Molecular Genetic Analysis for Malignant Hyperthermia

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ABSTRACT

Malignant hyperthermia (MH) is a rare pharmacogenetic disorder in humans caused by inhalational general anaesthetics and depolarising muscle relaxants. An MH reaction shows abnormal calcium homeostasis in skeletal muscle leading to a hypermetabolic state and increased muscle contracture. A mutation within the calcium release channel ryanodine receptor of skeletal muscle (*RYR1*) is one of the causes of MH leading to the abnormally high release of calcium ions into the cytosol during MH reactions.

The MH reaction can also be triggered by excess exercise, heat and stress. A New Zealand male, identified as M818, showed a fulminant MH reaction which resulted in death. The reaction was caused by exercise, and he did not have a family history of MH. As this individual did not have any of the mutations within *RYR1* found to date in New Zealand families, the entire *RYR1* cDNA was screened for a novel mutation that may result in susceptibility to exercise-induced MH. This patient may have had a novel *RYR1* mutation because exercise-induced MH is quite rare. Screening of this gene, however did not identify any mutations within *RYR1*, suggesting that the M818 patient may have a mutation in another gene because MH is a heterogeneous disorder with 40-50% of families showing linkage to alternative loci.

Heterogeneity of MH can result in discordance between genotype and phenotype. Some MH susceptible patients do not have a *RYR1* mutation that is found in other individuals with the same kindred. One or more other genes could be associated with MH for these individuals although alternative loci have not been studied in New Zealand families. A genome-wide scan was performed to search for other candidate loci using a large MH kindred known as the CH family within which discordance has been observed. Non-parametric linkage analysis across all chromosomes identified five weak linkages from one branch, and two strong linkages from another branch of the CH family. Secondary linkage analysis was performed on one candidate locus identified in the genome-wide scan, and a weak linkage and recombination was observed within the shorter region. No candidate genes with obvious relevance to calcium homeostasis or signalling were identified within this region. The existence of alternative causative loci in this family cannot be ruled out however, because the loci identified from the genome-wide scan are very large and contain many genes of unknown function.

ABBREVIATIONS

A	absorbance
ATP	adenosine triphosphate
bp	base pair
CCD	central core disease
cDNA	complementary DNA
cM	centimorgan
4-CmC	4-chloro-m-cresol
C-terminal	carboxy terminal
DEPC	diethylpyrocarbonate
DHPR	dihydropyridine receptor
DNase	deoxyribonuclease
DMSO	dimethyl sulphoxide
dNTPs	dinucleotide triphosphates
EC	excitation-contraction
EDTA	ethylene diamine tetra-acetate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[4-butanesulphonic acid]
IVCT	*in vitro* contracture test
kb	kilobase
MH	malignant hyperthermia
MHE	malignant hyperthermia equivocal (European protocol)
MHN	malignant hyperthermia negative (European protocol)
MHS	malignant hyperthermia susceptible (European protocol)
MH+	malignant hyperthermia positive (North American protocol)
MH-	malignant hyperthermia negative (North American protocol)
mRNA	messenger RNA

MOPS	3-[N-morpholino]propanesulphonic acid
NPL	non-parametric linkage
N-terminal	amino terminal
oligo(dT)	oligodeoxythymidine
pCO_2	carbon dioxide pressure
PCR	polymerase chain reaction
PCr	phosphocreatine
Pi	inorganic phosphorus
^{31}P NMR	phosphorus-31 nuclear magnetic resonance
RFLP	restriction fragment length polymorphism
RNase	ribonuclease
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
RYR	ryanodine receptor
SIDS	sudden infant death syndrome
SR	sarcoplasmic reticulum
SSCP	single stranded conformational polymorphism
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
Tm	melting temperature
Tris	tris (hydroxymethyl) aminomethane
T-tubule	transverse tubule
UV	ultraviolet light
VNTR	variable number of tandem repeat polymorphism

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

CHAPTER THREE : SCREENING OF MUTATIONS

CHAPTER FOUR : A GENOME-WIDE SCAN

CHAPTER ONE : INTRODUCTION

1.1 Overview of Malignant Hyperthermia

1.1.1 Introduction

Malignant Hyperthermia (MH) is an autosomal dominant inherited disorder of the skeletal muscle in humans as well as in different animals and is triggered by volatile anaesthetics and depolarising muscle relaxants used in general anaesthesia. MH leads to a hypermetabolic state and an abnormally high release of calcium ions from the sarcoplasmic reticulum of skeletal muscle (1). Palmerston North Hospital is the sole MH testing centre in New Zealand and also maintains a database of MH reactions and affected families. The incidence of MH is much higher in the lower North Island than other areas worldwide because there are some large families known to have the disorder. One patient will be diagnosed and treated as MH susceptible for every 100 patients in general surgery at Palmerston North Hospital (2). To date, about 40 families have been confirmed as MH susceptible in New Zealand from clinical reactions or *in vitro* contracture testing.

1.1.2 History

Inexplicable deaths and abnormal reactions during operations or in a postoperative stage were reported in the early 1900s. In 1960, it was reported that anaesthetics could cause hyperthermia and even death in several families (3). The disorder was found to be hereditary and the result of a dominant trait (4). Several years later, it was observed that individuals who had recovered from MH showed higher muscle contracture against halothane and caffeine in an *in vitro* test (5, 6). From these observations, the European MH Group developed a protocol of diagnostic testing using muscle specimens (7). The North American MH Group also developed a test protocol differing only in minor aspects (8). These protocols are still used today as the "gold standard" diagnostic tests for MH. The incidence of MH on a worldwide scale has been estimated to be 1:15,000 anaesthetics for children and 1:50,000 for adults (9). In the 1970s, two-thirds of affected patients died during or as a result of anaesthesia, usually from cardiac arrest. Dantrolene

sodium was introduced as an antidote for MH in 1979 (10) and as a result, mortality has dropped to less than 5 % today (11).

1.1.3 Clinical symptoms

Establishing the diagnosis of MH by clinical signs is difficult because none of the clinical symptoms can be regarded as specific signs of MH (12). MH reactions have variable intensity and time course. General signs observed from MH reactions include tachycardia, respiratory or metabolic acidosis, masseter spasm, generalised muscular rigidity, arrhythmias, rhabdomyolysis, skin mottling, and rapid temperature elevation (13, 14). Attempts have been made to predict MH susceptibility by observing these clinical indicators (15), however MH reactions can develop slowly, even in a postoperative onset without any clinical signs during the operation (16). Furthermore, some case reports have shown that MH susceptible individuals do not always show abnormal reactions against triggering drugs (17). These atypical forms of MH reactions may delay anaesthetists in establishing diagnoses and treatments of MH. Nevertheless, a clinical grading scale has been established to evaluate MH-like symptoms and is used to assess the relatively likelihood of clinical symptoms actually being MH (15).

1.1.4 Triggering agents

MH reactions can be triggered by anaesthetics widely used in general anaesthesia. These include non-halogenated anaesthetics such as ether and halogenated ones such as halothane, sevoflurane and desflurane (18). Halothane seems to be the most potent trigger. Depolarising muscle relaxants such as succinylcholine also induce MH although it has been reported that pure succinylcholine itself does not induce muscle contraction (19). Commercial succinylcholine solutions contain cresol derivatives such as 4-chloro-m-cresol (4-CmC) as a preservative, and it is thought that 4-CmC itself can be a trigger of MH reactions (20, 21). Chlorocresol derivatives are routinely added to commercial drugs as preservatives, and these drugs should not be used for MH susceptible patients. Some case reports have shown that psychotropic substances or tranquillisers like phenothiazines and tricyclic antidepressants may be triggers of MH although this is still controversial because of a low number of case reports (22, 23). These drugs should not be given to MH patients because they may induce symptoms

similar to MH and may hamper the establishment of a clinical diagnosis of MH.

Non-anaesthetic factors can also induce MH reactions. Exercise before the administration of a triggering agent increases the incidence and intensity of MH (24), and overheating alone can trigger MH reactions in swine (25). In fact, some case studies have reported MH reactions related to heat stroke in humans (26, 27). Some MH susceptible individuals show an abnormal response to graded exercise (28), and it has been confirmed that strenuous exercise can trigger MH in some individuals (29). Mental excitement may increase the intensity of reactions against drugs (30) and variance of intensity has been observed in an anxious patient (31). Hence, it has been suggested that MH may be a human stress syndrome (32). Overheating caused by viral infection has also been reported to induce MH (33).

1.1.5 Management and treatment

Any triggering anaesthetics should be avoided for MH susceptible individuals during anaesthesia. There are some suitable drugs for patients (34), and the non-triggering anaesthetic, propofol, is often used today for MH susceptible individuals. It is a non-water-soluble intravenous agent that does not trigger MH reactions (35). If MH reactions occur, it is critical to establish the diagnosis and terminate the administration of triggering drugs immediately. Dantrolene sodium is very effective in an MH reaction by preventing a hypermetabolic state. Immersion in an ice-cold bath is also effective for fulminant hyperthermia. Patients who suffer an MH reaction should be treated in an intensive care unit with body temperature and pulse being monitored. Patients who recover from MH, however may suffer sequeli such as muscle disability from rhabdomyolysis or impaired function of major organs (5).

1.2 Pathophysiology of MH

1.2.1 Introduction

Although the exact mechanism of variable MH reactions is not understood, malfunction of intracellular Ca^{2+} homeostasis has been shown to play an important role (36). MH susceptible individuals show higher concentrations of cytosolic Ca^{2+} during MH

reactions and also in normal conditions without exposure to triggering drugs (37). In addition, an abnormal sarcoplasmic reticulum (SR) ryanodine receptor in the skeletal muscle has been reported to be responsible for the abnormal calcium release in ~50% of affected families (38).

1.2.2 Excitation-contraction coupling

Intracellular Ca^{2+} regulates a wide range of events in all cells, such as cell growth, mitochondrial function, gene expression and muscle contraction. Ca^{2+} plays a fundamental role in excitation-contraction (EC) coupling of skeletal muscles (39). The wave of depolarisation from nerves is detected by an L-type voltage-dependent Ca^{2+} channel called the dihydropyridine receptor (DHPR) located in the wall of the transverse tubule (T-tubule). DHPR transmits the signal to the functionally coupled calcium release channel, ryanodine receptor (RYR), and activates it to release Ca^{2+} from the SR to intracellular cytosol (Figure 1-1). The released Ca^{2+} binds to troponin and activates actin, and muscle contraction occurs. The intracellular free Ca^{2+} is rapidly transferred back to the SR by ATP-ase calcium pumps and stored within the lumen, and muscle relaxation occurs (40).

1.2.3 Dihydropyridine receptor

The DHPR is located in the T-tubule membrane and detects membrane depolarisation. It is an L-type calcium channel passing Ca^{2+} through to the cytoplasm. It is also a voltage sensor, and if it detects a signal from nerves, it transmits the signal to the RYR leading it to open and releasing Ca^{2+} from the SR. The DHPR is an asymmetric protein which consists of five subunits (41) (Figure 1-1). The α1-subunit (185 kDa) consists of four domains I to IV and is located in the T-tubule membrane (42). This subunit contains positive charges and senses plasma membrane depolarisation. It complexes with a cytoplasmic subunit (β, 54 kDa) and two intra membrane subunits (γ, 30 kDa and δ, 26 kDa). An extracellular subunit (α2, 143 kDa) is linked via a disulfide bridge to the δ-subunit. The DHPR is clustered with the T-tubule and RYR forming the triad, and the signal is transmitted through the loop between the II and III domains to the RYR (Figure 1-1), controlling release of Ca^{2+} and following EC coupling via the RYR (43). The activated RYR also transmits a current-enhancing signal to the DHPR through the II-III

loop (44).

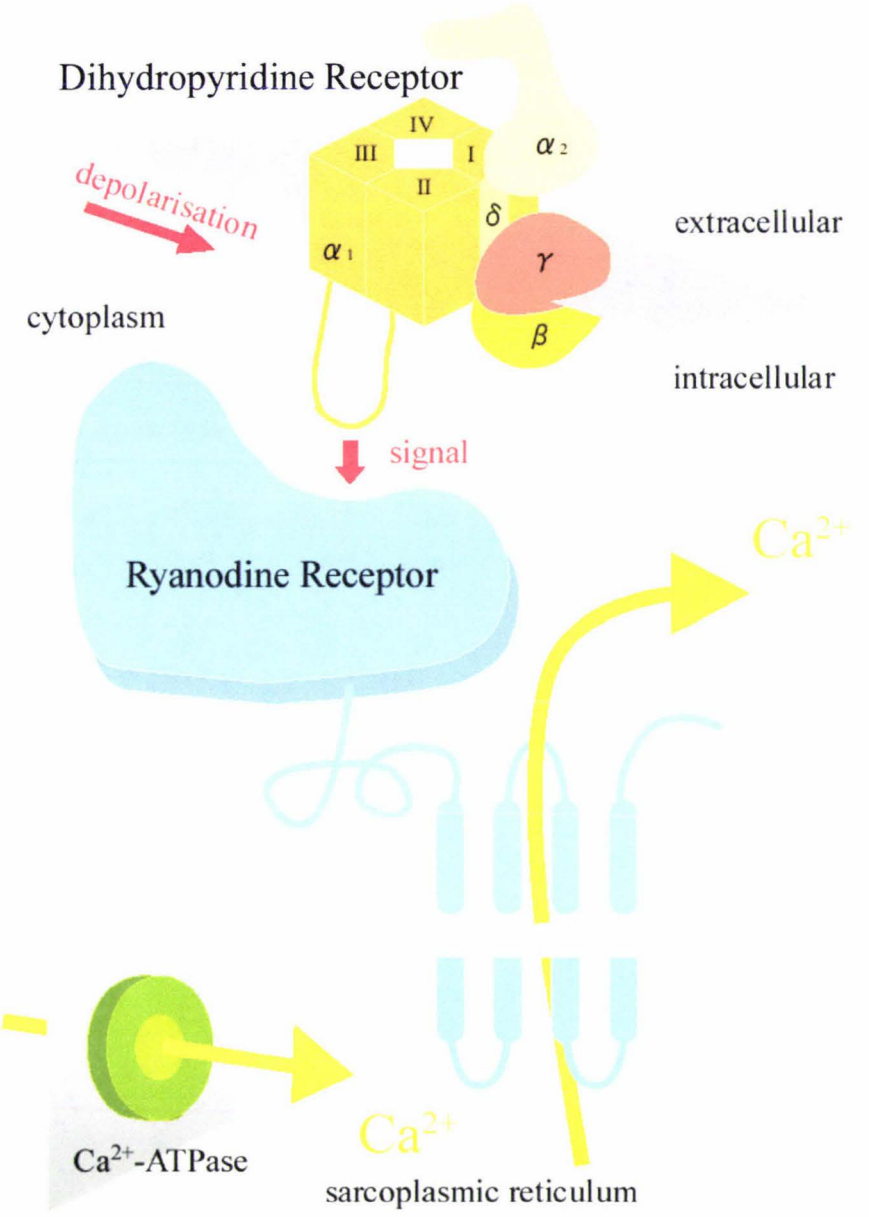

Figure 1-1 The excitation-contraction pathway of skeletal muscle

A depolarising signal is received by the DHPR and transmitted to the RYR through the II-III loop causing a release of calcium ions from the SR. One DHPR and one of four monomers of RYR are shown. Other associated proteins are omitted. The released calcium ions are pumped back to the SR lumen by a calcium transport ATPase. Diagram adapted from references (45) and (46).

1.2.4 Ryanodine receptor

The RYR is a large Ca^{2+} channel situated in the SR membrane (47) and is built up of four monomers, each with a molecular weight of about 560 kDa (48, 49). About 80 % of the protein is exposed in the cytoplasm forming the foot structure (50), and the remaining carboxyl terminal (C-terminal) region is embedded in the SR membrane (Figure 1-1). The RYR receives the signal from the activated DHPR, and opens the channel releasing Ca^{2+} from the SR lumen to cytoplasm. Three separate genes identified as *RYR1*, *RYR2* and *RYR3* have been found to encode the RYR (51), and each is predominantly expressed in skeletal muscle, cardiac muscle or brain and smooth muscle, respectively (52-54).

1.2.5 The proposed mechanism of MH reactions

As Ca^{2+} controls several important systems in cells, Ca^{2+} concentrations within the cytoplasm are under tight control. The activated RYR has a very short open time and is regulated by ATP, Mg^{2+} and Ca^{2+}. In addition, released Ca^{2+} within the cytoplasm is pumped back to the SR readily by a Ca^{2+}-ATPase (Figure 1-2, left). Hence, the metabolic state and muscle contracture do not last long. In contrast, an MH crisis shows dysfunctional control of Ca^{2+} release. In muscle specimens from MH susceptible swine, the RYR shows a prolonged duration of the open state and has a defect in a low-affinity Ca^{2+} binding site (55). In addition, Ca^{2+} release in MH susceptible muscles is reduced far later than in normal specimens (56). Hence, it is thought that the RYR does not close readily during MH reactions allowing large amounts of Ca^{2+} to pass through to the cytoplasm. The released Ca^{2+} induces muscle contraction and mitochondrial metabolism (Figure 1-2, right). Excessive muscle contraction causes membrane damage leading to rhabdomyolysis, and the overall higher metabolic activity causes increased CO_2 and heat production as well as increased O_2 consumption leading to acidosis, tachycardia and hyperthermia. These events may cause death because of cardiac arrhythmia or cardiac arrest.

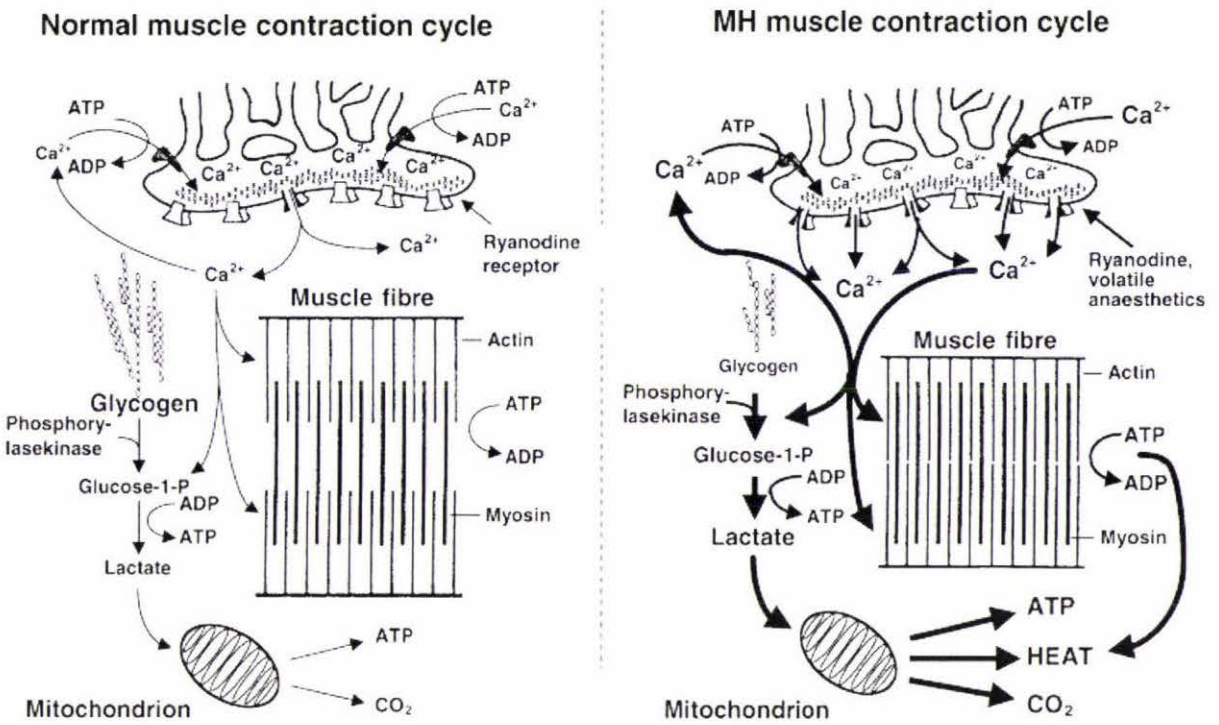

Figure 1-2 Proposed mechanism for induction of malignant hyperthermia

The activated RYR releases calcium ions from the SR to cytosol. Released intracellular Ca^{2+} within the muscle cell induces muscle contraction. The RYR closes immediately and Ca^{2+}-ATPase pumps rapidly transfer unbound Ca^{2+} back into the SR, and normalised Ca^{2+} concentration results in muscle relaxation (left). In MHS muscle cells, the RYR does not close readily causing an abnormally high release of Ca^{2+} into the cytoplasm. Hence, excess calcium ions induce a hypermetabolic state and excess muscle contraction (right). From reference (1).

1.3 Diagnosis of MH

1.3.1 The in vitro contracture test

As prediction of MH from clinical signs is difficult, attempts have been made to establish preoperative tests to diagnose MH susceptibility, but unfortunately, they have lacked reliability (57). To date, the gold standard and widely used method is the *in vitro* contracture test (IVCT), which is a method to determine the response of a patient's

muscle to the triggering agents halothane and caffeine *in vitro* (5, 6). The IVCT requires the surgical excision of a piece of quadriceps muscle to provide several muscle specimens for investigation. The fresh specimens are suspended in a bath, and the test drugs, halothane or caffeine, are added. Tension of the muscle is measured by a transducer before and after addition of the drugs.

The European MH Group developed a protocol for the IVCT in 1984. This protocol defines a positive response as 0.2 g or greater against 2 % v/v halothane or 2.0 mM caffeine. If both tests using halothane and caffeine show positive responses, the individual is diagnosed as MH susceptible (MHS). If both tests do not show positive responses at all, then the diagnosis is MH normal (MHN). If only the halothane test or only the caffeine test is positive, then the diagnosis is MH equivocal (MHE) although the person is regarded as clinically susceptible to MH. The North American MH Group has also developed an IVCT protocol in 1989 that differs slightly from the European protocol (8). It defines 0.5 and 0.3 g as a positive response to 3 % v/v halothane and 2.0 mM caffeine test, respectively. The North American protocol does not establish an MHE diagnosis. If one or both drug tests exhibit a positive response, then the diagnosis is MH positive (MH+), otherwise the patient is considered negative for MH (MH-). Therefore, it has been said that the North American protocol could result in more false positive or negative diagnoses than the European protocol (58).

1.3.2 Limitation of the IVCT

Unfortunately, the IVCT is not an absolute method for diagnosis of MH. It has been reported that the European protocol has 99 % sensitivity and 93.6 % specificity, and the North American protocol has 97 % sensitivity and 78 % specificity (59). This means that even the most reliable method, the European protocol, can produce 1 % false negative diagnoses and about 4 % false positive diagnoses. In fact, some cases of false negative diagnoses have already been reported (60). In addition, the IVCT is invasive and expensive, requiring minor surgery and temporary disability as well as a degree of uncertainty in diagnosis due to the equivocal nature of the test itself as well as the possibility of obtaining insufficient tissue. Hence, refinements in testing protocols or other methods for testing are required (61).

1.3.3 Other diagnostic methods

To improve reliability and discrimination between the MHS and MHN groups, the protocol for the IVCT has been modified with the introduction of drugs to replace or supplement halothane and caffeine. These include ryanodine, 4-chloro-m-cresol (4-CmC) and the phosphodiesterase-III inhibitor enoximone (62-64). 4-CmC may be a more reliable drug than halothane or caffeine. Multicentre evaluation has estimated its sensitivity and specificity as 96.1 % and 99 %, respectively (65). Other non-invasive diagnostic tests to replace the IVCT have also been developed.

Galloway *et al.* (1984) introduced phosphorus-31 nuclear magnetic resonance (^{31}P NMR) to determine relative concentrations of ATP, phosphocreatine (PCr) and inorganic phosphorus (Pi) in skeletal muscle (66). MHS muscle shows an abnormal metabolic state against triggering drugs, and hence, shows significantly higher Pi/PCr values than does MHN muscle (67). This method is not currently used for diagnostic testing, however because the value is changeable, and it is not sufficiently reliable to establish diagnoses (68). As abnormal Ca^{2+} release is an important phenomenon during MH reactions, Ca^{2+} release from the SR in skeletal muscle stimulated by drugs *in vitro* has been investigated. Censier *et al.* (1998) found that cultured skeletal muscle cells from MHS individuals showed abnormal intracellular calcium homeostasis in the presence of halothane (69). Later, coincidence of diagnoses from this method and from the IVCT was reported (70). This method does not replace the IVCT however, because it does not have higher sensitivity or specificity than can be obtained with the IVCT. It also requires skeletal muscle for cell culture, and is thus invasive and time consuming although the procedure could be developed from skeletal muscle obtained from needle biopsy and hence would be less invasive for patients than surgical excision for the IVCT (71). Klingler *et al.* (2002) have also introduced a diagnostic method using cultured skeletal muscle cells (72). These authors have determined H^+ release instead of Ca^{2+} after the administration of 4-CmC, although this method requires an expensive microphysiometer to detect secretion from cultured cells.

Blood samples have also been used to predict MH susceptibility. Anetseder *et al.* (2002) have introduced the *in vivo* caffeine test. After intramuscular injection of caffeine, MHS individuals show higher local carbon dioxide pressure (pCO_2) because of the high

metabolic state caused by caffeine, but MHN individuals do not (73). Sei *et al.* (1999) have found that *RYR1* is not expressed only in skeletal muscle, but also in B lymphocytes (74). As the type 1 RYR plays a critical role for calcium signalling in B lymphocytes, it is thought that these cells would show abnormal calcium homeostasis against drugs in MHS patients. In fact, a few reports have shown higher drug-induced Ca^{2+} release from MHS B lymphocytes isolated from blood (75, 76). These techniques do not require muscle biopsy and have the possibility of replacing the IVCT, although further investigation is required to confirm reliability, sensitivity and specificity.

1.4 Other disorders associated with MH

1.4.1 Central core disease

MH may be associated with other disorders especially myopathy. So far, the only disease that is clearly associated with MH is central core disease (CCD). CCD is a morphologically distinct myopathy with quite variable clinical features. It is characterised by muscle weakness and muscle cramps after exercise, and autosomal dominant inheritance is observed in most subjects. The association of this rare disorder with MH has been found from observation that most CCD patients have an abnormal response in the IVCT or show MH reactions during anaesthesia indicating that the two disorders can co-exist (77). Therefore, according to these observations, all patients with CCD must be considered at risk from MH unless the IVCT indicates otherwise.

1.4.2 Other disorders

Other disorders may also be associated with MH although evidence of association has still not been shown conclusively. King and Denborough (1973) reported an unusual syndrome within MHS patients in Australia and New Zealand (78). The syndrome, called King Syndrome is characterised by short stature, slowly progressive myopathy and an unusual facial appearance. The cause of the disease is unknown and the association with MH is still unclear, but some case reports have indicated co-existence of MH and King syndrome (79). Other myopathies including Duchenne muscular dystrophy, Evans myopathy and myoadenylate deaminase deficiency may have some relationship to MH (80) although this is still controversial.

The association of MH with sudden infant death syndrome (SIDS) has been indicated in the early 1980s because of the high rate of SIDS incidence within MHS families (81, 82). The cause and mechanism of SIDS is still unknown, but some reports have shown that overheating may be one of the causative factors (83, 84). MH can be triggered by overheating itself (25), and hence, it could be the cause of SIDS for some MHS infants.

1.5 Animal models

1.5.1 Porcine model

The homologous disorder to human MH has been found in some animals. Porcine MH was reported in 1966 and has been widely investigated as a model of human MH (85). It shows an autosomal recessive trait whereas human MH is autosomal dominant (86). Porcine MH is sometimes triggered by stress, such as fighting, and high temperature (87). It is called porcine stress syndrome indicating that human MH may be a human stress syndrome. The skeletal SR from MHS pigs shows an abnormal release of Ca^{2+} caused by halothane and caffeine (88, 89). In addition, linkage analysis has revealed that chromosome 6q is linked to the disorder and the susceptible locus encodes the RYR of skeletal muscle, *RYR1* (90). A major breakthrough occurred when the first mutation, R615C, was found to be associated with porcine MHS, which co-segregates with porcine MH phenotypes (91, 92). As porcine MH is associated only with the *RYR1* R615C mutation, a molecular genetic test was developed to predict porcine MH susceptibility by detecting this mutation within *RYR1* (93).

1.5.2 MH in other animals

MH has also been found in the dog (94), and Roberts *et al.* (2001) found a V547A mutation within canine *RYR1* which is linked to MH in dogs (95), although the homologous mutation has not been found in humans. MH is also found in the cat (96, 97) and in the horse (98, 99), however molecular genetic studies have not yet been performed in these animals.

1.6 Molecular genetics of human MH

1.6.1 RYR1 mutations

As with porcine MH, linkage analysis has shown that *RYR1* is a candidate gene associated with human MH and is located on chromosome 19q (100, 101). Later, an R614C mutation, homologous to porcine R615C, was found in human *RYR1*. This mutation co-segregates with the IVCT phenotype in some MH susceptible families, and both homozygous and heterozygous mutations clearly match the phenotype (102). Hence, *RYR1* is linked to MH and the heterozygous mutation within the gene can lead to susceptibility showing an autosomal dominant trait for this disorder. To date, over 60 mutations associated with MH and/or CCD have been found within *RYR1* (Table 1-1). A single-amino-acid deletion has also been reported to be associated with MH (103). *RYR1* mutations found so far are clustered in specific regions called "hot spots" – N-terminal (exon 2-17), central (exon 39-46) and C-terminal (exon 95-102) (Figure 1-3). The N-terminal and central hot spots are located in the foot region of the RYR exposed to the cytoplasm, and the C-terminal one is located in the channel region embedded in the SR membrane.

1.6.2 Functional effects of RYR1 mutations

Cultured skeletal muscle cells from an MHS individual show an abnormal response against halothane, and a mutant skeletal muscle RYR containing *RYR1* mutations also leads to high sensitivity to drugs (69). HEK-293 cells expressing the mutant RYR also show abnormal responses against drugs, although all mutations have not yet been investigated for their functional effect on the RYR (104, 105). Mutant RYR appears to be leaky resulting in higher resting Ca^{2+} concentrations in cytoplasm (106). Hence, causative mutations within *RYR1* may alter the RYR function as a calcium release channel. The difference of effect on RYR function between different mutations is still unclear. Some mutations, however appear to show higher sensitivity to drugs than do others (107).

	Exon	Nucleotide	Amino acid	Disorder	Proportion	Reference
RYR1	6	C487T	R163C	MH, CCD	2%	Quane *et al.*, 1993 (108)
mutations	9	G742A	G248R	MH	2%	Gillard *et al.*, 1992 (109)
	11	G1021A	G341R	MH	6%	Quane *et al.*, 1994 (110)
	39	C6487T	R2163C	MH	4%	Manning *et al.*, 1998 (111)
	39	G6502A	V2168M	MH	7%	Manning *et al.*, 1998 (111)
	40	C6617T	T2206M	MH	3%	Manning *et al.*, 1998 (111)
	45	G7303A	G2434R	MH	4%	Keating *et al.*, 1994 (112)
	100	C14477T	T4826I	MH	1 family	Brown *et al.*, 2000 (113)
	102	T14693C	I4898T	MH, CCD	4%	Lynch *et al.*, 1999 (114)
CACNA1S	26	G3256T	R1086C	MH	1 family	Jurkat-Rott *et al.*, 2000 (115)
mutations	26	G3257A	R1086H	MH	1 family	Monnier *et al.*, 1997 (116)

Table 1-1 Selected *RYR1* and *CACNA1S* mutations

Over 60 mutations within *RYR1* have been reported to date, which are associated with MH and/or CCD. Only two mutations have been identified within *CACNA1S* from MHS individuals. These two mutations are linked only to MH, and no *CACNA1S* mutations are known to be associated with CCD.

1.6.3 Molecular genetic testing

As *RYR1* mutations clearly co-segregate with MH susceptibility in some families, molecular genetic testing may be useful to predict susceptibility instead of the IVCT for certain families (117). It is possible to establish an MHS diagnosis by detecting a causative mutation because MHN individuals do not contain *RYR1* mutations, and individuals with mutations are always MHS (118, 119). This does not necessarily mean, however that all MHS patients have *RYR1* mutations. It has been reported that between 30 and 80 % of MHS families show linkage to *RYR1* (120). In fact, discordance between genotype and IVCT phenotype has been observed in some families (121, 122). Hence, molecular genetic testing may be useful in some individuals in specific families,

but it cannot be suitable for all cases. It may be useful to use as a supplemental method for the IVCT in some specific families (123).

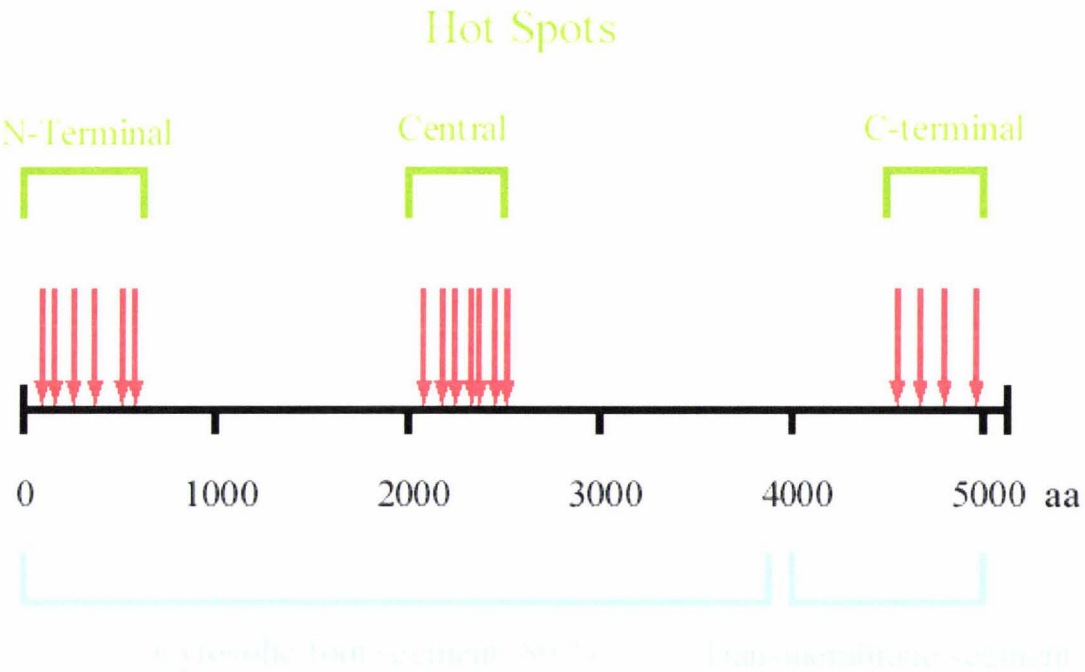

Figure 1-3 A linear representation of location of *RYR1* mutations

The RYR consists of 5,038 amino acids represented by a black line. Red arrows indicate the location of *RYR1* mutations found. They are clustered in N-terminal, central or C-terminal hot spots. Most hot-spot regions form the foot segment exposed in cytosol, and the C-terminal region is embedded in the SR membrane forming the transmembrane segment.

1.6.4 Other susceptible loci to MH

30-80 % linkage to *RYR1* indicates genetic heterogeneity of MH (120). This means that there must be other candidate loci associated with MH. In fact, linkage studies have identified other candidate loci on several chromosomes. Robinson *et al.* (1997) have performed a genome-wide scan of a MHS family, which does not have linkage on

chromosome 19q, encoding *RYR1* (124). These authors found strong linkage between MHS and chromosome 1q encoding the α-subunit of the DHPR. The gene (*CACNA1S*) was subsequently screened, and an R1086H mutation was found to co-segregate with MH susceptibility in this family (116). Later, a second mutation (R1086C) was found within this gene indicating that the *CACNA1S* gene may be a second candidate locus (115), although the functional effect of *CACNA1S* mutations is still unclear. Other candidate loci have been demonstrated on chromosome 3q (125), 5p (124), 7q (126) and 17q (127), however these are controversial because no mutations have been found within these loci, and potential candidate genes have not been identified for some of them.

1.7 Research goals

There were two separate and unrelated goals for the research described in this thesis. Both however, were aimed at identifying genetic factors causing MH in New Zealand families.

1.7.1 Screening RYR1 of the patient with exercise-induced MH

Recently, a male New Zealander suffered a fulminant MH crisis and died. The reaction was induced by exercise, but he did not have any signs of MH before the crisis and he did not have a family history of MH. Furthermore, molecular genetic analysis showed that he lacked *RYR1* mutations previously identified in New Zealand families. It is well known that MH reactions can be triggered by exercise, and in fact, mutations have been found in patients who showed exercise-induced MH-like reactions (128). It has not been confirmed, however that specific *RYR1* mutations are responsible for exercise-induced MH. It was possible that this patient had a novel *RYR1* mutation that is responsible for MH susceptibility caused by excess exercise. To determine this possibility, mRNA of the patient was extracted from his skeletal muscle followed by production of cDNA from mRNA using reverse transcriptase (RT). DNA fragments were amplified by PCR covering the entire *RYR1* cDNA. The fragments were purified and directly sequenced to detect any mutations and polymorphisms.

1.7.2 Linkage analysis within the CH family

To date, over 40 MH families have been identified in New Zealand. One of the largest MH families identified as the CH family has more than 1,400 individuals in total. Linkage analysis has shown that *RYR1* is responsible for MH susceptibility in this family (129), and in fact, the T4826I mutation in MHS patients was found to co-segregate with the phenotype in this family (113). It has also been observed, however that there is some discordance between genotype and phenotype showing that some MHS patients, as diagnosed by the IVCT do not have the T4826I mutation. Although the IVCT can produce false diagnoses, the rate of discordance in this family is higher than the probability of IVCT false diagnosis. Hence, it is thought that MH susceptibility in this family is not caused only by *RYR1*, but also by one or other traits in the discordant patients because of heterogeneity of MH. To search for other candidate loci that are susceptible to MH, a genome-wide scan was performed for patients selected from the CH family. Twenty-six individuals were selected from the family, and non-parametric analysis was adopted for the linkage analysis.