A Novel Mitochondrial DNA (mtDNA) Mutation Significantly Attenuates Transcription Termination In A Patient With A Mitochondrial Myopathy

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The scientist has a lot of experience with ignorance and doubt and uncertainty, and this experience is of very great importance, I think.

It is scientific only to say what is more likely and what less likely, and not to be proving all the time the possible and impossible.

Our imagination is stretched to the utmost, not, as in fiction, to imagine things which are not really there, but just to comprehend those things which are there.

Richard Feynman

What is science in the last analysis but the study and the love of Nature, displayed not in the form of abstract worship but in the practical form of seeking to understand Nature?

... the principal requisite for success in scientific research is not the maturity of knowledge associated with age and experience, but the freshness of outlook which is the natural attribute of youth.

Sir C.V.Raman

"*...what a long strange trip it's been*"

The Grateful Dead

Dedication

I dedicate this thesis to the memory of my mother, Lalitha Raghupathi (1941-2001) and my maternal grandmother, G. Ambujammal (1918-2010).

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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge or belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Ravinarayan Raghupathi

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love and support have carried me through the good times and the bad and she has been an incredible source of strength and inspiration.

Abbreviations

8-OHdG	8-hydroxy-2-deoxyguanosine
AD	Alzheimer's Disease
ADP	Adenosine diphosphate
ADPD	Alzheimer's Disease and Parkinson's Disease
adPEO	Autosomal Dominant Progressive External Ophthalmoplegia
ALS	Amyotrophic Lateral Sclerosis
ALT	Alanine transaminase
AP	Alkaline phosphatase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BN-PAGE	Blue-Native Polyacrylamide Gel Electrophoresis
bp	Base-pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CK	Creatine kinase
CNS	Central Nervous System
CoA	Coenzyme A
CoQ	Coenzyme Q
COX	Cytochrome *c* oxidase
CPEO	Chronic progressive external ophthalmoplegia
CRS	Cambridge Reference Sequence

CS	Citrate synthase
CSB	Conserved Sequence Blocks
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra-acetic acid
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
ETC	Electron Transport Chain
FMN	Flavin mononucleotide
GGT	gamma-glutamyl transferase
HCl	Hydrochloric acid
HD	Huntington's Disease
HMG	High mobility group
Hsp	Heat shock protein
HSP	Heavy strand promoter
IMS	Inter-membrane space
KCl	Potassium chloride
KCN	Potassium cyanide
KSS	Kearns-Sayre syndrome
LHON	Leber's Hereditary Optic Neuropathy
LIMM	Lethal Infantile Mitochondrial Myopathy

LSP	Light strand promoter
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms
MERRF	Myoclonic Epilepsy and Ragged-Red Fibre Disease
$MgCl_2$	Magnesium chloride
MMC	Maternally Inherited Myopathy and Cardiomyopathy
MND	Motor Neuron Disease
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
mtDBP	Mitochondrial displacement (D)-loop binding protein
mtDNA	Mitochondrial DNA
mTERF	Mitochondrial transcription termination factor
MTG	MitoTracker Green
mt-Hsp	matrix heat shock protein
mtRNA	Mitochondrial RNA
MTS	Matrix targeting sequences
mtSSB	mitochondrial single-stranded binding protein
mtTFA/TFAM	Mitochondrial transcription factor A
NAD	Nicotinamide adenine dinucleotide
NADH	reduced Nicotinamide adenine dinucleotide
NADH-TR	NADH-tetrazolium reductase
NARP	Neurogenic ataxia retinitis pigmentosa
NCR	Non-coding regions
nt	Nucleotide
OXPHOS	Oxidative phosphorylation

PAGE	Polyacrylamide gel electrophoresis
PAM	Presequence Translocase Associated Motor
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PCR-RSM	PCR-mediated restriction site modification
PD	Parkinson's Disease
PEG	Poly-ethylene glycol
PEM	Progressive encephalomyopathy
POLRMT/mtRPOL	Mitochondrial RNA Polymerase
PPR	Pentacotripeptide Repeat
PTP	Permeability Transition Pore
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
REST	Relative Expression Software Tool
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RRF	Ragged-Red Fibres
rRNA	Ribosomal RNAs
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SAM	Sorting and assembly machinery
SD	Standard deviation
SDH	Succinate dehydrogenase
SEM	Standard error of the mean

SSC	Saline sodium citrate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
tRNA	Transfer RNA
Wt	Wild type

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Abstract

This PhD study aimed to characterise the pathophysiology of a novel mitochondrial mutation in a patient with a mitochondrial myopathy. This mutation, an adenine insertion at nucleotide (nt) position 3230 of the human mitochondrial genome (RefSeq NC_012920), was shown to significantly disrupt transcription termination, leading to increases in the levels of both the genome-length mitochondrial DNA (mtDNA) polycistronic transcript and selected mRNAs encoding subunits of the respiratory chain complexes. A corresponding increase in the levels of two subunits of Cytochrome *c* oxidase (COX, Complex IV) was observed; however, there was no increase in the levels of any of the respiratory chain holocomplexes. Complex I and Complex IV activities were elevated in the proband's tissues. No evidence of DNA damage through apoptosis or necrosis was found and the proband's cells did not show elevated levels of 8-hydroxy-2-deoxyguanosine, a biomarker of oxidative stress. Attenuation of transcription termination in human mitochondria appears to be a novel mechanism of mitochondrial disease.

Preface

This thesis is divided into six chapters. Chapter I provides a comprehensive review of pertinent literature in the field of human mitochondrial disease. It covers the biogenesis of mitochondria, mitochondrial function and dysfunction and explores the approaches commonly used in the study of mitochondrial disease. It ends with the background to this project, including the clinical case study, and outlines the aims of the project.

Chapter II details the biological samples used in this study, which included lymphoblasts, cytoplasmic hybrids (cybrids) and skeletal muscle biopsy samples obtained from the proband and six controls and the experimental procedures used to obtain the results described.

In Chapter III, the initial molecular genetic analysis of the mutation is described. Using a PCR-RFLP assay, the pedigree and tissue distribution of the mutation and its load in different samples was studied. Mitochondrial mass and number were assayed using a mitochondrion-selective fluorophore, MitoTracker Green. The effect of this mutation on $tRNA^{Leu(UUR)}$ folding was analysed using mFOLD.

Chapter IV describes the study of the effects of the mutation on mtDNA transcription and translation. The levels of the polycistronic transcript and three mtDNA mature transcripts (ND1, ND2 and COX 1) were measured by Real-time RT-PCR and Northern blotting. Respiratory chain holocomplex levels were analysed by Blue-Native PAGE and mass spectrometry. The levels of COX sub-

units I, II and IV were measured by immunoblotting with monoclonal antibodies against each subunit.

The penultimate Chapter (V) details the study of the effects of this mutation on mitochondrial function. Standardised spectrophotometric assays were used to measure respiratory chain activity. DNA damage was analysed by gel electrophoresis to look for the typical DNA laddering associated with apoptosis. A commercial ELISA kit was used to measure 8-OHdG levels.

Chapter VI presents an in-depth discussion of the overall findings and looks at their implication in a novel mechanism of mitochondrial disease in humans. This project has paved the way for interesting future projects, some of which are introduced in this chapter.

Note: unless otherwise mentioned, all artwork presented in this thesis is original.