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Clinical Study

Enzyme Histochemical Assessment of Mitochondrial Functions in Patients with Myopathic form of Limb-Girdle Syndrome

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Isolated mitochondrial myopathy is characterized by slowly progressive limb-girdle muscle weakness and resembles other muscle disorders like muscular dystrophy or inflammatory myopathy on clinical grounds. Identification of abnormal mitochondria in the muscle tissue is required for the diagnosis of isolated mitochondrial myopathy. Therefore, this study was done with aim to identify patients with isolated mitochondrial myopathy among those with limb-girdle muscle syndromes of undefined cause. Forty-eight consecutive patients with limb-girdle muscle disease from 2008 to 2010 were screened for Duchenne/Becker muscular dystrophy gene deletion, metabolic myopathy, and drug-induced and endocrine causes. Twenty patients without an identifiable cause were subjected to muscle biopsy for hematoxylin and eosin staining and enzyme histochemistry. Clinical, biochemical, and electrophysiological features in all these patients with limb-girdle muscle disease were nonspecific, and no conclusion regarding the underlying cause could be drawn from these investigations. On hematoxylin and eosin staining, 12 patients were diagnosed as muscular dystrophy, inflammatory myopathy with characteristic appearance of polymyositis was diagnosed in 4 patients, and 3 patients had normal muscle histology. After enzyme histochemistry, one patient was identified having mitochondrial myopathy. A brief case summary of the only patient diagnosed as isolated mitochondrial myopathy in our study is presented.

1. Introduction

Mitochondrial syndromes are a group of heterogeneous disorders that affect multiple organ systems. Most mitochondrial syndromes like mitochondrial encephalopathy with lactic acidosis and stroke (MELAS) are clearly defined clinical syndromes which are easily identifiable and can be confirmed by identifying specific genetic mutations in the mitochondrial DNA. However, few newer mitochondrial syndromes like isolated mitochondrial myopathy are increasingly being [1]. These patients present with slowly progressive or fluctuating severity of limb girdle muscle weakness with or without lactic acidosis. The disorder is often sporadic and is acquired after normal birth and unremarkable early development. In the absence of a known underlying genetic defect in the mitochondrial DNA in these patients with isolated mitochondrial myopathy, the diagnosis requires identification of ragged red fibres on histochemical examination of muscle biopsy specimen [2]. The present study aims to recognise mitochondrial myopathy among patients of myopathic form of limb girdle weakness without an attributable cause by using enzyme histochemical examination of their muscle biopsy specimen and to describe the clinical, biochemical, and histopathological spectrum of these patients.

2. Material and Methods

All consecutive patients attending the outpatient department of Neurology at Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, from November, 2008 to October, 2010 with limb-girdle weakness attributable to muscle disease on the basis of clinical, biochemical, and electrophysiological tests were included in the study. Patients with characteristic clinical features suggestive of muscular dystrophy (e.g., facio-scapulo-humeral muscular dystrophy, congenital muscular dystrophy, congenital

myopathy, those with areas of hypertrophy and atrophy in the same muscle, and those with gene mutations positive for Duchenne/Becker muscular dystrophy), inflammatory myopathy (with characteristic skin rash of dermatomyositis, associated immunological disease or malignancy), drug-induced myopathy, osteomalacia associated myopathy, and endocrine myopathy (hypothyroid, hyperthyroid, steroid induced, Cushing's disease, and acromegaly) were excluded.

Forty-eight patients with limb girdle muscle disease were seen in the above-mentioned period. Twenty-eight patients were excluded (10 patients who tested positive for gene deletion study for Duchenne muscular dystrophy, 12 patients having osteomalacic myopathy, 4 with hypothyroidism-associated myopathy and 2 patients with steroid-induced myopathy). Twenty patients were included in the study.

All 20 patients included in the study were subjected to a muscle biopsy.

2.1. Muscle Biopsy. Muscle biopsy from either quadriceps (18) or gastrocnemius (2) muscle was obtained under local anaesthesia after obtaining written consent from the patients/relatives. Each muscle biopsy was received fresh in the department of pathology without any additive or fixative. One small piece was fixed in 10% buffered formalin for routine processing and paraffin embedding for hematoxylin and eosin stain. A second piece was immediately snap frozen in precooled isopentane at -80°C. 5-6 μm thick serial sections were obtained on poly-L-lysine coated slides for enzyme histochemistry, periodic acid Schiff stain and oil-red-O staining.

2.2. Staining. Apart from routine hematoxylin and eosin stain, various other histochemical stains were done to identify the muscle disease. These included modified gomori trichrome, oil-red-O, periodic acid Schiff, nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), succinic dehydrogenase (SDH), and cytochrome oxidase (COX).

3. Results

3.1. Clinical Examination. Patients ranged in age from 8 to 58 years with male to female ratio of 1 : 1. The duration of illness varied from 4 months to 15 years. In addition to progressive shoulder and hip girdle weakness, one patient each had prominent distal weakness, neck flexor weakness, ptosis with external ophthalmoplegia, and bulbar weakness. Other features like retinitis pigmentosa, cardiomyopathy, seizures, and muscle cramps were also seen occasionally (Table 1). Two patients had family history of similar myopathy in a sibling, suggesting an autosomal recessive pattern. Total creatine phosphofructo kinase (CPK) ranged from normal to as high as 7000 IU/mL. Resting state and postexercise lactate was normal in all the patients. None of the patients had evidence of rhabdomyolysis or myoglobinuria. All patients had normal nerve conduction tests. Electromyography showed small polyphasic motor unit action potentials (MUAPs) on minimal volition in all but two patients who had normal MUAPs. In addition, four patients had signs of muscle membrane irritability in form of positive sharp waves or fibrillations.

3.2. Pathological Examination. Fourteen patients showed maintained fascicular architecture. Fibre size variability was seen in 19 patients, which was more marked in three patients. Necrotic and regenerating fibres were seen in two patients and marked fibrosis was evident in three patients. Interstitial inflammation was seen in seven patients. Fibre type grouping was not identified in any biopsy. Ragged red fibres on modified Gomori Trichrome stain with subsarcolemmal dense staining on NADH/SDH and COX negative fibres were seen in one patient. Criteria used for diagnosis of mitochondrial myopathy were evidence of almost 5% ragged red fibres in 100x magnification field. Histopathological diagnosis was given as muscular dystrophy in 12 patients, inflammatory myopathy in 4 with characteristic appearance of polymyositis in one patient, mitochondrial myopathy in one patient, and normal muscle histology in 3 cases. A brief case summary of the only patient diagnosed as isolated mitochondrial myopathy in our study is presented.

4. Case Summary

A 35-year-old male complained of progressive symmetrical eyelid drooping, first noticed 20 years ago, and proximal limb weakness for last 5 years. Drooped eyelids covered half of sclera resulting in compensatory neck extension for distant vision. Limb weakness was progressive, producing difficulty in standing from a chair and running. Upper limb weakness was noticed for a year especially on lifting a weight overhead. There was no history of orthopnea, paroxysmal nocturnal dyspnoea, chest pain, pedal oedema, diurnal variation of muscle weakness, diplopia, thinning of limbs, or muscle cramps, impairment in vision, or hearing loss. Patient's younger sibling, who was 28-year-old, also had a similar drooping of both eyelids for 15 years and dyspnoea on exertion for two years. Dyspnoea was noticed at the beginning of uphill walking or climbing stairs and also after a brisk walk for fifty meters or more. He never noticed any limb weakness. Patient had three female siblings (24, 16, and 12 years old, resp.) who were normal. Patient's parents and their siblings did not have similar complaints. They had a consanguineous marriage. Patient's examination revealed incomplete ptosis, external ophthalmoplegia, normal pupil size and light reaction, and normal fundus examination. The rest of the cranial nerves were normal. Mild atrophy of bilateral deltoids, infraspinatus, biceps, first dorsal interossei, quadriceps, and gastrocnemius muscles was noticed. Bilateral winging of scapulae (rhomboids type) was present. Muscle power was grade 3/5 (Medical Research Council scale) in hip and shoulder girdle muscles and grade 4/5 in distal limb muscles. All deep tendon reflexes were reduced, and both plantars were flexors. The rest of the neurological and systemic examination was normal. Examination of patient's brother revealed similar ocular findings. Similar pattern of muscle atrophy was present, but winging of scapulae was not seen. His muscle power was grade 4/5 in

Table 1: Clinical, biochemical and electrophysiological characteristics of patients with limb-girdle muscle weakness.

N	Age (years)	Sex	Duration of illness (months)	Pattern of weakness	Family history	Others	CPK (IU/mL)	EMG
(1)	8	M	36	Proximal	Of IGE (AD)	Ret pig	75	Myopathic
(2)	14	M	96	Proximal + distal	AR	Iliotibial contractures	1243	Myopathic, fibs
(3)	33	M	120	Proximal > distal	—	DCMP	2439	Myopathic
(4)	20	F	60	Proximal + distal	—	Ankle contractures Cramps, GTCS	528	Myopathic
(5)	12	F	60	Mild proximal	—	Pes cavus	740	Normal
(6)	34	F	180	Proximal + distal	—	Elbow knee ankle contract.	179	Myopathic
(7)	16	M	108	Proximal + distal	—	—	1443	Myopathic
(8)	20	M	84	Proximal > distal	—	Skin changes	1441	Myopathic
(9)	58	F	24	Proximal + distal + neck flexor	—	—	600	Myopathic, fibs, PSW
(10)	48	F	120	Proximal	AR	—	1817	Myopathic
(11)	11	M	84	Proximal > distal	—	Ichthyosis, hyperkeratotic skin, contractures b/l knee, elbow, ankle joints, hepatomegaly	2500	Myopathic
(12)	35	M	36	Proximal > distal, external ophthalmoplegia	AR	HOCM	110	Myopathic
(13)	15	F	60	Mild proximal + distal	—	Muscle cramps (rest)	96	Myopathic
(14)	29	F	8	Proximal > distal	—	—	407	Myopathic, PSW. Fibs
(15)	24	M	5	Proximal > distal	Type 2 DM in father	—	2500	Myopathic
(16)	26	F	8	Proximal > distal, bulbar	—	Recurrent oral ulcers	191	Myopathic RNS −ve
(17)	45	F	4	Proximal > distal	—	—	1628	Myopathic, PSW, Fibs
(18)	37	F	12	Proximal > distal	—	—	1774	Normal
(19)	8	F	7	Proximal > distal	—	—	7128	Myopathic
(20)	55	M	10	Proximal > distal	—	Chronic alcoholic and smoker, hypertension 1 year	109	Myopathic

AR—autosomal recessive, AD—autosomal dominant, CPK—creatine phosphokinase, DM—diabetes mellitus, DCMP—dilated cardiomyopathy, F—female, fibs—fibrillations, GTCS—generalized tonic-clonic seizures, IGE—idiopathic generalised epilepsy, M—male, PSW—positive sharp waves, RNS—repetitive nerve stimulation.

proximal and grade 5/5 in distal limb muscles. Tachycardia and S3 gallop rhythm was noticed on cardiac auscultation. The rest of the systemic examination was normal. Investigations revealed normal haematological and biochemical blood parameters including total CPK and CPK-MB, and pre- and postexercise blood lactate. Nerve conduction tests were normal. Electromyography revealed small polyphasic motor unit action potentials without any spontaneous activity and early complete recruitment on forced voluntary muscle activity. Echocardiography showed presence of hypertrophic and restrictive cardiomyopathy with ejection fraction of 35%. Muscle biopsy findings (Table 2) were positive for ragged red fibers with several COX negative muscle fibers suggestive of mitochondrial myopathy (Figure 1).

5. Discussion

The first patient with isolated mitochondrial myopathy was recognized by Coleman in the year 1967 [3]. Since then, a number of patients have been described and different workers have recognized different mitochondrial gene mutations which have been considered as responsible for the disease of their patient/s. Some new gene mutations were also found in patients with isolated mitochondrial myopathy in addition to the already known mutations which are also seen in other well-defined mitochondrial diseases like mitochondrial encephalopathy with lactic acidosis and stroke (MELAS). Mitochondrial gene polymorphism in patients with isolated mitochondrial myopathy has limited the diagnostic utility of genetic testing in these patients. On consideration of clinical, biochemical and electrophysiological findings, the patients with genetically proven isolated mitochondrial myopathies (Table 3) [4–10] are similar to our patients (Table 1) who turned out to be either muscular dystrophy or inflammatory myopathy on histopathology. Exercise intolerance, myalgias, elevated lactate, and creatine kinase are nonspecific features seen in muscular dystrophies, inflammatory myopathies, aminoaciduria and mitochondrial disorders.

Table 2: Histopathological and enzyme histochemical profile of patients with limb-girdle muscle weakness.

No	H and E					NADH/SDH/COX	MGT	ORO/PAS	Diagnosis
	Architecture	Fiber size variation	Interstitial fibrosis	Central nuclei	Interstitial inflamm.	Fiber type grouping	Ragged red fibers	Fat/glycogen deposition	
1	Maintained	Present (foal)	Mild	Absent	Absent	Absent	Absent	Absent	Dystrophy
2	Maintained	Present	Absent	Absent	Mild	Absent	Absent	Absent	Dystrophy
3	Distorted	Marked	Present	Present	Absent	Absent	Absent	Absent	Dystrophy
4	Distorted	Moderate	Present	Absent	Absent	Absent	Absent	Absent	Dystrophy
5	Maintained	Absent	Occasionl	Absent	Absent	Absent	Absent	Absent	Normal
6	Maintained	Present	Mild	Absent	Absent	Absent	Absent	Absent	Dystrophy
7	Distorted	Marked	Marked	Absent	Absent	Absent	Absent	Absent	Dystrophy
8	Distorted	Marked	Marked	Few	Absent	Absent	Absent	Absent	Dystrophy
9	Maintained	Minimal	Minimal	Absent	Minimal	Absent	Absent	Absent	Inflammatory
10	Maintained	Present	Minimal	Absent	Absent	Absent	Absent	Absent	Dystrophy
11	Maintained	Present	Present	Absent	Absent	Absent	Absent	Absent	Dystrophy
12	Maintained	Present	Absent	Present	Absent	Hyperstained NADH, SDH fibers COX negative fibers	>5%	Absent	Mitochondrial myopathy
13	Distorted	Present	Present	Present	Present	Absent	Absent	Absent	Dystrophy
14	Maintained	Present	Absent	Absent	Present	Absent	Absent	Absent	Inflammatory
15	Maintained	Present	Absent	Few	Present	Absent	Absent	Absent	Inflammatory (polymyositis)
16	Maintained	Present	Minimal	Absent	Absent	Absent	Absent	Absent	Normal
17	Maintained	Mild	Absent	Absent	Present	Absent	Absent	Absent	Inflammatory
18	Maintained	Mild	Minimal	Absent	Absent	Absent	Absent	Absent	Dystrophy
19	Maintained	Present	Present	Present	Absent	Absent	Absent	Absent	Dystrophy
20	Maintained	Mild	Absent	Absent	Mild	Absent	Absent	Absent	Normal

H and E—hematoxylin and eosin, NADH— nicotinamide adenosine dehydrogenase, SDH—succinic dehydrogenas, COX—cytochrome oxidase, MGT—modified gomori trichrome, ORO—oil red O, PAS— periodic acid Schiff.

Clinical hallmarks traditionally described for identification of mitochondrial diseases like short stature, hearing loss, retinitis pigmentosa, and cardiac conduction disorders were rarely seen in any of the cases with genetically proven isolated mitochondrial myopathy (Table 3). Presence of fibre size variation, central nucleoli and fibrosis on H and E staining are nonspecific findings and were seen in our patient with mitochondrial myopathy as well as in patients with muscular dystrophy and inflammatory myopathy (Table 2). As of now, identification of ragged red fibres on enzyme histochemistry of muscle biopsy of patients with limb girdle muscle disease of unknown aetiology is the only reliable diagnostic test available to recognize these cases [11]. Gene sequencing of mitochondrial DNA in these patients can provide invaluable insight into the underlying genetic mechanism, but it remains largely a research exercise which is difficult to offer to patients in resource poor countries. Many mitochondrial DNA mutations have been reported in mitochondrial myopathy/encephalomyopathy [12]. However, mitochondrial myopathy with isolated skeletal muscle involvement and pathogenic mtDNA mutation is relatively rare [13–15]. There may be many patients with pure myopathy but unknown genetic defects in mtDNA.

Bilateral ptosis was seen in the patient described by Meulemans et al. [8], who also described the patient's mother as having proximal limb weakness, facial weakness, and short stature (Table 3). Phenotypically, the case resembles our prototype patient with isolated mitochondrial myopathy. Our patient had a superficial resemblance to Kearns Sayre syndrome (KSS) and progressive external ophthalmoplegia

Table 3: Clinical, pathological and biochemical characteristics of patients with isolated mitochondrial myopathy with proven mitochondrial mutations.

	Age/ Sex	DOI	Limb weakness	Other features	Family history	EMG	Biochemical	Histopathology	Mutation
Yang et al. [4]	55/F	—	Mild	Severe respiratory dysfunction	—	Myopathic	—	RRF on MGT	m.A3243G tRNA (Leu(UUR))
Hirata et al. [5]	70/M	20 Y	Proximal	Hypertension, hyperlipidemia	Brother, son	Myopathic	CK 4–10X	RRF on MGT, COX deficient, RC enzymes reduced	A-G transition at np8291 and 9bp del
	64/M	1Y	Proximal	AMI	Brother of case 1	Myopathic	CK 4X	Same	Same
	38/M	3Y	Proximal	Subcutaneous lipomas	Son of case 1	Myopathic	CK 2-3X	Same	Same
	71/F	3Y	Proximal	Hypothyroidism hyperlipidemia	Sister	—	CK 2X	Same	Same
	52/F	7Y	Proximal	—	Sister of case 4	—	CK 10X	Same	Same
	58/F	14Y	Proximal	—	—	—	CK 10X	Same	Same
	42/F	4Y	Proximal	—	—	—	CK 7X	Same	Same
Seneca et al. [6]	—/F	—	Exercise intolerance	—	Negative	—	—	RRF, RC enzymes I, IV reduced	m.A7526GtRNA (Asp)
Swalwell et al. [7]	46/F	18M	Proximal	Myalgia	Negative	Myopathic	CK 7X, Post ex Lactate 3X	RRF, COX negative fibers	m.5591 G > A tRNA(Ala)
Meulemans et al. [8]	48/F	—	Proximal	Facial weakness, mild b/l ptosis	Short stature in mother	—	—	RRF on MGT, COX deficient fibers, RC enzymes normal	m.5888 insA & m. 14639 A > G

Table 3: Continued.

	Age/ Sex	DOI	Limb weakness	Other features	Family history	EMG	Biochemical	Histopathology	Mutation
	34/F	2Y	Proximal	IDDM	Negative	Myopathic	CK 10 X Lactate N	RRF, COX negative, Mus CoQ ↓	ETFDH gene m.
	29/M	6M	Proximal	Scapular winging ↓DTR b-n-	Consanguinity in parents	Myopathic	CK 10X Post ex lactate 10X	Same	Same
Gempel et al. [9]	13/M	—	Proximal	—	Consanguinity in parents	—	CK 20X	Same	Same
	17/F	—	Proximal	—	—	—	CK 8X	Same	Same
	12/F	—	Proximal	—	Sister	Normal	CK 40X	Same	Same
	—/F	—	Proximal	Myalgia	Sister of above case	—	—	Same	Same
Leshinsky-Silver et al. [10]	13/F	—	Proximal	—	—	—	CK 5X	Same	Same
	—/—	—	Mild weakness		—	—	—	RRF	H90N m. In TK2 gene

DOI—duration of illness, F—female, M—male, Y—years, M—months, CK—creatine kinase, X—times normal, RRF—ragged red fibers, COX—cytochrome C oxidase, MGT—modified Gomori Trichrome, m. —mutation.

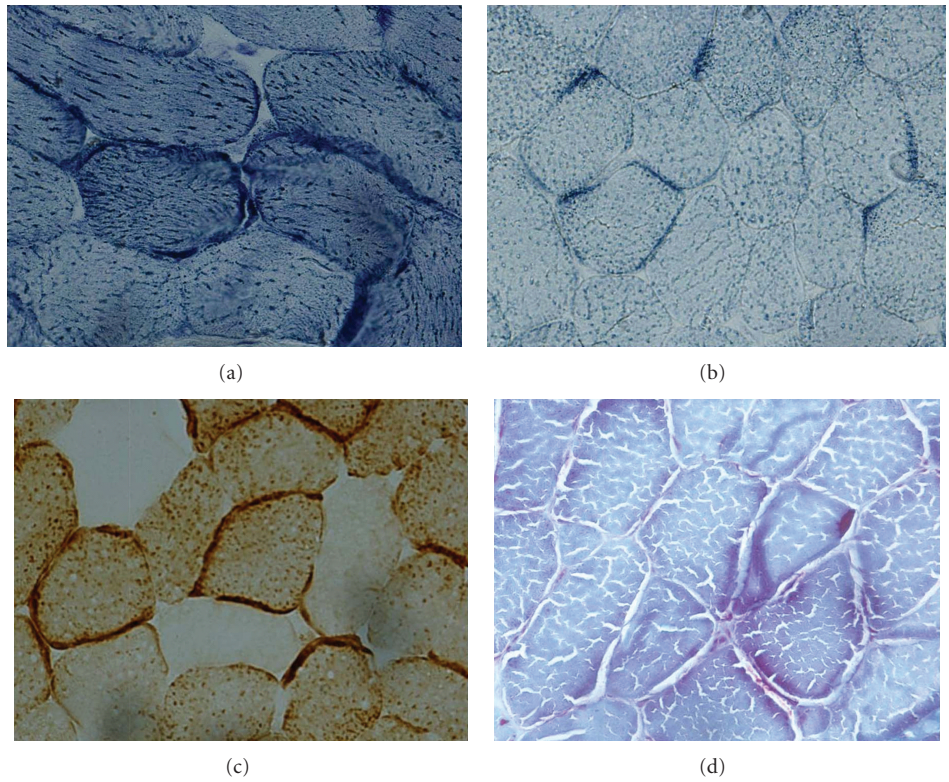

Figure 1: Biopsy from quadriceps muscle shows intense staining of fibre with NADH-TR and SDH stain (a, b) and appear white on cytochrome oxidase activity (c). These muscle fibres shows presence of ragged-red fibres with modified Gomori trichrome stain (d).

(PEO). Kearns Sayre syndrome is a widespread multiorgan system disorder with a defined triad of clinical findings: onset before age 20, chronic progressive external ophthalmoplegia (CPEO), and pigmentary retinopathy plus one or more of the following features: complete heart block, cerebrospinal fluid (CSF) protein >1.0 g/L, or cerebellar ataxia. This typical triad is not present in our case. Progressive external ophthalmoplegia is not known to be associated with hypertrophic obstructive cardiomyopathy (seen in the brother of our patient). Therefore, our patient with isolated mitochondrial myopathy might represent a group of mitochondrial myopathy with some novel genetic mutation in the nuclear or the mitochondrial DNA.

On histopathology, we classified our patients into four groups consisting of muscular dystrophy in 12, inflammatory myopathy in four, mitochondrial myopathy in one, and normal histology in three patients. All patients with muscular dystrophy had duration of illness in years except one patient who had a history of seven months. On the contrary, the patients diagnosed as inflammatory myopathy had duration of illness lasting few months. Other findings like limb weakness, creatine kinase levels, electromyography, and other clinical features like skin changes and so forth were not specific to either entity. Those with family history of proximal weakness proved to be muscular dystrophy on histopathology in our study. Three patients who had normal muscle histology possibly either had a muscle biopsy from a nonrepresentative muscle group or had some undetected metabolic myopathy with no characteristic appearance on histopathology.

6. Conclusion

Muscular dystrophy, inflammatory myopathy, and isolated mitochondrial myopathy have overlapping clinical, biochemical, and electrophysiological features. Enzyme histochemistry should be performed as a routine procedure in addition to Hematoxylin and Eosin stain especially in resource poor countries, where enzyme histochemical staining of muscle biopsy is performed in the selected cases with high clinical suspicion of mitochondrial disorders.

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