various muscular dystrophies (2). Of these, α-dystroglycan is a heavily glycosylated mucin-type glycoprotein on the surface of muscle cells (3–5). It is the key component of the DGC, providing a tight linkage between the cell and basement membranes by binding laminin via its carbohydrate residues (3–5). α-Dystroglycan plays an active role in the basement membrane assembly itself (6).

Fukuyama-type congenital muscular dystrophy (FCMD), one of the most common autosomal-recessive disorders in Japan, is characterized by congenital muscular dystrophy associated with brain malformation (polymicrogyria) due to a defect during neuronal migration (7). Patients with FCMD manifest

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