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Mitochondrial dysfunction in myofibrillar myopathy

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Abstract

Myofibrillar myopathies (MFM) are characterised by focal myofibrillar destruction and accumulation of myofibrillar elements as protein aggregates. They are caused by mutations in the *DES*, *MYOT*, *CRYAB*, *FLNC*, *BAG3*, *DNAJB6* and *ZASP* genes as well as other as yet unidentified genes. Previous studies have reported changes in mitochondrial morphology and cellular positioning, as well as clonally-expanded, large-scale mitochondrial DNA (mtDNA) deletions and focal respiratory chain deficiency in muscle of MFM patients. Here we examine skeletal muscle from patients with desmin (n = 6), ZASP (n = 1) and myotilin (n = 2) mutations and MFM protein aggregates, to understand how mitochondrial dysfunction may contribute to the underlying mechanisms causing disease pathology. We have used a validated quantitative immunofluorescent assay to study respiratory chain protein levels, together with oxidative enzyme histochemistry and single cell mitochondrial DNA analysis, to examine mitochondrial changes. Results demonstrate a small number of clonally-expanded mitochondrial DNA deletions, which we conclude are due to both ageing and disease pathology. Further to this we report higher levels of respiratory chain complex I and IV deficiency compared to age matched controls, although overall levels of respiratory deficient muscle fibres in patient biopsies are low. More strikingly, a significantly higher percentage of myofibrillar myopathy patient muscle fibres have a low mitochondrial mass compared to controls. We concluded this is mechanistically unrelated to desmin and myotilin protein aggregates; however, correlation between mitochondrial mass and muscle fibre area is found. We suggest this may be due to reduced mitochondrial biogenesis in combination with muscle fibre hypertrophy.

Keywords: Myofibrillar myopathy; Mitochondria; Mitochondrial DNA deletion; Immunofluorescence; Histochemistry; Cytochrome *c* oxidase deficiency

1. Introduction

Myofibrillar myopathies (MFMs) are a group of myopathies characterised by aggregation of the Z-disk proteins and focal myofibrillar destruction. MFMs or Z-disk diseases are clinically and genetically heterogeneous. Mutations causing MFM can be found in genes encoding a range of Z-disk proteins including; desmin (*DES*) [1], αB-crystallin (*CRYAB*) [2], myotilin (*MYOT*) [3], filamin C (*FLNC*) [4], ZASP (*LDB3/ZASP*) [5] and Bcl2-associated athanogene 3 (*BAG3*) [6]. Novel MFM causing genes are continuously being identified and at present only 50% of suspected MFM cases have a genetic diagnosis.

They are typically present in the third or fourth decade of life or later, though rare cases of adolescent onset have also been reported. Most commonly they present as distal myopathies initially, progressing to involve proximal limb muscles. However, myofibrillar myopathies can vary in clinical presentation and can lead to cardiomyopathy associated with skeletal muscle myopathy or isolated cardiomyopathy. Key clinical patterns in presentation can be noted for some of the causative genes; however, once more, these are not clear cut and there are exceptions.

Mitochondrial dysfunction is a common finding in many proteinopathies including protein aggregate myopathies (PAMs) [7–15], and some neurodegenerative conditions [16]. Cytochrome *c* oxidase (COX) deficiency has been found to be associated with clonally-expanded mtDNA rearrangements in sarcopenia, patients with single or multiple mitochondrial DNA (mtDNA) deletions and inclusion body myositis. However, mtDNA point mutations [17] and depletion of mtDNA copy number [18] may also lead to focal respiratory chain deficiency.

Previous reports have noted that both mitochondrial morphology and positioning are altered in muscle of MFM

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patients [19], as well as the presence of clonally-expanded large-scale mtDNA deletions [20,21] and focal respiratory chain deficiency [22,23]. However, there have only been a few previous publications to date and only small numbers of patients reported in these studies. As such it is unclear what role, if any, clonally expanded mtDNA deletion and mitochondrial respiratory chain deficiency may play in the pathogenesis of myofibrillar myopathy.

In this paper we investigate a cohort of nine genetically determined MFM patients for evidence of mitochondrial dysfunction. From this cohort, we further selected a group of six patients based on the frequency of COX-deficient fibres identified by sequential COX/SDH histochemistry to study for presence of clonally-expanded large-scale mtDNA deletions. We also subjected all patient muscle biopsies to a recently developed immunofluorescence assay [24] to assess NDUFB8 (complex-I) and COX-I (complex-IV) protein levels normalised to mitochondrial mass (Porin).

2. Materials and methods

2.1. Patient cohort

Muscle biopsies were taken for diagnostic purposes from patients with a suspected neuromuscular condition (see Table 1). Informed consent was obtained from all patients in the study. Ethical approval was granted by the Newcastle and North Tyneside local research and ethics committee (LREC2002/205). Inclusion criteria were the presence of protein aggregates on sections stained with anti-desmin and anti-myotilin antibodies and confirmed molecular diagnosis for MFM genes. We studied nine MFM patients with mutations in *DES* (n = 6), *MYOT* (n = 2) and *ZASP* (n = 1). A patient with compound heterozygous *RRM2B* variants, leading to a disturbance of mtDNA maintenance and multiple mtDNA deletions [25], was used as a positive control, as well as two healthy control muscle biopsies from individuals aged 52 and 63 years.

2.2. Histochemistry

Muscle was cryosectioned (10 μm) from transversely-orientated muscle blocks and subjected to histochemical reactions for the individual activities of COX, SDH and the sequential assay of COX/SDH activity [26]. COX-deficient fibres were counted using a stereomicroscope, by outlining the full muscle biopsy section and using the meander scan function in Stereo Investigator. Fibres were categorised by eye with blue fibres being categorised as COX negative, blue-grey fibres as intermediate negative and grey-brown fibres as intermediate positive. Intermediate categories were included to make comparison with immunofluorescent quantification easier. The minimum number of fibres counted was 127, which was for patient DES3 who had the smallest muscle biopsy section. Serial sections were stained with Haematoxylin and Eosin (H&E) to examine muscle morphology.

2.3. Quadruple immunofluorescence

A quadruple immunofluorescent method using antibodies recognising laminin (membrane marker), NDUFB8 (complex I marker), COX-I (complex IV marker), and porin as a mitochondrial mass marker, was employed to assess the degree of respiratory chain deficiency [24]. Briefly, one 10 μm cryosection from each patient and age matched controls was labelled with all four antibodies and a serial 10 μm section with just laminin to be used as a negative (no primary antibody) control. Sections were incubated with secondary antibodies coupled with Alexa Fluor 405, 488, 546 and 647 (Life Technologies). Using a Zeiss AxioImager and AxioCam MRm with AxioVision software tiled images scanning of the full section were acquired and stitched using ZEN (blue edition). Analysis using IMARIS version 7.6 involved creating a surface over the laminin staining, which was masked, allowing a second surface covering each fibre to be created and average fluorescent intensity for each muscle fibre to be collected.

Using values from the negative (no primary antibody) control for each case, non-specific background was subtracted and NDUFB8 and COX-1 intensity was normalised to porin intensity as the mitochondrial mass marker. Control case values then created a "normal population" for which the patient fibres could then be assigned a z-score describing their deviation from the normal population. Statistical analysis is described in more detail by Rocha et al. [24]. Total fibres analysed was dependent on section size and ranged from n = 111 to n = 1883 in patients and controls.

Table 1
Myofibrillar myopathy patient information (n = 9). Patients are organised by gene then age order and all mutation nomenclature uses the primary transcripts for *DES* (NM_001927) and *MYOT* (NM_006790) and for *ZASP* transcript variant 2 (NM_001080114.1).

Patient	Gender	Mutated gene	Mutation	CK	Biopsy site	Age at biopsy	Age at onset (if known)
DES 1	M	*DES*	Het. c.1069G > C, p.(Ala357Pro)	480	Quadriceps	31.2	29
DES 2	M	*DES*	Het. c.1069G > C, p.(Ala357Pro)	NK	NK	32.1	NK
DES 3	M	*DES*	Hom. c.46C > T, p.(Arg16Cys)	450	Quadriceps	33.8	20s
DES 4	F	*DES*	Het. c.735 + 20C > T (MAF = 0.01); c.638C > T, p.(Ala213Val)	NK	Quadriceps	51.3	Early childhood
DES 5	F	*DES*	Het. c.638 C > T, p.(Ala213Val)	74	Quadriceps	53	40s
DES 6	F	*DES*	Het. c.1346A > C , p.(Lys449Thr)	287	NK	64.3	46
MYOT 1	M	*MYOT*	Het. c.179C > G, p.(Ser60Cys)	446	Quadriceps	60.5	55
MYOT 2	M	*MYOT*	c.179C > G, p.(Ser60Cys)	400–500	Tibialis anterior	65.7	NK
ZASP 1	F	*ZASP*	Het. c.494C > T, p.(Ala165Val); Het. c.728C > T, p.(Pro243Leu)	300	Quadriceps	69.6	60s

DES: desmin; MYOT: myotilin; Hom.: homozygous; Het.: heterozygous; MAF: maternal allele frequency; CK: creatine kinase; NK: not known.