Journal of the Neurological Sciences 404 (2019) 91–100

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Genetic heterogeneity of mitochondrial genome in thiamine deficient Leigh syndrome patients

Shalini Mani[a,b,*], S. Narasimha Rao[c], M.V. Kranthi Kumar[b]

[a] Department of Biotechnology, Centre for Emerging Diseases, Jaypee Institute of Information Technology, Noida 201301, India
[b] Centre for Cellular and Molecular Biology, Hyderabad 500007, India
[c] Government Institute of Child Health, Niloufer Hospital for Women and Children, Red Hills, Hyderabad, India

ARTICLE INFO

Keywords:
Leigh syndrome
Thiamine deficiency
Mitochondrial genome
Complex I
Complex IV

ABSTRACT

In our previously published study, we cared for 165 thiamine deficient Leigh syndrome (LS) patients who presented in acute life threatening conditions with severe neurological abnormalities. However the molecular basis for this atypical phenotype was not explored. This study is an effort to undermine the possible molecular defects in mitochondria of those patients and put-forth an explanation towards this clinical presentation.

Protein coding genes of mitochondrial (mt) DNA were sequenced in total 165 LS patients and 94 age matched controls. To understand their pathogenic significance, nucleotide variations were also studied using various *in-silico* tools. Histochemical and electron microscopic analysis was also done in tissue samples obtained from 23 patients.

We observed a very high level of genetic heterogeneity across the mt DNA of all these patients. In the concordance of published literature we also observed a large number of variations in ND5 gene (hot spot for LS). We also observed a total 13 nucleotide variations across COX genes, which is otherwise not common in LS. As per *in-silico* analysis, many of these variations were suggested to be pathogenic. Histochemical and electron microscopic studies also suggested the defects in the mitochondria of these patients.

As these patients were thiamine deficient, hence we propose that genetic defects and thiamine deficiency may together severely affect the ATP levelof these patients, leading to acute and life threatening clinical presentation. Present study has opened up many avenues for further research towards understanding the genetic basis and possible role of thiamine deficiency in LS patients.

1. Introduction

Since its first description by Denis Archibald Leigh [1], Leigh syndrome (LS) has evolved as one of the defined clinical syndromes. It is a sub-acute/chronic progressive neurometabolic disease with characteristic pathological features and usually presents in infancy or early childhood. Hallmarks of the disease are symmetrical lesions in the basal ganglia or brain stem on MRI, and a clinical course with rapid deterioration of cognitive and motor functions. It is known to be caused by defect in mitochondrial metabolism, mostly due to mutations in mitochondria DNA (mt DNA).The human mitochondrial genome is a closed, circular molecule and located within the mitochondrial matrix. It is typically 16,569 bp in length and each mitochondrion is estimated to contain 2–10 mtDNA copies. As the mtDNA is present in close proximity of site for ROS generation and also not protected by histone like proteins hence the rate of mutations in mitochondrial genome is very high. It is generally believed that mitochondria and mtDNA are exclusively maternally inherited in humans but recently in year 2018 Luo et al. [2] have provided enough evidence to suggest in certain cases, the offspring may carry mtDNA of his/her father and thus have challenged the dogma of strict maternal mtDNA inheritance in humans.In last two decades, a large number of mutations in mitochondrial and nuclear genes, which encodes for different components of the oxidative phosphorylation system (OXPHOS), have been described in LS patients [3–7]. Thus these patients exhibit high level of genetic heterogeneity.

In addition to genetic heterogeneity, the phenotypic expression of LS is furtherknown to be modulated by deficiencies like vitamins and CoQ [8–11]. Thiamine (vitamin B1) deficiency is one of the common factors for occurrence of brain related abnormalities [12,13] and thiamine supplementation is also recommended as immediate treatment for LS [14]. In some cases, dysfunction in pyruvate dehydrogenase complex

* Corresponding author at: Department of Biotechnology, Centre for Emerging Diseases, Jaypee Institute of Information Technology, Noida 201301, India.
E-mail address: shalini.mani@jiit.ac.in (S. Mani).

https://doi.org/10.1016/j.jns.2019.07.007
Received 20 May 2019; Received in revised form 3 July 2019; Accepted 8 July 2019
Available online 10 July 2019
0022-510X/

is also reported to be associated with thiamine responsive Leigh syndrome [15]. Many of the time the study and cure of these LS patients is found to be complex mainly because of its high level of genetic and phenotypic heterogeneity.

LS in India have hardly been explored and very little data exists concerning its etiology and clinical course [16–18]. As an attempt towards the same, in our previous studies [19] we had clinically investigated a total of 165 LS patients from southern region of India. Most of these patients were infants with mean age of onset at 7 months. All the patients were analyzed on the basis of clinical picture, biochemical parameters, and radiological features. It was found that, most of the patients were thiamine deficient and presented with acute neurological manifestations and severe respiratory abnormalities, which are not the typical presentation of LS. Because of acute life threatening condition with respiratory disturbances, neurological abnormalities and thiamine responsiveness of these patients, their phenotype was proposed to be an atypical presentation of LS. However the molecular defect behind this atypical presentation was not investigated. Hence, in an attempt to understand the etiopathogenesis, structural and functional abnormality of mitochondria and the genetic defect in mtDNAof these thiamine responsive LS patients, the study was conducted further.

2. Materials and methods

Total 165 well characterized, thiamine responsive LS patients and 94 age as well as ethnicity matched control children were enrolled in the study. These patients were diagnosed based upon different clinical, biochemical and brain imaging based methods [19]. A detailed proposal with the design of the study and format for the informed consent was submitted to the Institutional ethics committee of Centre for Cellular and Molecular Biology, Hyderabad and Government Institute of Child Health, Niloufer Hospital for Women and Children, Hyderabad, India. Written informed consent was obtained from the parents in accordance with the IEC approved protocols.

2.1. Histochemical staining and electron microscopic analysis of muscle samples

As most of these patients were admitted in acute life-threatening condition, muscle samples were obtained from 23 patients only. A $1 \times 1\,cm^2$ piece of muscle was removed by open biopsy from the vastus-lateralis muscle of these patients and processed further for histochemical and electron microscopic analysis. The basic structure and organization of the tissue was evaluated by haematoxylin and eosin staining. The subsarcolemmal aggregation of mitochondria was evaluated by modified Gomori'strichrome (MGT) and Masson trichrome staining. The presence of excess amount of neutral lipids and polysaccharides was assessed by Oil red O and Periodic acid-schiff. Fiber typing was done by evaluating the activity of adenosine triphosphatase at pH 9.4 and 4.6. Other enzymes such as succinic acid dehydrogenase (SDH), NADH-tetrazoliumreductase (NADH-TR), and cytochrome *c* oxidase (COX) were also studied for their activity in these samples, as per standard protocol [20].

In orders to investigate the structural abnormality of mitochondria, a $1mm^2$ biopsy sample was also used for transmission electron microscopic analysis.

2.2. Genetic analysis

For genetic screening of coding region of mtDNA, total 5 ml of EDTA anticoagulated blood was mixed with erythrocyte lysis buffer (ELB) in 1:4 ratio and total volume was made up to 20 ml. This mix was incubated on ice for 15 min. The lysate was centrifuged at 3000 rpm for 10 min at RT and pellet was suspended in 5 ml of ELB supplemented with 1% SDS and Proteinase K (160 μg/ml of blood) and was incubated at 37 °C overnight. Further the total genomic DNA was precipitated and isolated using salting out method as described by Miller et al. in 1988 [21].

Because of the thiamine responsiveness of these patients, initially PDHE1αgene was screened in all the LS patients and controls. Following it, the protein coding region of entire mitochondrial genome was amplified using published primer sequences [22] and sequences of amplified fragments were confirmed using the Dideoxy method of DNA sequencing as developed by Sanger et al. [23]. For sequencing, ABI PRISM DNA sequencing kits were used as per manufacturer's protocol.The data was analyzed and compared with mitochondrial genome sequence obtained from NCBI and nucleotide variations in mitochondrial genome as reported at www.mitomap.org.

2.3. In-silico analysis of non-synonymous mutations

Several nucleotide changes found in our study were either novel or have already been reported in other mitochondrial disorders. In order to suggest the possible pathogenic effect of all these non synonymous changes on their respective proteins, various *in-silico* tools such as Clustal W, SIFT (Sorting Intolerant From Tolerant; http://blocks.fhcrc.org/sift/SIFT.html), PolyPhen (Polymorphism Phenotype; http://www.borl.embl-heidelberg.de/PolyPhen/) and GOR4 (http://workbench.sdsc.edu) were used to predict the potential of every non-synonymous mutations.

3. Results

3.1. Electron microscopic and histochemical analysis of muscle samples

Histological examination showed the differences in fiber shape, size and their distribution in several patients. Fifteen out of twenty three patients showed predominance of type I fibers and atrophy of type II fibers (Fig. 1). Two patients showed interstitial fibrosis on Masson's Trichrome stain and the interstitium of one patient showed increased adipose tissue after staining with Oil red O stain. Red Ragged Fibers (RRF) was not found in any of the patient. The enzymatic activity of SDH, NADH and COX was found to be normal in all the tissue samples (data not shown).On electron microscopic analysis, various structural abnormalities in mitochondria of these samples were observed. These abnormalities included diffused cristae, vacuoles, abnormally elongated and rudimentary mitochondria as well. Few samples showed increased number of mitochondria too, whereas some samples had no mitochondria at all (Fig. 2).

3.2. Absence of nucleotide variation in PDHE1α gene but mitochondrial genome was genetically highly heterogeneous

Though these patients were thiamine deficient and mutations in PDHE1α gene is commonly associated with thiamine deficient phenotypes. However in our study, these patients did not carry any of the reported/novel mutation in their PDHE1α gene (data not shown). On the other hand, a total of 100 non-synonymous (Table 1) and 296 synonymous changes (data not given) were identified across the protein-coding regions of mitochondrial genome of these patients. Out of 100 non-synonymous changes, 80 were reported and 20 were novel nucleotide variations. Most of the non-synonymous changes were homoplasmic in nature and absent in controls.The important variations across mtDNA of these patients are represented as Fig. 3a and b.

Approximately, 50% of the nonsynonymous variations were spread across different mitochondrial genome encoded subunits of complex I (ND1-ND6, ND4L). Variation found in ND1, ND2 and ND3 genes had also been reported in association with several other neurological disorders such as, Leber hereditary optic neuropathy (LHON) [24–26], Multiple Sclerosis (MS) [27,28], Parkinson's disease (PD) and Alzheimer's disease (AD) [29–31]. ND4 and ND5 genes, which are known to be the commonest hot spot for LS, were also found to have total 6 and

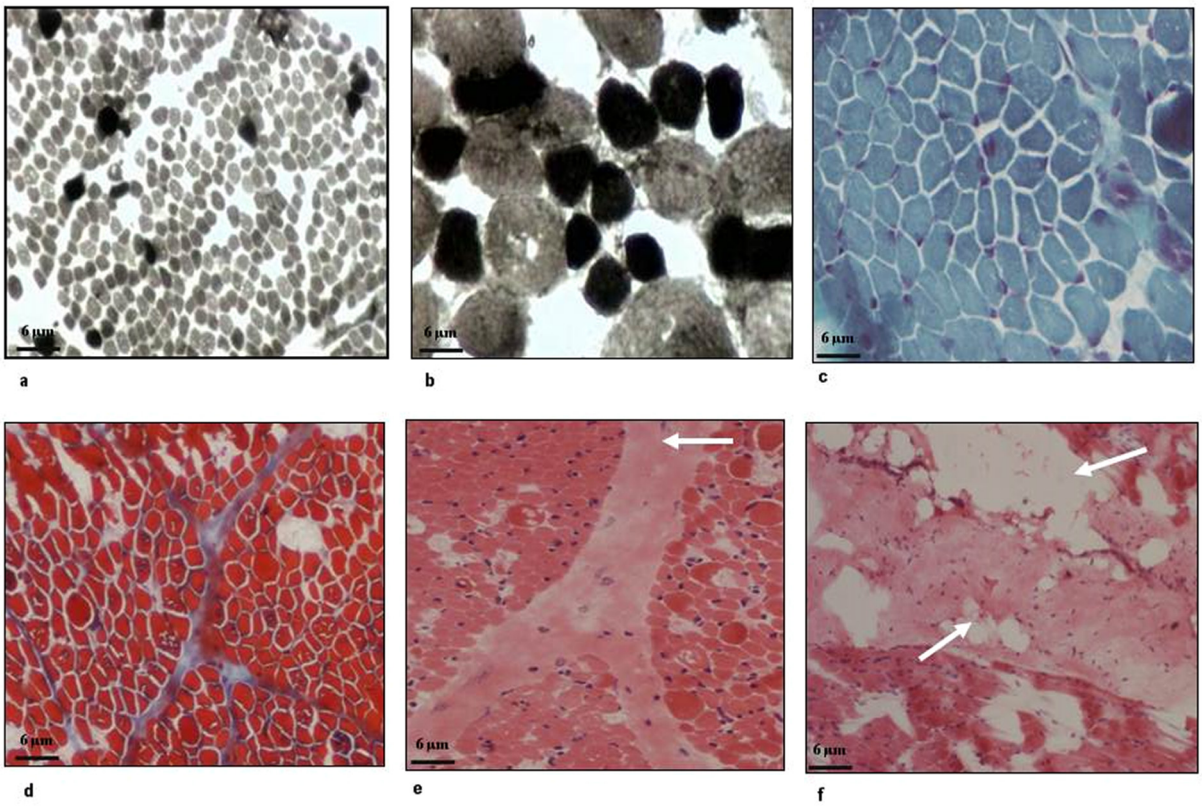

Fig. 1. Histochemical analyses showing functional defect of mitochondria in muscle biopsy samples of patients with LS. a. Type I predominance. b. Type II fiber atrophy. c. Granules on MGT. d. Masson Trichrome showing fibrosis. e. Interstitial fibrosis. f. Increased adipose tissues and fibrosis.

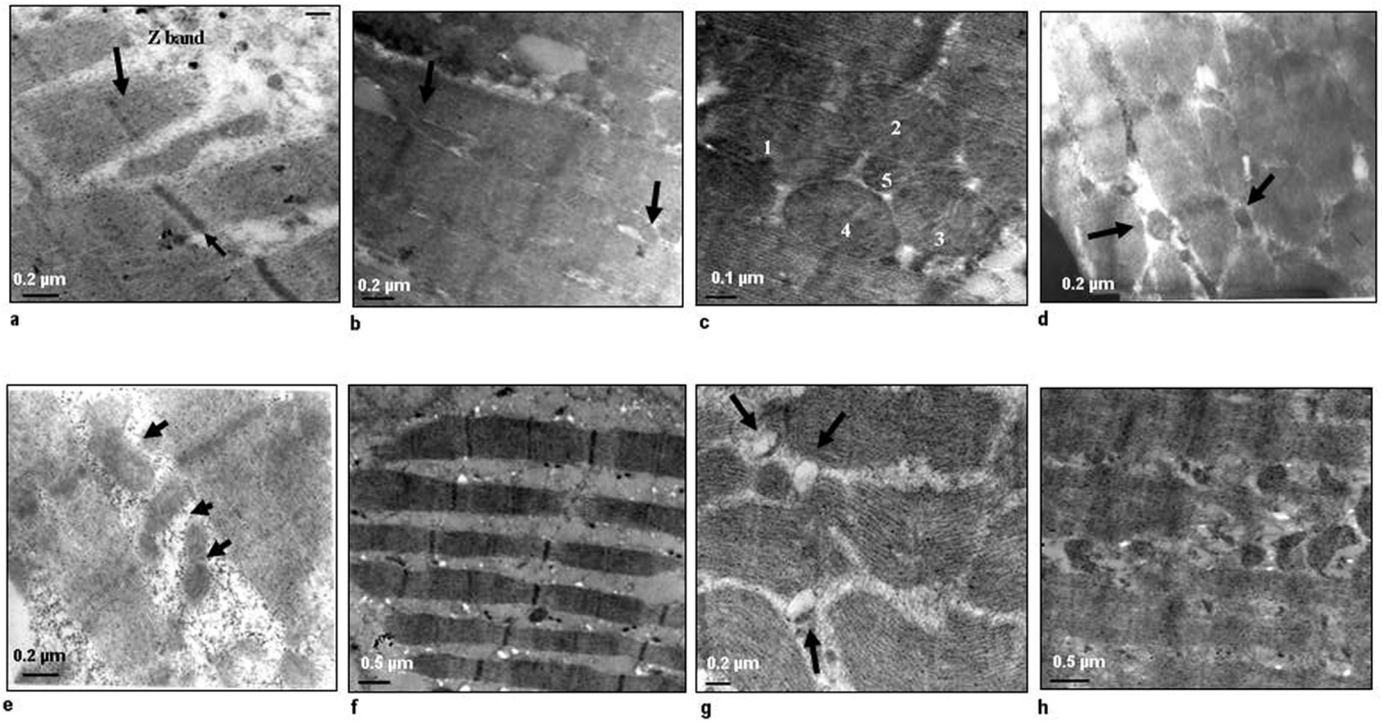

Fig. 2. Electron microscopic analyses showing structural defect of mitochondria in muscle biopsy samples of patients with LS. a. Normal mitochondrial length spanning between two Z bands of a sarcomere. b. Abnormally elongated mitochondria spanning through 3 z-bands of a sarcomere c. Increased number and elongated mitochondria. d. Rudimentary Mitochondria. e. Mitochondria with diffused cristae. f. No mitochondria between muscle fibers. g.Vacuolated mitochondria. h. Atrophic muscle fiber.

Table 1
Distribution of total nucleotide changes across coding part of mitochondrial genome of LS patients.

Enzyme	mt gene	Reported non-synonymous changes	Novel non-synonymous changes	Synonymous changes
Complex I	ND1	8	2	25
	ND2	11	1	20
	ND3	3	1	9
	ND4	6	0	44
	ND4L	0	0	6
	ND5	11	7	64
	ND6	1	0	11
Complex III	CYT B	11	0	29
Complex IV	COX I	3	3	30
	COX II	3	3	15
	COX III	1	0	28
Complex V	ATPase6	18	2	13
	ATPase8	4	1	2
Total changes		80	20	296

18 nucleotide variations in patients, respectively. Amongst 18 non-synonymous changes of ND5 gene, 7 were novel and 11 had been reported in other mitochondrial disorders. G13135A was the most common mutation of ND5 gene and present in 16 patients. The next common variations were A13966G and A14128G, which were interestingly always found together in 8 patients. Many of the patients were found to carry variations commonly associated with LHON phenotype (Table 2).

Next to ND genes, CytB gene was found to have total 11 variations in thesepatients. Except a common polymorphism G15326A, majority of them were found in patients only. G14831A which is a proven cause for LHON was also found to be present in two patients but none of the controls.

Though, not much report is available with respect to the mutations in mtDNA encoded components of complex IV and their association with LS phenotype. However, as per our study, total 13 nucleotide changes were found across COX I, COX II and COX III genes. Five out of thirteen mutations were novel and have never been reported in any of the mitochondrial disorders but remaining eight nucleotide changes have been reported either a proven cause or as polymorphisms in several mitochondrial disorders (Table 2).

Additionally, total 20 non-synonymous were identified in the ATPase6 gene (mtDNA encoded subunit of complex V) of these patients. Two out of 20 non-synonymous changes were novel and remaining has been reported mostly as polymorphisms in several mitochondrial

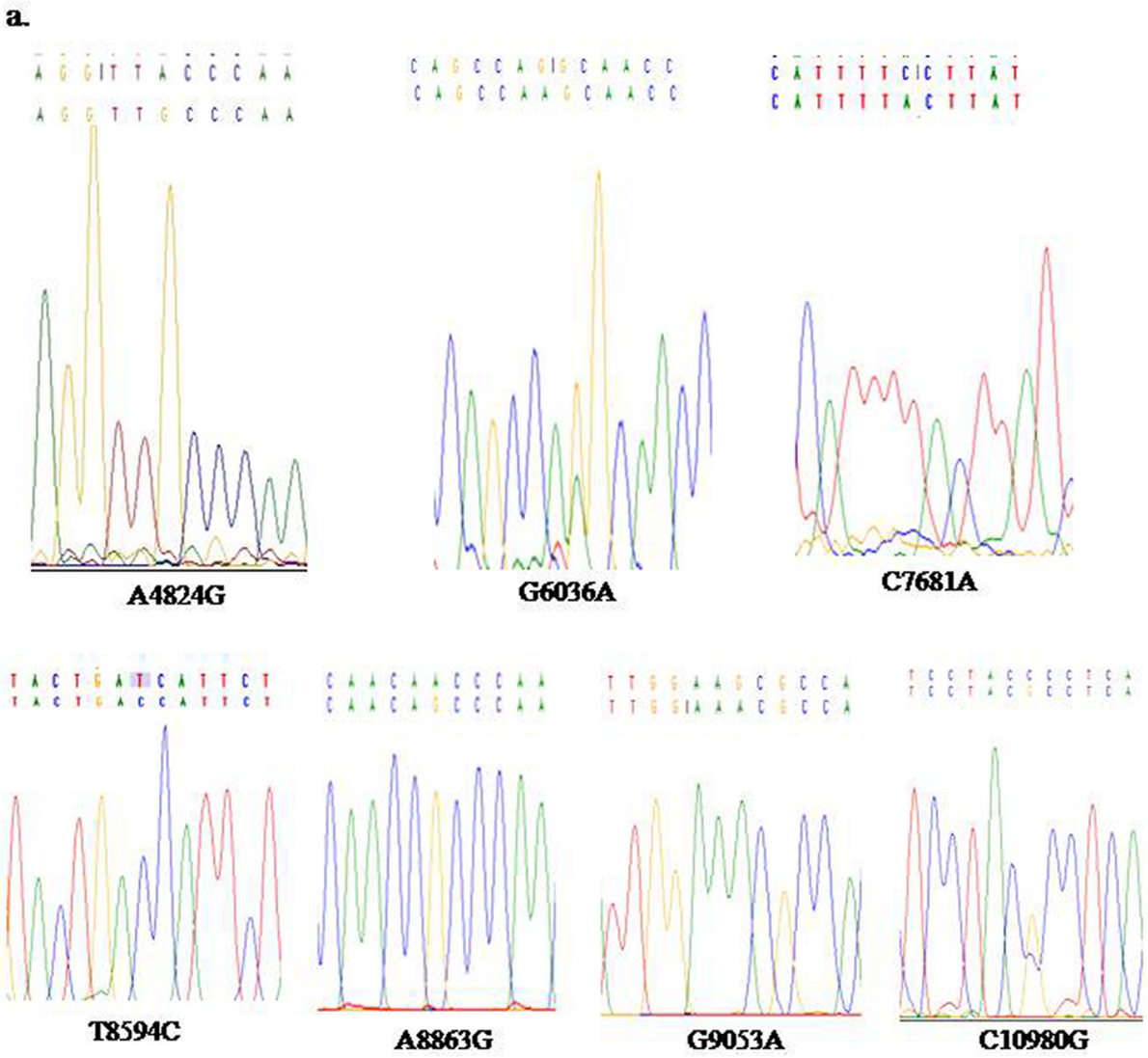

Fig. 3. Sequencing results showing important variations across protein coding region in mtDNA of LS patients. a. Variations across ND2, ND4, COX I, ATPase6 genes. b. Variations across ND5 gene (known hot spot gene in case of LS).

b.

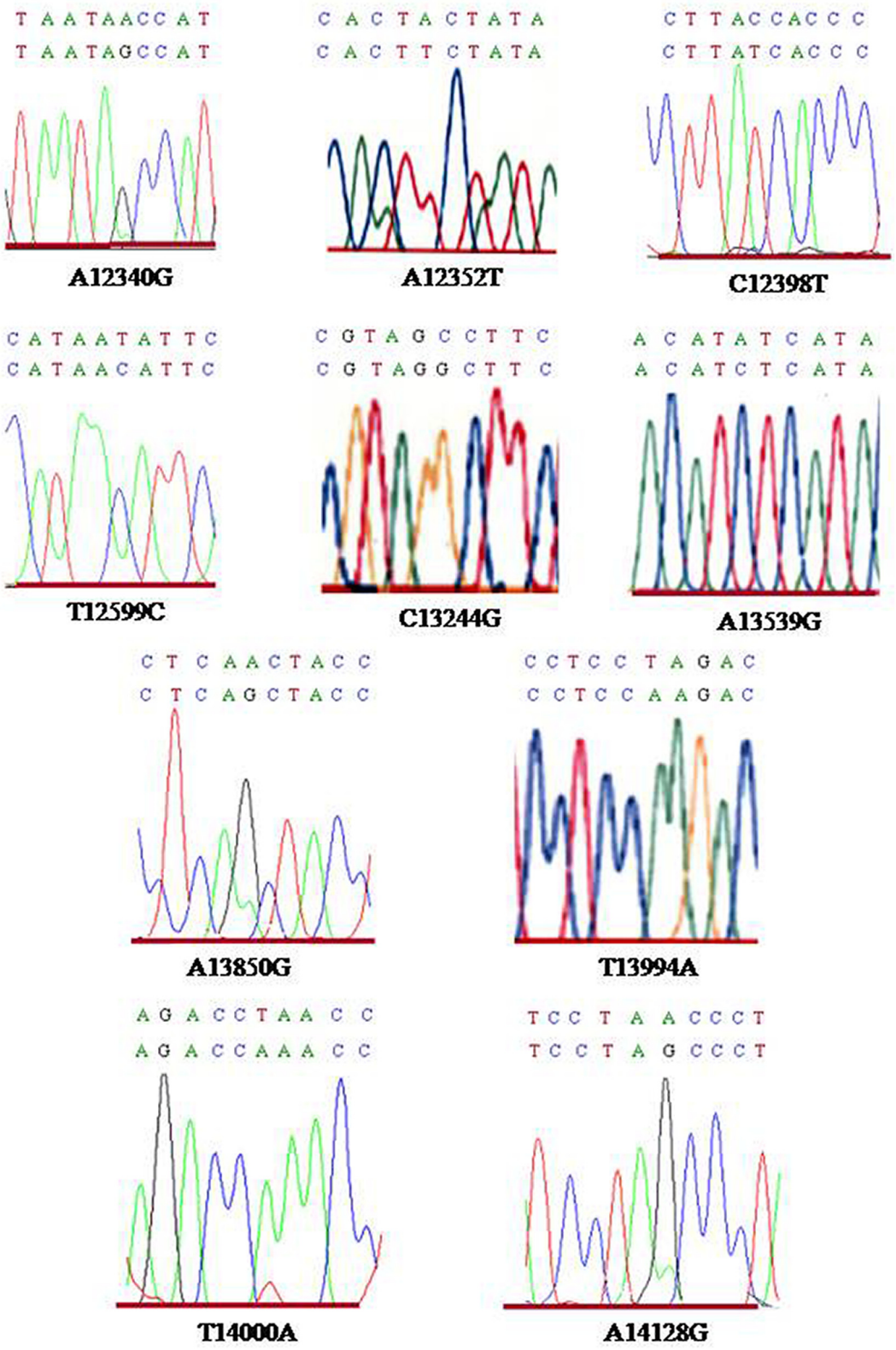

Fig. 3. (*continued*)

Table 2
All the non-synonymous mutations in protein coding mitochondrial genes of LS patients &controls and their *in-silico* analysis.

Mt Gene	nt change	AA change	Het/Homo	No. of patients (N = 165)	No. of control (N = 94)	Clustal W	*In silico analysis*		GOR 4	Association
							PolyPhen (Analysis Score)	SIFT (Analysis Score)		
Complex I										
ND1										
1	G3316A	A4T	Homo	1	1	NC	B (0.211)	TOL (0.78)	C-C	DM, LHON, PEO/Unclear
2	A3385G	I27V	Homo	1	0	NC	B (0.167)	TOL (1.00)	C-C	NR
4	C3476T	T57I	Homo	2	0	NC	B (0.092)	TOL (0.92)	C-H	NR
5	A3505G	T67A	Homo	1	1	PC	B (0.387)	INTOL (0.02)	C-C	MERRF/Unclear
3	G3640A	A112T	Homo	1	0	PC	B (0.138)	TOL (0.24)	C-C	POL
6	A3796G	T164A	Homo	1	0	PC	B (0.761)	TOL (0.69)	E-H	Adult onset dystonia/ Proven
7	C4025T	T240 M	Homo	1	0	NC	B (0.226)	TOL (0.09)	C-C	LHON, occipital stroke/ POL
8	G4048A	D248N	Het	1	0	NC	B (0.627)	TOL (0.47)	C-C	LHON/POL
9	A4093G	T263A	Homo	1	0	NC	B (0.486)	TOL (0.36)	C-H	LHON/POL
10	T4216C	Y304H	Homo	1	0	NC	B (0.839)	TOL (0.67)	E-C	PD, LHON, MS/POL
ND2										
1	G4659A	A64T	Homo	1	1	PC	B (0.613)	INTOL (0.00)	H-C	LHON/POL
3	A4824G	T119A	Homo	1	0	C	B (0.998)	TOL (0.08)	C-C	LHON, PD/POL
4	A4842G	T125A	Homo	2	0	NC	B (1.082)	TOL (0.28)	C-C	NR
5	A4965G	S166G	Homo	1	0	NC	B (0.989)	TOL (1.00)	C-C	POL
6	G5046A	V193I	Homo	2	0	PC	B (0.428)	TOL (1.00)	E-E	Hearing loss/POL
7	T5074C	I202T	Homo	1	0	PC	B (0.701)	TOL (0.08)	E-C	LHON/POL
8	A5301G	I278V	Homo	6	1	PC	B (0.285)	TOL (0.25)	E-H	PD/POL
9	A5319G	T284A	Homo	3	2	PC	B (1.4)	INTOL (0.02)	H-H	PD/POL
10	G5460A	A331T	Homo	5	4	NC	B (0.737)	TOL (0.42)	H-E	AZ, PD, LHON, PE/POL
11	T5493C	F342 L	Homo	2	0	PC	B (0.307)	TOL (0.68)	C-C	POL
12	T5503C	M345 T	Homo	2	0	NC	POS (1.503)	TOL (0.63)	E-E	POL
ND3										
1	T10084C	I9T	Homo	1	0	PC	B (0.146)	TOL (0.25)	H-H	ME/POL
2	A10188G	M44 V	Homo	1	0	PC	B (0.231)	TOL (0.07)	C-C	NR
3	T10237C	I60T	Homo	1	0	C	PRB (3.4)	TOL (0.38)	C-C	LHON/Proven
4	A10398G	T114A	Homo	83	35	PC	B (0.052)	TOL (0.88)	E-E	Breast cancer, AD, PD/Proven
ND4										
1	C10980G	P74R	Homo	1	0	C	PRB (2.96)	TOL (0.08)	H-H	Hearing loss/POL
2	A10988C	I77L	Homo	1	0	PC	B (0.595)	TOL (1.00)	H-H	Hearing loss/POL
3	C11061T	S101F	Homo	2	0	PC	B (0.168)	TOL (0.20)	H-H	POL
4	G11150A	A131T	Homo	1	0	PC	B (0.759)	TOL (0.18)	H-H	Ovarian carcinoma/POL
5	C11151T	A131V	Het	3	0	PC	B (0.756)	TOL (0.27)	H-H	POL
6	C11288G	L177V	Homo	3	0	PC	B (0.458)	TOL (0.50)	H-H	POL
ND4L	No mutation									
ND5										
1	A12340G	T2A	Het	1	0	PC	Unknown	TOL (0.53)	C-C	POL
2	A12352T	T6S	Homo	1	0	PC	Unknown	TOL (1.00)	E-C	NR
3	C12398T	T21I	Homo	1	0	PC	Unknown	TOL (0.41)	E-E	NR
4	G12406A	V24I	Het	2	0	NC	B (0.45)	TOL (0.59)	C-E	LHON, Hearing loss/POL
5	A12469G	I45V	Homo	1	0	PC	B (0.738)	TOL (0.10)	E-E	POL
6	T12599C	M88T	Homo	1	0	PC	B (0.998)	TOL (0.33)	C-C	NR
7	G12940A	A202T	Het	1	0	NC	B (0.182)	INTOL (0.05)	E-E	POL
8	G13135A	A267T	Het	16	0	PC	B (0.664)	TOL (0.28)	C-C	LHON, PD, ME, IC/POL
9	C13244G	A303G	Homo	1	0	C	B (1.528)	INTOL (0.03)	E-E	NR
10	A13539C	M401I	Het	1	0	PC	B (1.024)	TOL (0.12)	C-C	NR
11	G13708A	A458T	Het	5	3	PC	B (0.694)	TOL (0.35)	H-H	LHON, MS/POL
12	G13759A	A475T	Het	1	0	NC	B (0.566)	TOL (1.00)	C-C	Many mt disorders/POL
13	A13850G	N505S	Het	1	0	PC	B (0.465)	TOL (0.48)	H-H	NR
14	T13879C	S515P	Het	1	0	PC	B (0.407)	TOL (0.25)	C-C	LHON/POL
15	A13966G	T544A	Het	8	0	PC	B (0.539)	TOL (0.58)	C-H	LHON, PD, MS/POL
16	T13994A	L553Q	Homo	1	0	C	POS (1.68)	INTOL (0.00)	H-H	NR
17	T14000A	L555Q	Het	1	0	C	B (0.061)	TOL (0.68)	H-H	POL
18	A14128G	T598A	Het	8	3	PC	B (0.28)	TOL (0.52)	E-E	POL
ND6										
1	C14553T	V41I	Homo	1	0	NC	B (1.102)	TOL (0.11)	H-H	POL
Complex III										
CytB										
1	G14831A	A29T	Homo	2	0	PC	B (0.038)	TOL (0.12)	H-C	LHON/Proven
2	C14990T	L82F	Homo	2	1	PC	B (1.471)	INTOL (0.02)	E-E	POL
3	G15110A	A122T	Homo	2	1	PC	B (0.244)	TOL (0.63)	H-H	Deafness/POL
4	A15113G	T123A	Homo	1	0	PC	B (1.441)	INTOL (0.00)	H-H	POL
5	G15119A	A125T	Homo	2	0	C	B (0.398)	INTOL (0.00)	H-C	POL
6	T15287C	F181L	Homo	6	2	PC	B (0.877)	TOL (0.09)	C-H	Diabetes and deafness/POL
7	A15326G	T194A	Homo	114	89	PC	B (0.391)	TOL (0.20)	H-H	Many mt disorders/POL
8	G15431A	A229T	Homo	5	1	NC	B (0.208)	INTOL (0.04)	H-C	POL
9	C15452A	L236I	Homo	1	1	PC	B (0.25)	TOL (1.00)	H-H	Many mt disorders/POL

(*continued on next page*)

Table 2 (*continued*)

Mt Gene	nt change	AA change	Het/Homo	No. of patients (N = 165)	No. of control (N = 94)	Clustal W	*In silico analysis*		GOR 4	Association
							PolyPhen (Analysis Score)	SIFT (Analysis Score)		
10	T15479C	F245L	Homo	3	0	C	B (0.936)	INTOL (0.00)	C-C	POL
11	A15758G	I338V	Homo	2	0	C	B (0.619)	TOL (0.08)	C-C	Many mt disorders/POL
Complex IV										
COX I										
1	G6036A	G45S	Het	3	0	C	POS (1.824)	TOL (0.06)	H-H	NR
2	C6250T	A116V	Homo	1	0	PC	B (1.066)	TOL (0.45)	H-H	NR
3	T6253C	M117T	Homo	3	0	PC	B (0.951)	TOL (0.21)	H-H	Prostate cancer/Proven
4	G6267A	A122T	Homo	1	0	C	B (0.386)	TOL (0.08)	C-C	Pancreatic & Prostate cancer/Proven
5	G7269A	V456 M	Homo	1	0	PC	B (0.952)	INTOL (0.05)	C-C	NR
6	T7389C	Y496H	Homo	1	0	PC	B (0.647)	TOL (0.55)	E-C	Thyroid tumor/POL
COX II										
1	C7681A	F32 L	Homo	2	0	PC	B (1.299)	TOL (0.54)	H-H	POL
2	A7746G	N54K	Homo	1	0	PC	POS (1.64)	TOL (0.33)	C-E	NR
3	G7775A	V64I	Homo	1	0	PC	B (0.287)	TOL (1.00)	H-H	NR
4	G7830A	R82H	Homo	1	0	PC	POS (2.12)	TOL (0.07)	H-H	POL
5	G7859A	D92N	Homo	2	0	PC	B (0.124)	TOL (1.00)	C-C	PE/Proven
6	G8027A	A148T	Homo	1	0	PC	B (0.204)	TOL (1.00)	C-C	POL
COXIII	G9966A	V254I	Homo	3	0	PC	B (0.087)	TOL (0.57)	C-C	Many mt diseases/POL
Complex V										
ATPase 6										
1	A8537G	N4S	Homo	1	0	PC	B (1.051)	TOL (0.68)	C-C	POL
2	G8557A	A11T	Homo	3	0	PC	B (0.546)	TOL (0.73)	C-C	Metabolic diseases/POL
3	A8566G	I14V	Homo	1	0	PC	B (0.45)	TOL (0.54)	C-C	POL
4	G8581A	A19T	Homo	2	0	PC	B (0.37)	TOL (0.14)	H-C	Cardiomyopathy/POL
5	G8584A	A20T	Homo	5	3	C	B (0.227)	TOL (0.29)	H-C	LHON/POL
6	G8587A	V21 M	Homo	1	0	PC	B (0.524)	TOL (0.16)	H-H	Optic neuritis/POL
7	T8594C	I23T	Het (3), Homo (5)	8	0	PC	PRB (2.18)	TOL (0.27)	H-H	Many mt Disorders/POL
8	T8632C	Y36H	Het (1), Homo (2)	3	0	PC	B (0.644)	TOL (0.18)	C-H	NR
9	T8654C	I43T	Homo	1	0	PC	B (0.405)	TOL (0.60)	E-C	POL
10	A8659G	T45A	Homo	2	0	PC	B (0.4161)	TOL (0.14)	E-H	POL
11	A8701G	T59A	Homo	93	51	PC	B (0.223)	TOL (0.56)	E-H	Many mt Disorders/POL
12	A8812G	T96A	Homo	4	0	C	B (0.729)	INTOL (0.01)	C-H	Hearing loss/POL
13	A8860G	T112A	Homo	164	53	PC	B (0.601)	TOL (0.23)	H-H	Many mt Disorders/POL
14	G8863A	V113M	Homo	1	0	C	POS (1.657)	TOL (0.13)	H-H	NR
15	T8987C	M154T	Homo	1	0	PC	POS (1.621)	INTOL (0.02)	H-H	Many mt Disorders/POL
16	G9053A	S176N	Het (2), Homo (1)	3	0	PC	B (0.055)	INTOL (0.01)	C-C	Hearing loss, LHON/POL
17	C9094T	L190F	Homo	1	1	PC	B (0.283)	TOL (0.77)	C-C	POL
18	T9103C	F193L	Homo	1	0	PC	B (1.091)	TOL (0.88)	H-H	Glaucoma/POL
19	T9110C	I195T	Homo	1	2	PC	B (1.078)	TOL (0.18)	H-H	POL
20	T9128C	I201T	Homo	1	0	PC	B (1.053)	INTOL (0.03)	H-H	Hearing loss/POL
ATPase 8										
1	A8396G	T11A	Homo	1	2	PC	B (0.598)	TOL (0.10)	E-E	POL
2	C8472A	P36H	Het	3	0	PC	B (0.274)	TOL (0.77)	C-C	NR
3	A8502G	N46S	Homo	4	3	PC	B (0.189)	TOL (0.29)	C-C	POL
4	A8508G	N48S	Homo	1	0	PC	B (1.09)	TOL (0.34)	C-E	Hearing loss/POL
5	A8537G	I58V	Homo	1	0	C	B (1.061)	TOL (0.08)	E-E	POL

disorders. Out of two novel changes, T8632C and G8863Awerepresent in only three and one patient, respectively. A8860G and A8701G were present in total 164 and 93 patients, respectively, regarded as most common nucleotide change observed in present study (Table 2).

3.3. Several novel changes in coding region of mitochondrial genome found to be damaging by in-silico analysis

As a result of *in-silico* analysis of all the nonsynonymous nucleotide variations, total 48 non-synonymous changes across different mitochondrial genes were found to affect the respective mitochondrial proteins (Table 2). These 48 non-synonymous changes were present exclusively in patients and were comprised of 21, 7, 6 and 14 nucleotide changes in mitochondrial components of complex I, III, IV and V, respectively. Clustal W analysis showed that total 15 changes affected the highly conserved residues in respective proteins. SIFT and PolyPhen analysis together predicted total 30 changes to be intolerable for their respective proteins. GOR4 analysis showed that atleast 23 nucleotides changes affected the secondary structure of respective proteins in different ways.

The nucleotide changes T10237C (I60T) and C10980G (T114A) in ND3 and ND4 genes, respectively, altered a highly conserved amino acid residue and PolyPhen analysis showed both the changes to be “probably damaging” for the respective proteins. ND5 gene carried a novel nucleotide change T13994A (L553Q) in the patients. This change affected the highly conserved amino acid residue and was predicted to be INTOLERANT and “possibly damaging” for the protein by different tools. The only mitochondrial subunit of complex III, CytB also harbored a mutation, G15119A (A125T), which affected a highly conserved residue, altered secondary structure of protein from alpha helix to random coil and was shown to be INTOLERANT by SIFT analysis. Similarly, G6036A (G45S) a novel nucleotide change in COX I gene

changed the highly conserved amino acid residue and PolyPhen suggested this change to be "possibly damaging" for the COX I protein. Additionally, one mutation A7746G (N54K) in COX II gene could also be of significance as GOR4 analysis showed this mutation to affect the secondary structure of COX II protein and it was also shown to be "possibly damaging" for the protein by PolyPhen analysis. Other than the above-mentioned nucleotide changes, there were large number of nucleotide changes across the different coding region of the mitochondrial genome of these patients and were found to affect the respective protein by different *in-silico* analyses (Table 2).

4. Discussion

Study of muscle biopsy samples of 23 patients showed corroborative evidence of structural and functional defects of mitochondria in their electron microscopy and histochemistry, respectively. The mitochondrial structural abnormalities of these patients, such as diffused cristae, increased number of mitochondria and elongation of mitochondria are commonly reported in several mitochondrial diseases [32,33]. Histochemical analysis also showed various defects in terms of fiber type distribution and shape as well as size of mitochondria, which are known to be reported in association with different neurological disorders such as early onset hypotonia [33,34] and myopathies [35,36]. Along with difference in the fiber type distribution, presence of fibrosis and increase of adipose tissue in muscle fibers of these patients indicated towards a kind of cell damage due to inflammation of muscle fibers and a defective mitochondrial metabolism, respectively. Neither the RRF nor any specific enzyme deficiency was identified in the biopsy samples of these patients. However, relative paucity or absence of RRFs is known to be common in muscle biopsies of patients with mitochondrial cytopathiessuch as LHON, neuropathy ataxia and retinitis pigmentosa (NARP) [36–39]. Similarly, NADH-dehydrogenase stain rarely shows a pattern of decreased activity to suggest the underlying defect, as it has been reported in several cases with severe complex I defect [38,40]. The muscle biopsies are found to be frequently normal in mitochondrial disorders like LHON, which are associated with mutations in various mitochondrial genes [38].

On screening the entire protein coding region of mitochondrial genome, it was observed that in spite of having a very homogeneous clinical presentation these patients had a varied genetic spectrum. A large number of reported and novel nucleotide changes were observed in these patients. Out of total 100 non synonymous changes, 80 variations were exclusively present in patients and were able to project the variation in mtDNA of 143 patients. Remaining 20 non synonymous changes were identified in control as well but interestingly none of these variations were novel in nature.Across the entire mtDNA, the largest number of nucleotide variations was observed in the mt genes encoding subunits of complex I (ND1-ND6, ND4L).At the outset T4216C variation was observed only in one patient. However, thepublished studies have identified its association with different neurological disorders such as LHON, PD and MS, highlighting the significance of this variation in our patient too. Though T4216C is suggested to weakly associate with PD [41] and even in case of MS also it was not found to be significantly associated with the phenotype as per the reports published till 2015 [27]. However, as per the meta-analsyis and systemic reviews, it was suggestedto be one of the important variations, responsible for susceptibility for MS [28]. In addition to MS, the presence of this variation in LHON patients isalso known to increase the penetrance and expressivity of LHON associated mutations [42].Aheteroplasmic G13135A change was present in ND5 gene of 16 patients but absent in controls. This change has already been reported in association with LHON, PD, mitochondrial myopathy, idiopathic cardiomyopathy and many other mitochondrial disorders (Table 2) [43–45] and our study also suggested that G13135A may affect the partially conserved amino acid in ND5 protein. We further observed the co-existence ofA13966G and C14128T n ND5 gene of 8 patients. As per our analysis, A13966G mutation was present in heteroplasmic condition and predicted to affect the secondary structure of ND5 protein by altering it from random coil to beta sheet. The mutation, A13966G has been reported in many mitochondrial disorders like LHON, PD and multiple sclerosis [46,47]. This mutation along with other mtDNA mutations are also reported to help in metastasis of cancer cells [48]. On the other hand, the nucleotide change C14128T is reported as a polymorphism [49,50]. Based upon the published literatures as well our study, G13135A and A13966G/C14128T variations may be proposed to be a potential pathogenic change in patients suffering from different neurological disorders such as LHON, PD, MS mitochondrial myopathy, idiopathic cardiomyopathy including LS. Hence, these variations may be studied as a diagnostic marker for such phenotypes.

Another common mutation, G13708A was found to be present in total 5 patients in this study. This mutation has already been reported as a secondary mutation in more than 43% of the LHON patients and shown to be pathologically significant on a specific haplotype background [51]. G13708A was also reported in patients suffering from multiple sclerosis [52], further highlighting the significance of this variation in our patients. Two novel changes of ND5 gene C13244G and T13994A was also of significance in our study as it was predicted to affect the highly conserved amino acid residues and were found to be INTOLERANT by SIFT analysis. T13994A was further suggested to be "Possibly damaging" for ND5 protein structure by PolyPhen analysis. These predictions together indicated towards the pathogenic nature of these novel nucleotide changes in ND5 gene.

Other ND genes of complex I was also found to have several nucleotide changes. A3796G mutation in ND1 gene is a known pathogenic mutation for adult onset of dystonia [53] and our study also predicted this variation to alter the secondary structure of ND1 protein from beta sheet to alpha helix. It was further important to observe that T10237C change in ND3 gene which is a proven primary pathogenic mutation in patients of LHON and PD [54,55], was observed in our patient too. Further our *in-silico*analysis also predict this change to be "probably damaging" for respective protein. Hence the pathogenic nature of T10237C change may also be hypothesized for our patients.

Next to complex I, several mutations in CytB gene(mitochondrial subunit of complex III) has been reported in patients with myopathy, cardiomyopathy, exercise intolerance and myoglobinuria [56,57]. However, none of them has been reported for LS phenotype. In our study, a few patients were found to carry G14831A, G15110A and T15287C change in their CytB gene, which are known pathogenic mutation for LHON, deafness and diabetes, respectively [58–60]). One novel mutation G15119A which was present in patients only, is proposed to affect a highly conserved amino acid residue and alter the secondary structure of the protein from alpha helix to random coil. It was also predicted to be intolerant for protein function.

As per literature, complex IV deficiency is mostly due to mutations in nuclear genes which code for the proteins important for proper assembly and functioning of this enzyme, but they are not the integral component of the complex. However, in present study, several patients had mutations in their COX genes (COX I, II, III). G6267A change in COX I gene, which is a known pathogenic mutation for prostatecancer and other neurological disorder [61,62], was observed in one patient and proposed to affect a highly conserved amino acid residue. Further, a novel change, G6036A was identified in COX I gene of three patients that affected the highly conserved amino acid residue and predicted to be "possibly damaging" for the COX I protein. Similarly, COX II gene also harbored a novel change A7746G in the patients altered the secondary structure of COX II protein from random coil to beta sheet, at specific position. In addition, A7746G mutation was also predicted to be "possibly damaging" for COX II protein by PolyPhen analysis. Based on the predictions of *in-silico*analysis and their existence in patients only, both these novel changes are expected to affect the complex IV activity.

Several reported and novel nucleotide changes were also observed

in ATPase6 gene, which is an important mitochondrial subunit of complex V. Two novel changes, T8632C and G8863A were present in ATPase6 gene of three patients and were also predicted to alter the secondary structure of ATPase6 protein. Thus, based on their *in-silico* analysis and heteroplasmic status, both these changes seem to be pathogenic in the present study, which can be confirmed by further studies. Other than these two novel changes, several polymorphisms such asG8584A, T8594C, A8812G, T8987C G9053A and T9128C which have already been reported in LHON, several mitochondrial disorders and hearing loss [63–66] were also found in our patients. Similar to our study, several lines of evidences suggest the importance of mitochondrial mutations in patients suffering from stroke and ischaemic heart disease. Studies suggest that accumulation of mutations in mtDNA may affect thefunction of heart in aged individuals which may eventually lead to different cardiac related abnormalities [67,68]. It is proposed that mutated mtDNA may affect the process of OXPHOS and excessive accumulation of ROS can damage the cardiac cells leading to various abnormalities. For instance, it may affect the development and progression of left ventricular remodeling leading to myocardial infarction [69]. Based upon these evidences, we also propose that variations found in mtDNA of these thiamine responsive LS patientsmay lead to increased ROS in neuronal cells, affecting the structure and function of the same.

While comparing the entire mtDNA variations in patients and controls, we observed that certain variations such as A10398G, A 15326G, A8701G and A8860G were not only prevalent in patients but were equally common in most of the controls as well, though with different frequencies. Comparing the frequencies revealed that A15326G and A8860G was significantly high ($p = .0001$) in patients. However there was no significant difference in prevalence of A10398G and A8701G ($p = .0516$ and $p = .795$, respectively) amongst patients and controls. This analysis again emphasizes upon the possible pathogenic nature of A15326G and A8860G variations in these patients.

As per the genetic screening of mtDNA in all these patients and controls, a large number of variations are proposed to be pathogenic based upon different criteria such as their proven association with other disease phenotype, heteroplasmic nature, their absence in controls and predictions of *in-silico* analyses. Out of all the 100 non-synonymous nucleotide changes, several reported variations such as A3796G, T6253C, G6267A, G7830A G7859A, T8594A, T8987C T10237C, A10398G, C10980G, G14831A and few novel variations such as G8863A, A7746G, G7269A, G6036A, and T13994A may be proposed to be most pathogenic variations in our study. After doing the comprehensive analysis of all these important variations, we could further observe that these nucleotide changes may possibly explain the molecular basis of this syndrome in 66.06%(109) of our patients. Based on their *in-silico* predictions, these variations may possibly affect the activity of respective protein and in turn the ATP level too.However, functional assays (cybrid generation) are an important and final step to validate their role in the disease.

As per literature, 1/5th cases of thiamine responsive LS are found to carry mutations in their *PDHE1α* gene [70]. But interestingly none of the patient in our study had any mutation in *PDHE1α* gene. Additionally, majority of patients had low erythrocyte transketolase levels and they exhibited a dramatic recovery by thiamine supplementation [19]. It is proven that thiamine in the form of thiamine pyrophosphate acts like a cofactor for several mitochondrial enzymes like transketolase, pyruvate dehydrogenase, α-ketoglutarate and branched-chain α-ketoacid dehydrogenases, which are directly and indirectly involved in mitochondrial metabolism. By affecting these enzymes, thiamine deficiency may significantly affect the level of ATP production. Thus, both the factors, genetic and thiamine deficiency in combination, may severely affect the respiratory chain enzyme activity in these patients, which may lead to acute and life threatening clinical presentation of these patients.Hence over the huge diversity in terms of genetic abnormality, a pleiotropic effect of thiamine deficiency remains a distinct possibility in them.

5. Conclusion

The present study highlights the high level of genetic heterogeneity in the mt DNA of these patients. Though these mutations were spread across all the mt genome, however many potential and novel pathogenic mutationswere observed to be present in genes encoding different subunits of complex I and IV. Most of these nucleotide changesare known to be associated with several mitochondrial disorders. It may further strengthen the concept of phenotypic heterogeneity of mitochondrial DNA mutations and suggest that on a different haplotype background or with different mutant load, these mutations might also contribute to the phenotype of LS in the study population.The present study has also opened up many avenues for further research towards understanding the genetic basis and possible role of thiamine deficiency in LS patients.

Funding

This work was supported by CSIR, Govt of India and CCMB Hyderabad.

Declaration of Competing Interest

The authors have declared no competing interests.

Acknowledgement

Authors acknowledge Dr. G R Chandak, Scientist, Centre for Cellular and Molecular Biology, Hyderabad for guiding the project. Authors also acknowledge Dr KarunaPottala, Government Institute of Child Health, Niloufer Hospital for Women and Children, Red Hills, Hyderabad, India for helping in sample collection.

References

[1] D. Leigh, Subacute necrotizing encephalomyelopathy in an infant, J. Neurol. Neurosurg. Psychiatry 14 (3) (1951) 216–221.
[2] S. Luo, C.A. Valencia, J. Zhang, L. Ni-Chung, S. Jesse, G. Baoheng, et al., Biparental inheritance of mitochondrial DNA in humans, Proc. Natl. Acad. Sci. U. S. A. 115 (51) (2018) 13039–13044.
[3] Y. Tatuch, J. Christodoulou, A. Feigenbaum, J.T. Clarke, J. Wherret, C. Smith, et al., Heteroplasmic mtDNA mutation (T——G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high, Am. J. Hum. Genet. 50 (4) (1992) 852–858.
[4] V. Tiranti, M. Munaro, D. Sandonà, E. Lamantea, M. Rimoldi, S. Di Donato, et al., Nuclear DNA origin of cytochrome c oxidase deficiency in Leigh's syndrome: genetic evidence based on patient's-derived rho° transformants, Hum. Mol. Genet. 4 (11) (1995) 2017–2023.
[5] S. Rahman, R.B. Blok, H.H. Dahl, D.M. Danks, D.M. Kirby, C.W. Chow, et al., Leigh syndrome: clinical features and biochemical and DNA abnormalities, Ann. Neurol. 39 (3) (1996) 343–351.
[6] R. Horvath, A. Abicht, E. Holinski-Feder, A. Laner, K. Gempel, H. Prokisch, et al., Leigh syndrome caused by mutations in the flavoprotein (Fp) subunit of succinate dehydrogenase (SDHA), J. Neurol. Neurosurg. Psychiatry 77 (1) (2006) 74–76.
[7] I.S. Ruhoy, R.P. Saneto, The genetics of Leigh syndrome and its implications for clinical practice and risk management, Appl. Clin. Genet. 7 (2014) 221–234.
[8] L.C. López, M. Schuelke, C.M. Quinzii, T. Kanki, R.J. Rodenburg, A. Naini, et al., Leigh syndrome with nephropathy and CoQ10 deficiency due to decaprenyldiphosphate synthase subunit 2 (PDSS2) mutations, Am. J. Hum. Genet. 79 (6) (2006) 1125–1129.
[9] P.K. Rai, O.M. Russell, R.N. Lightowlers, D.M. Turnbull, Potential compounds for the treatment of mitochondrial disease, Br. Med. Bull. 116 (1) (2015) 5–18.
[10] D. Lehmann, R. McFarland, Overview of approaches to mitochondrial disease therapy, J. *Inborn. Errors.* Metab. *Screen.* 6 (2018) 1–7.
[11] E. Rinninella, M. Pizzoferrato, M. Cintoni, S. Servidei, M.C. Mele, Nutritional support in mitochondrial diseases: The state of the art, Eur. Rev. Med. Pharmacol. Sci. 22 (13) (2018) 4288–4298.
[12] R.F. Butterworth, Effects of thiamine deficiency on brain metabolism: implications for the pathogenesis of the Wernicke-Korsakoff syndrome, Alcohol Alcohol. 24 (4) (1989) 271–279.
[13] R. Nardone, Y. Holler, M. Storti, M. Christova, F. Tezzon, S. Golaszewski, et al., Thiamine deficiency induced neurochemical, neuroanatomical, and neuropsychological alterations: a reappraisal, Sci. World J. 2013 (2013) 309143.

[14] M. Di Rocco, L.D. Lamba, G. Minniti, U. Caruso, E. Naito, Outcome of thiamine treatment in a child with Leigh disease due to thiamine-responsive pyruvate dehydrogenase deficiency, Eur. J. Pediatr. Neurol. 4 (3) (2000) 115–117.
[15] E. Naito, M. Ito, I. Yokota, T. Saijo, J. Matsuda, H. Osak, et al., Biochemical and molecular analysis of an X-linked case of Leigh syndrome associated with thiamin-responsive pyruvate dehydrogenase deficiency, J. Inherit. Metab. Dis. 20 (4) (1997) 539–548.
[16] S. Pradhan, D. Ghosh, S. Kumar, Leigh's disease: analysis of clinical and imaging characteristics with possible therapeutic implications, Neurol. India 45 (3) (1997) 171–181.
[17] A.A.S.R. Mannan, M.C. Sharma, P. Shrivastava, A.M. Ralte, V. Gupta, M. Behari, et al., Leigh's syndrome-clinical brief, Indian J. Pediatr. 71 (11) (2004) 1029–1033.
[18] L. Pandit, G. Narayanappa, L. Shetty, S. Krishna, Adult onset Leigh syndrome, Ann. Indian. Acad.Neurol. 10 (1) (2007) 55–57.
[19] S. Narasimha Rao, S. Mani, K. Madap, M.V. Kranthi Kumar, L. Singh, G.R. Chandak, High prevalence of infantile encephalitic beriberi with overlapping features of Leigh's disease, J. Trop. Pediatr. 54 (5) (2008) 328–332.
[20] H.J. Manz, Pathology of skeletal muscle: principles of reaction patterns and histochemistry and experience with 195 biopsies, Virchows. Arch. A Pathol. Anat. Histol 386 (1) (1980) 1–19.
[21] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acids Res. 16 (3) (1988) 1215.
[22] M.D. Brown, A. Torroni, C.L. Reckord, D.C. Wallace, Phylogenetic analysis of Leber's hereditary optic neuropathy mitochondrial DNA's indicates multiple independent occurrences of the common mutations, Hum.Mutat. 6 (4) (1995) 311–325.
[23] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. U. S. A. 74 (12) (1997) 5463–5467.
[24] F. Zhao, M. Guan, X. Zhou, M. Yuan, M. Liang, Q. Liu, et al., Leber's hereditary optic neuropathy is associated with mitochondrial ND6 T14502C mutation, Biochem. Biophys. Res. Comm. 389 (3) (2009) 466–472.
[25] E. Dombi, A. Diot, K. Morten, J. Carver, T. Lodge, C. Fratteretal, The m. 13051G > A mitochondrial DNA mutation results in variable neurology and activated mitophagy, Neurology 86 (20) (2016) 1921–1923.
[26] S. Emperador, M. Vidal, C. Hernández-Ainsa, C. Ruiz-Ruiz, D. Woods, A. Morales-Becerra, et al., The decrease in mitochondrial DNA mutation load parallels visual recovery in a leber hereditary optic neuropathy patient, Front. Neurosci. 12 (2018) 61.
[27] S. Andalib, M. Talebi, E. Sakhinia, M. Farhoudi, H. Sadeghi-Bazargani, A. Gjedde, et al., Mitochondrial DNA T4216C and A4917G variations in multiple sclerosis, J. Neurol. Sci. 356 (1–2) (2015) 55–60.
[28] S. Andalib, M. Emamhadi, S. Yousefzadeh-Chabok, A. Salari, A.E. Sigaroudi, M.S. Vafaee, et al., MtDNA T4216C variation in multiple sclerosis: a systematic review and meta-analysis, Acta. Neurolo. Belg. 116 (4) (2016) 439–443.
[29] F.H. Lin, R. Lin, H.M. Wisniewski, Y.W. Hwang, I. Grundke-Iqbal, G. Healy-Louie, et al., Detection of point mutations in codon 331 of mitochondrial NADH dehydrogenase subunit 2 in Alzheimer's brains, Biochem. Biophys. Res. Commun. 182 (1) (1992) 238–246.
[30] D.C. Wallace, J.M. Shoffner, M.D. Brown, A. Torroni, M.T. Lott, M. Cabell, Mitochondrial DNA mutations associated with Alzheimer's and Parkinson's disease, Am. J. Hum. Genet. 51 (1992) A30.
[31] J.M. Shoffner, M.D. Brown, A. Torroni, M.T. Lott, M.F. Cabell, S.S. Mirra, et al., Mitochondrial DNA variants observed in Alzheimer disease and Parkinson disease patients, Genomics. 17 (1) (1993) 171–184.
[32] B. Schwartzkopff, S. Zierz, H. Frenzel, M. Block, E. Neuen-Jacob, K. Reiners, et al., Ultrastructural abnormalities of mitochondria and deficiency of myocardial cytochromec oxidase in a patient with ventricular tachycardia, Virchows. Arch. A Pathol. Anat. Histopathol. 419 (1) (1991) 63–68.
[33] A.E. Vincent, Y.S. Ng, K. White, T. Davey, C. Mannella, G. Falkous, The spectrum of mitochondrial ultrastructural defects in mitochondrial myopathy, Sci. Rep. 6 (2016) 30610.
[34] T. Kyriakides, J.M. Silberstein, S. Jongpiputvanich, E.P.J. Silberstein, P.J. Walsh, S.S. Gubbay, et al., The clinical significance of type 1 fiber predominance, Muscle Nerve 16 (4) (1993) 418–423.
[35] A. Genge, G. Karpati, D. Arnold, E.A. Shoubridge, S. Carpenter, Familial myopathy with conspicuous depletion of mitochondria in muscle fibers: a morphologically distinct disease, Neuromuscul.Disord. 5 (2) (1995) 139–144.
[36] I. Pant, S. Chaturvedi, K. Bala, S. Kushwaha, Muscle histopathology in today's era of molecular genetics: role and limitations, Ann. Indian. Acad. Neu. 18 (4) (2015) 398–402.
[37] C. Darryl, The expanding clinical spectrum of mitochondrial diseases, Brain and Development 15 (1) (1993) 1–22.
[38] M.A. Tarnopolsky, S.K. Baker, T. Myint, C.E. Maxner, J. Robitaille, B.H. Robinson, Clinical variability in maternally inherited leber hereditary optic neuropathy with the G14459A mutation, Am. J. Med. Genet.Part A 124 (4) (2004) 372–376.
[39] C.S. Chi, Diagnostic approach in infants and children with mitochondrial diseases, Pediatr. Neonatol. 56 (1) (2015) 7–18.
[40] J.M. Bourgeois, M. Tarnopalsky, Pathology of skeletal muscle in mitochondrial disorders, Mitochondrion. 4 (5–6) (2004) 441–452.
[41] D. Otaegui, C. Paisan, A. Saenz, I. Marti, M. Ribate, J.F. Marti-Masso, et al., Mitochondrial polymporphisms in Parkinson's Disease, Neuro. Sci. Lett. 370 (2–3) (2004) 171–174.
[42] X. Zhou, H. Zhang, F. Zhao, Y. Ji, Y. Tong, J. Zhang, et al., Very high penetrance and occurrence of Leber's hereditary optic neuropathy in a large Han Chinese pedigree carrying the ND4 G11778A mutation, Mol. Genet. Metab. 100 (4) (2010) 379–384.
[43] T. Ozawa, M. Tanaka, S. Sugiyama, H. Ino, K. Ohno, K. Hattori, et al., Patients with idiopathic cardiomyopathy belong to the same mitochondrial DNA gene family of Parkinson's disease and mitochondrial encephalomyopathy, Biochem. Biophys. Res. Commun. 177 (1) (1991) 518–525.
[44] Y. Qian, X. Zhou, Y. Hu, Y. Tong, R. Li, F. Lu, et al., Clinical evaluation and mitochondrial DNA sequence analysis in three Chinese families with Leber's hereditary optic neuropathy, Biochem. Biophys. Res. Commun. 332 (2) (2005) 614–621.
[45] X. Zhou, Y. Qian, J. Zhang, Y. Tong, P. Jiang, M. Liang, Leber's hereditary optic neuropathy is associated with the T3866C mutation in mitochondrial ND1 gene in three Han Chinese families, Invest. Ophthalmol. Vis. Sci. 53 (8) (2012) 4586–4594.
[46] S. Kösel, E.M. Grasbon-Frodl, U. Mautsch, R. Egensperger, U. von Eitzen, D. Frishman, et al., Novel mutations of mitochondrial complex I in pathologically proven Parkinson disease, Neurogenetics 1 (3) (1998) 197–204.
[47] N. Povalko, E. Zakharova, G. Rudenskaia, Y. Akita, K. Hirata, M. Toyojiro, et al., A new sequence variant in mitochondrial DNA associated with high penetrance of Russian Leber hereditary optic neuropathy, Mitochondrion. 5 (3) (2005) 194–199.
[48] H. Imanishi, K. Hattori, R. Wada, K. Ishikawa, S. Fukuda, K. Takenaga, et al., Mitochondrial DNA mutations regulate metastasis of human breast cancer cells, PloS.one. 6 (8) (2011) e23401.
[49] D. Mishmar, E. Ruiz-Pesini, P. Golik, V. Macaulay, A.G. Clark, S. Hosseini, et al., Natural selection shaped regional mtDNA variation in humans, Proc. Natl. Acad. Sci. U. S. A. 100 (1) (2003) 171–176.
[50] A. Olivieri, M. Achilli, V. Pala, S. Battaglia, N. Fornarino, Al-Zahery, et al., The mtDNA legacy of the Levantine early Upper Palaeolithic in Africa, Science 314 (5806) (2006) 1767–1770.
[51] D.R. Johns, J. Berman, Alternative simultaneous complex I mitochondrial DNA mutations in Leber's hereditary optic neuropathy, Biochem. Biophys.Res.Commun. 174 (3) (1991) 1324–1330.
[52] S. Andalib, M. Talebi, E. Sakhinia, M.F. Arhoudi, H. Sadeghi-Bazargani, M.R. Emamhadi, et al., Mitochondrial DNA G13708A variation and multiple sclerosis: is there an association, Rev. Neurol. 173 (3) (2017) 164–168.
[53] D.K. Simon, J. Friedman, X.O. Breakefield, J. Jankovic, M.F. Brin, J. Provias, et al., A heteroplasmic mitochondrial complex I gene mutation in adult-onset dystonia, Neurogenetics. 4 (4) (2003) 199–205.
[54] J. Horvath, R. Horvath, V. Karcagi, S. Komoly, D.R. John, Sequence analysis of Hungarian LHON patients not carrying the common primary mutations, J. Inherit. Metab. Dis. 25 (4) (2002) 323–324.
[55] D. Ghezzi, C. Marelli, A. Achilli, S. Goldwurm, G. Pezzoli, P. Barone, et al., Mitochondrial DNA haplogroup K is associated with a lower risk of Parkinson's disease in Italians, Eur. J. Hum. Genet. 13 (6) (2005) 748–752.
[56] A.L. Andreu, C. Bruno, T.C. Dunne, K. Tanji, S. Shanske, C.M. Sue, et al., A nonsense mutation (G15059A) in the cytochrome b gene in a patient with exercise intolerance and myoglobinuria, Ann. Neurol. 45 (1) (1999) 127–130.
[57] A.L. Andreu, N. Checcarelli, S.O. Iwata, S. Shanske, S. Dimauro, A missense mutation in the mitochondrial cytochrome b gene in a revisited case with histiocytoid cardiomyopathy, Pediatr. Res. 48 (3) (2000) 311–314.
[58] F.M. Reid, G.A. Vernham, H.T. Jacobs, Complete mtDNA sequence of a patient in a maternal pedigree with sensorineural deafness, Hum. Mol. Genet. 3 (8) (1994) 1435–1436.
[59] S. Fauser, J. Luberichs, D. Besch, B. Leo-Kottler, Sequence analysis of the complete mitochondrial genome in patients with Leber's hereditary optic neuropathy lacking the three most common pathogenic DNA mutations, Biochem. Biophys. Res. Commun. 295 (2) (2002) 342–347.
[60] G.M.C. Janssen, A. Neu, C.M.T. van de Sande, J.A. Maassen, Novel mitochondrial DNA length variants and genetic instability in a family with diabetes and deafness, Exp. Clin. Endocrinol. Diabetes 114 (4) (2006) 168–174.
[61] J.A. Petros, A.K. Baumann, E. Ruiz-Pesini, M.B. Amin, C.Q. Sun, J. Hall, et al., mtDNA mutations increase tumorigenicity in prostate cancer, Proc. Natl. Acad. Sci. U. S. A. 102 (3) (2005) 719–724.
[62] J. Zhang, Z.X. Zhang, P.C. Du, W. Zhou, S.D. Wu, Q.L. Wang, et al., Analyses of the mitochondrial mutations in the Chinese patients with sporadic Creutzfeldt–Jakob disease, Eur. J. Hum. Genet. 23 (1) (2015) 86–91.
[63] S. Finnilä, I.E. Hassinen, K. Majamaa, Phylogenetic analysis of mitochondrial DNA in patients with an occipital stroke: evaluation of mutations by using sequence data on the entire coding region, Mutat. Res. 458 (1–2) (2001) 31–39.
[64] T. Pulkes, D. Liolitsa, I.P. Nelson, M.G. Hanna, Classical mitochondrial phenotypes without mtDNA mutations: the possible role of nuclear genes, Neurology. 61 (8) (2003) 1144–1147.
[65] M. Rodriguez-Ballesteros, M. Olarte, L.A. Aguirre, F. Galan, R. Galan, L.A. Vallejo, et al., Molecular and clinical characterisation of three Spanish families with maternally inherited non-syndromic hearing loss caused by the 1494C→ T mutation in the mitochondrial 12S rRNA gene, J. Med. Genet. 43 (11) (2006) e54.
[66] R. Bi, A.M. Zhang, X. Jia, Q. Zhang, Y.G. Yao, Complete mitochondrial DNA genome sequence variation of Chinese families with mutation m. 3635G > A and Leber hereditary optic neuropathy, Mol. Vis. 18 (2012) 3087–3094.
[67] S. Andalib, A.A. Divani, T.M. Michel, P.F. Høilund-Carlsen, M.S. Vafaee, A. Gjedde, Pandora's Box: mitochondrial defects in ischaemic heart disease and stroke, Expert Rev. Mol. Med. 19 (2017) e5.
[68] M. Hayakawa, S. Sugiyama, K. Hattori, M. Takasawa, T. Ozawa, Age-associated damage in mitochondrial DNA in human hearts, Mol. Cell. Biochem. 119 (1–2) (1993) 95–103.
[69] T. Ide, H. Tsutsui, S. Hayashidani, D. Kang, N. Suematsu, K.I. Nakamura, et al., Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction, Circ. Res. 88 (5) (2001) 529–535.
[70] W. Lissens, L. De Meirleir, S. Seneca, C. Benelli, C. Marsac, B.T. Poll-The, et al., Mutation analysis of the pyruvate dehydrogenase E1α gene in eight patients with a pyruvate dehydrogenase complex deficiency, Hum. Mutat. 7 (1) (1996) 46–51.