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Muscular dystrophy with marked Dysferlin deficiency is consistently caused by primary dysferlin gene mutations

Running title: Marked Dysferlin deficiency is primary

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ABSTRACT

Dysferlin is a 237-kDa transmembrane protein involved in calcium-mediated sarcolemma resealing. Dysferlin gene mutations cause limb-girdle muscular dystrophy (LGMD) 2B, Miyoshi myopathy (MM), and distal myopathy of the anterior tibialis. Considering that a secondary Dysferlin reduction has also been described in other myopathies, our original goal was to identify cases with a Dysferlin deficiency without dysferlin gene mutations. The dysferlin gene is huge, composed of 55 exons that span 233,140 bp of genomic DNA. We performed a thorough mutation analysis in 65 LGMD/MM patients with ≤20% Dysferlin. The screening was exhaustive, since we sequenced both genomic DNA and cDNA. When required, we used other methods, including real-time PCR, long PCR and array CGH.

In all patients we were able to recognize the primary involvement of the dysferlin gene. We identified 38 novel mutation types. Some of these, such as a dysferlin gene duplication, could have been missed by conventional screening strategies. Nonsense mediated mRNA decay was evident in six cases, in three of which both alleles were only detectable in the genomic DNA, but not in the mRNA. Among a wide spectrum of novel gene defects, we found the first example of a “nonstop” mutation causing a dysferlinopathy.

This study presents the first direct and conclusive evidence that an amount of Dysferlin ≤ 20% is pathogenic and always caused by primary dysferlin gene mutations. This demonstrates the high specificity of a marked reduction of Dysferlin on western blot and the value of a comprehensive molecular approach for LGMD2B/MM diagnosis.
Keywords: DYSF: dysferlin, LGMD: Limb-Girdle Muscular Dystrophy; MM: Miyoshi Myopathy; NMD: Non sense Mediated mRNA Decay; CGH: Comparative Genomic Hybridization
INTRODUCTION

Mutations in the dysferlin gene are responsible for three main dystrophic phenotypes: limb-girdle muscular dystrophy type 2B (LGMD2B; MIM# 253601), Miyoshi myopathy (MM; MIM# 254130) and distal myopathy with anterior tibialis onset (DMAT; MIM#606768). During the course of the disease, the phenotypes show a substantial amount of overlap with weakness extending from the proximal to distal muscles and vice versa. Even if clinical differences are reported, they may not be so striking at the pathological level. A unique finding within the spectrum of muscular dystrophies is that the majority of Dysferlin-deficient patients appear to have no initial muscle weakness. Indeed, they often show a good performance at sport or have jobs that require physical activity suggesting that exercise may be a disease-triggering factor. A secondary Dysferlin reduction has been also observed in calpain 3 (LGMD2A), caveolin-3 (LGMD1C). A similar phenotype has been described also in patients with anoctamin 5 gene (LGMD2L) mutations.

The dysferlin gene is huge and routine mutation detection is a long and expensive process. In addition, there are no prevalent mutations, but, instead, 415 different allelic variants have been identified thus far (www.dmd.nl). The cost-effectiveness of a dysferlin gene mutation screening using DHPLC approaches has been reported in large groups of patients with the identification of many mutations, so validating the efficacy of genomic mutational screening for routine diagnosis.

Most authors have been able to identify the majority of dysferlin gene mutations (but not all), when Dysferlin was absent from the muscle. Several mutational screenings have been reported, many of them based on isolated cases/families and the identification of private mutations. The biggest cohort was analyzed by Krahn et al., composed
of 134 patients, including a group that had previously been described. The authors identified at least one pathological allele in 119/134 patients (88%). The success rate was higher than in other LGMD cases, but not all cases were solved, indicating either that other genes causing secondary defects of Dysferlin are rarer, or that the mutation analyses have been inaccurate in that they have missed some of the defects.

Our original aim was to focus on LGMD cases with a marked Dysferlin reduction and no mutation. To achieve this objective we performed a complete mutation analysis of the dysferlin gene in a group of 65 LGMD/MM patients with a marked reduction or absence of the protein detected by western blot. We used all available screening methods starting from genomic DNA and mRNA. Here we show the high specificity of the absence or marked reduction of dysferlin expression on muscle biopsy and the high specificity and sensitivity of a comprehensive molecular approach in the diagnosis of LGMD2B/MM.
MATERIALS AND METHODS

Patient recruitment

The patients were between 26 and 77 years of age. They had serum levels of creatine kinase between 1,200 U/L and 8,000 U/L (with an average value of 3,507 U/L). These patients showed an absence or marked reduction (5-20% of the normal amount) of Dysferlin in their skeletal muscle. Ten out of 65 were affected by Miyoshi myopathy, while the other 55 had an LGMD2B phenotype. In our study we analysed 8 pairs of siblings. Only three of these patients are wheelchair-confined in accordance with the slow progression rate of the dysferlinopathies. In particular, the age at which they began to use a wheelchair was very variable (22, 39 and 49 years of age respectively), again in accordance with the phenotypical variability of the dysferlinopathies. Biological samples were obtained from 65 patients (61 of which Italians) from different centres: Naples (16/65), Rome (5/65), Genoa (8/65), Padua (31/65), Ankara (Turkey) (3/65) and Buenos Aires (Argentina) (1/65). For all these patients genomic DNA and/or mRNA from blood samples or muscle biopsies were available.

Muscle biopsy

At the time of diagnosis, an open biopsy from the quadriceps femoris muscle was obtained under local anesthesia after written informed consent. Muscle biopsy specimens were frozen in isopentane, cooled in liquid nitrogen and stored at −80°C until processed.

Multiple Western blot analysis of muscle proteins and quantification
Western blot (WB) analysis and detailed protein analyses were performed as previously described using a mixture of monoclonal antibodies against Calpain-3 (Calp12A2, diluted 1:800), alpha-Sarcoglycan (diluted 1:300), beta-Sarcoglycan (diluted 1:300), Dystrophin (Dys-2, diluted 1:1000), and Dysferlin (Hamlet, diluted 1:1000), all purchased from Novocastra (UK). In brief, the quantity of muscle proteins in the samples from the controls and patients was determined by densitometry using ImageJ software v.1.34n and normalised to the amount of tissue loaded in each lane, using the skeletal myosin bands in the post-transfer Coomassie blue-stained gels. The values in each patient were expressed as percentages of the mean of the controls.

**PCR conditions from gDNA**

The dysferlin gene (NM_001130987.1, 55 coding exons) was amplified by PCR from genomic DNA. All the exons and flanking intron sequences were amplified using specific primer pairs (Supplementary Table S1). In a final volume of 25 µl, 60-75ng of genomic DNA were combined with 0.6 µM of each primer, 0.14mM dNTPs, buffer LB1X (20mM Tris, 10mM Hepes, 2.5mM magnesium sulphate, 10mM potassium chloride, 10mM ammonium sulphate) or buffer LC1X (20mM Tris, 10mm Hepes, 2.5M magnesium sulphate, 20mM ammonium sulphate, 5% glycerol), and 0.9 U of AmpliTaq-Gold (Perkin Elmer, Massachusetts, USA). After polymerase activation 7 min at 95°C, reactions were then carried out for 30 seconds at 95 °C, 1 minute at Tm (see Supplementary Table S1) and 1 minute at 68 °C, for 30 cycles.

**DHPLC Analysis**
We performed comparative mutation scanning to select amplicons for aberrant DHPLC profiles not shared by the normal controls. DHPLC analysis was performed on a WAVE DNA fragment analysis system (Transgenomic Inc., San Jose, CA) equipped with a DNASEp column (3,500 High Throughput [HT]) employing a UV-C scanner to detect eluted DNA.

**mRNA extraction and cDNA preparation**

We used a TRIzol® reagent (Invitrogen Carlsbad, California) according to the manufacturer’s instructions to extract RNA from the muscle biopsies and the PAXgene™ Blood RNA Kit (Qiagen, Hilden, Germany) to extract RNA from the blood.

The retrotranscription reaction was performed using 2μg of total mRNA according to the SuperScript® III kit (Invitrogen Carlsbad, California).

**RT-PCR and long PCR**

We amplified the dysferlin cDNA in 13 overlapping fragments (Supplementary Table S2). The reaction was performed in a final volume of 25μl using: 1μl of cDNA, 1μM of each primer, 0.8mM of dNTPs, Buffer JD1X, 0.5U of LA-Taq DNA polymerase (Takara BIO Inc.) and 1U.I. of Pfu polymerase (Stratagene). Thermocycling was then carried out for 30 seconds at 94 °C, 1.30 minute at Tm (see Supplementary Table S2) and 2 minute at 68 °C, for 30 cycles.

Fragments were recovered from agarose gels by using the Mini Elute™ Gel Extraction Kit (Qiagen, Hilden, Germany) and then sequenced.
Sequencing

BigDye® Terminator sequencing chemistry and ABI3130XL automatic DNA sequencer (Applied Biosystems, Foster City, CA) were used. Each nucleotide change was verified by reverse sequencing and, in addition, by the sequencing of an overlapping PCR product obtained with different primers. Mutations were numbered based on protein (GenBank NP_003485) and cDNA sequence (GenBank NM_003494). Nucleotides were numbered according to international recommendations.

Array CGH

A custom array CGH (MotorChip 2.0) was developed using the Agilent 8X60K format (SurePrint G3 arrays). All dysferlin exons, both the 5’- and 3’-UTR, 2,000 bp at the 5’ end of the gene (covering the dysferlin promoter) were included. Probes were designed based on the exon and flanking intron sequences. Array CGH results were confirmed by independent assays, such as real time PCR, long PCR and MLPA.

Bioinformatic software

Splice View software was useful to verify the effect of intronic variants on mRNA splicing (http://bioinfo.itb.cnr.it/oriel/splice-view.html). Conservation analysis was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). To assess intronic and exonic mutations leading to splicing defects, the Human Splicing Finder website (http://www.umd.be/SSF/) was consulted.
RESULTS

To select patients with a Dysferlin deficiency from a heterogeneous population of patients affected with an unclassified form of LGMD or MM, we previously analyzed muscle samples by a multiple WB. A group of 65 patients that showed a marked reduction or absence of Dysferlin were included in the study (fig. 1A, B). We excluded cases having more than 20% Dysferlin using a quantitative WB assay. The average Dysferlin level was 5% ± 5. We combined different screening methods to identify the causative alleles (fig. 1C).

From genomic DNA - DHPLC

Despite the incomplete DHPLC sensitivity and the noise of the variants and polymorphisms, this first step was chosen for its cost-effectiveness. We screened by DHPLC and targeted a sequencing of all the 55 exons and flanking introns from the genomic DNA. DHPLC analysis was performed on a first group of 52 patients. We identified 47 causative mutations, 28 of which were new (Supplementary Table S3). A full molecular diagnosis (both alleles) was provided for 35 out of 52 patients (67%).

mRNA analysis

We next performed mRNA analysis in cases without mutations or with ambiguities (one allele, new mutations, possible splice defects, etc.). We first confirmed on cDNA the splice mutations. We also extended the analysis to an additional group of 13 patients. Two sources of mRNA were considered: 1) skeletal muscle that, when available, is the first choice for the expression level and canonical splicing of muscle dysferlin; 2) leukocytes that are easy to collect and representative of non muscle dysferlin, with some
differences in the alternative splicing of exons 5a, 17 or 40a\textsuperscript{35,36}. For this analysis we performed 16 muscle biopsies and, when not possible, we collected blood samples. The cDNA was amplified by PCR in 13 overlapping fragments and then sequenced (fig.S1). We identified 15 mutations, eight of which were novel. mRNA analysis was useful to demonstrate the effect of two intronic variations on RNA splicing (fig. 2A-B). Patient X311 carrying the homozygous mutation 906+4A>G showed a smaller amplification product of fragment 3 (including approximately exons 7 to 12) of the dysferlin cDNA compared to the control (fig. 2A). Direct sequencing showed that exon 9 was skipped (fig. 2A’). At the protein level, exon-9 skipping causes the loss of a part of the C2B domain, important for the protein function. Therefore, this variation should be considered as pathological.

The transversion 1639-6T>A, found in a homozygosity in patient X389, inactivated the donor splice site (with the reactivation of a new donor site in intron 18) resulting in retention of 4bp from intron 18 sequence (fig. 2B) and a frame-shift (Supplementary Table S3). Patient X546 (classified as having severe LGMD2B) showed < 5% Dysferlin. Molecular analysis confirmed the presence of a frameshift mutation, 1-bp deletion (g.2077delC) on exon22 (fig. 2C), and the heterozygous deletion of the entire exon 17 (fig. 2D). Exon 17 skipping did not affect the open reading frame, but the amino acid at the exon junction changed (GAG>GTG, g.1481_1522del, p.E494V+ex17skipping). The alternative splicing of the exon 17 had previously been identified and characterized as blood cell isoform\textsuperscript{36}, but it had never been detected in muscle.

DNA re-sequencing
All-exon and flanking intron resequencing was used to confirm all the mutations. We identified three additional mutations, the diagnosis being completed in 58/65 patients (Table 1). Surprisingly, six apparently homozygous alleles were not confirmed by the DNA analysis. These alleles were heterozygous and for 3 out of 6 patients a second nonsense mutation was identified in other regions of the gene. This can be explained by the nonsense mediated decay (NMD) of the mRNA (fig. 2E). For the other three patients with NMD the promoter region was not mutated.

**Array CGH, real time PCR and long PCR**

In all cases with an incomplete gene testing (0 or 1 mutated allele) we performed a CGH array to identify possible heterozygous intragenic rearrangements, such as deletions or duplications (Table 1). We used a minimum of three probes per exon plus promoter probes covering a genomic region of 220kb using a total of 196 probes. We identified a heterozygous duplication involving the 5’ end of the gene from exon 1 to 22 (3484), and a heterozygous deletion (X583). In addition, we performed a long PCR on the mRNA and/ or gDNA using primers located at distant positions in the transcript to confirm the alterations.

**Mutation spectrum**

The exhaustive molecular analysis of the dysferlin gene led to the identification of 65 different mutations, 38 of which (60%) had not previously been described (Supplementary Table S3 and fig.3). Thirty-one patients showed homozygous alleles (47.7%), most confirmed by segregation studies. Mutation scanning methods used in our study led to the identification of 19 additional novel non-pathological variants.
(Supplementary Table S4). The present study confirmed the absence of a mutational hot-spot region as well as the spreading of mutations along the entire gene. We detected 9 (14%) nonsense mutations, 28 (43%) missense mutations, 5 (8%) splicing mutations, 20 (30.5%) frame shift mutations, one nonstop mutation (1.5%), and two large genomic rearrangements (3%) (Table 2).

For the novel mutations their absence was demonstrated in > 1,000 control chromosomes from healthy individuals of matched ethnic origin. In all 10 patients with 10-20% of Dysferlin we always found at least one missense mutation (100%), while among 24 patients with 0% Dysferlin a missense mutation was only found in 7 cases (29%).
DISCUSSION

The present study demonstrates that the marked reduction of Dysferlin observed in LGMD/MM patients is not genetically heterogeneous. Given the location of Dysferlin, close to the muscle membrane, its interactions with other proteins, and cumulative data about the existence of secondary dysferlinopathies, this is surprising, because in other membrane complexes, such as sarcoglycans, a marked secondary reduction of each component is common. A Dysferlin reduction has been observed in primary calpain 3 deficiency (LGMD2A) or Caveolin 3 deficiency (LGMD1C). An LGMD/MM phenotype has also been observed in patients carrying mutations in the Anoctamin 5 gene (LGMD2L).

Our results derive from an exhaustive analysis of RNA and DNA from 65 patients having a severe Dysferlin reduction. In theory, it has been claimed that a DNA analysis is necessary to diagnose a primary dysferlinopathy. In practice, however, it is not easy. The dysferlin gene is huge and composed of 55 exons. It spans 233,140bp of genomic DNA and generates a 6.9 kb-wide transcript. In our mutation screening flowchart (fig.1), the DNA analysis was first carried out by a DHPLC of all exons and flanking introns. DHPLC is cheaper, but it can give false-negative/positive results and therefore it can only have a screening value. A second problem with DHPLC consists in the huge number of polymorphisms and variants that are present in this gene (Supplementary Table S4). These are located in all exons and confound an interpretation of the results with many heteroduplex shifts per patient. We therefore used a second technique based on the sequence analysis of the dysferlin cDNA. When a muscle biopsy was not available, we were able to analyze the patients’ mRNA from blood, since Dysferlin is highly expressed in the monocytes. This method is less
invasive and can provide an adequate amount of mRNA: the analysis helped us to
understand the pathogenic role of the two intronic variants identified by DNA analysis,
both leading to an alteration of the splicing mechanism (fig. 2A-B).

Despite the larger number of cases identified by mRNA analysis, this method alone can
be faulty when the mutated allele is not expressed. We showed that the mechanism of
nonsense-mediated mRNA decay (NMD) also occurred in dysferlinopathy\textsuperscript{16}. In six out
of 65 patients (fig. 2D) we identified a homozygous mutation from cDNA, a mutation
which was heterozygous from gDNA. By direct sequencing of gDNA we identified in
3/6 (X584, X674, X676) an additional frameshift mutation missed by DHPLC. For the
three other patients (X267, X268, X675), we failed to identify the primary cause of the
missing mRNA expression of the second allele, but the NMD anyhow confirmed the
primary involvement of the dysferlin gene.

This confirms that mRNA analysis alone can be faulty, since true homozygote patients
cannot be distinguished from compound heterozygote patients with important
consequences in respect of genetic counselling.

Thirdly, we resequenced all the relevant genomic regions and three additional mutations
were found. Furthermore, we used three additional methods: long PCR, real time PCR
and array CGH.

Particularly noteworthy was the first evidence of a non stop mutation as a new
pathological mechanism involved in the dysferlinopathies. Patient X295 carries a
homozygous 8-bp (g.6233\_6240del, p. P2078fsNON STOP) deletion that was identified
in exon 55 of the dysferlin gene. The deletion led to a frameshift in the reading frame
with the loss of the stop codon (fig. 4A, B). We supposed that the new reading frame
could give rise to the translation of 97 additional amino acids through the 3’end of the
mRNA. The patient showed a residual expression of <10% of larger sized Dysferlin in the skeletal muscle (fig. 4C). We hypothesize that the mutation could cause a mechanism of nonstop mRNA decay. Indeed, it was demonstrated that in the eukaryotes there is a mechanism of degradation of mRNA lacking the stop codon \(^{41-44}\). Non stop mutation has previously been identified in the ACTA1 gene\(^{45}\).

Many groups have questioned the value of protein analysis in carrying out a correct diagnosis. Fanin et al.\(^{26}\) observed that the levels of Dysferlin were reduced to 50% of those of the controls in the carriers of LGMD2B. They showed that a reduction of 50% indicated both familial and isolated LGMD2B heterozygotes, and suggested the use of Dysferlin protein testing to select muscle biopsies from suspected carriers for a subsequent mutation analysis\(^{26}\).

Our data support the dysferlin gene as the unique cause of Dysferlin deficiency between 0% and 20% by WB analysis. Although for three patients (3484, 4132 and X147) the second allele was not identified, this was only due to incomplete testing for insufficient DNA. However, this does not affect the main conclusions of the study, because these patients show sure causative alleles (frame-shift/duplication) that cannot be coincidental.

This marked reduction is necessary to affect muscle membrane repair. We cannot exclude the presence of other functional mutations, but a direct proof of pathogenicity is always required, since the dysferlin gene shows a large number of variants and polymorphisms that can be misleading. In these cases, the possible lack of mutations in the dysferlin gene may be due to incomplete genetic testing.

The results obtained in this present paper have an immediate diagnostic application: a Dysferlin reduction to 20% (that can also be measured from blood monocytes \(^{28,35}\)) can
be used to identify LGMD2B with 100% accuracy. In the case of LGMD2B this observation is noteworthy. When a rapid Dysferlin blood testing will be available, important decisions will derive, such as to avoid steroids (that are ineffective/deleterious in LGMD2B in contrast with other forms of muscular dystrophy) and any distressing sport activities in children\textsuperscript{7,46}.

Although, it is generally agreed that an extensive molecular analysis has a high cost, a precise determination of the DYSF gene is, however, particularly important from a diagnostic/counselling perspective and in view of the development of a future therapeutic strategy. A successful recognition of all the mutations demonstrates the power of a combined diagnostic strategy. More importantly, a complete genetic testing should be applied to all other LGMD cases\textsuperscript{47}.

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Conflict of interest
The authors declare that they have no conflict of interest.
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TITLES AND LEGENDS TO FIGURES

Figure 1: Mutational Scanning. **A.** The picture shows the western blot on muscle lysate from patients (1, 2) and control (C). As observed, patient 1 showed a complete absence of the dysferlin-specific band, while sample 2 shows a residual expression (5%) of dysferlin protein, compared to the control (as indicated by the arrow). **B.** An exemplary image of a multiple western blot assay used in this study to screen and select the patients for further molecular analyses (samples 1 and 2 show a complete dysferlin deficiency). For both A and B, the skeletal myosin bands in the post transfer Coomassie blue staining gels were used to normalize the amount of loaded protein. **C.** The flow chart shows the general design of the mutation analysis.

Figure 2 Elusive/peculiar mutations: **A:** Exon 9 skipping in a patient with the variation 906+4A>G in homozygous status. RT-PCR products between DYSF exons 7 and 12 showing the aberrantly-spliced transcript. **A:** Graphical presentation of the sequence composition of the wild-type transcript and the aberrantly-spliced transcript with the exon 9 skipping. **B.** Partial intron 18 retention in a patient with the variation 1639-6T>A in homozygous status. RT-PCR products sequencing between DYSF exons 12 and 19 showing a 4 bp retention of the intron 18, which is predicted to result in a truncated protein. **C-D:** Analysis of the cDNA sequence of patient X546 for C fragment 5 (including exon 22) and D fragment 4 (including exon 17). Both mutations produce a frameshift. **E:** Sequence analysis performed on specific fragments of dysferlin obtained from cDNA and DNA. The same mutation is shown for both cDNA and DNA. The mutated base is highlighted in blue. All the patients showed the mutation in homozygous status on cDNA and heterozygous status on DNA.
Figure 3: Mutation spectrum. The picture shows the position of all the mutations identified in this study. Δ identifies missense mutations; ○ identifies the frameshift (del/ins) mutation; ◊ identifies the mutations affecting the splicing mechanism; ● identifies the non sense mutation.

Figure 4: Non stop mutation. A-B: Analysis of DNA sequence obtained from a control (A) and patient (B). The mutated base is highlighted in blue. The sequence of exon 55 shows a homozygous deletion of 8bp (g. 6233_6240del; p. Pro2078LeufsNON STOP). The frameshift skips the stop codon and produces hypothetically the translation of 97 additional amino acids. Both nucleotide and translated sequences are reported for both samples. C: The picture shows the western blot on muscle lysate from a patient (X295) and control (C). As observed, patient X295 showed a residual expression (<10%) of larger sized Dysferlin, compared to the control (as indicated by the arrow).
Table 1: Number of causative alleles identified by the different techniques

| mutations  | DHPLC DNA sequencing | mRNA sequencing | gDNA sequencing | Array CGH Real Time/long PCR |
|------------|----------------------|-----------------|----------------|-----------------------------|
| 0 alleles  | 5                    | 0               | 0              | 0                           |
| 1 allele   | 12                   | 8               | 5              | 5                           |
| 2 alleles  | 35                   | 18              | 4              | 0                           |
| Samples examined | 52            | 26              | 9              | 5                           |

Table 2: Classification of all mutation types identified (Het: heterozygote, Hom: homozygote). Numbers indicate how many mutation types were discovered

| Class of Mutation    | Mutations (%) | Status |
|----------------------|---------------|--------|
|                      |               | Het    | Hom    |
| Missense             | 28 (43%)      | 27     | 12     |
| Nonsense             | 9 (14%)       | 9      | 5      |
| Splice site          | 5 (8%)        | 2      | 5      |
| Frameshift           | 20 (30.5%)    | 24     | 8      |
| Non-Stop             | 1 (1.5%)      | 0      | 1      |
| Genomic Rearrangements| 2 (3%)        | 2      | 0      |
