X-linked Dilated Cardiomyopathy with Mutation in the 5′ Splice Site Intron 1 of Dystrophin Gene with Utrophin Upregulation

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J Pediatr Neurol 2018;16:29–34.

Abstract

We report a teenage boy presented with dilated cardiomyopathy (DCM) with no initial skeletal involvement and initial normal creatine kinases. One year after the heart transplantation, he had exercise-induced transient muscle weakness with elevated creatine kinases (CKs). Muscle biopsy showed normal structures and normal dystrophin immunohistochemical labeling, but utrophin, which is an autosomal homologue of dystrophin, was overexpressed at sarcolemma. Sanger sequencing confirmed a heterozygous c.31 + 1G > A, 5′ splice site point mutation at the first intron of dystrophin gene. A review of previous reports of patients with different point mutations in the same region, the first exon–intron boundary that involved the muscle promotor to exon 1, confirmed a high correlation of cardiospecific phenotype sparing the muscles with this specific site of mutations. The confirmation of upregulation of brain and Purkinje isoforms of dystrophin protein in the skeletal muscles but not in the heart in past studies help to explain the skeletal sparing presentation. X-linked DCM (XLDCM) is an important cause of isolated cardiomyopathy. Routine immunohistochemical staining study including dystrophin in cardiac muscle biopsy, and dystrophin and utrophin labelling on skeletal muscle biopsy for patients with subsequent muscle symptoms or raised creatine kinases, help in the early diagnosis of the XLDCM. Future experimental study to determine the aberrant pre-mRNA splicing of this specific splice site mutation involving exon 1 and intron 1 will help to understand better the underlying complex mechanism of the splicing regulation.

Keywords
- dilated cardiomyopathy
- X-linked dilated cardiomyopathy
- dystrophin gene
- utrophin upregulation
- splice site mutation

Introduction

Dilated cardiomyopathy (DCM) often leads to progressive heart failure with left ventricular dilatation, contractile dysfunction and early death, thereby making it a major indication for heart transplantation. Acquired causes include infection, myocarditis, exposure to toxins or cardiotoxic drugs, and metabolic and endocrine disturbances. Genetic mutations account for one third of cases and involve over 50 known gene defects that encode cytoskeletal, sarcomere, and

received
January 6, 2017
accepted after revision
May 22, 2017
published online
August 24, 2018

DOI https://doi.org/10.1055/s-0037-1603997.
ISSN 1304-2580.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

nuclear envelope proteins, among others, with different mutation changes.[1]

X-linked dilated cardiomyopathy (XLDCM) is caused by dystrophin gene mutation and has a cardiospecific phenotype characterized by selective cardiac involvement without overt skeletal myopathy. Previous studies in Japan and Italy have reported different percentage of patients with sporadic dilated cardiomyopathy having XLDCM that ranges from 3% to 13.6%.[2,3] X-linked dilated cardiomyopathy can be caused by different type of mutations across the whole dystrophin gene. The precise mechanism of how these different mutations in the dystrophin gene cause isolated cardiomyopathy sparing the skeletal muscles in the affected male patients and even asymptomatic female carriers are not fully understood.[4] On the other hand, different mutations of the dystrophin gene can also cause Duchenne muscular dystrophy or Becker muscular dystrophy with early presentation of skeletal myopathy with or without dilated cardiomyopathy when they enter adolescent age. While a precise relationship of the different dystrophin mutations with the exact mechanism causing XLDCM, a cardiospecific phenotype, remains unclear, a clear pathomechanism with absent dystrophin protein in the cardiac muscles causing DCM has been well proven.

We report a boy with isolated DCM, normal CK, and no sign of skeletal muscle involvement upon initial presentation. Subsequently, he developed transient muscle weakness and myalgia after exercise and infection with persistently raised CK leading to muscle biopsy study. The utrophin upregulation supports the suspicion of dystrophinopathy. Subsequent genetic diagnosis confirmed a novel 5′ splice site missense mutation in intron 1 that had not been reported at the time of dignosis. A review of the previous case reports of affected patients having mutations in the same region confirmed a highly consistent genotypic–phenotypic relationship with all patients having a cardiospecific phenotype and a unique dystrophin isoform expression in cardiac and skeletal muscles.

Case Report

The boy was born at full term with good past health. He loves sport and was an active member of his school rugby team. He complained of progressive exertional dyspnea since early teenage years and was subsequently diagnosed to have DCM. He received heart transplantation in our hospital when he was 15.5 years old in September 2009. His CK level measured 4 months before heart transplantation (CK: 93 U/L; normal $<$ 308 U/L) and 1 month after transplantation (CK: 109 U/L; normal $<$ 308 U/L) were both normal. Endomyocardial biopsy showed hypertrophic cardiomyocytes and focal interstitial fibrosis (►**Fig. 1A**). Immediately postcardiac transplantation, he was put on cyclosporine, mycophenolate mofetil and prednisolone, and subsequently switched to tacrolimus due to the side effects of cyclosporine. He had never been on statin. His cardiac function remained stable after the transplantation.

One year after the cardiac transplantation, he had an episode of severe myalgia associated with weakness after prolonged exercise in school. His muscle ache and weakness improved after rest for 2 days. The initial CK levels were raised persistently during admission at 2,534 and 1,759 U/L (normal $<$ 300 U/L). Two months later, he had another episode of limb weakness after onset of fever with coryzal symptoms. CK level was elevated up to 6,700 U/L but rapidly decreased down to 1,304 U/L when the weakness subsided after 2 days. Cardiac assessment including troponin I, electrocardiogram, echocardiogram, and chest X-ray were all normal. Metabolic workup including blood for lactate, pyruvate, carnitine and acylcarnitine profile, free fatty acid and amino acid pattern, as well as urine for organic acid, and autoimmune markers were all normal. Infective workup including cytomegalovirus pp65 was negative. When he recovered, he did not have any skeletal muscle weakness and examination did not show muscle atrophy or pseudohypertrophy. In view of the persistent raised CK level, neuromuscular workup was arranged. Nerve conduction study was normal. Muscle biopsy from left quadriceps showed only mild variation in fiber size and shape with occasional small fibers and internal nuclei (►**Fig. 1B**). There was no necrotic or regenerating fiber or inflammatory infiltrate, with only a very mild focal increase in interstitial fibrous tissues. Immunohistochemical staining showed preserved sarcolemmal staining with mouse monoclonal antibodies raised against the three domains of dystrophin including the C-terminal (NCL-DYS2, clone Dy8/6C5, Novocastra) (►**Fig. 1C**), rod domain (NCL-DYS1, clone Dy4/6D3, Novocastra) (►**Fig. 1D**) and N-terminal (NCL-DYS3, clone Dy/12B2, Novocastra) (►**Fig. 1E**). The insets represent normal controls for each of the three antibodies. There was, however, diffuse upregulation of utrophin at the sarcolemma (►**Fig. 1F**) when compared with normal control, in which the utrophin should only be present in blood vessels and not at the sarcolemma (►**Fig. 1F**, inset). Preserved membrane staining was also observed for merosin, alpha-sacroglycan, and beta-sacroglycan. Nuclear staining for emerin was normal. Ragged red fibers were not seen in Gomori trichrome and COX negative fibers were absent. Ultrastructural study showed preserved myofibrillar framework and normal-looking mitochondria.

Despite the dystrophin immunohistochemical staining findings being normal, possible defect in the dystrophin gene was suspected due to the significant utrophin upregulation and current clinical presentation. As a result, mutation analysis was arranged. There was no exon deletion or duplication from Multiplex ligation-dependent probe amplification of the dystrophin gene. Direct Sanger sequencing showed a hemizygous c.31 + 1G > A (Ref. sequence: NM_004006.2) change at the 5' splice site of intron 1 of the dystrophin gene.

When last examined on February 2016, the patient reported no skeletal muscle weakness. Examination by neurologist and physiotherapist confirmed normal muscle power of upper and lower limbs with no calf pseudohypertrophy. He could jump and run without any reported problem. His forced vital capacity in sitting position was 3680 cc (83% predicted). The latest CK level remained mildly elevated at

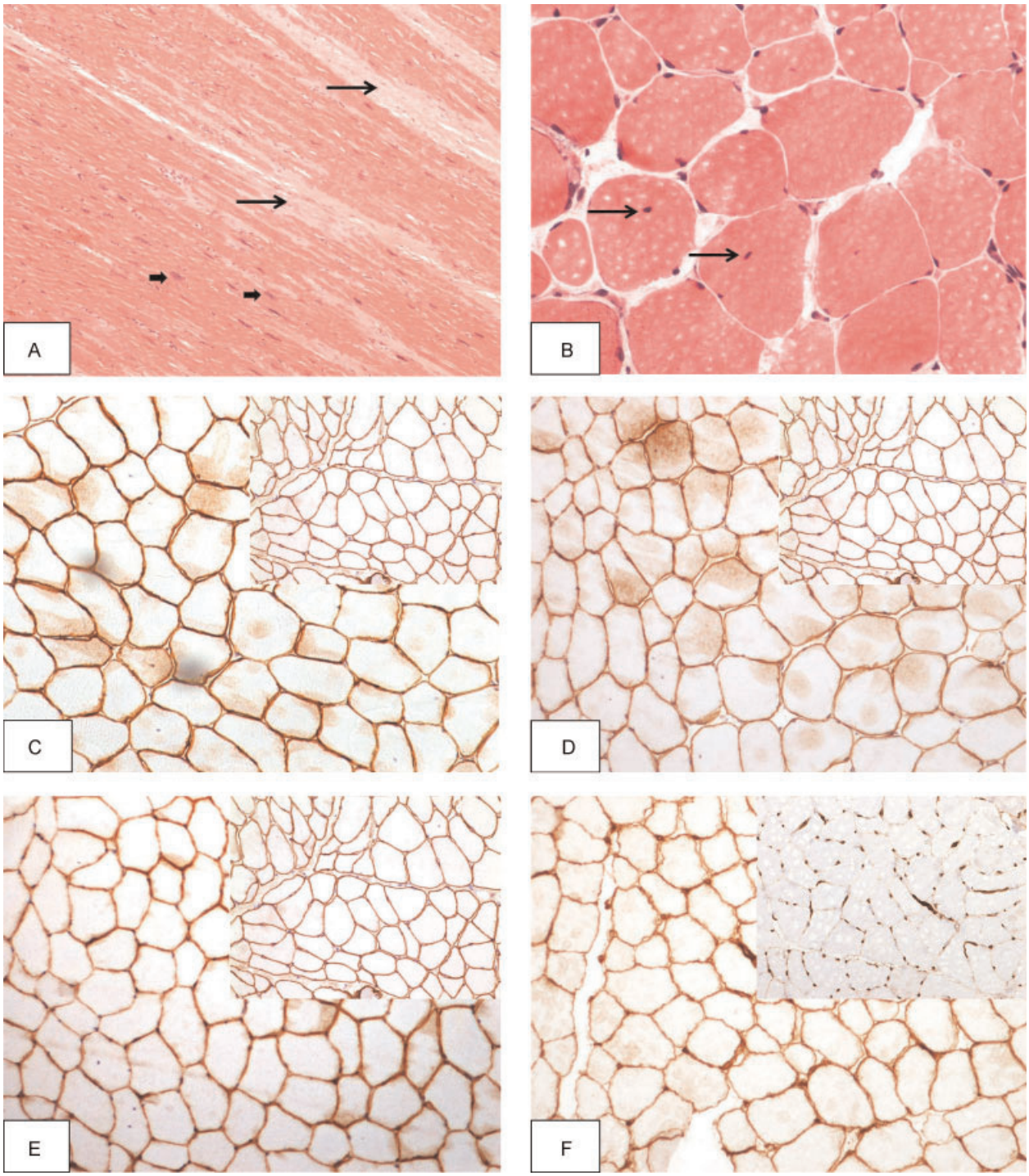

Fig. 1 (**A**) The explant heart showed myocyte hypertrophy (short arrows) and interstitial fibrosis (long arrows). (**B**) The haematoxylin and eosin (H&E) section of the left quadriceps biopsy showed mild myopathic changes with mild variation in fibre size and increased internal nuclei (long arrows) (400× magnification). (**C–E**) Immunostaining with antibodies raised against the C-terminal, rod domains, and N-terminal of dystrophin was all preserved and looked identical (200× magnification), as with control (inset). (**F**) There was diffuse upregulation of utrophin at the sarcolemma, which was only seen in vessels in normal control (inset) (200× magnifications).

1658U/L. The mother was confirmed as the asymptomatic carrier of the dystrophin mutation and her CK level was normal. The whole family has received genetic counseling including discussion of family screening to further identify the surrogate carriers and possible prenatal diagnosis for those confirmed carriers, to prevent recurrence in future pregnancies.

Discussion

We presented a boy with initial presentation of DCM requiring heart transplantation with no clinical muscle weakness, no calves pseudohypertrophy, and normal CK level. The two episodes of transient skeletal muscle weakness with myalgia, followed by the mildly elevated CK lead

to further neuromuscular workup. The significant upregulation of utrophin in the muscle biopsy despite the normal immunohistochemical staining with dystrophin antibodies, led to the suspicion of an underlying cause of XLDCM. Subsequent Sanger sequencing confirmed a splice site mutation in intron 1 of the dystrophin gene c.31+1G>A in our patient and the mutation had not been reported in literature at the time of diagnosis. Until recently in 2015 and 2016, this mutation has been reported in 2 unrelated patients with isolated cardiomyopathy.[5,6] Milasin et al also identified a mutation at the same site of the dystrophin gene mutation as our patient, but with a different nucleotide change, c.31+1G>T, in two adult males from the same family.[7] These two patients presented with idiopathic dilated cardiomyopathy, without obvious signs and symptoms of skeletal muscle involvement. Kimura et al again identified a mutation in the first exon-intron boundary of the muscle dystrophin isoform gene, c.31+5G>A, of which the site of mutation is very close to our patient (c31+1G>A) in a 13 year-old boy with dilated cardiomyopathy without skeletal myopathy.[8] The endomyocardial biopsy of these three patients showed absent dystrophin. The skeletal muscle biopsy performed for these 3 patients due to raised CK showed positive immunoreactivity with continuous labelling to antibodies targeting the N-terminus, mid-rod, and C-terminus of dystrophin but all with reduced intensity (►**Table 1**).

Muntoni and his group[9] studied the normal human muscles and found that only the muscle dystrophin isoform was transcribed and expressed in both the skeletal muscles and the myocardium. There was also brain dystrophin isoform expressed in the skeletal muscle but at a lower level compared with that in the brain. Bastianutto et al, on the other hand, observed the expression of Purkinje isoforms and brain isoforms in the skeletal muscles.[10] Holder et al demonstrated that up to 20% of the mRNA transcripts in the skeletal muscle were Purjinje isoforms.[11]

Early in 1993, three patients with severe cardiac dysfunction who had absence of significant muscle weakness were reported to have deletion limited to the 3′-end of exon 1 and part of intron 1 of the dystrophin gene by two different groups.[12,13] Muntoni and his group studied the two patients who had mutations in similar regions with deletion of the 3′-end of exon 1 and part of intron 1 with isolated dilated cardiomyopathy and noted activation of expression for both brain and Purkinje dystrophin isoforms in skeletal muscle.[9] Milasin et al, again studied the dystrophin isoforms expression in both endomyocardial and skeletal muscle biopsies of his patient with XLDCM with mutation c.31 +1G>T. This was performed through isolation of the total RNA from frozen endomyocardial and skeletal biopsies, followed by reverse transcription and polymerase chain reaction (PCR) amplification using the three isoform specific primers, to study the expression of major dystrophin mRNA isoforms (muscle-, brain-, Purkinje-dystrophin isoforms from the muscle-, brain-, and Purkinje cell-promoters). The group found complete absence of all the 3 dystrophin isoforms in the endomyocardial muscles of the affected patient. The brain- and Purkinje cell dystrophin mRNA isoforms, however, were detected in the skeletal muscles but not the muscle dystrophin isoform.[7] Kimura et al again performed similar study on his patient with XLDCM with a mutation in the first exon-intron boundary of the muscle dystrophin isoform gene, c.31+5G>A. Reverse transcription-PCR analysis again showed that all muscle, brain, and Purkinje isoforms were absent in the cardiac muscle but the brain and Purkinje muscle dystrophin isoforms were found in the skeletal muscle of the patient, but not the muscle dystrophin isoform.[8] These findings suggest that the brain and Purkinje dystrophin isoforms have an important ability in maintaining the function of skeletal muscles, and they appear to be crucial in preventing a skeletal myopathy.

Bastianutto et al tried to study how these non-muscle dystrophin isoforms (brain and Purkinje dystrophin isoforms) are upregulated in skeletal muscles through the non-muscle promoters. The team studied two patients with XLDCM who had deletions that extended through muscle (M) promotor and exon 1 of the dystrophin gene, with preserved first exons of the brain and Purkinje isoforms flanking muscle exon 1. With the deletions taking away the M promoter and exon 1, the group observed that preserved dystrophin muscle enhancer 1 (DME1) located in intron 1 led to an increase in brain and Purkinje promoter activity in the skeletal muscles, but not in the cardiac muscles. Sequences essential for dystrophin gene expression in cardiac muscle is predicted to lie within the region deleted in these 2 patients, while the enhancing elements involved in brain and Purkinje promoter activation in skeletal muscle must be located outside the deletion.[10] What specific regulatory mechanism is involved in this skeletal-muscle-specific activation, and whether other enhancers in addition to DMEI are involved, are important questions that have yet to be answered.

►**Table 1** summarizes more detailed findings of the patients who were reported in the previous studies[6–8,14] and have mutations in dystrophin gene involving N-terminal region abolishing the 5′ splice site consensus sequence of the first intron. All of them presented as an isolated dilated cardiomyopathy with apparent no skeletal muscles involvement on initial presentation. The creatine kinase levels were often within normal limits initially. There was no calf pseudohypertrophy. Some patients developed exercise-induced myalgia, myoglobinuria, or transient weakness, some years after the cardiac presentation. Muscle biopsies of skeletal muscle with immunohistochemical staining showed continuous labelling with the anti-dystrophin antibodies against the N-terminal (DYS3), mid-rod domains (DYS1), and the C-terminal (DYS2). However, on immunofluorescent labelling the immunoreactivity appeared evidently less intense compared to the control. Utrophin expression was not studied.[6–8] In contrast, our patient has clear near-normal immunohistochemical staining of dystrophin using antibodies raised against the three domains (►**Fig. 1C–E**). It was the utrophin upregulation shown in the muscle biopsy of our patient that led to the genetic diagnosis. Utrophin is the protein product of an autosomal homologue of the dystrophin gene. In normal skeletal muscle, it is expressed in vascular smooth muscle, endothelium and nerves, and in selected mature

Table 1 This table illustrates all previous reported mutations in families of different ethnic origin and our patient with mutation in the 5′ splice site intron 1 of the dystrophin gene causing X-linked dilated cardiomyopathy

Subject	II1	II2 (brother of II 1)	DCM 27	Son of DCM 58	3	38	Our patient
Status	Affected male (Italian)	Affected male (Italian)	Affected male (African)	Affected male (African)	Affected male (Caucasian)	Affected twin 2 (Polish)	Affected male (Chinese)
First symptom	Heart failure	Chest pain	Heart failure	Heart failure	Heart failure	Heart Failure	Heart failure
Age at Dx of DCM	24	32	11	12	13	5	15
Serum CK	Normal	244–488 (ref:<170) U/L	Not mentioned	Not mentioned	969 (ref: 57–284) U/L	Not mentioned	Normal initially. ↑ since age 16 1658–2545 (ref.:65–355) U/L
Endomycardial biopsy	Dystrophin not done	Absent of dystrophin	–	–	Absent of dystrophin	–	Dystrophin not done
Skeletal biopsy	—	Minimal changes Continuous labeling on all fibers for IHC using antibodies toward DYS3, DYS1, DYS2, but the intensity of fluorescent labeling was obviously paler compared with control muscle	–	–	Moderate variation in fiber size Continuous labeling on all fibers for IHC using antibodies toward DYS3, DYS1, DYS2, but the intensity of fluorescent labeling was clearly paler compared with control muscle.	Unspecific change	Minimal changes Continuous labeling on all fibers for IHC using antibodies toward DYS3, DYS1, DYS2 was clearly similar to control muscle Utrophin upregulation
Reason for muscle biopsy	—	↑ CK urine pigmentation after physical exercise, sporadic myalgias	–	–	↑ CK	–	↑ CK, one year after heart transplantation: also transient muscle weakness and myalgia after physical exercise or infection
Persistent muscle weakness	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Calves pseudohypertrophy	Nil	Nil	Nil	Nil	Nil	Not mentioned	Nil
Management	Heart transplantation Died 2 years later	Medical treatment Long term outcome not known	Medical treatment Died suddenly	Died suddenly	Not mentioned	Heart transplantation	Heart transplantation Post transplantation, well for past 8 years
Genetic findings	c.311+1G>T	c.31 +1G>T	c.31 +1 G>T	c.31 +6 T>C	c.31 +5G>A	c.31 +1G>A	c.31 +1 G>A (confirmed 2013)
Reference	Milasin J et al 1996[7]	Milasin J et al 1996[7]	Feng J et al 2002[14]	Feng J et al 2002[14]	Kimura S et al 2007[8]	Pronicka E et al 2016[6]	This case report

Abbreviations: Dx of DCM, diagnosis of dilated cardiomyopathy; DYS3, DYS1, DYS2, antidystrophin antibodies against N-terminal (DYS3), rod-domain (DYS1), and C-terminal (DYS2) on muscle biopsy; IHC, immunohistochemical staining.

muscle fibers mainly at the neuromuscular and myotendinous junctions. It is also expressed on the sarcolemma of fetal muscle fibers and regenerating fibers. Utrophin upregulation or over-expression on mature fibres has frequently been reported in muscular dystrophy and is regarded as a diagnostically helpful secondary immunohistochemical marker for the detection of dystrophinopathies. It can also be seen in inflammatory myopathies and some other disorders.[15] Interpretation must therefore take into account the patient's age, clinical presentation, and the presence or absence of fibre regeneration.

For all patients with isolated dilated cardiomyopathy even with normal CK levels, we recommend routine dystrophin immunohistochemical staining on cardiac muscle biopsy when patients undergo heart transplantation to facilitate the early diagnosis of XLDCM. The absence of dystrophin in the endomyocardial biopsy will confirm the diagnosis. For those patients with intermittent muscle symptoms or raised creatine kinases, both dystrophin and utrophin immunohistochemical staining study for the muscle biopsy study is recommended if XLDCM is suspected. As in our patient, dystrophin immunohistochemical staining could appear normal, and the utrophin upregulation or over-expression, as a secondary helpful diagnostic marker, gave important diagnostic clue. An early confirmed diagnosis guides the long term medical care and further assist in identifying the surrogate carriers and genetic counseling in the family.

Our patient continued to enjoy good general health with full participation of regular physical activities 8 years after heart transplantation. Having said this, it is uncertain whether he will develop more muscle weakness later in life as a mild Becker muscular dystrophy phenotype[16] and this can only be confirmed with further follow-up. However, there is still a significant discrepancy between the cardiac and skeletal muscle involvement, as clinical weakness affecting daily physical activities are not noted for this group of patients.

In conclusion, XLDCM is a unique phenotype of dystrophinopathy with predominant cardiac specific involvement without skeletal myopathy. The high correlation of dystrophin gene mutation in the first exon and intron boundary with a cardiospecific phenotype having upregulated expression of the brain and Purkinje dystrophin isoforms in the skeletal muscle but not in heart, suggests that the deleted sequences from the aberrant splicing of these splice site mutations with the removal of the M promotor control the shutdown of muscle dystrophin isoform expression in both skeletal and cardiac muscles. It is not precisely understood how the observed selective 'rescue' transcription of the brain and Purkinje cell-specific dystrophin isoforms in skeletal muscles - as opposed to in the heart - is secondary to the absence of muscle isoform in the skeletal muscles or the activation of both brain and Purkinje promoters through the Dystrophin muscle enhancer 1 (DME1) or other enhancers. Further experimentation to identify and characterise the missing sequences from pre-mRNA splicing for these splice site mutations will give insight to the development of potential gene editing intervention as possible therapeutic approach.

Conflict of Interest

None.

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