

Detection of mtDNA mutations in Egyptian patients with mitochondrial respiratory chain disorders

A thesis Submitted for partial fulfilment of PhD degree of Science in Biochemistry

Submitted by

Ghada Mahmoud Metwally Al-Etribi Al-Hessi

Research Assistant

Medical Molecular Genetics Department

Human genetics and genome research division

National Research Centre

Under the supervision of

Prof. Dr. Amr Mahmoud Karim

Professor of Biochemistry

Biochemistry Department

Faculty of Science

Ain Shams University

Dr. Gamila Mohamed Labib Shanab

Assistant Prof. of Biochemistry

Biochemistry Department

Faculty of Science

Ain Shams University

Prof. Dr. Laila Kamal Al-Din Effat

Professor of Molecular Genetics

Medical Molecular Genetics

department

Human genetics and genome

research division

National Research Centre

Prof. Dr. Hala Al-Tabei Al-Bassyouni

Professor of Clinical Genetics

Clinical Genetic Department

Human genetics and genome

research division

National Research Centre

Biochemistry Department

Faculty of Science

Ain Shams University

2012

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

"يَا أَيُّهَا النَّاسُ قَدْ جَاءَكُمْ مَوْعِظَةٌ مِنْ رَبِّكُمْ وَشِفَاءٌ لِمَا فِي
الْصُّدُورِ وَهُدًى وَرَحْمَةٌ لِّلْمُؤْمِنِينَ (57) قُلْ بِفَضْلِ اللَّهِ
وَبِرَحْمَتِهِ فَبِذَلِكَ فَلْيَفْرَحُوا هُوَ خَيْرٌ مِّمَّا يَجْمَعُونَ (58)"

(سورة يونس)

Praise and gratitude be to Allah, The One, The Self Sufficient, The Impregnable, The All Glorious, The Owner of all sovereignty, The All Beneficent, The Most Merciful, and The Forbearing.

Deep thanks to my LOVELY father, mother, sisters and brother.

I declare that this thesis has been composed by me and the work therein has not been submitted for a degree at this or other university.

Ghada Mahmoud Metwally Al-Etribi Al-Hessi

Contents:

Title	Page
Abstract	I
Acknowledgement	II
List of abbreviations	III
List of figures	IX
List of tables	XIV
List of charts	XVI
Introduction and aim of the work	1
1. Review of literature	4
1.1 Mitochondria	5
1.2 Structure of mitochondria	5
1.3 Functions of mitochondria	6
1.4 Respiratory Chain complexes and Oxidative Phosphorylation	9
1.5 Basic concepts of mitochondrial genetics	10
1.5.1 Structure of mitochondrial DNA	13
1.5.2 Mitochondrial DNA replication transcription and translation	13
1.5.2.1 MtDNA replication	18
1.5.2.2 MtDNA transcription	18
1.5.2.3 MtDNA translation	21
1.5.3 Rules of mitochondrial genetics	22
1.5.3.1 Homoplasmy, heteroplasmy and threshold	23
1.5.3.2 Mitotic segregation	23
1.5.3.3 Maternal transmission and bottleneck	25
1.6 Human mtDNA haplogroups	26
1.7 MtDNA mutations	27
1.7.1 Definition and types	30

1.7.2 Pathogenicity	32
1.2 Mitochondrial diseases	38
1.2.1 Definition and importance	38
1.2.2 Classification of mitochondrial diseases	38
1.2.2.1 Clinical classification of mitochondrial diseases	38
1.2.2.2 Molecular genetics classification of mitochondrial diseases	39
1.2.2.2.1 Molecular genetics classification of MRC diseases	44
1.2.2.2.1.1 MRC diseases due to mutations in mitochondrial genome	44
1.2.2.2.1.2 MRC diseases due to mutations in nuclear genome	47
1.2.3 Diagnosis of mitochondrial diseases	50
1.2.3.1 Diagnostic criteria for mitochondrial diseases	51
1.2.3.2 Current practice in diagnosis of mitochondrial disorders	53
1.2.3.2.1 Clinical evaluation	53
1.2.3.2.2 Clinical investigations	58
1.2.3.2.3 Neuroimaging	59
1.2.3.2.4 Skeletal muscle biopsy	61
1.2.3.2.5 Biochemistry	63
1.2.3.2.6 Molecular genetic analysis	65
1.2.3.2.6.1 Importance and caveats	65
1.2.3.2.6.2 Establishing the molecular diagnosis of mitochondrial diseases.	66
1.2.3.2.6.3 Appropriate tissues to be used for mtDNA molecular diagnosis	68
1.2.3.2.6.4 Molecular methodologies used for diagnosis of mitochondrial disorders	69
1.2.3.2.7 Family history studies	70
1.2.4 Genetic counselling	71

1.2.4.1 Inheritance and risks	71
1.2.4.2 DNA banking.	73
1.2.4.3 Prenatal Testing	73
1.2.5 Pathogenesis of mtDNA disease	74
1.2.6 Potential therapeutic options	74
1.3 Classical mtDNA diseases	77
1.3.1 Leber Hereditary Optic Neuropathy (LHON).	77
1.3.1.1 History of LHON	77
1.3.1.2 Epidemiology of LHON.	77
1.3.1.3 Clinical manifestations of LHON.	77
1.3.1.4 Molecular basis of LHON	80
1.3.1.5 Phenotypic variability of LHON mutations.	82
1.3.1.6 Incomplete penetrance and gender bias in LHON	84
1.3.1.7 Factors affecting disease expression	85
1.3.1.7.1 Heteroplasmy	85
1.3.1.7.2 MtDNA haplogroup	86
1.3.1.7.3 Nuclear genetic factors	88
1.3.1.7.4 Hormonal factors	88
1.3.1.7.5 Environmental factors.	89
1.3.1.8 Diagnosis of LHON	90
1.3.1.9 Pathophysiology of LHON	91
1.3.2 Mitochondrial Myopathy, Encephalopathy, Lactic acidosis, and Stroke-like episodes (MELAS)	94
1.3.2.1 Definition	94
1.3.2.2 History	94
1.3.2.3 Clinical manifestations of MELAS	94
1.3.2.4 Molecular basis of MELAS	97
1.3.2.5 Phenotypic variability of the 3243A>G mutation	100

1.3.2.6 Factors affecting disease expression	100
1.3.2.6.1 Heteroplasmy and tissue distribution	100
1.3.2.6.2 Mitochondrial haplogroups.	102
1.3.2.7 Diagnosis of MELAS	103
1.3.2.7.1 Clinical criteria	103
1.3.2.7.2 Laboratory evaluation	104
1.3.2.7.3 Histology and histochemistry on muscle biopsy.	104
1.3.2.7.4 Neuroimaging.	105
1.3.2.7.5 Molecular testing	106
1.3.2.8 Pathogenesis of MELAS	107
1.3.3 Myoclonus Epilepsy and Ragged Red Fibres (MERRF).	110
1.3.3.1 Definition and clinical manifestations of MERRF	110
1.3.3.2 Molecular basis of MERRF	112
1.3.3.3 Phenotypic heterogeneity of the 8344A>G mutations	113
1.3.3.4 Factors affecting disease expression	114
1.3.3.5 Diagnosis of MERRF	115
1.3.3.6 Pathophysiology of MERRF	116
1.3.4 Leigh Syndrome (LS) and Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP)	118
1.3.4.1 Epidemiology of LS and NARP	118
1.3.4.2 Clinical manifestation of MILS	119
1.3.4.3 Clinical features of NARP	121
1.3.4.4 Molecular basis of MILS and NARP	122
1.3.4.5 The clinical heterogeneity of the mtDNA mutations	124
1.3.4.6 Genotype-Phenotype Correlations	124
1.3.4.7 Diagnosis of LS and NARP	127
1.3.4.7.1 Laboratory evaluation	127
1.3.4.7.2 Histology and histochemistry on muscle biopsy	128

1.3.4.7.3 Biochemical Findings.	128
1.3.4.7.4 Electrophysiology and neuroimaging	129
1.3.4.7.5 Genetics	131
1.3.4.8 Pathogenesis of LS and NARP.	131
2. Patients and methods	134
2.1 Patients	134
2.2 Samples	134
2.3 Clinical evaluation	135
2.4 Reagents and preparations.	135
2.4.1 Reagents used in genomic DNA extraction from blood, using salting-out procedure of <i>Miller et al., 1988</i> .	135
2.4.2 Reagents for PCR	136
2.4.3 Reagents used in RFLP analyses	136
2.4.4 Reagents used in agarose gel electrophoresis	137
2.4.5 Preparation of 50 ml total volume of 1.5 % agarose ge	138
2.4.6 Reagents used in polyacrylamide gel preparation	138
2.4.7 Preparation of 50 ml total volume of 8 and 10 % non-denaturing polyacrylamide gel	139
2.4.8 Reagents used in silver staining	139
2.4.9 Reagents used in automated DNA Sequencing	139
2.5 Molecular Studies	139
2.5.1 Extraction of genomic DNA From blood samples using salting-out procedure of <i>Miller et al., 1988</i>	139
2.5.2 Measurement of DNA concentration and purity (<i>Sambrook et al., 1989</i>)	140
2.5.3 Polymerase Chain Reactions (PCR)	141
2.5.4 Restriction Endonuclease digestions	146
2.5.5 SSCP analyses	155
2.5.6 DNA Sequencing	155
2.5.6.1 Applied biosystems automated DNA sequencing	155

2.5.6.2 The DNA sequencing work	157
3. Results	159
3.1 Clinical Data	159
3.2 Molecular Studies Results	185
3.2.1 Results of PCR amplifications	185
3.2.2 Results of RFLP analyses	186
3.2.3 Results of SSCP analyses	187
3.2.4 Results of DNA sequencing	187
4. Discussion	222
Summary	233
References	237
Arabic summary	
Arabic abstract	

Abstract

Mitochondrial respiratory chain (MRC) diseases are a group of genetically and clinically heterogeneous diseases, caused due to mutations in either the nuclear or the mitochondrial genes that are responsible for oxidative phosphorylation (OXPHOS). The current study aimed to investigate the presence of eleven common mtDNA point mutations in thirty six Egyptian patients with suspicion of having a mitochondrial disease.

PCR-RFLP analysis was pursued for the detection of the 3243A>G, 3271T>C, 8334A>G, 8993T>G/C, 3256 C>T, 4332 G>A, and 12147 G>A mitochondrial DNA (mtDNA) point mutations in all patients. SSCP followed by DNA direct sequencing was pursued for the detection of the 11778G>A, 3460G>A and 14484T>C mtDNA point mutations in eight patients who manifested with optic atrophy.

The molecular analysis did not reveal any of the common mtDNA mutations in the Egyptian patients. DNA sequence analysis of the 11778 and the 3460 fragments for the eight patients with optic atrophy showed 4 mtDNA variants (silent polymorphisms) named 11467A>G, 11719G>A, 3348A>G and 3357G>A in six of them. DNA sequence analysis of the 8344 fragment showed another variant (silent polymorphism) named 8251G>A. It was detected in a homoplasmic state in one patient (P12) and in a heteroplasmic state in all the other patients.

Mitochondrial disorders are caused and influenced by a variety of genetic and racial factors. The negative results of this study indicate that the chosen mutations might not be specific in Egyptians. Another explanation might be the low heteroplasmic levels of the mtDNA mutation that hinder their detection. A registry for different mtDNA mutations in Egyptian patients is highly recommended.

Acknowledgement

I would like to express my deep gratitude and sincere appreciation to **Dr. Amr Mahmoud Karim**, Professor of Biochemistry, Biochemistry Departement, Ain Shams University, for his kind supervision, precious guidance, helpful instructions, abounding patience, and powerful support.

I would like to express my great thanks to **Dr. Laila Kamal Al-Deen Effat**, Professor of Molecular Genetics, Medical Molecular Genetics Department (MMGD), Human Genetics and Genome Research division (HGGRD), National Research Center (NRC), for her sincere guidance, great support, invaluable advice, kind encouragement, and great help.

My profound and sincere thanks to **Dr. Gamila Mohamad Shanab**, Assistant Professor of Biochemistry, Biochemistry Department, Ain Shams University, for her sincere guidance, valuable discussion, great efforts, and time she spent for this thesis.

A word of thanks to **Dr. Hala Al-Bassyouni**, Professor of Clinical Genetics, Clinical Genetics Department, Human Genetics and Genome Research division (HGGRD), National Research Center (NRC), for her valuable guidance, great support, strong encouragement, and kind help in providing us with the blood samples and in interpreting clinical data of the patients.

A word of thanks to **Dr. Maha Saad Zaki**, Professor of Clinical Genetics, Clinical Genetics Department, Human Genetics and Genome Research division (HGGRD), National Research Center (NRC), for her valuable guidance, great support, strong encouragement, and kind help in providing us with the blood samples and in interpreting clinical data of the patients.

I would like to thank all the patients and their family members who participated in this work.

List of abbreviations

<u>Abbreviations</u>	<u>Full term</u>
1H-MRS:	The proton magnetic resonance spectroscopic
8-oxoG:	8-oxo-7,8-dihydroxyguanine
ADP:	Adenosine diphosphate
Ala:	Alanine
AMD:	Age-related macular degeneration
ANT:	Adenine nucleotide translocator
ANT1:	Adenosine nucleoside translocator 1
APS:	Asparagine
Arg:	Arginine
ARMS:	Allele refractory mutation system
ASO:	PCR/allele-specific Oligonucleotide
ATP:	Adenosine 5'-triphosphate
ATPase6:	Adenosine triphosphate synthase subunits 6
ATPase8:	Adenosine triphosphate synthase subunits 8
BER:	Base excision repair
Bp:	Base pair
CACT:	Carnitine–acylcarnitine translocase
CGH:	Comparative genomic hybridization
CI:	Complex I: NADH-coenzyme Q reductase
CI:	Conservation index
CII:	Complex II: succinate-coq reductase
CIII:	Complex III: ubiquinol-cytochrome c reductase
CIV:	Complex IV: cytochrome c oxidase
CNS:	Central nervous system
CoA:	Acetyl–coenzyme A
CoQ:	Coenzyme Q
COXI-III:	Cytochrome c oxidase subunits 1-3
CPEO:	Chronic progressive external ophthalmoplegia
CPK:	Creatine phosphokinase
CPT:	Carnitine palmitoyltransferase
Cr:	Reduced creatine
CRS:	Cambridge reference sequence
CS:	Citrate synthase
CSB:	Conserved sequence blocks
CSF:	Cerebrospinal fluid
CT:	Computed tomography

CV:	Complex V: ATP synthase
CVS:	Chorionic villus sampling
Cyt. B:	Complex III: ubiquinol-cytochrome c reductase
Cyt. C:	Cytochrome c
ddNTPs:	dideoxynucleotides
DF:	Dilution Factor
DIC:	Dicarboxylate carrier
D-Loop:	Displacement loop
DNA:	Dioxy ribo nucleic acid
DQ:	Digital Quotient
DWI:	Diffusion-weighted imaging
EAAT1:	Excitatory amino acid transporter 1
ECG:	Electrocardiogram
ECHO:	Echocardiography
EDTA:	Ethylenediaminetetraacetic acid
EEG:	Electroencephalography
EFTu	Elongation factor Tu
EFTs	Elongation factor Ts
EFG1	Elongation factor G1
EFG2	Elongation factor G2
EMG:	Electromyography
ETC:	Electron transfer chain
ETF:	Electron-transfer flavoprotein
ETFDH:	Electron-transfer dehydrogenase
FADH2:	Reduced flavin-adenine dinucleotide
Gly:	Glycine
H strand:	Heavy strand
HCL:	Hydrochloric acid
His:	Histidine
HRE:	Hormone response element
HSP:	Heavy strand transcription
HUGO:	Human genome organisation
HVR:	Hyper variable regions
IF1, IF2:	MITOCHONDRIAL initiation factors
IMM:	Inner mitochondrial membrane
IQ:	Intelligence Quotient
KCl:	Potassium chloride
kDa:	Kilo dalton
KSS:	Kearns-Sayre syndrome

L strand:	Light strand
Leu:	Leucine
LHON:	Leber's hereditary optic neuropathy
LS:	Leigh's syndrome
LSP:	Light-strand transcription
MELAS:	Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF:	Myoclonic epilepsy and ragged red fibers
Met:	Methionine
MgCl₂:	Magnesium chloride
MIDD:	Maternally inherited diabetes and deafness
MILS:	Maternal inherited Leigh's syndrome
MIM:	Mendelian Inheritance in Man
MIMDSs:	Multiple mtdna deletion syndromes
MNGIE:	Mitochondrial neurogastrointestinal encephalomyopathy
MRC:	Mitochondrial respiratory chain
MRCA:	Matrilineal most recent common ancestor
MRP:	Mitochondrial ribosomal proteins
MRS:	Magnetic resonance spectroscopy
MSL:	Multiple systemic lipomatosis
MT-ATPase 6:	The mitochondrial ATP synthase subunit 6
MT-CO1:	The mitochondrial Cytochrome c oxidase subunits 1
MT-CYB:	The mitochondrial cytochrome-b
mtDNA:	Mitochondrial deoxyribonucleic acid
mTERF:	Mitochondrial transcription termination factor
MT-NC1, 2, 7, 8:	the mitochondrial non-coding nucleotides 1, 2, 7, 8
MT-ND1-6 and 4L:	The mitochondrial NADH reductase subunits 1-6 and 4L
MtRF1a:	Termination release factor
MtRNAGln:	The mitochondrial transfer RNA Glutamine
mtRNAHis:	The mitochondrial transfer RNA Histidine
mtRNALeu (UUR) :	The mitochondrial transfer RNA Leucine1
mtRNALeu1:	The mitochondrial transfer RNA Leucine1
mtRNALys:	The mitochondrial transfer RNA Lysine
MT-RNR2:	The mitochondrial 16s rna
MT-RNR3:	The mitochondrial 5S-like sequence
MT-TC:	The mitochondrial transfer RNA cystiene
MT-TER:	Mitochondrial transcription terminator