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Phenotypic spectrum of α-dystroglycanopathies associated with the c.919T>A variant in the FKRP gene in humans and mice

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Abstract:	Mutations in the fukutin-related protein gene, FKRP, are the most frequent single cause of α-dystroglycanopathy. Rare FKRP mutations are clinically not well characterized. Here we review the phenotype associated with the rare c.919T>A mutation in FKRP in humans and mice.

Phenotypic spectrum of α-dystroglycanopathies associated with the c.919T>A variant in the *FKRP* gene in humans and mice

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Author contributions

FM: assessment of patients; study design; drafting the manuscript.

SCB: generation and analysis of mouse model, drafting the manuscript.

MFF: Western blotting and immunocytochemistry.

JV: assessment of patients; study design; drafting the manuscript.

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Abstract

Mutations in the fukutin-related protein gene, *FKRP,* are the most frequent single cause of α-dystroglycanopathy. Rare *FKRP* mutations are clinically not well characterized. Here we review the phenotype associated with the rare c.919T>A mutation in *FKRP* in humans and mice.

We describe clinical and paraclinical findings in six patients, two homozygous and four compound heterozygous for c.919T>A, and compare findings with a mouse model we generated, which is homozygous for the same mutation. In patients, the mutation at the homozygous state is associated with a severe congenital muscular dystrophy phenotype invariably characterized by severe multisystem disease and early death. Compound heterozygous patients have a severe limb-girdle muscular dystrophy phenotype, loss of ambulation before age 20 and respiratory insufficiency. By contrast, mice homozygous for the same mutation show no symptoms or signs of muscle disease. Evidence therefore defines the *FKRP* c.919T>A as a very severe mutation in humans. The huge discrepancy between phenotypes in humans and mice suggests that differences in protein folding/processing exist between human and mouse Fkrp. This emphasizes the need for more detailed structural analyses of FKRP and shows the challenges of developing appropriate animal models of dystroglycanopathies that mimick the disease course in humans.

Introduction

The secondary dystroglycanopathies are a genetically and clinically heterogeneous group of muscular dystrophies characterized by a defective glycosylation of α-dystroglycan (α-DG), a critical component of the dystrophin-glycoprotein complex. The wide spectrum of clinical phenotypes in the dystroglycanopathies span from patients with the congenital muscular dystrophy (CMD) variants Muscle-Eye-Brain Disease and Walker-Warburg Syndrome that involve brain, eyes, heart and skeletal muscles, to limb-girdle muscular dystrophies (LGMDs), where only skeletal muscle and sometimes heart are involved (1).

The two subunits of dystroglycan, α-DG and β-DG, are encoded for by the *DAG1* gene, the transcript of which is postranslationally cleaved to generate the α and β subunits. α-DG is a peripheral membrane glycoprotein that binds via its glycosylated domains to extracellular matrix components that include laminin α2, agrin and perlecan. β-DG is a transmembrane protein that binds to both α-DG and cytoskeletal actin via dystrophin or utrophin (2). The dystroglycan complex therefore links the cytoskeleton with the extracellular matrix and plays an essential role in the maintenance of muscle fibre integrity in addition to contributing to the role of the dystrophin-glycoprotein complex as an important signalling platform (3). α-DG also plays a pivotal role in basement membrane formation and maintenance, a role which has been shown to be important in brain and eye development (4).

The defective glycosylation of α-DG reduces the functional binding capacity with laminin α2 and other α-DG ligands of the extracellular matrix, thereby impairing the structural stability of the complex, and in skeletal muscle the ability of the muscle fibre to withstand repeated cycles of contraction and relaxation. An increasing number of genes are now known to be involved in the glycosylation pathway of α-DG, either by directly acting on dystroglycan itself or indirectly by way of delivering donor substrates. In addition to the 6 classical genes described several years ago (*POMT1, POMT2, POMGNT1, fukutin, FKRP* and *LARGE*), the recent identification of at least 11 new genes has significantly expanded the clinical phenotypes and increased the complexity of genetic diagnosis of dystroglycanopathies (4-10).

Fukutin-related protein was initially identified as a homolog of fukutin (11). Fukutin and fukutin-related proteins are known to be sequentially acting ribitol 5-phosphate transferases (12-14). Mutations in the fukutin gene cause Fukuyama Muscular Dystrophy, which is common in Japan, but rare elsewhere, and in rare cases can also result in a milder LGMD phenotype (type 2N). Mutations in fukutin-related protein gene, *FKRP,* is the most frequent single cause of α-dystroglycanopathy, and were first described in patients with a severe form CMD type 1C (11). However, it is now known to be associated with more severe forms of CMD with structural brain and eye involvment and is also one of the most frequent recessive forms of LGMD (type 2I) in Northern Europe (15-19). To date, more than 150 different mutations have been described in the *FKRP* gene (20), but a common founder mutation, c.826C>A, underlies most cases of LGMD2I (17,21).

In 2003, our group reported the first case associated with the *FKRP* c.919T>A mutation in a 19-year-old patient with a Duchenne muscular dystrophy (DMD)-like phenotype (16). Since then, only few patients sharing the same mutation have been described (17,21-24). In this report, we describe a second homozygous patient and review the cases previously published to establish a possible genotype-phenotype correlation and confirm the severity of this mutation in humans. By way of comparison, we also revisit a mouse model with this mutation, which shows no discernible phenotype (25). These observations imply a structural difference in FKRP between the two species that has implications for the generation of future models intended to recapitulate the human disorder.

Patients

We describe 6 unrelated patients with the c.919T>A mutation in the *FKRP* gene, who were sequenced as previously described (16,17,23,24, 26). Some data on patients 2-6 have been previously reported by us (16,17,23,24, 26). The new patient has been carefully assessed and additional data have been added for the remaining patients. Informed consent was obtained from all patients or parents.

Clinical data, included age at onset, presenting symptoms, maximal motor ability acquired, muscle weakness distribution, loss of ambulation and current clinical status, respiratory, cardiac, eye and CNS involvement, mental retardation and other complications were assessed. Plasma CK levels were obtained in all patients and brain MRI was performed in four.

Muscle biopsies from the six patients were re-evaluated. Muscle biopsies were processed according to standard protocols. Muscle samples were screened by immunohistochemistry

(IHC) for dystrophin, utrophin and laminin α2. α-DG was assessed by IHC in patients 4, 5 and 6. No muscle was available to complete the analysis of α-DG in the remaining patients.

Generation of the mouse model

Mice were generated with the Fkrp^{Tyr307Asn} knock-in mutation as previously described (25). In brief, a targeted ES clone was microinjected into blastocysts from 129J mice. The resulting male chimeras were then bred with female C57BL6 mice to generate germ line-transmitted heterozygous mice. These mice were then crossed to generate mice homozygous for the mutation. In order to remove the neomycin cassette (introduced as part of the targeting process) heterozygotes were crossed with β-actin Cre transgenic mice Cre–mediated chromosome loss in mice. Genotyping of newborn mice was performed on gDNA prepared from tail tissue (25). Genotyping was performed by PCR and sequencing of the Fkrp gene was used to confirm the point mutation (c.919 T > A). Homozygous wild-type, homozygous Tyr307Asn missense mutants or heterozygous mice were identified using the Vector NTi advance10 software (Invitrogen). After initial heterozygote crossings, the colony was maintained as a homozygous line. All procedures were approved by the Royal Veterinary College local Ethics and welfare committee and were performed under Home Office Project license (PPL 70/7988) according to the Animal (Scientific Procedures) Act 1987 of the United Kingdom.

Mouse muscle processing

Muscle was collected from 20-week old mice following cervical dislocation. Quadriceps and the tibialis anterior muscles were dissected, mounted on cork and immediately frozen in liquid nitrogen cooled isopentane. Twelve µm sections were cut using a Bright OTF5000 cryostat (Bright Instruments, Huntington, UK).

For general histology, tissues were stained with haematoxylin and eosin, and sections digitally imaged using a DM4000B upright microscope (Leica, Germany) interfaced with an Axiovision monochrome mRM camera (Zeiss, UK). For immunocytochemistry, sections were immunolabelled with either anti laminin α2 (4H8, Abcam) for 1 hour at room temperature followed by anti-rat Alexa 488 (Molecular Probes) for 30 minutes, or IIH6 (which recognizes a glycosylated epitope of α-DG) (Millipore) overnight at 4°C followed by anti-IgM biotinylated antibody (30 minutes) and streptavidin conjugated with Alexa 488 or 594 (30 minutes). Nuclei were stained with Hoechst 33342 (Sigma). All dilutions and washings were made in phosphate buffered saline. Sections were mounted in aqueous mountant and viewed with epifluorescence using a DM4000B upright microscope (Leica, Germany). Images were digitally captured with an Axiovision mRM monochrome camera, (Zeiss, UK) with equal exposure and equal scaling.

Western blotting and laminin overlay assay of mouse muscle tissue was performed as previously described (25).

Clinical features

The main clinical features of the six patients are summarized in the Table 1. Patients are classified into three groups according to the mutation: the first group comprised two patients homozygous for the c.919T>A mutation, and the second group of three patients who are compound heterozygous for the c.919T>A and the common c.826C>A mutations. The third group consisted of one patient compound heterozygous for c.919T>A and a rare c.1012C>T mutation. All patients were unrelated and were of Northern European origin.

Group 1: patients 1 and 2 homozygous for c.919T>A mutation showed the most severe phenotype consistent with Muscle-Eye-Brain disease. Both presented at birth with a muscle weakness, hypotonia and respiratory distress. Motor development was severely delayed, they never walked and the maximal motor ability acquired was sitting with support in patient 1. They developed progressive respiratory insufficiency requiring invasive ventilation, and both had recurrent respiratory infections. Patient 2, developed left ventricle hypertrophy, and both patients died of respiratory complications at age of 7 (patient 2) and 14 (patient 1) years, respectively. Both had a profound mental retardation with severe CNS involvement on brain MRI, including lissencephaly type II (cobblestone), polymicrogyria, pons and vermis hypoplasia, cerebellar cysts and Dandy-Walker malformation (Fig. 1). Severe bilateral congenital myopia was observed in both patients. Retinal dysplasia with detachment and rarefaction of the pigmented epithelium was also observed in patient 2.

Group 2: patients 4, 5 and 6, compound heterozygous for c.826C>A and c.919T>A, presented with a milder phenotype than group 1, consistent with a LGMD without mental retardation. Time of onset was during childhood, ranging from before the age of 3 for patients 4 and 5 with a DMD-like phenotype to age 7 for patient 6, who had a severe Becker muscular dystrophy (BMD)-like phenotype. Clinical picture at presentation included proximal weakness and walking difficulties. Patients were never able to run and they lost ambulation before the age of 12 years in the two DMD-like patients and at 20 years in the BMD-like patient. All patients developed an early respiratory involvement requiring non-invasive ventilation (BiPAP, bilevel positive air pressure) in the second decade. Patient 6 died of respiratory complications at age 42 years. Tongue hypertrophy was present in all cases and calf hypertrophy in patients 5 and 6. Mild-moderate left ventricle hypertrophy was identified in patients 4 and 6.

Group 3: the patient compound heterozygous for a non-common c.1012C>T mutation (patient 3), showed an intermediate phenotype between groups 1 and 2, compatible with a severe CMD with eye and cerebellar malformation, but no mental retardation. He presented with severe neonatal hypotonia, poor head control and feeding difficulties. He acquired the ability to stand with support but never independent ambulation. He had significant tongue hypertrophy, scoliosis and respiratory involvement requiring BiPAP at age of 14 and died at 16 due to respiratory complications. Myopia, rarefaction of the pigmented epithelium and bilateral retinal detachment were also present in this patient. Brain MRI revealed severe posterior fossa structural defects with pons and cerebellar hypoplasia and frequent cerebellar cysts. Cognitive development was normal.

Muscle pathology

All human muscle biopsies showed a classic dystrophic pattern of variable severity (Figs. 1 and 2).

Group 1: the two patients with the Muscle-Eye-Brain phenotype (patients 1 and 2) presented a severe dystrophic pattern with a significant degree of fibrosis, adipose tissue substitution and atrophy (patient 1 in Fig. 1C).

Group 2: biopsies from these three patients showed more moderate changes and less degree of fibrosis than patients in group 1. Central nuclei, split fibres, round atrophic fibres, hypertrophic fibres were frequent features (patient 5 in Fig. 2C and patient 6 in Fig. 2D).

Group 3: the biopsy from patient 3 displayed the most severe lesions. Performed at the age of three years, only few round atrophic fibres were present scattered within the extensive endomysial connective tissue infiltration and adipose replacement (patient 3 in Fig. 2B).

No rimmed vacuoles, inflammatory infiltrates, or other distinctive structural features were identified in any biopsy. Muscle biopsies were performed in all patients for diagnostic purpose, however a complete workup with α-DG immunostaining could only be done in the three patients with a milder phenotype (patients 4, 5 and 6), revealing a moderate reduction in all these cases. Dystrophin and utrophin were normal in all cases and laminin α2 showed only an irregular immunolabelling in patient 1 (fig. 1F).

Mouse model

Haematoxylin and eosin stained sections of the quadriceps and the tibialis anterior muscles of $Fkrp^{Tyr307Asn}$ mice were indistinguisable from wild-type (Fig. 3A and 3C), with no evidence of an abnormal variation in fibre size, presence of internal nuclei, vacuolation, necrosis, ragged red fibers or inflammation. Histology of heart did not show any abnormalities. Western blotting with the IIH6 antibody, showed no difference in glycosylation in the quadriceps and tibialis anterior between mutant and wild-type controls (Fig. 3C).

Discussion

More than 150 mutations have been described in the *FKRP* gene (20), but the c.826C>A mutation is by far the most common mutation in Northern Europe with an allele frequency ranging between 1/116 and 1/600 (15,17-19). This mutation is associated with a relatively mild phenotype, and accounts for a great majority of patients with LGMD2I (18,19,25,26). A much broader range of clinical phenotypes, including Walker-Warburg Syndrome and Muscle-Eye-Brain Disease, and diverse types of CMDs, have been associated with other mutations (11,16,22,23,27,28). Regardless of this genetic variability, few reports have been published focusing on the rare *FKRP* variants.

Here we review a cohort of six patients carrying the c.919T>A (Tyr307Asn) *FKRP* mutation, an infrequent mutation affecting a highly conserved Tyrosine residue at position 307. The mutation seems to confer a very severe phenotype, because patients homozygous for this variant and the patients with this variant and another rare mutation predicted to disrupt FKRP (c.1012C>T), all presented with severe multisystem disease, never acquired independent ambulation and died before the end of their second decade of life. Apart from the six cases we present here, the c.919T>A variant has only been reported previously in two other patients, who were compound heterozygous for the mutation in combination with the common c.826C>A mutation. They were included in two large reviews of LGMD2I carried out in Germany (19) and France (29) and were reported to have a BMD-like (19) and DMD-like (29) course, very similar to the three heterozygous patients of our study. References to a third heterozygous patient, also with the common mutation was published in another review, but neither the phenotype nor any additional clinical information were discussed (30).

The first patient homozygous for the c.919T>A (Tyr307Asn) mutation (patient 2) was reported in 2004 (23) and presented with a severe CMD/ MEB phenotype. Since then, only 6 patients carrying this variant in a heterozygous state have been described (17,19, 24,26,29), and although they were consistent with the hypothesis that this mutation confers a severe phenotype, further verification of this was required. The new homozygous patient for the c.919T>A mutation reported here (patient 1) displayed a similar severe phenotype as found in patient 2. The clinical picture of both patients was characterized by extreme weakness, hypotonia, and motor delay with a congenital onset and profound mental retardation. In addition to severe eye abnormalities, lissencephaly, polymicrogyria, cerebellar malformations with cysts and pons hypoplasia in posterior fossa on brain MRI were also present. CNS involvement is often associated with non-common *FKRP* mutations and a congenital onset. The clinical phenotype described here is consistent with a Muscle-Eye-Brain Disease phenotype, which has also been described in other patients with a variety of non-common mutations in *FKRP* (11,16,22,23). The new homozygous case described here, in addition to the first case previously reported, reinforces the hypothesis that this mutation is associated with CNS structural involvement and the resulting severe clinical phenotype.

Regarding the severity of the mutation, it is also noteworthy that the six patients (100%), required ventilatory support before the age of twenty, and three out the six (50%), including the two that were compound heterozygous for the mutation, died from respiratory complications during the progression of the disease. All these data are consistent with previously published smaller series, showing that while in homozygous state the common mutation seems to be associated with a relatively benign and homogenous course, in heterozygous patients the severity and clinical course of the disease depend mainly on the second mutation (18,31-33).

The c.1012C>T mutation found at compound heterozygous state in this study has never been reported in other patients, but seems also to confer a severe phenotype, when comparing with the LGMD phenotype of the other three compound heterozygous patients with the common c.826C>A mutation (patients 4-6). The muscle biopsy from this patient showed the most severe changes of all patients, the patient had brain and eye involvement and died at age 16 years. Taken together, the findings presented here strongly support the notion that the c.1012C>T mutation results in a particularly severe phenotype.

Although clinical severity in human patients affected by pathogenic variants in *FKRP* appears to relate to the specific mutation, the introduction of the $\mathrm{Fkrp}^{\mathrm{Tyr307Asn}}$ mutation in mice surprisingly failed to generate a disease phenotype (25 and present report). However, mice homozygous for the missense P448L mutation do display a muscle pathology demonstrating that the mouse is a useful model but only for specific mutations (34). Our report of the absence of a phenotype after introduction of the $\mathrm{Fkrp}^{\mathrm{Tyr307Asn}}$ mutation strongly suggests that differences in protein folding/processing must exist between human and mouse Fkrp, which are differentially altered by specific mutations. Interestingly, this has also been observed with respect to a mutation in the sarcoglycan gene, which in human patients is commonly associated with LGMD, but that fails to give rise to a disease phenotype in the mouse (35, 36). A knock-down of Fkrp expression levels is sufficient to give rise to a Muscle-

Eye-Brain phenotype in the mouse as we previously reported (25, 37) and it has been proposed that a wide range of mouse models may be generated by combining a knock-down and missense mutation approach (34). Interestingly, central nervous system involvement was reported in a mouse model, which had both a knock-down and P448L missense mutation (38) whereas in the presence of just the missense mutation, CNS involvement was eliminated and the severity of the muscular dystrophy reduced (34).

Overall, our observations strongly suggest that the c.919T>A mutation in *FKRP* confers a severe disease phenotype in humans but that there appears to be a huge discepancy between pathogenicity of this variant in humans vs. mice, which highlights the need for developing appropriate animal models of dystroglycanopathies that mimick the disease course in humans.

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Figure Legends

Figure 1: Saggital (A) and horizontal (C) brain MRI of patient 1 at age 1 year, showing lissencephaly type II (cobblestone), polymicrogyria, pons and vermis hypoplasia, cerebellar cysts and Dandy-Walker malformation. Muscle biopsy from the same patient at age 1 year shows (B) severe dystrophic features with hypercontracted fibres, cell necrosis, increased connective tissue and wide fiber size variability. Laminin α2 staining in the patient (D) shows irregular immunolabelling.

Figure 2: Muscle histology of a healthy 1.5-year-old child (A), patient 3 (B), patient 5 (C) and patient 6 (D) stained by haematoxylin and eosin and their corresponding stains for α-dystroglycan glycosylation using the IIH6-antibody (E-H).

Figure 3: Haematoxylin and Eosin stained sections of tibialis anterior (TA) of wild-type (A) and $Fkrp^{Tyr307Asn}$ (B) mice showing normal histology with no abnormal variation in fibre size and no evidence of regeneration or necrosis. Western blot of tibialis anterior (TA) and quadriceps (Q) muscle from wild-type (Lanes 1 and 2) and 4 individual $Fkrp^{Tyr307Asn}$ mice (lanes 3-10). The Coomassie stained running gel confirms equal loading of the lanes.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age / Gender	12 years/M	Died at 7 years/M	Died at 16years /M	29 years/M	22 years/F	Died at 43 years/M
Origin	Danish	German	English	German	Danish	Danish
***FKRP* Mutation**	c.919T>A / c.919T>A homozygous	c.919T>A / c.919T>A homozygous	c.919T>A /c.1012C>T heterozygous	c.919T>A / c826C>A heterozygous	c.919T>A / c826C>A heterozygous	c.919T>A / c826C>A heterozygous
Phenotypic Classification	MEB	MEB	CMD-CRB*	LGMD-no MR (DMD-like)	LGMD-no MR (DMD-like)	LGMD-no MR (BMD-like)
Age of onset	Neonatal	Neonatal	1.5 months	1.5 years	3 years	7 years
Symptoms at onset	Hypotonia, respiratory distress	Hypotonia, respiratory distress	Hypotonia, poor head control	walking difficulties	walking difficulties	walking difficulties
Maximum motor ability	Sitting with support	Never head control or sit	Standing with support	Walking, never ran	Walking, never ran	Walking, never ran
Loss of ambulation	_	_	_	12 years	8 years	20 years
Muscle hypertrophy	NR	NR	tongue	tongue, thighs	tongue, calves	tongue, calves
Joint contractures	NR	Achilles, hips	scoliosis	Achilles, hips	No	Achilles, elbow, scoliosis
Respiratory involvement	Respirator	Recurrent infections	BiPAP (14 years)	BiPAP (16 years)	BiPAP (12 years)	BiPAP-respirator (32 years)
Cardiac involvement	NR	LVH	NR	DCM	Normal	LVH mild
Eye involvement						
Myopia	Severe	Severe	Severe	No	No	No
Retinal abnormalities	No	Detachment, RPE	Detachment, RPE	No	No	No
Mental retardation	Yes	Yes	No	No	No	No
Brain MRI	Yes	Yes	Yes	Yes	ND	ND
Cortical malformation	Polymicrogyria	Pachygyria, lissencephaly	No	No	_	_
Cerebellar malformations	Hypoplasia, cysts	Vermis hypoplasia, cysts	Dysplasia-Hypoplasia, cysts	No	_	_
Others	Dandy Walker	Pons hypoplasia, Dandy Walker	Pons hypoplasia	No	_	_
Average CK levels (U/l)	4000	3000-4000	2000-3000	1700-3300	1500	2000
Muscle biopsy (location / age)	quadriceps / 1 year	NR / 1 year	quadriceps / 3 years	NR / NR	Tibial ant. / 13 years	Tibial ant. / 35 years
Muscle atrophy / fibrosis	Dystrophic +++ / +++	Dystrophic +++ / +++	Dystrophic ++++ / ++++	Dystrophic ++ / ++	Dystrophic ++ / ++	Dystrophic ++ / ++
Alpha-dystroglycan (IHC)	ND	ND	ND	Reduced	Reduced	Reduced
Reference	Not reported	[17]	[19]	[14]	[18, 21]	[18, 21]

Table 1: **Clinical features and laboratory findings in 6 patients homozygous or compound heterozygous for the c.919T>A variant in the fukutin-related protein gene.** ND= not done; NR= not reported; DCM= dilated cardiomyopathy; LVH= left ventricular hypertrophy; IHC= immunohistochemistry; + mild, ++ moderate, +++ severe, ++++ end stage

Phenotypic spectrum of α-dystroglycanopathies associated with the c.919T>A variant in the *FKRP* gene in humans and mice

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Author contributions

FM: assessment of patients; study design; drafting the manuscript.

SCB: generation and analysis of mouse model, drafting the manuscript.

MFF: Western blotting and immunocytochemistry.

JV: assessment of patients; study design; drafting the manuscript.

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Abstract

Mutations in the fukutin-related protein gene, *FKRP,* are the most frequent single cause of α-dystroglycanopathy. Rare *FKRP* mutations are clinically not well characterized. Here we review ~~In this study, we describe~~ the phenotype associated with the rare c.919T>A mutation in *FKRP* in humans and mice.

We describe clinical and paraclinical findings in six patients, two homozygous and four compound heterozygous for c.919T>A, and compare findings with a mouse model we generated, which is homozygous for the same mutation. In patients, the mutation at the homozygous state i~~wa~~s associated with a severe congenital muscular dystrophy phenotype invariably characterized by severe multisystem disease and early death. Compound heterozygous patients have~~d~~ a severe limb-girdle muscular dystrophy phenotype, loss of ambulation before age 20 and respiratory insufficiency. By contrast, mice homozygous for the same mutation show no symptoms or signs of muscle disease. Evidence therefore ~~This study~~ defines the *FKRP* c.919T>A as a very severe mutation in humans. The huge discr~~e~~epancy between phenotypes in humans and mice suggests that differences in protein folding/processing exist between human and mouse Fkrp. This emphasizes the need for more detailed structural analyses of FKRP and shows the challenges of developing appropriate animal models of dystroglycanopathies that mimick the disease course in humans.

Introduction

The secondary dystroglycanopathies are a genetically and clinically heterogeneous group of muscular dystrophies characterized by a defective glycosylation of α-dystroglycan (α-DG), a critical component of the dystrophin-glycoprotein complex. The wide spectrum of clinical phenotypes in the dystroglycanopathies span from patients with the congenital muscular dystrophy (CMD) variants Muscle-Eye-Brain Disease and Walker-Warburg Syndrome that involve brain, eyes, heart and skeletal muscles, to limb-girdle muscular dystrophies (LGMDs), where only skeletal muscle and sometimes heart are involved (1).

The two subunits of dystroglycan, α-DG and β-DG, are encoded for by the *DAG1* gene, the transcript of which is postranslationally cleaved to generate the α and β subunits. α-DG is a peripheral membrane glycoprotein that binds via its glycosylated domains to extracellular matrix components that include laminin α2, agrin and perlecan. β-DG is a transmembrane protein that binds to both α-DG and cytoskeletal actin via dystrophin or utrophin (2). The dystroglycan complex therefore links the cytoskeleton with the extracellular matrix and plays an essential role in the maintenance of muscle fibre integrity in addition to contributing to the role of the dystrophin-glycoprotein complex as an important signalling platform (3). α-DG also plays a pivotal role in basement membrane formation and maintenance, a role which has been shown to be important in brain and eye development (4).

The defective glycosylation of α-DG reduces the functional binding capacity with laminin α2 and other α-DG ligands of the extracellular matrix, thereby impairing the structural stability of the complex, and in skeletal muscle the ability of the muscle fibre to withstand repeated cycles of contraction and relaxation. An increasing number of genes are now known to be involved in the glycosylation pathway of α-DG, either by directly acting on dystroglycan itself or indirectly by way of delivering donor substrates. In addition to the 6 classical genes described several years ago (*POMT1, POMT2, POMGNT1, fukutin, FKRP* and *LARGE*), the recent identification of at least 11 new genes has significantly expanded the clinical phenotypes and increased the complexity of genetic diagnosis of dystroglycanopathies (4-10).

Fukutin-related protein was initially identified as a homolog of fukutin (11). Fukutin and fukutin-related proteins are known to be sequentially acting ribitol 5-phosphate transferases (12-14). Mutations in the fukutin gene cause Fukuyama Muscular Dystrophy, which is common in Japan, but rare elsewhere, and in rare cases can also result in a milder LGMD phenotype (type 2N). Mutations in fukutin-related protein gene, *FKRP,* is the most frequent single cause of α-dystroglycanopathy, and were first described in patients with a severe form CMD type 1C (11). However, it is now known to be associated with more severe forms of CMD with structural brain and eye involvment and is also one of the most frequent recessive forms of LGMD (type 2I) in Northern Europe (15-19). To date, more than 150 different mutations have been described in the *FKRP* gene (20), but a common founder mutation, c.826C>A, underlies most cases of LGMD2I (17,21).

In 2003, our group reported the first case associated with the *FKRP* c.919T>A mutation in a 19-year-old patient with a Duchenne muscular dystrophy (DMD)-like phenotype (16). Since then, only few patients sharing the same mutation have been described (17,21-24). In this

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report~~study~~, we describe~~report~~ a second homozygous patient and review the cases previously published to establish a possible genotype-phenotype correlation and confirm the severity of this mutation in humans. By way of comparison, we also revisit a mouse model with this mutation, which shows no discernible phenotype (25). These observations imply a structural difference in FKRP between the two species that has implications for the generation of future models intended to recapitulate the human disorder.

~~Materials and methods~~

Patients

We describe 6~~Six~~ unrelated patients with the c.919T>A mutation in the *FKRP* gene, who were sequenced as previously described (16,17,23,24, 26)~~ are included in this investigation~~. Some data on patients 2-6 have been previously reported by us (16,17,23,24, 26). The new patient has been carefully assessed and additional data have been added for the remaining patients. Informed consent was obtained from all patients or parents.

Clinical data, included age at onset, presenting symptoms, maximal motor ability acquired, muscle weakness distribution, loss of ambulation and current clinical status, respiratory, cardiac, eye and CNS involvement, mental retardation and other complications were assessed. Plasma CK levels were obtained in all patients and brain MRI was performed in four.

M

~~Muscle biopsy~~

~~Previously performed diagnostic m~~uscle biopsies from the six patients were re-evaluated. Muscle biopsies were processed according to standard protocols. Muscle samples were screened by immunohistochemistry (IHC) for dystrophin, utrophin and laminin α2. α-DG was assessed by IHC in patients 4, 5 and 6. No muscle was available to complete the analysis of α-DG in the remaining patients.

~~Molecular genetic studies~~

~~Genomic DNA was extract from peripheral blood and genetic analyses were performed as previously reported (16,17,23,24,26).~~

Generation of the mouse model

Mice were generated with the Fkrp^{Tyr307Asn} knock-in mutation as previously described (25). In brief, a targeted ES clone was microinjected into blastocysts from 129J mice. The resulting male chimeras were then bred with female C57BL6 mice to generate germ line-transmitted heterozygous mice. These mice were then crossed to generate mice homozygous for the mutation. In order to remove the neomycin cassette (introduced as part of the targeting process) heterozygotes were crossed with β-actin Cre transgenic mice Cre–mediated chromosome loss in mice. Genotyping of newborn mice was performed on gDNA prepared from tail tissue (25). Genotyping was performed by PCR and sequencing of the Fkrp gene was used to confirm the point mutation (c.919 T > A). Homozygous wild-type, homozygous Tyr307Asn missense mutants or heterozygous mice were identified using the Vector NTi advance10 software (Invitrogen). ~~Offspring were genotyped by PCR analysis using mouse tail/ear biopsies.~~ After initial heterozygote crossings, the colony was maintained as a homozygous line. All procedures were approved by the Royal Veterinary College local Ethics and welfare committee and were performed under Home Office Project license (PPL 70/7988) according to the Animal (Scientific Procedures) Act 1987 of the United Kingdom.

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Mouse muscle processing

Muscle was collected from 20-week old ~~adult~~ mice following cervical dislocation. Quadriceps and the tibialis anterior muscles were dissected, mounted on cork and immediately frozen in liquid nitrogen cooled isopentane. Twelve µm sections were cut using a Bright OTF5000 cryostat (Bright Instruments, Huntington, UK).

For general histology, tissues were stained with haematoxylin and eosin, and sections digitally imaged using a DM4000B upright microscope (Leica, Germany) interfaced with an Axiovision monochrome mRM camera (Zeiss, UK). For immunocytochemistry, s

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~~Immunocytochemistry~~

~~S~~ections were immunolabelled with either anti laminin α2 (4H8, Abcam) for 1 hour at room temperature followed by anti-rat Alexa 488 (Molecular Probes) for 30 minutes, or IIH6 (which recognizes a glycosylated epitope of α-DG) (Millipore) overnight at 4°C followed by anti-IgM biotinylated antibody (30 minutes) and streptavidin conjugated with Alexa 488 or 594 (30 minutes). Nuclei were stained with Hoechst 33342 (Sigma). All dilutions and washings were made in phosphate buffered saline. Sections were mounted in aqueous mountant and viewed with epifluorescence using a DM4000B upright microscope (Leica, Germany). Images were digitally captured with an Axiovision mRM monochrome camera, (Zeiss, UK) with equal exposure and equal scaling.

Western blotting and laminin overlay assay of mouse muscle tissue was performed as previously described (25).

~~Western blotting and laminin overlay assay.~~

~~Protein extracts were obtaining after crushing muscle tissues in liquid nitrogen and placing them in sample buffer consisting of 75 mM Tris–HCl, 1% SDS, 2-mercaptoethanol, plus a cocktail of protease inhibitors (Roche). Thirty μg of protein was resolved using a NuPage Pre-cast gel (3–8% Bis–Tris; Invitrogen, USA) and then transferred to PVDF membrane (Hybond-ECL, GE Healthcare, UK). Membranes were blocked in 5% dried non-fat milk in phosphate-buffered saline buffer, and then probed with the primary antibodies: anti mouse~~

~~α-DG IIH6 (Millipore UK,cat,05-593) anti-mouse β-DG (Vector Labs,UK), anti-mouse V5 (Invitrogen, USA) at room temperature for 1 hour. After washing, they were incubated with the appropriate biotinylated secondary antibody: anti IgM (Dako,Denmark), anti mouse IgG (GE Healthcare, UK) followed by a HRP-streptavidin (Dako, Denmark). All the incubations were for 1 hour at room temperature. After washing, membranes were visualized using chemiluminescence (ECL+Plus,GE Healthcare, UK). For the laminin overlay assay, PVDF membranes were blocked for 1 hour in laminin binding buffer (LBB: 10 mM triethanolamine, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, pH 7.6) containing 5% non-fat dry milk followed by incubation of mouse Engelbreth-Holm-Swarm laminin (Invitrogen,USA) overnight at 4°C in laminin-binding buffer. Membranes were washed and incubated with anti rabbit laminin (Sigma, USA) followed by HRP-anti rabbit IgG (Jackson ImmunoResearch, USA). Blots were visualized using chemiluminescence (ECL+Plus, GE Healthcare,UK).~~

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~~Results~~

Clinical features

The main clinical features of the six patients are summarized in the Table 1. Patients a~~we~~re classified into three groups according to the mutation: the first group comprised two patients homozygous for the c.919T>A mutation, and the second group of three patients who a~~we~~re compound heterozygous for the c.919T>A and the common c.826C>A mutations. The third group consisted of one patient compound heterozygous for c.919T>A and a rare c.1012C>T mutation. All patients were unrelated and were of Northern European origin.

Group 1: patients 1 and 2 homozygous for c.919T>A mutation showed the most severe phenotype consistent with Muscle-Eye-Brain disease. Both presented at birth with a muscle weakness, hypotonia and respiratory distress. Motor development was severely delayed, they never walked and the maximal motor ability acquired was sitting with support in patient 1. They developed progressive respiratory insufficiency requiring invasive ventilation, and both had recurrent respiratory infections. Patient 2, developed left ventricle hypertrophy, and both patients died of respiratory complications at age of 7 (patient 2) and 14 (patient 1) years, respectively. Both had a profound mental retardation with severe CNS involvement on brain MRI, including lissencephaly type II (cobblestone), polymicrogyria, pons and vermis hypoplasia, cerebellar cysts and Dandy-Walker malformation (Fig. 1). Severe bilateral congenital myopia was observed in both patients. Retinal dysplasia with detachment and rarefaction of the pigmented epithelium was also observed in patient 2.

Group 2:~~-~~ patients 4, 5 and 6, compound heterozygous for c.826C>A and c.919T>A, presented with a milder phenotype than group 1, consistent with a LGMD without mental retardation. Time of onset was during childhood, ranging from before the age of 3 for patients 4 and 5 with a DMD-like phenotype to age 7 for patient 6, who had a severe Becker muscular dystrophy (BMD)-like phenotype. Clinical picture at presentation included proximal weakness and walking difficulties. Patients were never able to run and they lost ambulation before the age of 12 years in the two DMD-like patients and at 20 years in the BMD-like patient. All patients developed an early respiratory involvement requiring non-invasive ventilation (BiPAP, bilevel positive air pressure) in the second decade. Patient 6 died of respiratory complications at age 42 years. Tongue hypertrophy was present in all cases and calf hypertrophy in patients 5 and 6. Mild-moderate left ventricle hypertrophy was identified in patients 4 and 6.

Group 3: the patient compound heterozygous for a non-common c.1012C>T mutation (patient 3), showed an intermediate phenotype between groups 1 and 2, compatible with a severe CMD with eye and cerebellar malformation, but no mental retardation. He presented with severe neonatal hypotonia, poor head control and feeding difficulties. He acquired the ability to stand with support but never independent ambulation. He had significant tongue hypertrophy, scoliosis and respiratory involvement requiring BiPAP at age of 14 and died at 16 due to respiratory complications. Myopia, rarefaction of the pigmented epithelium and bilateral retinal detachment were also present in this patient. Brain MRI revealed severe posterior fossa structural defects with pons and cerebellar hypoplasia and frequent cerebellar cysts. Cognitive development was normal.

Muscle pathology

All human muscle biopsies showed a classic dystrophic pattern of variable severity (Figs. 1 and 2).

Group 1: the two patients with the Muscle-Eye-Brain phenotype (patients 1 and 2) presented a severe dystrophic pattern with a significant degree of fibrosis, adipose tissue substitution and atrophy (patient 1 in Fig. 1C).

Group 2: biopsies from these three patients showed more moderate changes and less degree of fibrosis than patients in group 1. Central nuclei, split fibres, round atrophic fibres, hypertrophic fibres were frequent features (patient 5 in Fig. 2C and patient 6 in Fig. 2D).

Group 3: the biopsy from patient 3 displayed the most severe lesions. Performed at the age of three years, only few round atrophic fibres were present scattered within the extensive endomysial connective tissue infiltration and adipose replacement (patient 3 in Fig. 2B).

No rimmed vacuoles, inflammatory infiltrates, or other distinctive structural features were identified in any biopsy. Muscle biopsies were performed in all patients for diagnostic purpose, however a complete workup with α-DG immunostaining could only be done in the three patients with a milder phenotype (patients 4, 5 and 6), revealing a moderate reduction in all these cases. Dystrophin and utrophin were normal in all cases and laminin α2 showed only an irregular immunolabelling in patient 1 (fig. 1F).

~~**Molecular genetic studies**~~

~~The c.919T>A [p.(Tyr307Asn)] mutation was identified in 6 patients (table 1), two in homozygous and four in compound heterozygous, of which three with the common c.826C>A [p.(Leu276Ile)] mutation. The remaining compound heterozygous patient carries a very rare c.1012C>T [p.(Arg404Cys)] mutation in *FKRP* gene.~~

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Mouse model

Haematoxylin and eosin stained sections of the quadriceps and the tibialis anterior muscles of Fkrp^{Tyr307Asn} mice were indistinguisable from wild-type (Fig. 3A and 3C), with no evidence of an abnormal variation in fibre size, presence of internal nuclei, vacuolation, necrosis, ragged red fibers or inflammation. Histology of heart did not show any abnormalities. Western blotting with the IIH6 antibody, showed no difference in glycosylation in the quadriceps and tibialis anterior between mutant and wild-type controls (Fig. 3C).

Discussion

More than 150 mutations have been described in the *FKRP* gene (20), but the c.826C>A mutation is by far the most common mutation in Northern Europe with an allele frequency ranging between 1/116 and 1/600 (15,17-19). This mutation is associated with a relatively mild phenotype, and accounts for a great majority of patients with LGMD2I (18,19,25,26). A much broader range of clinical phenotypes, including Walker-Warburg Syndrome and Muscle-Eye-Brain Disease, and diverse types of CMDs, have been associated with other mutations (11,16,22,23,27,28). Regardless of this genetic variability, few reports have been published focusing on the rare *FKRP* variants.

Here we review ~~In this study, we present~~ a cohort of six patients carrying the c.919T>A (Tyr307Asn) *FKRP* mutation, an infrequent mutation affecting a highly conserved Tyrosine residue at position 307. The mutation seems to confer a very severe phenotype, because patients homozygous for this variant and the patients with this variant and another rare mutation predicted to disrupt FKRP (c.1012C>T), all presented with severe multisystem disease, never acquired indepndent ambulation ~~-~~and died before the end of their second decade of life. Apart from the six cases we present here, the c.919T>A variant has only been reported previously in two other patients, who were compound heterozygous for the mutation in combination with the common c.826C>A mutation. They were included in two large reviews of LGMD2I carried out in Germany (19) and France (29) and were reported to have a BMD-like (19) and DMD-like (29) course, very similar to the three heterozygous patients of our study. References to a third heterozygous patient, also with the common mutation was published in another review, but neither the phenotype nor any additional clinical information were discussed (30).

The first patient homozygous for the c.919T>A (Tyr307Asn) mutation (patient 2) was reported in 2004 (23) and presented with a severe CMD/ MEB phenotype. Since then, only 6 patients carrying this variant in a heterozygous state have been described (17,19, 24,26,29), and although they were consistent with the hypothesis that this mutation confers a severe phenotype, further verification of this was required. The new homozygous patient for the c.919T>A mutation reported here (patient 1) displayed a similar severe phenotype as found in patient 2. The clinical picture of both patients was characterized by extreme weakness, hypotonia, and motor delay with a congenital onset and profound mental retardation. In addition to severe eye abnormalities, lissencephaly, polymicrogyria, cerebellar malformations with cysts and pons hypoplasia in posterior fossa on brain MRI were also present. CNS involvement is often associated with non-common *FKRP* mutations and a congenital onset. The clinical phenotype described here is consistent with a Muscle-Eye-Brain Disease phenotype, which has also been described in other patients with a variety of non-common mutations in *FKRP* (11,16,22,23). The new homozygous case described here, in addition to the first case previously reported, reinforces the hypothesis that this mutation is associated with CNS structural involvement and the resulting severe clinical phenotype.

Regarding the severity of the mutation, it is also noteworthy that the six patients (100%), required ventilatory support before the age of twenty, and three out the six (50%), including the two that were compound heterozygous for the mutation, died from respiratory complications during the progression of the disease. All these data are consistent with previously published smaller series, showing that while in homozygous state the common mutation seems to be associated with a relatively benign and homogenous course, in heterozygous patients the severity and clinical course of the disease depend mainly on the second mutation (18,31-33).

The c.1012C>T mutation found at compound heterozygous state in this study has never been reported in other patients, but seems also to confer a severe phenotype, when comparing with the LGMD phenotype of the other three compound heterozygous patients with the common c.826C>A mutation (patients 4-6). The muscle biopsy from this patient showed the most severe changes of all patients, the patient had brain and eye involvement and died at age 16 years. Taken together, the findings presented here strongly support the notion that the c.1012C>T mutation results in a particularly severe phenotype.

Although clinical severity in human patients affected by pathogenic variants in *FKRP* appears to relate to the specific mutation, the introduction of the $\mathrm{Fkrp}^{\mathrm{Tyr307Asn}}$ mutation in mice surprisingly failed to generate a disease phenotype (25 and present report). However, mice homozygous for the missense P448L mutation do display a muscle pathology demonstrating that the mouse is a useful model but only for specific mutations (34). Our report of the absence of a phenotype after introduction of the $\mathrm{Fkrp}^{\mathrm{Tyr307Asn}}$ mutation strongly suggests that differences in protein folding/processing must exist between human and mouse Fkrp, which are differentially altered by specific mutations. Interestingly, this has also been observed with respect to a mutation in the sarcoglycan gene, which in human patients is commonly associated with LGMD, but that fails to give rise to a disease phenotype in the mouse (35, 36). A knock-down of Fkrp expression levels is sufficient to give rise to a Muscle-

Eye-Brain phenotype in the mouse as we previously reported (25, 37) and it has been proposed that a wide range of mouse models may be generated by combining a knock-down and missense mutation approach (34). Interestingly, central nervous system involvement was reported in a mouse model, which had both a knock-down and P448L missense mutation (38) whereas in the presence of just the missense mutation, CNS involvement was eliminated and the severity of the muscular dystrophy reduced (34).

Overall, our observations strongly suggest that the c.919T>A mutation in *FKRP* confers a severe disease phenotype in humans but that there appears to be a huge discepancy between pathogenicity of this variant in humans vs. mice, which highlights the need for developing appropriate animal models of dystroglycanopathies that mimick the disease course in humans.

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Figure Legends

Figure 1: Saggital (A) and horizontal (C) brain MRI of patient 1 at age 1 year, showing lissencephaly type II (cobblestone), polymicrogyria, pons and vermis hypoplasia, cerebellar cysts and Dandy-Walker malformation. Muscle biopsy from the same patient at age 1 year shows (B) severe dystrophic features with hypercontracted fibres, cell necrosis, increased connective tissue and wide fiber size variability. Laminin α2 staining in the patient (D) shows irregular immunolabelling.

Figure 2: Muscle histology of a healthy 1.5-year-old child (A), patient 3 (B), patient 5 (C) and patient 6 (D) stained by haematoxylin and eosin and their corresponding stains for α-dystroglycan glycosylation using the IIH6-antibody (E-H).

Figure 3: Haematoxylin and Eosin stained sections of tibialis anterior (TA) of wild-type (A) and Fkrp^{Tyr307Asn} (B) mice showing normal histology with no abnormal variation in fibre size and no evidence of regeneration or necrosis. Western blot of tibialis anterior (TA) and quadriceps (Q) muscle from wild-type (Lanes 1 and 2) and 4 individual Fkrp^{Tyr307Asn} mice (lanes 3-10). The Coomassie stained running gel confirms equal loading of the lanes.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age / Gender	12 years/M	Died at 7 years/M	Died at 16years /M	29 years/M	22 years/F	Died at 43 years/M
Origin	Danish	German	English	German	Danish	Danish
***FKRP* Mutation**	c.919T>A / c.919T>A homozygous	c.919T>A / c.919T>A homozygous	c.919T>A /c.1012C>T heterozygous	c.919T>A / c826C>A heterozygous	c.919T>A / c826C>A heterozygous	c.919T>A / c826C>A heterozygous
Phenotypic Classification	MEB	MEB	CMD-CRB*	LGMD-no MR (DMD-like)	LGMD-no MR (DMD-like)	LGMD-no MR (BMD-like)
Age of onset	Neonatal	Neonatal	1.5 months	1.5 years	3 years	7 years
Symptoms at onset	Hypotonia, respiratory distress	Hypotonia, respiratory distress	Hypotonia, poor head control	walking difficulties	walking difficulties	walking difficulties
Maximum motor ability	Sitting with support	Never head control or sit	Standing with support	Walking, never ran	Walking, never ran	Walking, never ran
Loss of ambulation	_	_	_	12 years	8 years	20 years
Muscle hypertrophy	NR	NR	tongue	tongue, thighs	tongue, calves	tongue, calves
Joint contractures	NR	Achilles, hips	scoliosis	Achilles, hips	No	Achilles, elbow, scoliosis
Respiratory involvement	Respirator	Recurrent infections	BiPAP (14 years)	BiPAP (16 years)	BiPAP (12 years)	BiPAP-respirator (32 years)
Cardiac involvement	NR	LVH	NR	DCM	Normal	LVH mild
Eye involvement						
Myopia	Severe	Severe	Severe	No	No	No
Retinal abnormalities	No	Detachment, RPE	Detachment, RPE	No	No	No
Mental retardation	Yes	Yes	No	No	No	No
Brain MRI	Yes	Yes	Yes	Yes	ND	ND
Cortical malformation	Polymicrogyria	Pachygyria, lissencephaly	No	No	_	_
Cerebellar malformations	Hypoplasia, cysts	Vermis hypoplasia, cysts	Dysplasia-Hypoplasia, cysts	No	_	_
Others	Dandy Walker	Pons hypoplasia, Dandy Walker	Pons hypoplasia	No	_	_
Average CK levels (U/l)	4000	3000-4000	2000-3000	1700-3300	1500	2000
Muscle biopsy (location / age)	quadriceps / 1 year	NR / 1 year	quadriceps / 3 years	NR / NR	Tibial ant. / 13 years	Tibial ant. / 35 years
Muscle atrophy / fibrosis	Dystrophic +++ / +++	Dystrophic +++ / +++	Dystrophic ++++ / ++++	Dystrophic ++ / ++	Dystrophic ++ / ++	Dystrophic ++ / ++
Alpha-dystroglycan (IHC)	ND	ND	ND	Reduced	Reduced	Reduced
Reference	Not reported	[17]	[19]	[14]	[18, 21]	[18, 21]

Table 1: **Clinical features and laboratory findings in 6 patients homozygous or compound heterozygous for the c.919T>A variant in the fukutin-related protein gene.** ND= not done; NR= not reported; DCM= dilated cardiomyopathy; LVH= left ventricular hypertrophy; IHC= immunohistochemistry; + mild, ++ moderate, +++ severe, ++++ end stage

Figure 1

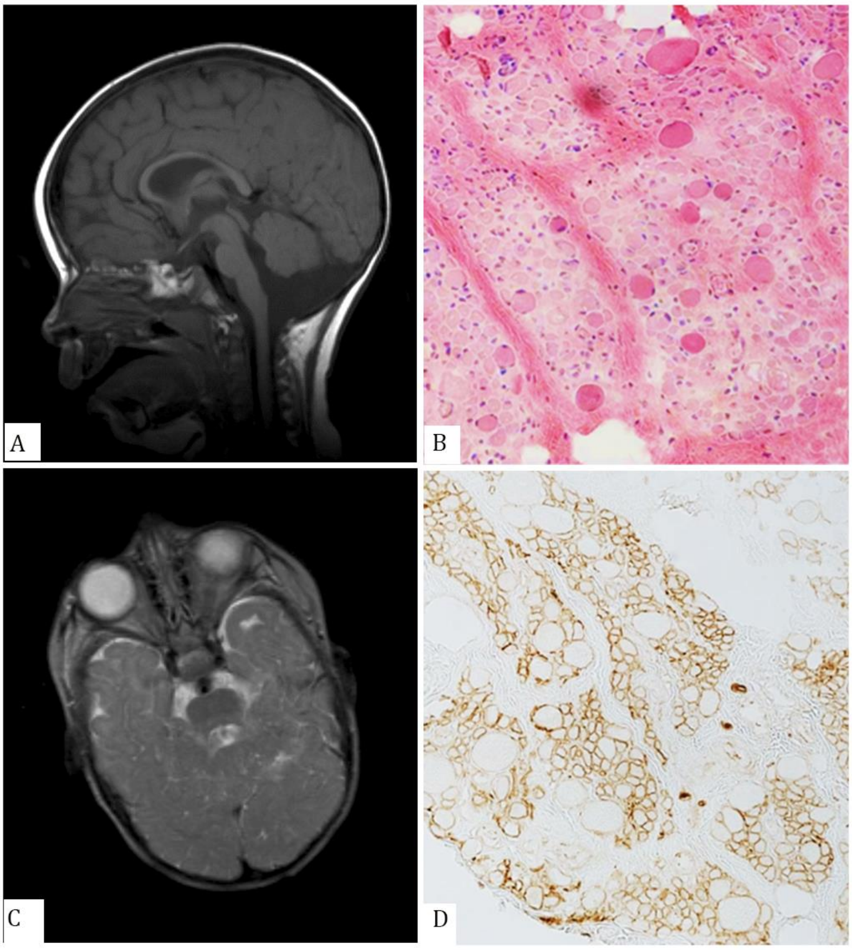


Figure 2

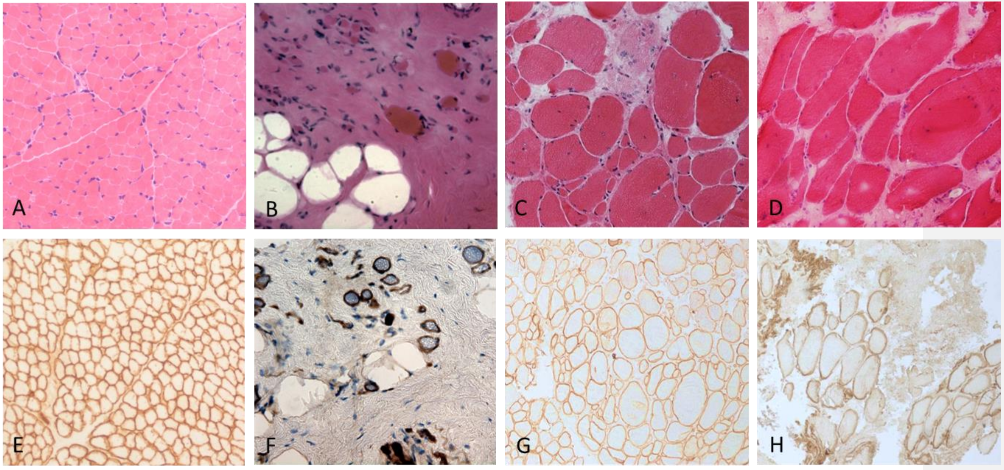
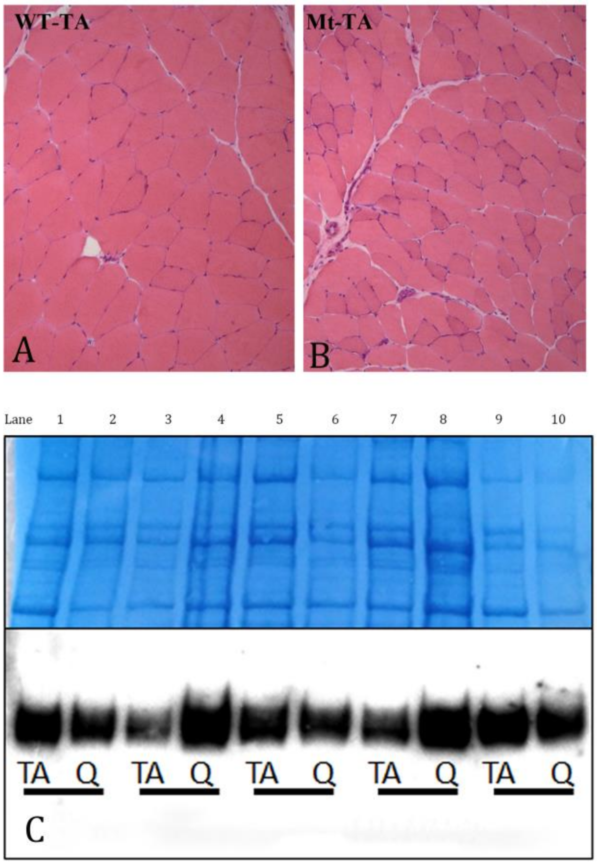


Figure 3



John M. Lee, MD, PhD
Editor-in-Chief
Journal of Neuropathology and Experimental Neurology

Dear Dr. Lee

Thank you for a helpful review of our manuscript entitled "Phenotypic spectrum of α-dystroglycanopathies associated with the c.919T>A variant in the FKRP gene in humans and mice", ID: JNEN 20-103.

We have now submitted the revised article. We appreciate the constructive and helpful comments and suggestions from the reviewers and have revised the manuscript according to their suggestions. We think the revision has improved the manuscript. As requested, the paper has been reformatted to a review paper.

In the following, we provide a point-by-point response to the reviewers and indicate where changes have been made in the revised manuscript. A clean copy and a copy with tracked changes in Word of the revised manuscript have been uploaded.

We hope that the paper is now acceptable for publication in Journal of Neuropathology and Experimental Neurology.

Yours sincerely,

John Vissing, MD

Response to reviewer Comments:

Reviewer #1: The authors present a thoroughly conducted study dealing with the spectrum of clinical phenotypes in human LGMD due to the rare homozygous or compound heterozygous c.919T>A mutation in the fukutin-related protein gene. The results are compared with a murine model likewise carrying the Tyr307ASN mutation as knock-in mutation.
The study is of clinical importance. Methods describe the human phenotypes and the microphotographs are of high quality and exhibit all important features. Finally, the discussion highlights all important aspects regarding human phenotypes.

However, there is some major aspect as well as some minor aspects which should be explained or included.

Major aspect:
The comparison with the murine model is not really satisfying. The model has been described thoroughly for the first time in 2009 (reference #25). At this time skeletal muscles as well as the brain has been investigated comprehensively. Thus, at this time, there seemed to be no further information. Since 50 % of patients which had been included in this study suffered from cardiac complication it would have been nice to investigate heart muscle of mice, e.g. with regard left ventricular hypertrophy. This should be included.

Response: The paper has now been reformatted to a review paper as also requested by the editors and reviewer # 2. We think the concern of the reviewer has been met by this revision. For the cardiac involvement in the mice, we did collect material, but the histology showed no abnormalities in adult mice, i.e. no evidence of any necrosis or fibrosis, so we did not investigate this aspect further. This has been stated in the revised manuscript (page 11).

Minor aspects:
In the section of material & methods, the mouse strain(s) used to generate this model should be specified.

Response: The methodology used to generate this mouse models has been described in the revised manuscript as requested (page 7).

In the paragraph of "Mouse muscle processing" not only adult mice but a precise age should be included.

Response: We have now included the precise age of the mice (20 weeks) in the revised paper (page 7).

In addition, there are some minor aspects which should be included to improve reading of the manuscript.
In the abstract section as well as in the last paragraph of the discussion section there is "discepancy" instead of "discrepancy".

Response: Thank you for picking up this typo which has been corrected.

Reviewer #2: This is an interesting clinical and pathological study of muscle disorders, but from pathological point of view, authors' description may not be sufficient. Since five out of six cases were already reported, one homozygous case is the new report, but only confirms the previously reported cases. Moreover, it is not clear how mouse model is genetically confirmed for knock in.

Response: *The paper has now been reformatted to a review paper as also requested by the editors. We think the concern of the reviewer may have been met by this revision. For the genetic confirmation of the knock-in, this has been described in reference 25 of the paper, but we have inserted a brief description, which reads; "Genotyping of newborn mice was performed on gDNA prepared from tail tissue.*[25] *Genotyping was performed by PCR and sequencing of the Fkrp gene was used to confirm the point mutation (c.919 T > A). Homozygous wild-type, homozygous Tyr307Asn missense mutants or heterozygous mice were identified using the Vector NTi advance10 software (Invitrogen).*

Major focus of this report is clinical phenotype and may not be suitable for JNEN readers. Review format may be more suitable for type of publication.

Response: We agree and have reformatted the revised manuscript to a review paper.

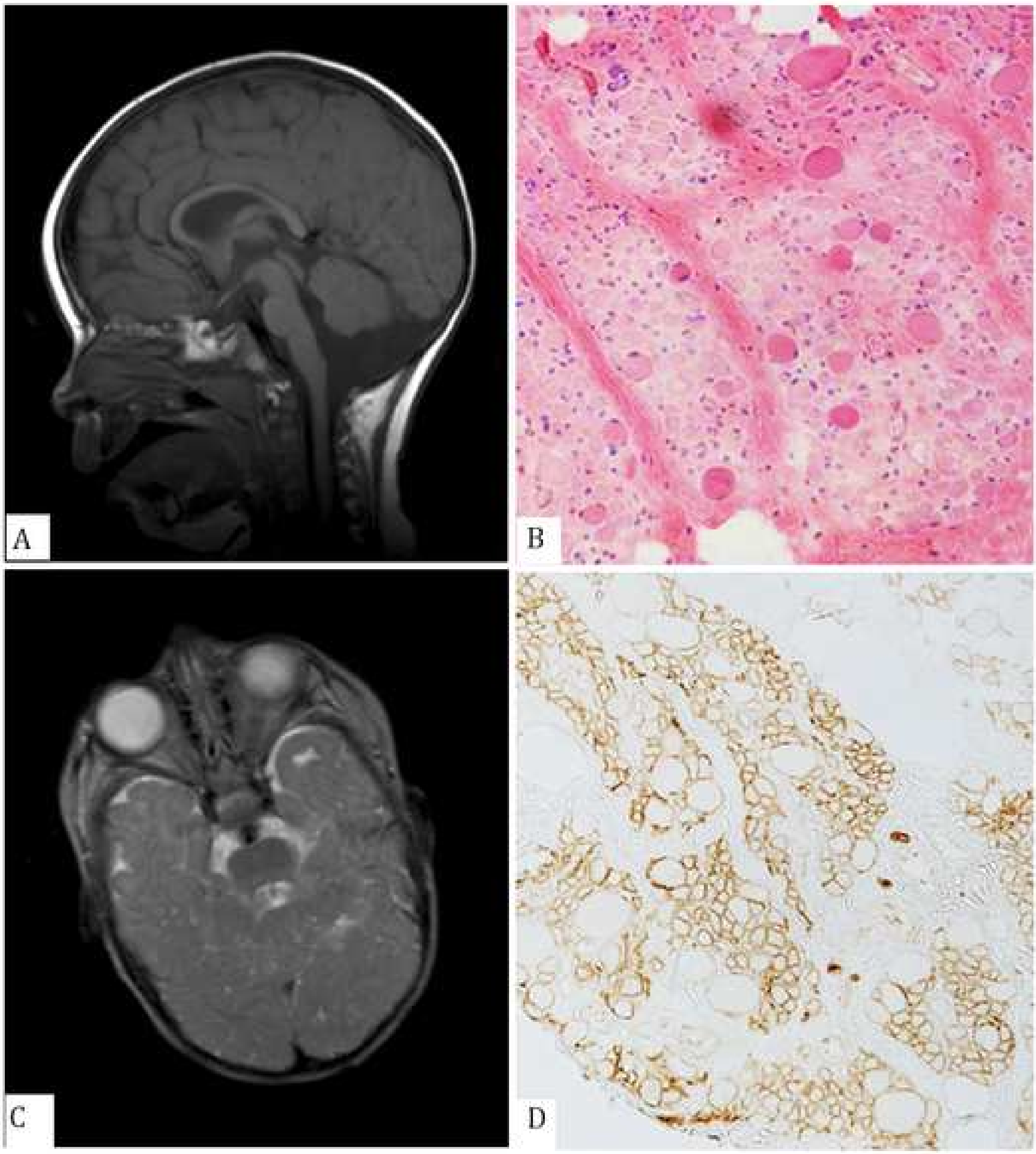
Editorial Reviewer:

1. Based on the suggestion of Reviewer #1, I agree that this may be better suited as a Review Article including both pathology (human and mouse) as well as clinical phenotype. Please resubmit the manuscript as a Review Article rather than an Original Article.

Response: We agree and have reformatted the revised manuscript to a review paper.

2. Please provide the figures as separate, individual files.

Response: Done.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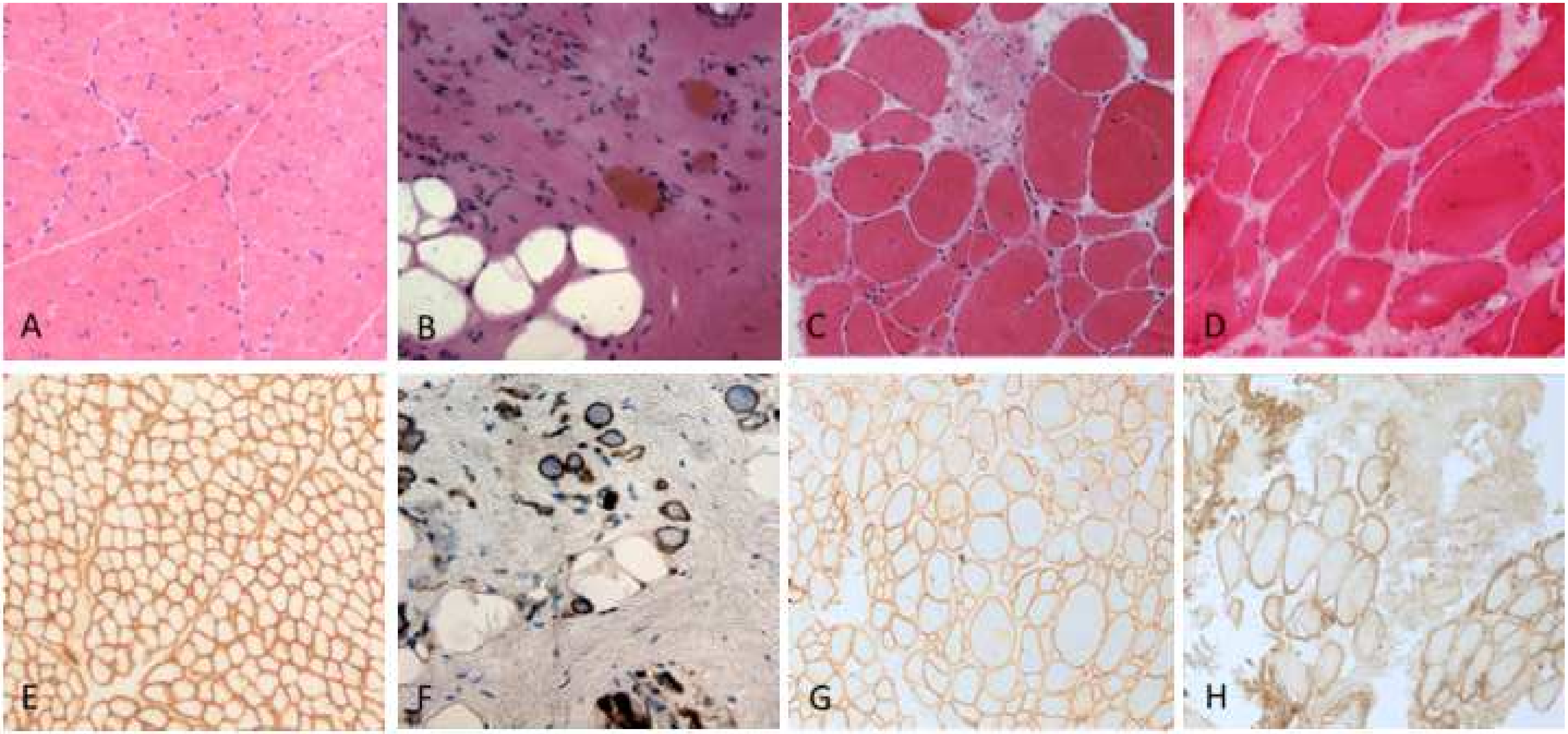
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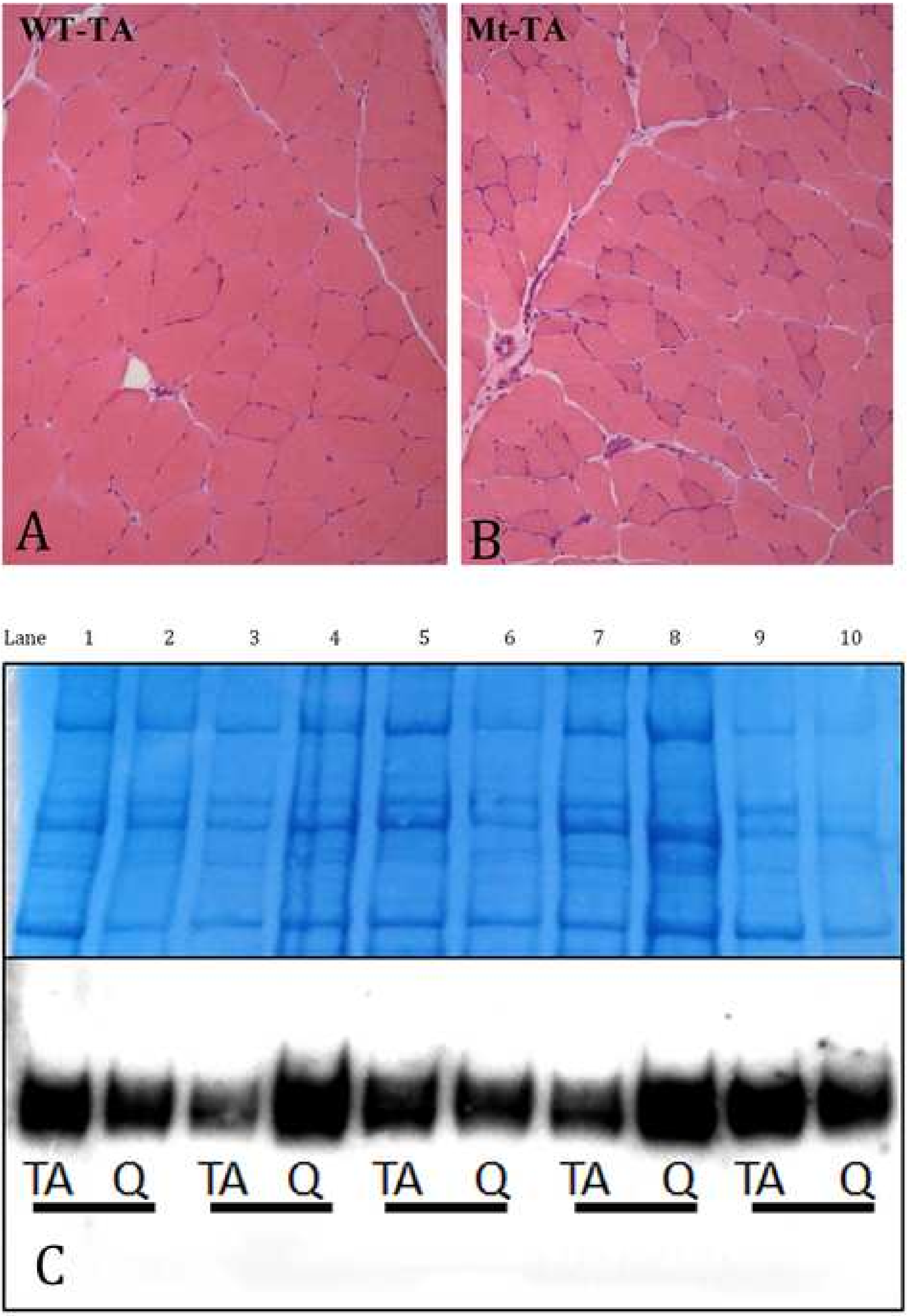
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