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SHORT COMMUNICATION

Private dysferlin exon skipping mutation (c.5492G > A) with a founder effect reveals further alternative splicing involving exons 49–51

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The allelic muscle disorders known as limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy and distal anterior compartment myopathy result from defects in dysferlin—a sarcolemma-associated protein involved in membrane repair. Mutation screening in the dysferlin gene (*DYSF*) enabled the identification of seven Portuguese patients presenting the variant c.5492G > A, which was observed to promote skipping of exon 49 (p.Gly1802ValfsX17). Several residually expressed products of alternative splicing also involving exons 50 and 51 were detected in the leukocytes and muscle of both patients and normal controls. Quantitative transcript analysis confirmed these results and revealed that Δ49/Δ50 transcripts were predominant in blood. Although the patients were apparently unrelated, the c.5492G > A mutation was found in linkage disequilibrium with a particularly rare haplotype in the population, corroborating the hypothesis of a common origin. Despite the presence of the same mutation on the same haplotype background, onset of the disease was heterogeneous, with either proximal or distal muscle involvement.

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INTRODUCTION

Dysferlin is involved in calcium-dependent vesicle trafficking and membrane fusion, playing an important role in muscle fibre repair.[1,2] Mutations in the dysferlin gene (*DYSF*, MIM*603009) lead to a clinically heterogeneous set of muscular dystrophies collectively known as dysferlinopathies. These may present as a predominantly proximal limb-girdle muscular dystrophy type 2B[1] or as predominantly distal forms, with early involvement either of the gastrocnemius, as in Miyoshi myopathy,[3] or of the anterior tibial muscles, as in distal anterior compartment myopathy.[4] Despite these distinctions, there appears to be no strict correlation between the type or location of the mutation and the clinical phenotype, as the same mutation has been described in both proximal and distal forms, even in an intrafamilial context.[5–8]

Over 400 variants have been reported to date, with pathogenic mutations widespread across this large gene. Moreover, an alternative promoter and alternatively spliced transcripts have recently been described,[9–11] which add complexity to mutation screening and interpretation.

Here we describe the characterization of the novel exon-skipping mutation c.5492G > A, with an apparent founder effect in our population, in which studies revealed further alternative splicing in this region of the gene, even in normal controls.

MATERIALS AND METHODS

Patients

The seven unrelated male patients had elevated serum creatine kinase levels and compatible clinical phenotype and/or muscle pathology (data summarized in Table 1). Informed consent was obtained for molecular studies in all the patients.

Mutation screening

In genomic DNA, all 56 exons and flanking intronic regions were cycle-sequenced using the BigDye Terminator Cycle Sequencing Kit V1.1 and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

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Table 1 Clinical–pathological data of the seven dysferlinopathy patients

Patient/ gender	*Onset/current age (years)*	*Presenting symptoms*	*Muscle involvement and progression*	*CK ($U\,l^{-1}$)*	*Muscle dysferlin*	*Cons.*	*Genotype*
I/M	18/34	Difficulty running and climbing stairs	Proximal tetraparesis	7960	Absent	N	c.[5492G>A]+ [5492G>A]
II/M	14/48	Unknown	Proximal muscular weakness in the lower limbs	8280	Absent	N	c.[5492G>A]+ [5492G>A]
III/M	12/23	Difficulty running	Proximal weakness and extreme pseudohypertrophy of the calves	14340	Reduced	U	c.[5492G>A]+ [5657delG]
IV/M	13/19	Fatigue; hyperCKemia; cardiac arrhythmia	Proximal muscular weakness in the lower limbs	5730	Absent	Y	c.[5492G>A]+ [5492G>A]
V/M	18/30	Difficulty walking on toes	Distal muscular weakness in the lower limbs	4950	ND	Y	c.[5492G>A]+ [5492G>A]
VI/M	18/25	Difficulty running and climbing stairs	Proximal weakness in the upper and lower limbs; generalized atrophy of the lower limbs	4470	Absent	Y	c.[5492G>A]+ [5492G>A]
VII/M	18/21	Muscle pain following exercise	Proximal muscular weakness in the lower limbs	8460	Absent	Y	c.[5492G>A]+ [5492G>A]

Abbreviations: CK, creatine kinase; Cons., consanguinity; M, male; N, no; ND, not determined; U, unknown; Y, yes.
Muscle biopsies were subjected to routine histochemical evaluation as well as immunohistochemical staining for dystrophin, merosin, dysferlin, and α-, β- and γ-sarcoglycans.

Analysis was aided by the SeqScape V2.5 software (Applied Biosystems) using the reference cDNA sequence filed under GenBank Accession Number NM_001130978.1.

Transcript analysis

Total RNA was extracted from peripheral blood and/or muscle biopsies of patients and controls using TRIzol isolation reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcribed using either Superscript One-Step RT-PCR with Platinum *Taq* (Invitrogen) or the High Capacity RNA-to-cDNA Kit (Applied Biosystems).

Gross changes around exon 49 were detected using the following primers designed in exons 47 and 53, respectively: c.(47)F-5′-CAGCAGCATAGAGTCAAG-3′ and c.(53)R-5′-CACCAGCCCTTCACTGTTTT-3′. Amplicons resolved on 1% w/v agarose gels were eluted and sequenced in both directions, as described above.

For quantitative transcript analysis, six TaqMan probes and primers were used: (i) human glyceraldehyde-3-phosphate dehydrogenase gene-specific set, as an endogenous control, (ii) a target region spanning the exon 48–49 junction (assay ID Hs01002534_m1; Applied Biosystems) and (iii) specifically designed probes and primers for alternatively spliced transcripts, namely for exon 48–50, 48–51, 49–51 and 48–52 junctions (Supplementary Table S1). Multiplex reactions were prepared in triplicate using TaqMan Gene Expression Master Mix and amplified on a 7500 Fast Real-Time PCR System (Applied Biosystems). Quantification was done by using the comparative ΔΔCt method.[12]

Haplotyping

Haplotypes were constructed using intragenic and flanking microsatellite markers (Cy172-H32 and 104-sat;[2,3] D2S2113, D2S2604, D2S291, D2S2112 and D2S2111) as well as intragenic single-nucleotide polymorphisms (c.1827T>C, c.2583T>A, c.3972C>T, c.4008C>A, c.4068C>T, c.4950-37C>T, c.5831-16T>C and c.5922A>C). Control samples consisted of 20 anonymized, unrelated and non-consanguineous singleton families from a randomized population sample. Only the parents were considered for ascertainment of haplotype frequencies (offspring were used to determine allele phase), providing a total of 80 control chromosomes for analysis. Data were analysed with Arlequin 3.01 software using the haplotype inference option and by the estimation of allele frequency at the loci.[13]

RESULTS AND DISCUSSION

Mutation analysis

Direct sequencing of *DYSF* led to the detection of the novel mutation c.5492G>A, which was found in homozygosity in all patients except in patient III. This variant, which was not detected in 240 control alleles (Supplementary Figure S1), could be either an amino acid substitution (p.Arg1831Lys) or a splicing mutation, as it coincided with the last nucleotide of exon 49. The possible effect was predicted with the aid of the Genscan program (http://genes.mit.edu/GENSCAN.html) and ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), and the scores of normal and mutated donor splicing sequences were further calculated according to Shapiro and Senapathy.[14] These algorithms favoured exon 49 excision due to the abolishment of a donor splice site and/or the disruption of an ESE element responsive to SRp40.

Transcript analysis in muscle tissue and/or peripheral blood revealed a smaller fragment, present in all seven patients, which was found to be missing in the entire sequence of exon 49 (Δ49). The aberrantly spliced transcript (r.5404_5492del) determines a shift in the reading frame with subsequent peptide truncation (p.Gly1802ValfsX17).

Alternative splicing of exons 49, 50 and 51

In addition to the predominant Δ49 transcript, weak smaller bands were detected in muscle and leukocyte mRNA samples, both in patients and in controls. These were found to correspond to alternatively spliced products involving also exons 50 and 51 (Figures 1a and b). Quantitative analysis confirmed the presence of these transcripts, although with different expression levels in blood and muscle (Figure 1c). Comparison of expression levels across groups and specimen types showed that the Δ49/Δ50 isoform was more frequent in blood, in patients and in controls, whereas Δ49/Δ50/Δ51, an in-frame isoform, was detected mostly in control muscle.

Indeed, exons 50 and 51 seem to be amenable to modulation as suggested by the low splice probability scores of the donor and acceptor sites (according to Shapiro and Senapathy[14]). Modulation-prone splicing was first described in this gene for an in-frame Δ17 isoform, which appears to be tissue- and differentiation-specific.[10] More recently, Pramono *et al.*[11] described transcript variants with further in-frame splicing combinations, including the newly identified exons 5a and 40a, generated under either of the two promoters. Despite the maintenance of the reading frame, all of these variants were far more abundant in blood, as was observed in our case.

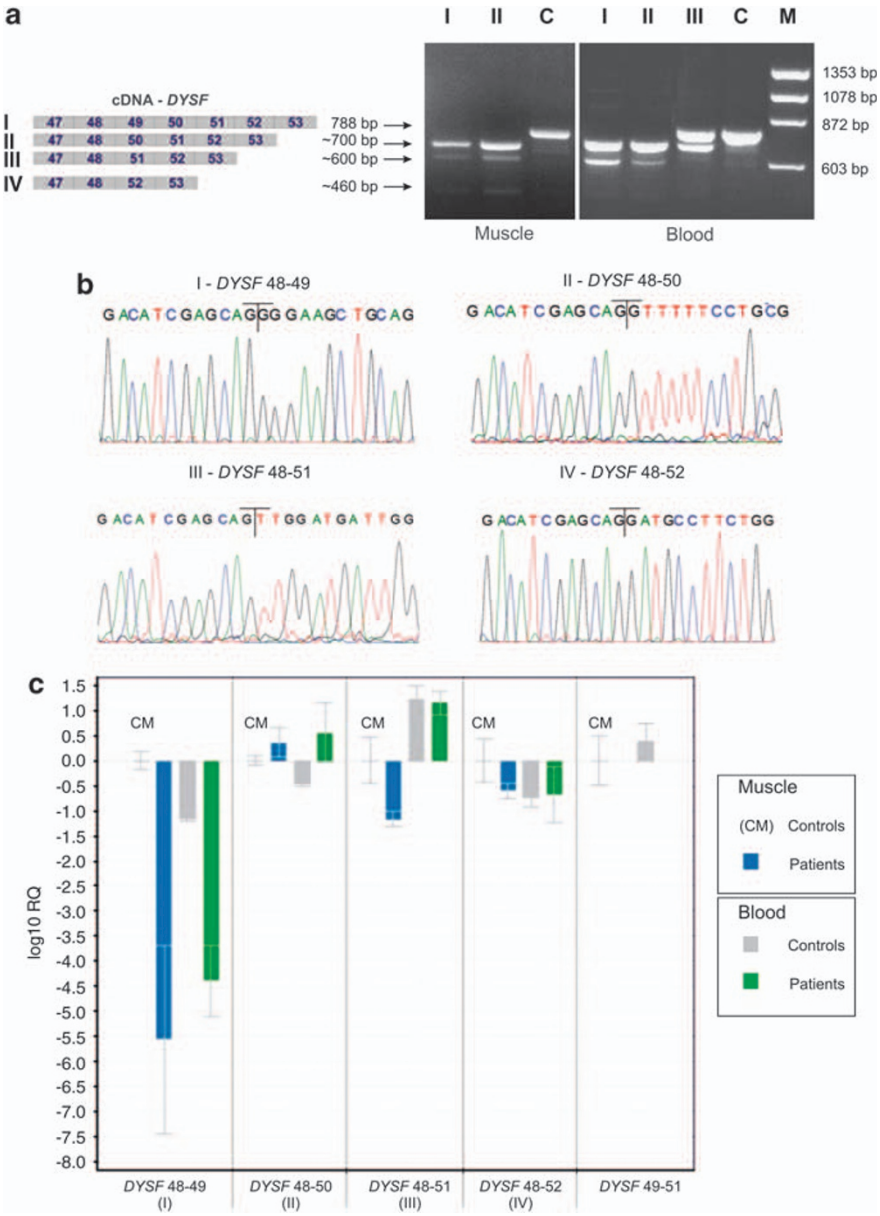

Figure 1 Dysferlin transcript analysis in the muscle and blood of patients and controls. (**a**) Conventional RT-PCR analysis of transcripts in patients (I, II and III) and a normal control (C). Grey bars represent the results observed by sequencing. (**b**) Electropherograms of junction sequences in the alternatively spliced RT-PCR products depicted in (**a**). (**c**) Quantitative real-time PCR using different Taqman probes designed to specifically detect junction fragments in normal (*DYSF* 48–49) and alternatively spliced transcripts (*DYSF* 48–50, 48–51, 48–52, 49–51). For Δ50 transcripts (*DYSF* 49–51), no signal was obtained in the patient samples. Quantification analysis was performed by the comparative ΔΔCt method using the muscle control samples (CM) as a calibrator.

Haplotype analysis

As all of the patients reside or have ancestry in a confined region in the northern interior part of Portugal, we tested the hypothesis of a founder effect for the c.5492G>A mutation. This mutation was found to be associated with the same *DYSF* gene haplotype in a stretch encompassing the gene and spanning approximately 1.4 cM (Figure 2). Sixty distinct haplotype blocks were distinguished in the 77 control chromosomes with informativity (Supplementary Table S2) and only one control chromosome shared the haplotype associated with the c.5492G>A mutation, thereby demonstrating its very low frequency (1.3%) in the population. Moreover, when analysis was extended to include the closest flanking extragenic markers D2S2604 and D2S291, none of the 77 control chromosomes presented the mutation-associated haplotype, thereby corroborating the hypothesis of a single mutational event leading to a founder effect. Previous reports on founder mutations in the *DYSF* gene include a nonsense mutation (p.R1905X) in the historically isolated Spanish town of Sueca and a missense mutation (p.R959W) in Italy.[8,15]

Concluding remarks

Although all our patients developed essentially a limb-girdle phenotype, some degree of heterogeneity was observed, especially at onset, with patient V initially presenting with distal muscle weakness in the lower limbs. Patient IV was being investigated by cardiology for

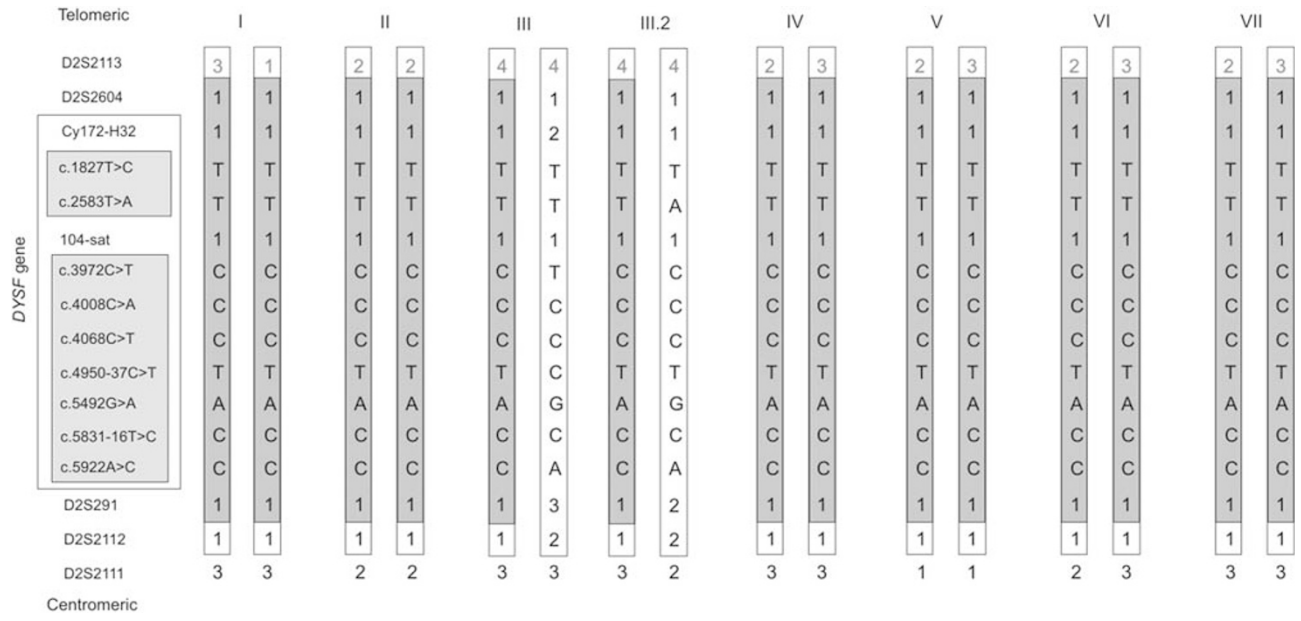

Figure 2 Haplotypes associated with the c.5492G>A mutation, detected in the studied patients. The mother of patient III is also shown (III-2). A full colour version of this figure is available at the *Journal of Human Genetics* journal online.

cardiac arrhythmia at the time of diagnosis, and only began to manifest proximal lower limb weakness at the age of 16.

Although splice mutation leakage and possibly alternative splicing may explain the varying degrees of severity, other modifying factors must necessarily determine the different manifesting patterns of the disease. Elucidation of the parallel and interacting mechanisms involved in membrane repair should provide insight into the clinical heterogeneity observed among dysferlinopathy patients with the same mutation, even on the same haplotype background, as reported here.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)