MALIGNANT HYPERTHERMIA

ALLELE SPECIFIC EXPRESSION AND MUTATION SCREENING OF THE RYANODINE RECEPTOR 1

A dissertation presented to Massey University in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry

Hilbert Grievink

2009

To be conscious that you are ignorant is a great step to knowledge

Benjamin Disraeli (1804-1881)

ACKNOWLEDGEMENTS

I would like to take this opportunity to first and foremost thank both my supervisors Assoc. Prof Max Scott and Assoc. Prof. Kathryn Stowell for their expertise, guidance and support during the last three years.

Kathryn, I will be forever grateful for the opportunities you have offered me and helped me realize. I sincerely thank you for presenting me with the possibility to do a PhD after my internship in your research group. You have always been enthusiastic, inspiring and supportive in your advice and guidance. Writing my thesis made me not only realize how much I have learned during these three years but also made me thankful for how much I enjoyed them.

Furthermore I would like to thank everybody in the Twilite Zone for the great times, elaborate pot-luck dinners and awesome lab outings. Will, thanks for all those great games of squash(ing). And Robyn, thank you for an awesome trip around Europe, the good times in the lab, and the much needed distraction (by Robert's emails) from the seemingly endless thesis writing.

I would also like to acknowledge Elaine Langton (Wellington Hospital) and Neil Pollock (Palmerston North Hospital) for supplying blood samples for genomic DNA extractions used for HRM. Thanks also goes to, Jo-Anne Stanton and Chris Mason from the Department of Anatomy and Structural Biology at University of Otago, for their contribution to the 454 data analysis. In addition, I am most grateful to Anthony Thrush and Paula Shields from Roche Diagnostics New Zealand, for their constant technical support over the last three years. Finally, this work would not have been possible without the financial support from the Royal Society of New Zealand Marsden Fund given to Assoc. Prof. Kathryn Stowell.

I am also greatly indebted to my family who have always shown me, love, support and understanding even when I decided to move to the other side of the world. I am looking

forward to being a bit closer to home again. Last but not least I would especially like to thank my wife Liat. Thank you for all your understanding, patience and encouragement over the last three years, all while undertaking your own PhD. I am really sorry for all those times I bored you with my far too detailed monologs of how cool or frustrating my results were. I wish I could promise you it won't happen again.

ABSTRACT

Malignant hyperthermia (MH) is a dominant skeletal muscle disorder caused by mutations in the ryanodine receptor skeletal muscle calcium release channel (RyR1). Allele-specific differences in RyR1 expression levels might provide insight into the observed incomplete penetrance and variations in MH phenotypes between individuals.

Firstly, an H4833Y allele-specific PCR (AS-PCR) assay was designed that allowed for the relative quantification of the two *RYR*1 mRNA alleles in heterozygous samples. In four MHS skeletal muscle samples and two lymphoblastoid cell lines (LCLs), the wild type allele was found to be expressed at higher levels than the mutant RyR1 allele. These differences were not caused by variations in *RYR*1 mRNA stabilities. Secondly, high-throughput amplicon sequencing was employed for the quantification of both the T4826I and H4833Y causative MH mutations in heterozygous MHS samples. With the exception of one, all detected H4833Y and T4826I mutation frequencies were about 50%. This included a control, which was constructed and proven to have a 3:1 ratio of the wild type (H4833) versus the mutant (Y4833) *RYR*1 allele. This suggested that that the high-throughput amplicon sequencing approach as used here, was not suitable for accurate quantification of the two RyR1 alleles in heterozygous H4833Y MHS samples.

To detect possible variations in RyR1 alleles at the protein level, the RyR1 was to be isolated from microsomes prepared from a H4833Y MHS frozen skeletal muscle tissue. Microsomes isolated from MHS skeletal muscle tissues lacked the immunoreactive band that was believed to be the full length RyR1. Poor muscle quality, due to long term storage was believed to be the main cause of RyR1 depletion.

Faster and less expensive screening methodologies are required for the identification of genetic variants in MH research. Thus, in an additional project inexpensive and high-throughput high-resolution melting (HRM) assays were developed to allow screening of the *RYR*1 gene, for mutations associated with MH and/or central core disease (CCD).

ABBREVIATIONS

ACTA1	Skeletal muscle α-actin
apoCaM	apo-calmodulin
AS1	Allele-specific primer 1
AS2	Allele-specific primer 2
AS-PCR	Allele-specific PCR
ATP	Adenosine triphosphate
AVA-CLI	Amplicon Variant Analyser Command Line Interface
CaM	Calmodulin
CCD	Central core disease
cDNA	Complementary DNA
CICR	Calcium-induced Ca^{2+} release channel
CLI	Command Line Interface
CSQ	Calsequestrin
Ct values	PCR crossing points
CV	Coefficient of variance
DEPC	Diethylpyrocarbonate
DHPR	Dihydropyridine receptor
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleoside triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
E	Amplification efficiency
ECCE	Excitation-coupled Ca^{2+} entry
EC-coupling	Excitation-contraction coupling
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic initiation factor 4E
EMHG	European Malignant Hyperthermia Group
emPCR	Emulsion PCR
ER	Endoplasmic reticulum
FKBP-12	12 kDa FK506 binding protein

FKBP12.6	12.6 kDa FK506 binding protein
gDNA	Genomic DNA
GUI	Graphical user interface
HPRT	Hypoxanthine-guanine-phosphoribosyltransferase
HRC	Histidine-rich calcium binding protein
HRM	High-resolution melting
IP_3R	Inositol 1,4,5-triphosphate receptors
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IRE	Iron-responsive element
IRES	Internal ribosome entry sites
IVCT	*In vitro* contracture test
JFM	Junctional face membrane
KcsA	Bacterial K^+ channel
LCL	Lymphoblastoid cell line
m7G	7 methylguanosine
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MH	Malignant Hyperthermia
MHE	Malignant hyperthermia equivocal
MHN	Malignant hyperthermia negative
MHS	Malignant hyperthermia susceptible
MHS1 to 5	Malignant hyperthermia loci 1 to 5
miRNA	MicroRNA
MmD	Multi minicore disease
mRNA	Messenger RNA
MthK	*Methanobacterium autotrophicum* potassium channel
MYH7	Beta-myosin heavy chain
NAMHG	North American Malignant Hyperthermia Group
NTC	Non template control
ORF	Open reading frame
PABP	Poly(A)-binding protein
PMCA	Plasma membrane calcium ATPase
PPi	Pyrophosphate

RT-PCR	Reverse transcription-PCR
RyR1	Skeletal muscle ryanodine receptor 1 isoform
RyR2	Cardiac muscle ryanodine receptor 2 isoform
RyR3	Brain ryanodine receptor 3 isoform
SEPN1	Selenoprotein gene
SERCA	Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase
SNP	Single nucleotide polymorphism
SOCE	Store-operated Ca^{2+} entry
SR	Sarcoplasmic reticulum
sstDNA	Single strand template DNA
T_m	Melting temperature
TRPC	Transient receptor potential channel
T-tubule	Transverse-tubule
UTR	Untranslated region
XALD	X-linked adrenoleukodystrophy
X-Gal	5-Bromo-4-Chloro-3-Indolyl-BD-Galactopyranoside

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS