Short Report

Isolated cardiomyopathy caused by a DMD nonsense mutation in somatic mosaicism: genetic normalization in skeletal muscle


X-linked dilated cardiomyopathy is a pure cardiac dystrophinopathy phenotype mainly caused by DMD mutations that present a specific transcription effect in cardiac tissue. We report a 26-year-old male who presented with severe dilated cardiomyopathy and high creatine kinase. The patient did not complain of skeletal muscle weakness. A muscle biopsy showed mild dystrophic changes and a low proportion of dystrophin-negative fibres. A molecular study identified a nonsense DMD mutation (p.Arg2098X) in somatic mosaicism. The ratio of mutant versus normal allele in blood and skeletal muscle suggests selective pressure against mutant muscle cells, a process known as genetic normalization. We hypothesize that this process may have mitigated skeletal muscle symptoms in this patient. This is the second report of a DMD somatic mosaic with evidence of genetic normalization in muscle. Somatic DMD mutations should be considered in patients presenting with idiopathic dilated cardiomyopathy.

Conflict of interest

All the authors state that they have no interests which might be perceived as posing a conflict or bias.

Dystrophinopathies are progressive degenerative diseases of muscle caused by mutations in the DMD gene. The most frequent and most severe form of the disease is Duchenne muscular dystrophy (DMD), which is characterized by onset in childhood and rapid progression. Becker muscular dystrophy (BMD) is a less common and milder form that has a later onset. Clinical phenotype in BMD ranges from severe DMD-like forms to mild subclinical forms with little or no muscle weakness (1). A third form, X-linked dilated cardiomyopathy (XLCM), is characterized by rapidly progressive cardiomyopathy without muscular weakness. Most patients, however, have high creatine kinase (CK) levels and mild dystrophic abnormalities in muscle biopsies, making difficult to distinguish between sub-clinical BMD and XLCM (2, 3). Cardiomyopathy also occurs in DMD patients but this develops late in the course of the illness. In BMD, cardiomyopathy can be the presenting symptom and represents the first cause of morbidity and mortality (4, 5).

In most dystrophinopathy patients, the clinical phenotype can be predicted according to mutation type by the reading-frame rule. Truncating mutations cause...
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absence of dystrophin expression in muscle originating a severe DMD phenotype. By contrast, mutations that maintain the normal reading frame allow the expression of a semi-functional dystrophin in muscle, and clinically manifest as BMD phenotype (6, 7). The reading-frame rule, however, does not occur in DMD mutations causing an XLCM phenotype. Mutations in these patients present a specific transcription effect only in cardiac tissue but skeletal muscle maintains dystrophin synthesis via exon skipping or alternative splicing (3).

The DMD gene shows a high mutation rate with one third of patients presenting de novo mutations. It has been described that mothers of sporadic cases present 14–20% risk to carry the mutation in germinal mosaicism (8). Despite the high meiotic mutation rate of the DMD gene and the existence of germinal mosaicism, few male patients with somatic mosaicism have been reported to date (9–15).

We describe a patient who presented with isolated severe dilated cardiomyopathy (DCM) caused by a nonsense DMD mutation in somatic mosaicism. We also provide data consistent with a genetic normalization process in skeletal muscle confirming that this process could be a common ameliorating mechanism in DMD mosaic patients.

Patient and methods

We present a 26-year-old man with asymptomatic persistent hyperCKaemia (1400 IU/l) and no family history of neuromuscular disorders. Clinical examination showed normal strength (5/5 MRC). Muscle wasting, atrophy and hypertrophy were absent. Muscle magnetic resonance imaging (MRI) showed a mildly increased signal in adductor magnus, sartorius and semimembranous muscles of the right leg (Fig. 1). An echocardiogram showed a severe DCM with a decreased ejection fraction (20%). At 3-year follow-up the serum CK level remained elevated and the patient continued to show normal skeletal muscle function. Cardiac function was stabilized with calcium antagonist drugs. The patient was included in a cardiac transplantation program, but no compatible heart donor has been found to date.

Muscle biopsy

A muscle biopsy taken from deltoid muscle was immediately frozen in liquid nitrogen-chilled isopentane and processed by routine histological and histochemical techniques. Immunohistochemical and Western-blot analyses were performed by standard procedures using monoclonal antibodies against dystrophin (C-terminal, Rod-domain and N-terminal), α, β, γ and δ sarcoglycans, caveolin-3, dysferlin, utrophin and emerin (Novocastra, Newcastle upon Tyne, UK).

Genetic analysis

Genomic DNA was extracted from the muscle biopsy using phenol-chloroform isolation. Molecular analysis of intragenic deletions and duplications in the DMD gene was performed using the MLPA technique (MRC-Holland, Amsterdam, Netherlands). Total mRNA was extracted and purified from approximately 30 mg of muscle biopsy using RNeasy Fibrous Tissue Mini kit (Qiagen, Hilden, Germany). Muscle RNA was retrotranscribed to cDNA by RT-PCR using polythymine primers (Invitrogen, Carlsbad, NM). Dystrophin cDNA was then amplified in 20 overlapping fragments, analyzed by electrophoresis on a 2.5% agarose gel and direct sequenced using a Big Dye Terminator-1 kit (Applied Biosystems, Foster City, CA). All changes detected by mRNA analysis were confirmed in blood genomic DNA. Segregation analysis was performed using several microsatellite markers located at Xp21 (DXS1238, DXS1237, DXS1236, DXS1235, DXS1234, DXS992, AF209163, DXS1242).

Because the mutation identified in this patient creates an HphI restriction site in exon 44, we calculated the ratio of normal vs mutated alleles using HphI digestion and multiplex QF-PCR. Different genomic DNA samples were used: blood and muscle DNA from the patient, and nine blood DNAs from normal controls. Three replicates were made for each sample. 1 μg of each replicate was digested with the HphI restriction endonuclease (New England Biolabs, Ipswich, MA). DMD exons 43 and 44 were then amplified in digested DNA products using multiplex PCR with fluorescent-labeled primers. PCR fragments were sized and quantified by capillary electrophoresis using ABI3130 equipment and Genemapper software (Applied Biosystems). Because the exon 43 amplicon

Fig. 1. Muscle magnetic resonance imaging (MRI) T1-sequences of lower limbs. (a) Arrows indicate an increased signal in the adductor magnus, sartorius and semimembranous muscles of the right upper leg. (b) No changes were found in the lower legs.
Fig. 2. (a) Dystrophin immunostaining of muscle biopsy sections using C-terminal antibody: stars indicate dystrophin-negative fibers. (b) Western blot of muscle samples against dystrophin using C-terminal and Rod-domain antibodies: lane 1 corresponds to a normal control and lane 2 to the patient. A load control (200 kDa band) corresponding to Myosin heavy chain. Dystrophin was reduced with C-terminal antibody but not with Rod-domain antibody.

lacked HphI restriction sites, it was used as a reference fragment to establish a normal 43 : 44 ratio from HphI digested normal controls. This ratio was used to normalize the patient’s DNA samples and to establish a relative ratio of normal vs mutated alleles (Fig. 3b–d).

The QF-PCR conditions used were: 100 ng of DNA, 4 mM dNTPs, 10 pmol of each PCR primer, 1× reaction buffer, 2 mM MgCl₂, and 0.5 U Taq DNA polymerase. Cycling conditions consisted of 25 cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 1 min), followed by a final extension (72°C, 10 min).

Primer sequences for exon 43 were FAM-GCTACAGGAAGCTCTCTCC and TTCAGCTCATTTGTCTGATTG and, for exon 44 were TTGTGTGTACATC TAGGTGTG and FAM-CAAATCAAAGACTTACCTTAAGATACC.

Results

Pathological findings

Skeletal muscle showed mild variability in fibre size with some atrophic and hypertrophic fibres and a few necrotic fibres. Dystrophin immunostaining showed a population of negative fibres (Fig. 2a). The percentage of dystrophin-negative fibres oscillated from 4 to 12%, with an average of 8% in 900 fibres of different fascicles. Dystrophin-negative fibres showed secondary reduction of sarcoglycans and utrophin overexpression. Caveolin-3, dysferlin and emerin were well preserved. Western-blot analysis showed a reduction of dystrophin, particularly using the C-terminal antibody (Fig. 2b). A secondary reduction was observed for γ- and α-sarcoglycans.

Molecular findings

No deletions or duplications of the 79 exons of the DMD gene were identified in blood genomic DNA from the patient using the MLPA technique. Complete muscle cDNA sequencing detected two transcript species: a wild-type and a transcript carrying a c > u transition at position 6292 (r.[=,6292c>u]) (Fig. 3a). This change is predicted to cause a nonsense mutation (p.Arg2098X) in exon 44 and has been previously reported in 11 DMD and 1 BMD patients (www.dmd.nl). We detected both wild-type and mutated sequences in blood genomic DNA (Fig. 3a). The mutation was not observed in either the patient’s mother or sister genomic DNA.

Microsatellite markers showed a hemizygous haplotype in the patient and this was shared with his mother and sister. Patient karyotype was performed showing normal 46,XY pattern. These findings together with a mosaic dystrophin immunostaining pattern in muscle provide evidence of somatic mosaicism for the mutation.

The relative proportion of normal vs. mutant DMD alleles in blood and muscle DNA was calculated according to the method described above. Muscle DNA presented a lower proportion of mutant alleles (30%) than blood DNA (51%) (Fig. 3d).

Discussion

We describe a young male who presented high CK levels and severe DCM without accompanying skeletal muscle weakness. Immunostaining analyses of muscle biopsy showed a patchy dystrophin pattern and normal caveolin-3, dysferlin and emerin patterns, prompting the specific study of DMD gene mutations. Molecular analysis revealed a somatic mosaicism for a nonsense mutation in exon 44 (p.Arg2098X). This case is of special interest because of the combination of a pure DCM phenotype and the somatic mosaicism for a nonsense mutation in the DMD gene.

Seven males carrying somatic DMD mosaics have been reported to date, two of them ascertained after autopsy studies (9, 10). The five alive patients presented a mild or atypical BMD phenotype even though they carried truncating mutations. Muscle symptoms and severity were heterogeneous and included mild muscle weakness, calf hypertrophy and contractures. Muscle
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weakness was asymmetric in three of the patients and a predominant DCM was detected in four. The mutant allele ratio found in these patients suggests a mild correlation between the proportion of mutant cells in skeletal muscle and clinical severity (11–15).

Kesari et al. (11) reported a patient who presented elevated CK levels, mild muscle weakness and acute heart failure as a result of DCM. The proportion of the mutated allele was lower in muscle DNA (20%) than in blood DNA (50%). The authors referred to the loss of dystrophin-negative fibres and regeneration by dystrophin-positive stem cells as a genetic normalization process. They suggested it might explain the mild muscular phenotype and the difference in allele ratio between blood and muscle observed in their patient.

This genetic normalization process was first described in manifesting female carriers (17).

The clinical presentation in our patient only involved the heart. Clinical examination did not reveal any sign of muscle weakness in either upper or lower extremities. However, MRI studies showed mild asymmetric involvement of adductor magnus, sartorius and semimembranosus muscles and skeletal muscle biopsy revealed mild dystrophic changes. These findings, together with high elevated CK levels, indicate a subclinical pathologic process also involving the skeletal muscle.

The relative quantification of normal vs mutant alleles revealed discordance between blood (51%) and skeletal muscle cells (30%). This could be explained by random segregation of DMD-mutant stem cells during

Fig. 3. Molecular analysis of DMD gene. (a) Automated sequence analysis of DMD gene exon 44 in muscle cDNA and blood genomic DNA. Arrows indicate the mutation c.6292C>T (p.Arg2098X). Both wild-type and mutant sequences are present. (b) HphI digested PCR products of exon 44. The mutation creates an HphI restriction site, generating two additional bands in the patient’s DNA. (c) Multiplex QF-PCR electropherograms of HphI digested DNA. Amplicons corresponding to DMD exons 43 and 44 are shown. Note the different proportion of non-cleaved exon 44 in muscle and blood DNA from the patient compared to a normal control. (d) Relative ratios of normal vs. mutant alleles. These ratios were established normalizing QF-PCR peak areas with a normal 43 : 44 ratio obtained from HphI digested normal controls.
development or, as Kesari et al. proposed, by genetic normalization. In a genetic normalization process, dystrophin-negative fibres would present a negative selective pressure causing necrosis and consequent activation of satellite cells. Fusion of wild-type satellite cells and diffusion of dystrophin from normal nuclei could allow the progressive positivization of fibres and prevent myopathic symptoms. The amount of mutant DNA in skeletal muscle (30%) and the proportion of negative fibers (8%) in our patient may reflect this normalizing process.

A genetic normalization process would not occur in blood. As white blood cells regenerate but do not express muscular dystrophin (Dp427) a negative selective pressure against mutant blood cells would not exist. This correlates with the balanced ratio of normal vs mutant alleles (49 : 51) found in blood DNA. In contrast, although cardiac tissue expresses dystrophin it would not be able to develop genetic normalization because cardiomyocytes cannot regenerate. Both processes, differential segregation of mutant stem cells and genetic normalization in skeletal muscle, could explain the absence of limb weakness and the severe cardiomyopathy observed in our patient. We performed allele quantification in the only two available tissues, blood and skeletal muscle. Further analysis of DNA from other tissues, mainly from the heart, could help to determine the plausibility of a genetic normalization process mitigating skeletal muscle symptoms and to distinguish it from random segregation of stem cells during development.

Our patient presents similar features to Kesari’s patient (11). Both cases presented severe cardiomyopathy caused by somatic nonsense DMD mutations located in the rod-domain. The levels of mosaicism found in muscle and blood were very similar in both patients and suggest a genetic normalization in skeletal muscle. However, minor differences were observed concerning muscle weakness: Kesari’s patient presented mild weakness of deltoids and hip flexors muscles and no weakness was found in our patient. This slight difference could be attributed to differences in age, ethnic group and lifestyle.

The present case indicates that the XL-DCM phenotype can be caused by DMD mutations in somatic mosaicism. It also confirms that genetic normalization is a plausible mechanism to prevent or mitigate muscle symptoms in these patients. We suggest that genetic normalization requires a minimum amount of DMD-normal cells in skeletal muscle to progress towards positivization of muscle. Somatic mosaicism in dystrophinopathy is probably more frequent than reported but may go undetected because of asymptomatic or atypical clinical presentation.

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