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MUTATION IN BRIEF

Dysferlin Mutations in LGMD2B, Miyoshi Myopathy, and Atypical Dysferlinopathies

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***DYSF* encoding dysferlin is mutated in Miyoshi myopathy and Limb-Girdle Muscular Dystrophy type 2B, the two main phenotypes recognized in dysferlinopathies. Dysferlin deficiency in muscle is the most relevant feature for the diagnosis of dysferlinopathy and prompts the search for mutations in *DYSF*. *DYSF*, located on chromosome 2p13, contains 55 coding exons and spans 150 kb of genomic DNA. We performed a genomic analysis of the *DYSF* coding sequence in 34 unrelated patients from various ethnic origins. All patients showed an absence or drastic decrease of dysferlin expression in muscle. A primary screening of *DYSF* using SSCP or dHPLC of PCR products of each of 55 exons of the gene was followed by sequencing whenever a sequence variation was detected. All together, 54 sequence variations were identified in *DYSF*, 50 of which predicting either a truncated protein or one amino-acid substitution and most of them (34 out of 54) being novel. In 23 patients, we identified two pathogenic mutations, while only one was identified in 11 patients. These mutations were widely spread in the coding sequence of the gene without any mutational "hotspot."** © 2005 Wiley-Liss, Inc.

KEY WORDS: *DYSF*; dysferlin; dysferlinopathies; LGMD2B; Miyoshi myopathy

INTRODUCTION

The term “dysferlinopathies” is an emerging concept which pertains to a group of autosomal recessive muscular dystrophies caused by mutations in the dysferlin gene (*DYSF, MIM*603009*) (Bashir et al., 1998; Liu et al., 1998).

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In such conditions, dysferlin, the protein encoded by *DYSF*, is deficient in the sarcolemma as demonstrated in muscle sections analyzed by immunocytochemistry. To date, reduced or absent expressions of dysferlin are the most relevant features leading to the diagnosis of dysferlinopathy and prompt molecular geneticists to search for mutations at the genomic or transcriptional level in *DYSF* (Anderson et al., 1999). This genetic screening is of particular interest for the accuracy of diagnoses and therefore for more reliable genetic counseling and appropriate medical care. Dysferlinopathies can manifest either as limb-girdle muscular dystrophy type 2B (LGMD2B), or distal myopathy (Miyoshi-type distal myopathy, MM). Moreover, the same mutations in *DYSF* can lead to both phenotypes within a single family (Illarioshkin et al., 2000; Weiler et al., 1999) or overlapping phenotypes in a single patient. Such clinical heterogeneity has already been reported in other human genetic disorders and is of particular importance in muscular dystrophies. The *DYSF* gene is located on chromosome 2p13, contains 55 coding exons and spans 150 kb of genomic DNA. The transcript is 6.3 kb large and is mainly expressed in skeletal muscle. All mutations in the *DYSF* gene found to date are point mutations or small deletions or insertions distributed all over the entire coding sequence. No hotspot has been identified, and missense as well as nonsense or frameshift mutations have been reported (Aoki et al., 2001; Takahashi et al., 2003). Consequently, mutation analysis of *DYSF* remains a time-consuming challenging task accounting for the paucity of large series. A majority of mutations have been identified in Japanese patients, where Miyoshi myopathy was first reported. However, recent reports indicate that both phenotypes, Miyoshi myopathy and LGMD2B, can be found everywhere in the world (Argov et al., 2000; Linssen et al., 1997).

Here we report a series of 54 sequence variations in the *DYSF* gene, 34 of which are novel, in a large group of patients with various ethnic origins. Our study also highlights the large number of non-pathogenic polymorphisms disseminated along the *DYSF* gene, which could, in some instances, lead to misdiagnosis in patients in whom the levels of expression of dysferlin have not been checked.

PATIENTS AND METHODS

Patients

All the patients included in the study were examined by neurologists from the Institut de Myologie, Hôpital la Pitié Salpêtrière, Paris and the Service des maladies neuromusculaires, Hôpital Timone, Marseille, France. Detailed clinical information was retrieved retrospectively from patients' medical records and then compiled. The inclusion criteria prior to gene analysis were as follows: i) clinical phenotype consistent with LGMD or distal myopathy, ii) loss or strong reduction of dysferlin expression evidenced by Western-blotting and/or immunohistochemistry on muscle biopsy, iii) in case no biopsy was available, confirmed dysferlin deficiency in the sibship, and/or linkage to chromosome 2p13. At least 2 criteria, including i) were needed prior to enrollment of patients. A peripheral blood sample was taken after an appropriate informed consent following rules set by local IRB.

Protein analysis

Immunohistochemistry and multiplex Western-blotting on muscle biopsy were carried out as previously described (Anderson and Davison, 1999; Matsuda et al., 1999), using antibodies to dysferlin (NCL-Hamlet antibody, Novocastra Newcastle upon Tyne, UK).

Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes by using standard protocols (Sambrook J, 1989). Briefly, 20 ng of total genomic DNA was used as a template for PCR amplification of each of the 55 exons of the *DYSF* gene. For each reaction, specific primers located in the flanking introns were used. PCR conditions and primers were previously described for exons 18 to 55 (available at the Neurology's Web site: http://www.neurology.org) (Aoki et al., 2001) and novel primer pairs were designed for amplifying exons 1 to 17. A combination of "Single Strand Conformation Polymorphism" (SSCP) analysis and dHPLC (WAVE - Transgenomic®) was used to detect potential variations in each of the PCR amplified fragments. Each sample behaving with an abnormal migration pattern or whose peak pattern was suggestive of a sequence variation was further sequenced, by using an automated sequencer ABI310 (Applied Biosystems) using standard protocols. Both strands were sequenced and analyzed by using Sequencher® software. The specific procedures for WAVE and ABI

sequencing were performed according to the manufacturers' instructions. Mutation numbering is based on the *DYSF* cDNA sequence (Genbank access n° NM_003494.2) using a 'c.' symbol before the sequence position. All positions refer the A at the first ATG as being the nt number 1. The SIFT program (Ng and Henikoff, 2001) was used to predict the consequences of the amino acid substitutions on the protein function and is available at: http://blocks.fhcrc.org/sift/SIFT.html.

RESULTS

Mutational analysis of the DYSF gene

We performed a genomic analysis of the *DYSF* coding sequence in 34 unrelated patients. Among these patients, 33 were included in the study on the basis of a marked or total loss of dysferlin expression in muscle, demonstrated either by Western-blotting or immunohistochemistry on muscle sections. In one patient (dysLE), the diagnosis of dysferlinopathy was less solid, since he showed a mild reduction of dysferlin expression on Western-blot along with a significant increase of Creatine Kinases in the serum, and remained asymptomatic at age 11. This case is discussed below.

All together, 54 sequence variations were identified in *DYSF*, 50 of which predicting either a truncated protein or one amino-acid substitution. All these mutations were ordered according to their type, position or the associated phenotype and are reported in Table 1 and schematically represented according to their position along the protein in Fig. 1. Eight patients carried a single homozygous mutation; 15 patients had two compound heterozygous mutations. In seven patients, only one heterozygous mutation could be identified while three patients presented more than two sequence variations. Briefly, 13 mutations are frameshift, 13 are nonsense, 17 are missense, 7 are substitutions or small deletions located at splice sites, 1 is an intronic one-base-pair substitution and 3 are inframe insertions. The pathogenic effect of these mutant alleles will be discussed later.

Most mutations (34 out of 54) identified in this study are novel. Five mutations were identified in more than one patient and are likely to represent recurrent mutations rather than founder mutations since they were found in patients from different ethnic origins. Fourteen mutations are homozygous and forty are heterozygous. Three mutations were present as either a homozygous or a heterozygous change. All consanguineous patients carried homozygous mutations. Two patients of French descent carried homozygous mutations although not born to consanguineous parents. The mutations were widely distributed in the coding sequence of the gene without any mutational "hotspot".

In seven patients, we identified only one heterozygous mutation in the *DYSF* gene despite an extensive screening of the gene by SSCP or dHPLC. In three additional patients (dysCAN, dysVA, dysBR), the second mutation is likely to be non-pathogenic; and in one patient (dysFI,), the second mutation is an intronic variation for which a potential pathogenic effect remains to be confirmed. Therefore, in eleven patients as a whole, our strategy allowed identifying only one heterozygous mutation that is certainly pathogenic and causal for the disease. Unexpectedly, additional mutations were identified in addition to the 2 required mutations in autosomal recessive disorders. Indeed, 3 patients (dysAM, dysBEN and dysJOI) carried a 3rd mutation. Finally, some of the identified mutations are suspected to be causal while some others might be polymorphisms. The consequences of the amino acid substitutions were evaluated using the SIFT program (Table 2).

Clinical analysis

Among the 34 patients analyzed in this study, 21 are male and 13 female. Ethnic or geographical origins are various: 21 patients are French by descent, nine originated from North Africa (six from Algeria, two from Morocco, and one from Tunisia), one patient is Iranian, one patient is African (Ivory Coast), one is from Belgium and one patient is North-American. Nine patients are consanguineous (parents are first-degree cousins).

Eleven patients had a distal phenotype of Miyoshi myopathy and six patients presented with a LGMD2B phenotype as reported in previous studies (Linssen et al., 1997; Mahjneh et al., 2001). In two patients, clinical data were not available. Three patients had a clinical presentation that first suggested a metabolic myopathy and were thus classified as « pseudo-metabolic » muscular dystrophy. These patients had painful calf swelling, whereas weakness or atrophy was absent at the beginning of the clinical course. They experienced recurrent episodes of rhabdomyolysis with massive elevation of CKs and dark urines following minimal physical exercises. For nine additional patients, it was not possible to distinguish between a distal phenotype of MM and a limb-girdle phenotype, even when they were examined at the onset. Indeed, these patients presented with weakness of

pelvifemoral muscles along with wasting of calf muscles. Therefore, we classified these atypical patients in a distinct « proximodistal » phenotypic group. A detailed clinical description of the atypical phenotypes will be provided elsewhere (Nguyen et al, in preparation). Finally, three patients, classified as "isolated hyperCKaemia", had no weakness or atrophy at the time of the study. In those cases, muscle biopsy performed because of very high serum CK activity allowed diagnosing dysferlinopathy.

DISCUSSION

All patients except one (dysLE) in whom a mutation analysis of the *DYSF* gene was carried out most probably had primary dysferlinopathy, based on dysferlin deficiency in muscle. A drastic or complete protein deficiency was the required criterion to include the patients in the analysis. We have identified 54 variations in the *DYSF* gene, most of them being novel. The first seven patients presented in Table 1 are consanguineous and carry homozygous frameshift mutations. We have no doubt that these mutations are causal for the phenotype since they predict a truncated protein and probably result in a complete loss of function of the protein. Within this group of "null-mutants" patients, the phenotype is heterogeneous (Miyoshi myopathy, LGMD2B or "pseudometabolic"). This suggests the absence of relationship between the type of mutation and the phenotype, as previously put forward (Argov et al., 2000). Five additional patients carry compound heterozygous truncating mutations (nonsense, frameshift and/or splice site mutations), including four mutations previously reported and related to LGMD2B or MM (Tagawa et al., 2003) (Leiden mutation database available at: http://www.dmd.nl/). One non-consanguineous French patient carries a homozygous nonsense mutation (c.3832C>T). This mutation is suspected to be recurrent in the French population since it was identified in two other unrelated French patients. Alternatively, a large deletion may be present in the second allele and not detected by our screening. Further studies will be required to testthis hypothesis. Therefore, in 13 patients in the whole group, we identified two truncating mutations certainly responsible for the disease. Eight mutations affect acceptor or donor consensus splicing sites. These variations lie either in introns or in exons and are likely to produce aberrantly spliced transcripts. Meanwhile, in order to confirm their pathogenic consequences on the transcript, these mutations remain to be further investigated by RT-PCR from muscle tissues that were not available at the time of the study. However, various splicing mutations have been reported so far in the *DYSF* gene and were demonstrated to be pathogenic elsewhere (McNally et al., 2000; Saito et al., 2002).

Furthermore, six patients carried compound heterozygous mutations including at least one missense mutation. Several arguments suggest these mutations have a deleterious effect: first, each of them was found in only one patient, but was never present in any other chromosome explored in this study, thus they cannot be considered as neutral polymorphisms; second, these mutations are located in a highly conserved domain during inter-species evolution and among the FERLIN group of proteins (Britton et al., 2000); finally, some of these mutations have been reported as being causal (Ueyama et al., 2002). Ninety additional DNAs (180 chromosomes) from individuals of various ethnic origins, served as controls and none of them harbored any of the 14 different missense mutations reported in this study.

In contrast, one sequence variation reported in this study, an in-frame insertion 3191_3196dupGAGGCG, is likely to be a common polymorphism and has been reported as such in the literature (Kawabe et al., 2004). In three patients, we have identified more than two defects in the *DYSF* gene. Patient dysJOI carries a c.3992G>T (p.R1331L) missense mutation found as a third variation although he is compound heterozygous for a splicing and a frameshift mutations, both predicted to be truncating. c.3992G>T is expected to be a polymorphism as previously discussed since the arginine residue, present in the human protein at this position, has diverged among mammals where it can also be a lysine. Likewise, c.3992G>T has recently been reported as a polymorphism (Cagliani et al., 2003). Additionally, *in silico* predictions were consistent with our analyses, providing additional arguments for the pathogenic or polymorphic nature of the mutants identified.

Patient dysAM, born to a consanguineous union, carries two different homozygous missense mutations and both could have pathogenic effects on the dysferlin protein. The first one, c.1663C>T (p.R555W) is novel and has been found recurrently in two other French patients in this series. Nevertheless, it changes an arginine residue that is highly conserved during evolution from *fer-1* to other ferlin proteins. This mutation is associated with a pathogenic truncating mutation in the patient dysGR with no other variation in the entire coding sequence, and with another missense mutation in dysNI. The second missense mutation in dysAM, c.4052A>G (p.N1351S), is novel and was found only once in our series. Meanwhile, although no formal argument can be raised to definitely conclude on the pathogenic effect of this variation, it is predicted to be a polymorphism by our *in silico* analysis. However, further

functional analyses of the missense mutations will be necessary to draw firm conclusions regarding their disease-causing effect.

Finally, in patient dysBEN, who also carries three homozygous mutations, only the nonsense mutation is expected to be causal and the missense mutations identified in this patient might be both non-pathogenic polymorphisms although one of them, c.565C>G (p.L189V), was previously described as a pathogenic mutation in LGMD2B (Leiden mutation database). The causality of this mutation in the latter case should be infirmed in the light of our new data.

Consequently, in 23 patients, we identified at least two mutations that are likely pathogenic. For eleven patients, our strategy combining a complete screening of the gene by SSCP or WAVE, and sequencing of the detected mutations, allowed identifying a single heterozygous mutation. The diagnosis of dysferlinopathy was performed based on a dysferlin loss of expression. Several arguments can be raised to explain the absence of a second mutation in the gene: first, the screening approach by SSCP or even dHPLC is not sensitive enough; second, the mutation may lie outside the explored sequences, such as introns or within the promoter region, which were not submitted to an initial screening; third, our strategy does not allow identifying large exon deletions or duplications. Such defects have been reported in *DYSF* but seem to be uncommon as compared to point mutations (Liu et al, 1998). By contrast, since all patients show an absence or strong reduction of dysferlin expression, changes at the DNA level, particularly missense and intronic variations may have unexpected consequences at the RNA level, including variations previously considered as polymorphisms at the protein level. In patient dysFI, in whom we found one heterozygous recurrent splicing mutation (c.855+1delG), the full set of coding exons was sequenced, but did not allow identifying a second exonic mutation. Meanwhile, we identified a variation located in intron 12 (c.1180+11C>T) possibly associated to the disease, although reported as a polymorphism (Leiden mutation database). In patient dysLE, we carried out the gene analysis although the level of expression of the dysferlin protein was slightly decreased. Indeed, this patient presented with an isolated hyperCKaemia at age 11. This patient might have either a primary dysferlinopathy at a pre-symptomatic stage, or a secondary dysferlin deficiency on muscle biopsy. We identified a homozygous missense mutation but its causality, although likely, cannot be formerly determined. This case illustrates that, currently, the protein studies on muscle biopsy remain necessary for a clear diagnosis of primary dysferlinopathy, before the gene analysis is undertaken (Pogue et al., 2001).

Concerning the mutations found in both our patients and in the literature, this study does not suggest any clear correlation between phenotype and genotype, as already mentioned by others (Illarioshkin et al., 2000; Mahjneh et al., 2001; Nakagawa et al., 2001; Takahashi et al., 2003; Ueyama et al., 2002; Walter et al., 2003; Weiler et al., 1999). For instance, one mutation (c.5594delG) found in two patients of our series (dysVE from Belgium presenting with a Miyoshi myopathy and dysLA from Morocco presenting with a severe proximodistal myopathy) was previously described in two Spanish families presenting, at least one of them, with a particular phenotype of distal anterior compartment myopathy (Illa et al., 2001; Liu et al., 1998). Obviously, this mutation is not related to a particular ethnic group, neither does it correlate with a particular phenotype. In addition, we did not observe that patients with missense mutations presented with a more severe course than those with truncating mutations, as previously suggested (Ueyama et al., 2002). Oppositely, the patient with the more severe course of the disease is homozygous for the recurrent frameshifting mutation c.5594delG (dysLA) whereas the patient having an isolated hyperCKaemia late in adulthood (dysNI) carries compound heterozygous missense mutations (dysNI).

Obviously, many more mutations have to be compiled before genotype/phenotype relationships can emerge. However, other factors and other genes acting as modifiers help explain the clinical heterogeneity in dysferlinopathies (Davis et al., 2000; Illarioshkin et al., 2000; Weiler et al., 1999).

Moreover, although classical Miyoshi myopathy and the LGMD2B phenotype are the most prevalent phenotypes associated to *DYSF* mutations, a spectrum of various phenotypes, ranging from isolated hyperCKaemia, even late in adulthood, to severe proximodistal phenotype have been observed, some of them being specifically reported in the present study. In the future, the number of reported *DYSF* mutations will probably increase substantially, due to the improvement of mutation detection approaches.

In conclusion, regarding the molecular strategy dedicated to the complete exploration of large coding sequences such as the *DYSF* gene, it seems not rational to perform a complete sequencing of the gene when no mutations or only one mutation is primarily observed. Indeed, in the present context of routine analyses available in the vast majority of molecular diagnostics laboratories, gene analysis is expensive and time consuming. And although it is used to confirm the clinical diagnosis, which for dysferlinopathies is primarily based on the protein analysis, it is critical for genetic counseling in affected families. In our laboratory, dHPLC is now used as the only pre-initial

screening procedure to search for mutations in the *DYSF* gene. Such a strategy will undoubtedly facilitate our approach by providing a gain of time and a better sensitivity. In the future, an evaluation of DNA chips and expression microarrays could also help the molecular orientation towards a specific genetic screening in muscular dystrophies in general and dysferlinopathies in particular.

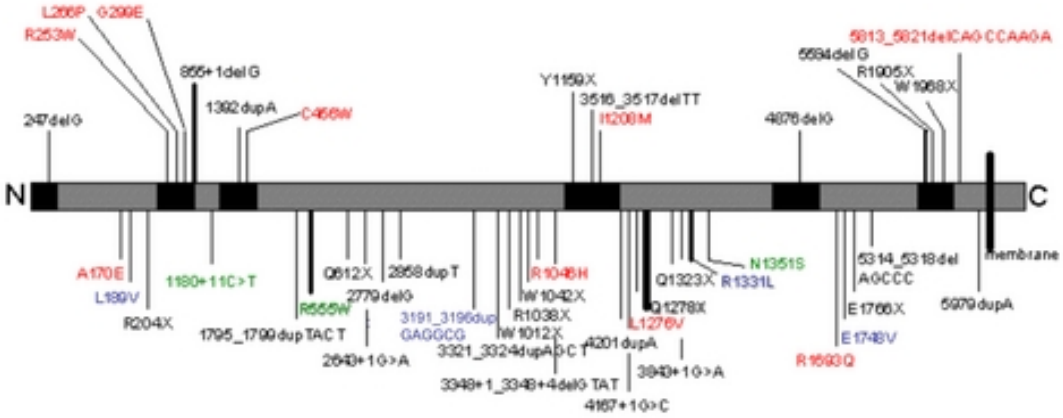

Figure 1. Schematic representation of the mutations identified in this study, along the Dysferlin protein sequence. The locations of the six C2 domains are indicated by painted-over boxes. The vertical lines indicate the position of mutations. Above the protein are indicated mutations inside C2 domains; below the protein are indicated the mutations outside C2 domains. The thick lines represent recurrent mutations. In black, truncating mutations; in red, missense mutations ; in blue, polymorphisms; in green, uncertain variants.

Table 1. Summary of mutations identified in 34 patients affected with dysferlinopathy. The previously reported mutations are italicized while recurrent mutations are indicated in bold. Mutations numbering is based on the *DYSF* cDNA (sequence position 1 is the A at the first ATG codon). The nomenclature sequence uses a 'c.' or 'p' symbol when referring to the cDNA or the protein respectively. LMDD: Leiden Muscular Dystrophy Database at http://www.dmd.nl/ Genbank access n° NM_003494.2

Patients	Geographical origin	Exon	Nucleotide change	Proteic change	Protein domain	Mutational event	State	Reference	Phenotype
dysBE	Tunisia	15	c.1392dupA		C2 d 3	frameshift	homozygous	this study	proximodistal
dysGH	Algeria	29	c.3035G>A	p.W1012X		nonsense	homozygous	this study	LGMD2B
dysAL	Morocco	27	c.2858dupT			frameshift	homozygous	this study	LGMD2B
dysZA	Iran	26	c.2779del G			frameshift	homozygous	this study	Pseudometabolic
dysLA	***Morocco***	***50***	***c.5594delG***		***C2 d 6***	***frameshift***	***homozygous***	***Liu,1998***	***proximodistal***
dysDA	Algeria	39	c.4201dupA			frameshift	homozygous	this study	proximodistal
dysDAR	Algeria	20	c.1795_1799dupTACT			frameshift	homozygous	this study	Miyoshi myopathy
dysFR	***France***	***34***	***c.3832C>T***	***p.Q1278X***		***nonsense***	***homozygous***	***LMDD***	-
dysLE	France	7	c.757C>T	p.R253W	C2 d 2	missense	homozygous	this study	isolated hyperCKaemia
dysAB	**France**	**8**	**c.855+1delG**		**C2 d 2**	**splice site**	**heterozygous**	**this study**	**proximodistal**
		29	c.3126G>A	p.W1042X		nonsense	heterozygous	this study	
dysGR	**France**	**19**	**c.1663C>T**	**p.R555W**		**missense**	**heterozygous**	**this study**	**LGMD2B**
		34	***c.3832C>T***	***p.Q1278X***		***nonsense***	***heterozygous***	***LMDD***	
dysGRA	*France*	*6*	*c.610C>T*	*p.R204X*		*nonsense*	*heterozygous*	*LMDD*	*Miyoshi myopathy*
		8	**c.855+1delG**		**C2 d 2**	**splice site**	**heterozygous**	**this study**	
dysOR	France	29	c.3112C>T	p.R1038X		nonsense	heterozygous	this study	Miyoshi myopathy
		34	***c.3832C>T***	***p.Q1278X***		***nonsense***	***heterozygous***	***LMDD***	
dysCA	France	15	c.1368C>G	p.C456W	C2 d 3	missense	heterozygous	this study	Miyoshi myopathy
		51	*c.5713C>T*	*p.R1905X*	*C2 d 6*	*nonsense*	*heterozygous*	*LMDD*	
dysVI	France	34	c.3826C>G	p.L1276V		missense	heterozygous	this study	proximodistal
		34	c.3843+1G>A			splice site	heterozygous	this study	
dysOU	*Africa*	*38*	*c.4167+1G>C*			*splice site*	*heterozygous*	*LMDD*	*proximodistal*
		25	*c.2643+1G>A*			*splice site*	*heterozygous*	*Tagawa, 2003*	
dysGA	France	8	c.797T>C	p.L266P	C2 d 2	missense	heterozygous	this study	Pseudometabolic
		44	c.4876delG		C2 d 5	frameshift	heterozygous	this study	
dysLAE	France	32	c.3477C>A	p.Y1159X	C2 d 4	nonsense	heterozygous	this study	-
		29	*c.3137G>A*	*p.R1046H*		*missense*	*heterozygous*	*Aoki, 2001*	
dysNI	*France*	*6*	*c.509C>A*	*p.A170E*		*missense*	*heterozygous*	*LMDD*	*isolated hyperCKaemia*

		19	**c.1663C>T**	**p.R555W**		**missense**	**heterozygous**	**this study**	
dysZI	*Algeria*	*20*	*c.1834C>T*	*p.Q612X*		*nonsense*	*heterozygous*	*LMDD*	*LGMD2B*
		37	c.3967C>T	p.Q1323X		nonsense	heterozygous	this study	
dysCAN	France	32	c.3516_3517delTT		C2 d 4	frameshift	heterozygous	LMDD	Miyoshi myopathy
		37	***c.3992G>T***	***p.R1331L***		***missense***	***heterozygous***	***Cagliani, 2003***	
dysVA	France	4	c.247delG		C2 d 1	frameshift	heterozygous	this study	LGMD2B
		30	*c.3191_3196dupGAGGCG*			*inframe*	*heterozygous*	*Kawabe, 2004*	
dysFI	**France**	**8**	**c.855+1delG**		**C2 d 2**	**splice site**	**heterozygous**	**this study**	**proximodistal**
		12	*c.1180+11C>T*			*splicing?*	*heterozygous*	*LMDD*	
dysBR	France	9	c.896G>A	p.G299E	C2 d 2	missense	heterozygous	this study	Miyoshi myopathy
		30	*c.3191_3196dupGAGGCG*			*inframe*	*heterozygous*	*Kawabe, 2004*	
dysON	USA	46	c.5078G>A	p.R1693Q		missense	heterozygous	this study	Miyoshi myopathy
dysGAB	France	30	c.3321_3324dupAGCT			frameshift	heterozygous	this study	isolated hyperCKaemia
dysJO	France	52	c.5903G>A	p.W1968X	C2 d 6	nonsense	heterozygous	this study	Pseudometabolic
dysBA	France	52	c.5813_5821delCAGCCAAGA		C2 d 6	inframe	heterozygous	this study	Miyoshi myopathy
dysMA	*France*	*53*	*c.5979dupA*			*frameshift*	*heterozygous*	*Cagliani, 2003*	*proximodistal*
dysGAU	France	33	c.3624C>G	p.I1208M	C2 d 4	missense	heterozygous	this study	LGMD2B
dysVE	***Belgium***	***50***	***c.5594delG***		***C2 d 6***	***frameshift***	***heterozygous***	***Liu, 1998***	***Miyoshi myopathy***
dysJOI	France	30	c.3348+1_3348+4delGTAT			splice site	heterozygous	this study	Miyoshi myopathy
		47	**c.5314_5318delAGCCC**			**frameshift**	**heterozygous**	**this study**	
		37	***c.3992G>T***	***p.R1331L***		***missense***	***heterozygous***	***Cagliani, 2003***	
dysAM	**Algeria**	**19**	**c.1663C>T**	**p.R555W**		**missense**	**homozygous**	**this study**	**Miyoshi myopathy**
		38	c.4052A>G	p.N1351S		missense	homozygous	this study	
dysBEN	*Algeria*	*6*	*c.565C>G*	*p.L189V*		*missense*	*homozygous*	*LMDD*	*proximodistal*
		47	c.5296G>T	p.E1766X		nonsense	homozygous	this study	
		47	c.5243A>T	p.E1748V		missense	homozygous	this study	

Table 2: In silico predictions of consequences of the missense mutations according to the SIFT program (Ng and Henikoff, 2001). SIFT (Sorting Intolerant From Tolerant) classifies substitutions as tolerated or deleterious. The reference sequence of the human dysferlin is GI4503431. For the SIFT prediction, statistical value is $p<0.05$ in favor of the pathogenic nature of the mutation. The conservation score represents the percentage of amino-acid conservation among ten different ferlin sequences that have been aligned.

Missense mutations	SIFT prediction	Conservation score (%)	Splicing	Consequence
c.757C>T p.R253W	0,00	78	no modification	probably pathogenic
c.1663C>T p.R555W	0,00	78	no modification	probably pathogenic
c.1368C>G p.C456W	0,00	89	no modification	probably pathogenic
c.3826C>G p.L1276V	0,00	89	activation of cryptic donor?	probably pathogenic (+ splice?)
c.797T>C p.L266P	0,09	78	no modification	probably pathogenic
c.3137G>A p.R1046H	0,00	89	no modification	probably pathogenic
c.509C>A p.A170E	0,12	67	no modification	probably pathogenic
c.3992G>T p.R1331L	1,00	89	no modification	polymorphism?
c.896G>A p.G299E	0,00	67	no modification	probably pathogenic
c.5078G>A p.R1693Q	0,00	78	no modification	probably pathogenic
c.3624C>G p.I1208M	0,02	89	no modification	probably pathogenic
c.4052A>G p.N1351S	1,00	89	no modification	polymorphism
c.565C>G p.L189V	0,34	67	activation of cryptic donor?	polymorphism?
c.5243A>T p.E1748V	0,00	78	activation of cryptic donor?	probably pathogenic (splice)

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