A GENETIC ANALYSIS OF MALIGNANT HYPERTHERMIA

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University of Manitoba

In partial fulfilment of the requirements for the
Degree of Master of Science

by

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- Modern 0582
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- Nuclear 0552
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A GENETIC ANALYSIS OF MALIGNANT HYPERTHERMIA

by

KIMBERLY D. SERFAS

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Abstract

Malignant hyperthermia (MH) is an inherited neuromuscular, pharmacogenetic disease which most commonly presents as a sustained muscle contracture accompanied by high fever following exposure to a halogenated inhalational anesthetic ± succinylcholine. MH susceptibility (MHS) is currently diagnosed using the *in vitro* caffeine/halothane contracture test (CHCT) in fresh muscle biopsy specimens. The objectives of this project were 1) to identify the genetic basis for MH in 16 unrelated individuals, who have had a documented MH crisis or a positive CHCT, and 2) to compare CHCT with DNA analysis in predicting MHS. None of the individuals studied had an underlying myopathy or systemic disease. In 4 of the 16 individuals screened, a mutation within the ryanodine receptor gene (*RYR1*) was identified: 1 individual had an amino acid arginine to cysteine replacement at position 614 (R614C) and 3 individuals had an amino acid glycine to arginine replacement at position 2433 (G2433R).

We were able to screen DNA from 68 family members of the individual with the R614C mutation for the presence of this *RYR1* mutation using PCR amplification followed by *Rsa*1 digestion. Twenty-two family members were found to be heterozygous for this mutation while 46 were found to be homozygous for the normal allele. Sixteen family members also had previously undergone a muscle biopsy for CHCT. MH status assignments using CHCT were in agreement with DNA results in 14 individuals but discordant in 2.

We screened DNA from 11 family members of one of the individuals with the G2433R mutation using PCR amplification followed by *Dde*1 digestion. Six family members were found to be heterozygous for this mutation, while 5 were homozygous for the normal allele. Six individuals also underwent a muscle biopsy for CHCT. The CHCT MH assignments were concordant in 4 individuals and discordant in 2.

We propose that the observed discrepancies between the DNA test results and CHCT assignment in these two families result from false positive assignments by CHCT which has a reported sensitivity of nearly 100% but a specificity of only 80%.

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A special note of thanks to the individuals and families who participated in this study. Without their interest in MH research and their generosity, this research project would not have been possible.

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List of Figures

List of Tables

List of Abbreviations

A	Adenine
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ADP	Adenosine Diphosphate
$\alpha^{32}P$	Alpha Labelled Phosphorous
Arg or R	Arginine
ASO	Allele-Specific Oligonucleotide
ATP	Adenosine Triphosphate
bp	Base Pairs
BRL	Gibco BRL Life Technologies
C	Cytosine
CCD	Central Core Disease
cDNA	Complementary Deoxyribonucleic Acid
CHCT	Caffeine/Halothane Contracture Test
Ci	Curie
CK	Creatine Kinase
cM	Centimorgan
CSC	Caffeine Specific Concentration
Cys or C	Cysteine
Da	Dalton
ddH_2O	Distilled Deionized Water
ddNTP	Dideoxynucleoside Triphosphate
dNTP	Deoxynucleoside Triphosphate
ds	Double Stranded
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
FA	Forward Mutant Primer
FG	Forward Normal Primer
fmol	Femtomole
$\gamma^{32}P$	Gamma Labelled Phosphorous
g	Gram
G	Guanine
Gly	Glycine
GPI	Glucose Phosphate Isomerase
HAL	Halothane gene
His	Histidine
HRP	Horseradish Peroxidase
Ile	Isoloeucine
kb	Kilobase
kV	Kilovolts
LB	Luria-Bertani medium
leu	Leucine
lod	Log of the Odds
Met	Methionine
MH	Malignant Hyperthermia
MHE	Malignant Hyperthermia Equivocal
MHN	Malignant Hyperthermia Normal
MHS	Malignant Hyperthermia Susceptibility
ng	Nanogram
N/n	Heterozygous State
N/N	Homozygous Normal
OD_{600}	Optical Density at 600 nm
µf	Microfarad
µg	Microgram
µL	Microlitre
min	Minute
mM	Milimolar
NBL	New England Bio-Labs
nM	Nanomolar
nm	Nanometer
PCR	Polymerase Chain Reaction
pg	Picogram
PGD	6-Phosphogluconate Dehydrogenase
PNK	Polynucleotide Kinase
poly(A)+	Polyadenylated (mRNA)
PSS	Porcine Stress Syndrome
θ	Recombination Fraction
RE	Restriction Endonuclease
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions Per Minute
RYR1	ryanodine receptor gene
SDS	Sodium Dodecyl Sulfate
Ser	Serine
SR	Sarcoplasmic Reticulum
ss	Single Stranded
SSC Citrate	Sodium Chloride/Sodium Buffer
SSCP	Single Stranded Conformational Polymorphism
T	Thymine
TAE	Tris/Acetate/EDTA Buffer
TBE	Tris/Boric Acid/EDTA Buffer
TE	Tris/EDTA
TEMED	N,N,N',N'-tetramethylethylene-diamine
thr	Threonine
Tris	Tris(hydroxymethyl)-aminomethane
Tyr	Tyrosine
U	Unit
UV	Ultraviolet
v/v	Volume Per Volume
w/v	Weight Per Volume

Table of Contents

INTRODUCTION

1.0 General Introduction

General anesthesia poses a low risk for most individuals, but to those who are genetically predisposed to malignant hyperthermia (MH), exposure to a combination of potent halogenated inhalational anesthetics and depolarizing skeletal muscle relaxants may be hazardous (Britt, 1991). In MH susceptible (MHS) individuals, the commonly used anesthetic combination of halothane and succinylcholine can trigger a life threatening crisis characterized by high fever and muscle rigidity. The morbidity and mortality associated with unrecognized or irreversible MH crises is well appreciated. MH crises can result in neurological, liver and kidney damage and are fatal unless the conditions are reversed. The routine practice of monitoring for early symptoms of an MH crisis and responding to such symptoms by terminating the anesthetic process and administering the clinical antidote, dantrolene, has lowered the death rate for such MH episodes from $>80\%$ to $<7\%$ in recent years (MacLennan, 1992). However, neurological or kidney damage still leads to the significant morbidity related to MH episodes.

Malignant hyperthermia is found not only in man but also in pigs, and other mammalian species such as cattle, greyhounds, racehorses and giraffes (Britt, 1985). In swine, malignant hyperthermia is known as the porcine stress syndrome (PSS). PSS occurs naturally in domestic pigs and serves as a useful animal model. PSS is considered similar to human MH.

2.0 Clinical Description of Human Malignant Hyperthermia

2.1 History

Malignant Hyperthermia (MH) is an inherited neuromuscular, pharmacogenetic disease which is most commonly manifested in humans as a halogenated general anesthetic-induced sustained muscle contracture accompanied by high fever. Clinical manifestations of human malignant hyperthermia were described as early as the 1900s. Denborough and Lovell (1962), however, were the first to fully describe this syndrome. They and their colleagues were treating a twenty-one year old male patient who required surgery to repair a fractured tibia. Their patient expressed a fear of anesthesia prompted by the death of 10 of 24 relatives who had received general anesthesia. Despite the man's concerns, the decision was made "to proceed cautiously avoiding ether and to be ready to stop if there was any untoward reaction" (Denborough and Lovell,1962). Unfortunately, the patient did experience an MH episode but survived due to early diagnosis and prompt termination of anesthesia. This initial report alerted the world to the risks of genetic susceptibility to certain anesthetic drugs.

2.2 Triggering agents

An MH crisis can be triggered by any of the potent halogenated inhalational anesthetics (e.g. halothane) and depolarizing neuromuscular blocking agents (e.g. succinylcholine). Stress can also be the cause of an MH crisis. Prolonged strenuous exercise or extensive skeletal muscle injury in hot and humid weather, or emotionally stressful situations, can lead to an MH stress reaction. This kind of reaction closely resembles heat stroke. In the winter, shivering may induce an MH reaction which, if

severe, can result in renal failure and rapid muscle wasting. A mild reaction is typified by muscle and joint aches and pains. It is believed, not surprisingly, that this "winter-type" reaction is more common in Canada than in the USA. Stress before and after anaesthesia is also a factor. If an MHS individual is very apprehensive prior to surgery, or has suffered trauma or infection before surgery, the individual risk of developing MH reaction increases (Britt, 1985). Also, if an MHS patient experiences pain, agitation, or shivering after surgery, an MH reaction can ensue despite the use of safe anesthetic agents (Britt, 1985; Duncan,1987).

2.3 MH and Its Association with Other Diseases

There have been many reports of patients with other muscle diseases who have been subject to MH episodes or to positive caffeine/halothane contracture test (CHCT) (Saidman *et al.*, 1964; Karpati *et al.*, 1986; Brownell, 1988; Heiman-Patterson *et al.*, 1988; Brownell *et al.*, 1993). Individuals with central core disease, myotonia congenita, myotonia dystrophica, limb-girdle muscular dystrophy, Brody's disease or Duchenne or Becker muscular dystrophy have had MH reactions or have been diagnosed as having MHS by CHCT.

2.4 MH crisis

The classical presentation of an MH crisis is a progressive rise in body temperature at the rate of 2°C or more per hour, potentially exceeding 43°C, and muscle rigidity (Britt, 1991). These symptoms are accompanied by hypermetabolism, leading to hyperventilation, hypoxia, and lactic acidosis. Tachycardia, dysrhythmia and unstable blood pressure are also associated with an MH crisis, likely secondary to cellular damage.

Damage to the skeletal muscle cells leads to electrolyte imbalance and elevation in the serum and urine levels of muscle enzymes and myoglobin (Britt, 1988). The earliest sign of a impending MH crisis however, is an increase in end tidal CO_2 concentration. Many susceptible patients present with some, but not necessarily all, of the classical MH signs when exposed to the triggering agents, and the reactions can be of variable intensity. If an MH crisis is left untreated, death rapidly ensues from cardiac arrest. If the patient survives an MH crisis, he/she may experience extensive muscle damage (rhabdomyolysis) with accompanying myoglobinuria. Acute renal failure and short term neurological disturbances can result from the release of muscle proteins into the circulation.

2.5 MH Treatment and Management

When MH was first described, treatment for an MH crisis consisted of termination of the anesthetic, ventilation with 100% oxygen, and active cooling in the form of an ice bath. Sodium bicarbonate was given intravenously to control the profound respiratory and metabolic acidemia. Approximately 90% of MH crises resulted in death. However in 1979, dantrolene sodium was introduced as the clinical antidote for MH crises (Harrison, 1975; Britt,1984;1991). It proved to be a fast acting, safe treatment of MH crisis in most cases. As a counteragent for an acute MH reaction, dantrolene is administered intravenously at a starting dose of 2.5 mg/kg up to a final dose of 10mg/kg or until the MH reaction is controlled. Procainamide may also be given intravenously to control dysrhythmias, while glucose or insulin may also be administered to treat hyperkalemia. A diuretic such as mannitol may be given to maintain urine output to prevent renal failure.

The introduction of dantrolene revolutionized the treatment of MH crisis and together with earlier diagnosis of the biochemical abnormality, made possible by end tidal capnometry, has caused a sharp decline in morbidity and mortality of MH. Today, mortality resulting from an MH crisis is less than 10%. This remaining mortality is felt to be related to avoidable errors in the management of an MH crisis. Neurological or renal damage can still occur. Survival is the expected outcome of an MH crisis with correct therapy including the use of dantrolene (Harrison, 1988).

In cases where the patient has been identified as high risk because of a positive family history, the anesthetic routine is altered to exclude triggering anesthetics. In a modern well-equipped operating room, the patient's heart rate, body temperature and end tidal CO_2 production are constantly monitored regardless of their MH status. Increases in any one of these may be the first clue of an impending MH crisis.

Once a crisis has occurred, the patient and his/her family should be investigated. Investigation of the proband's family members includes: documentation of the patients' medical histories, physical examination, blood samples for creatine kinase levels and, whenever possible, a muscle biopsy for CHCT and pathology.

3.0 Epidemiology

An MH crisis does not occur with every administration of anesthesia to susceptible individuals and some individuals may have several exposures before a crisis ensues (Duncan, 1987; Britt, 1991). The record is twelve previous uneventful general anesthetics using triggering drugs, with a fatal crisis with the thirteenth exposure (Britt, 1985).

Patient and anesthetic conditions can influence the development of an MH crisis. It is difficult to determine the actual frequency of MH susceptibility in the general population because of: 1) difficulty in defining mild clinical reactions; 2) the care and caution now taken by anesthesiologists; and 3) incomplete penetrance of the gene(s). The overall reported incidence of MH is approximately 1 per 15,000 cases of induced anesthesia in children and 1 per 50,000 exposures in adults (Britt, 1990). The number of susceptible patients is thought to be considerably higher than the quoted figures indicate for the reasons outlined above.

Crises occur most often in individuals between the ages of 3 and 30 years; but have been reported up to 78 years of age and in newborns. Crises are more common in male than in female teenagers. This may be due to the larger muscle mass and increased frequency of trauma in males. The overall sex ratio of MH is 1:1 when biopsy results and CK levels are considered. MH occurs in all racial groups (Britt, 1985; McPherson and Taylor 1982; Bevan et. al. 1988).

4.0 Genetics

Malignant hyperthermia is a familial disease. However, the pattern of inheritance has not been clearly understood. In their original report, Denborough and Lovell (1962) postulated an autosomal dominant mode of inheritance with incomplete penetrance. Sex linkage was ruled out because there was male to male transmission. Frequently observed vertical transmission made autosomal recessive inheritance very unlikely (Gronert, 1980; McPherson and Taylor, 1982). In a critical analysis of 133 cases of MH patients who

belonged to 93 different kindreds, no single pattern of inheritance could be established (McPherson and Taylor, 1982). Autosomal dominant, autosomal recessive and polygenic models as well as *de novo* mutations were suggested. The best model, however, is still that of autosomal dominant inheritance. This pattern is apparent in approximately one half of the families studied. Other cases are commonly labelled as sporadic or multifactorial (McPherson and Taylor, 1982).

5.0 Pathogenesis of Malignant Hyperthermia - Current Concepts

Skeletal muscle contraction and relaxation is achieved by an elaborate and powerful intracellular protein apparatus, consisting mainly of actin and myosin filaments. Calcium (Ca^{2+}) plays an important role in this process, acting on the accessory proteins, troponin and tropomyosin, which, in turn bind to actin. When the muscle is at rest, the concentration of Ca^{2+} in the sarcoplasm is low. The tropomyosin-troponin complex is oriented so that the inhibitory component of troponin covers the potential binding site for the globular head of myosin on actin. The muscle is stimulated to contract by the depolarization of the nerve resulting in the release of acetylcholine. This causes an electrical activation of the skeletal muscle fibers. This action potential is conducted into the interior of the muscle fiber across the membrane of the transverse tubules. This then stimulates the release of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum (SR) through the Ca^{+2} release channel. Ca^{2+} immediately attaches to troponin causing a change in its structure. The conformational change in troponin causes its attached tropomyosin to shift position in the actin filament, thus exposing the binding sites for the myosin globular head. ATP is hydrolyzed by the myosin

ATPase as the myosin head binds to actin. The hydrolysis of ATP provides the energy for the thin actin filament to slide past the thick myosin filaments thus contracting the muscle. When the action potentials cease, Ca^{2+} is transported back into the lumen of the SR by Ca^{2+} pumps, initiating muscle relaxation. Ca^{2+} is stored in association with calsequestrin and localized back to the junctional terminal cisternae. As the muscle relaxes, actin releases the myosin globular head and tropomyosin moves into its inhibitory position. (Alberts *et al.*, 1983; Tortora *et al.*, 1982)

As previously mentioned, muscle contraction requires ATP as an energy source. The immediate source of ATP is that which is stored in the muscle cell and available for short bursts of activity. The second source is phosphocreatine which is a high energy molecule which can convert ADP to ATP. Thirdly, ATP is regenerated by the metabolism of carbohydrates through glycogen breakdown (Tortora *et al.*, 1982). Ca^{2+} is also involved in this process through its binding and activation of phosphorylase kinase, thereby initiating glycolysis and ATP production to replenish the ATP stores utilized during muscle contraction (MacLennan and Phillips, 1992).

Abnormalities in the metabolism of the skeletal muscle can be detected in the early stages of an MH episode. Since 40% of body mass is comprised of skeletal muscle, these changes have profound effects upon the whole body, inducing severe metabolic acidosis. This leads to increased demand for oxygen which adds to the heat increase. A defect in the regulation of calcium resulting in calcium overload within the skeletal muscle has been hypothesized as the possible physiological basis for MH (Ball *et al.*, 1993).

There are many proteins involved in the regulation of Ca^{2+}, the junction between

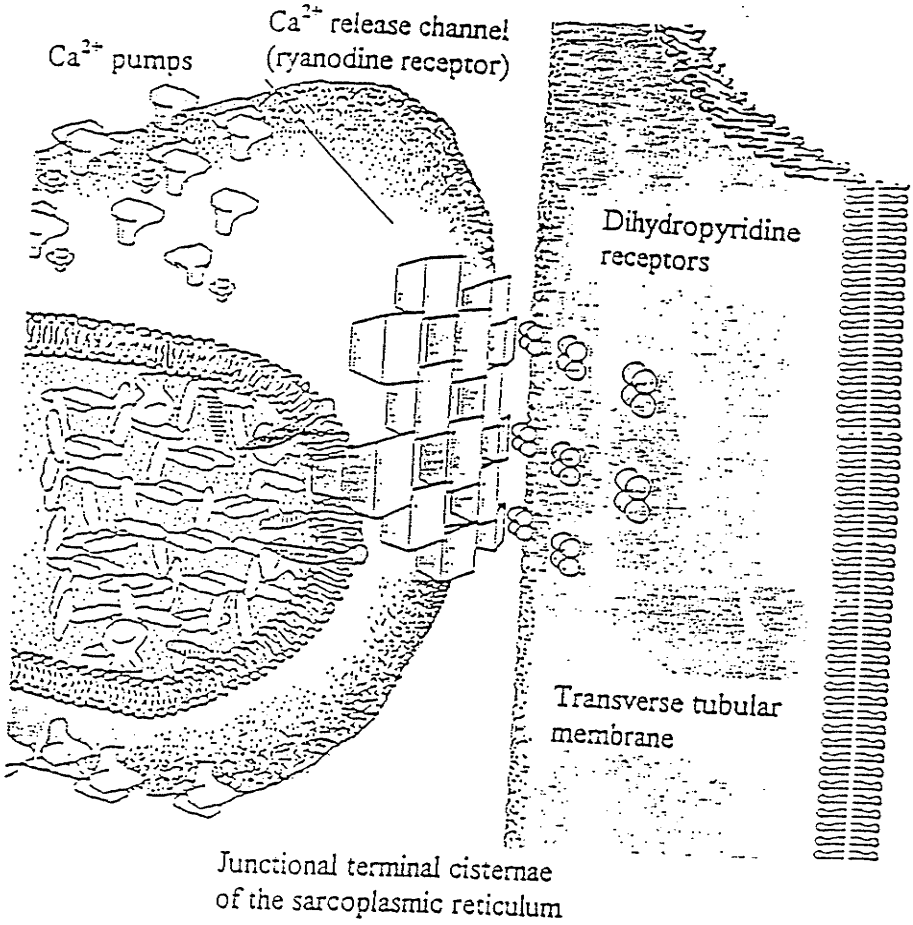


Figure 1:

The proposed relationship of the calcium release channel to the sarcoplasmic reticulum and the transverse tubular membranes. The junctional terminal cisternae of the sarcoplasmic reticulum are shown abutting the transverse tubular membrane. The calcium release channel (ryanodine receptor) is seen as a square pyramidal structure at the junctional face of the terminal cisternae. The Ca^{2+}-ATPase pump is the major protein within the longitudinal sarcoplasmic reticulum. This figure has been adapted from MacLennan *et al.*, 1992.

the SR, and transverse tubular membrane (Figure 1). Studies on the induction of MH have focused on some of these proteins. Abnormalities in the Ca^{2+} pump itself have been ruled out in biochemical studies (Nelson,1988). Recent studies have implicated the Ca^{2+} release channel (also called the ryanodine receptor, because it binds to the plant alkaloid, ryanodine) as a causative factor in MH. Differences in the Ca^{2+} induced Ca^{2+} release from human skeletal muscle fibers from normal and MH individuals have been demonstrated (Endo *et al*, 1983). Ryanodine binding studies have revealed that SR from MH swine remain open longer than normal swine SR (Mickelson *et al*, 1988). An amino acid sequence difference between normal and MHS Ca^{2+} release channels was demonstrated by digestion of the protein with trypsin (Knudson *et al*, 1990). Research therefore has focused on a possible defect in the Ca^{2+} release channel itself, hypothesizing that the abnormal Ca^{2+} release channel would: 1) be sensitive to lower concentrations of stimulators which open the channel; 2) release Ca^{2+} at increased rates and; 3) remain open longer. The Ca^{2+} pump would not be able to keep up with the excessive release of Ca^{2+} into the cell and thus Ca^{2+} overload occurs. This continual presence of Ca^{2+} then leads to contracture of the muscle and enhanced glycolytic and anaerobic metabolism. These processes may deplete ATP, oxygen and glucose, generating excess lactic acid, CO_2, and heat. The damage to the cell membranes and imbalance of ion transport could result in the life threatening systemic problems that arise during an MH crisis (MacLennan and Phillips, 1992).

6.0 Diagnostic Tests For Malignant Hyperthermia

MH does not usually threaten or incapacitate susceptible individuals in their daily lives but may result in an MH crisis when they are exposed to triggering agents. Therefore, the major goal of MH research has been to identify MH susceptible individuals prior to administration of anesthetics. If the MH status is known, alternative anesthetic and non-depolarizing muscle relaxant combinations can be used to avoid an MH episode or crisis. To date, no single test has been devised which is sufficiently inexpensive and noninvasive to permit application to the general population (Britt, 1991). Described below are several of the tests which have attempted to distinguish MH susceptible individuals from normal individuals.

6.1 Serum Creatine Kinase

Serum creatine kinase (CK) values are elevated in 80% of MHS individuals. Low or normal CK does not, however, rule out MH because 20% of MH crisis survivors have normal CK levels (McPherson and Taylor, 1982). CK elevation may also result from trauma or exercise around the time of testing. Therefore, to obtain an accurate estimate of an individual's CK value, a series of three CK measurements at monthly intervals is recommended. Many other medical conditions that are unrelated to MH also have elevated serum CK levels, such as underlying myopathies and hypothyroidism (Britt, 1985). CK elevation is not specific to MH, and it therefore cannot be utilized in the diagnosis of MH but it does serve as ancillary test.

6.2 *In Vitro* Caffeine Halothane Contracture Tests (CHCT)

The most reliable test at the present time is the *in vitro* contracture test which

permits the identification of the MH susceptible relatives of patients who have had an MH crisis. MH susceptibility is determined by the contracture response of biopsied viable skeletal muscle tissue upon *in vitro* exposure to halothane and caffeine. The *in vitro* halothane caffeine contracture test (CHCT) was developed on the assumption that the muscle from MHS individuals would contract in the presence of smaller amounts of either caffeine (Kalow, 1970) or halothane (Ellis, 1973) than normal muscle. Two tests have been standardized: North America test protocol (Larach, 1989) and the European test protocol (European MH Group, 1984). The two tests have slight variations in methodology and interpretations.

In North America, the muscle for this test is obtained from the vastus lateralis or previously from the rectus abdominus. The muscle fascicle is secured by clamp or silk suture to a plastic electrode frame and immersed in Krebs-Henseleit solution. The other end of the muscle is then attached to a force displacement transducer and isometric tension is recorded with a polygraph. Once stable twitch and baseline tensions are established, caffeine is added directly to the bath to attain doses from 0.5 to 32 mM. Four minutes after the addition of caffeine, the contracture is measured in grams of tension. Grams of tension induced by 2 mM caffeine is the most sensitive and specific measurement. An increase in tension of 0.2 g or more at 2 mM caffeine is associated with MH susceptibility (CAFF 2 mM). The amount of caffeine required to raise the resting tension by 1 gram is also measured and called the caffeine specific concentration (CSC). The patient may also be considered to be MHS if 1 g of tension is generated with 4 mM of caffeine or less. The contraction response to 3% halothane is measured in separate muscle strips in separate

baths. If, in response to 3% halothane, the muscle strips produce a tension greater than 0.2 g to 0.7 g (depending on the laboratory), the individual is defined as MHS.

The European protocol is somewhat different. Halothane is added in consecutive concentrations to the bath at 0.5, 1.0, 2.0, and 3.0% by volume. A contraction of 0.2 g at 2% halothane or less indicates MH susceptibility. In a separate bath, caffeine is added at concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 mM or until a threshold of 0.2 g contracture is obtained. At 2 mM caffeine (or less), MH susceptibility is indicated if 0.2 g (or more) of tension has been generated. To ensure viability of the muscle, 32 mM of caffeine is added once the additions of the caffeine or halothane have been completed. In the European protocol, an individual is considered MHS only if their muscle samples respond to both caffeine and halothane. Individuals are considered MH equivocal (MHE) if the muscle only react with one, but not the other.

The CHCT is a valuable clinical test (Larach, 1993). However, CHCT is both invasive and expensive and thus the test is not widely available. The Health Sciences Centre, Winnipeg, is one of only three centres in Canada which perform CHCT. The procedure itself can be quite painful as it is invasive. Sufficient sample is often not obtained to perform the proper control investigations or the muscle becomes damaged in transport. When the test is carefully executed and appropriate cutoff points are used, it achieves 92-95% sensitivity. Sensitivity is defined by Larach (1993) as the percentage of positive test results in the diseased population and calculated from the formula: 100 x [true positives/(true positives + false negatives)]. The test achieves 53-75% specificity, defined by Larach (1993) as the percentage of negative test results in the absence of disease and

calculated from the formula: 100 x [true negatives/(true negatives + false positives)]. Since failure to detect MHS individuals can result in a serious or fatal outcome, sensitivity approaching 100% is more important for clinical diagnosis than is specificity (Larach, 1993). In spite of its value as a clinical test, the lack of 100% specificity in the CHCT reduces its value as a predictor of phenotypic carriers of MH. In view of the limitations in accuracy of using CHCT for MH diagnosis, researchers at the North American Malignant Hyperthermia Registry have spent several years trying to optimize the North American CHCT protocols (Larach, 1992). These studies are helping to resolve the problems concerning differentiation of MHS and normal individuals in the population.

6.3 Other Experimental Tests

a) Attempts have been made to separate MHS and normal individuals on the basis of cytosolic free Ca^{2+}concentration in lymphocytes, both in the presence and absence of halothane (Klip *et al.*, 1987). It was theorized that the gene (*RYR1*, which will be discussed later in Section 8.4) which is defective in Ca^{2+} metabolism, resulting in an MH crisis, might be expressed in the lymphocytes. It is evident from these studies that defects may be present in both cellular and intracellular membranes. Defects in the *RYR1* gene encoding the Ca^{2+} release channel of the sarcoplasmic reticulum have been implicated in some MH families (Gillard, 1991; Hogan, 1992; Zhang, 1993; Quane, 1993; Serfas, 1994). However, as this gene seems to be expressed only in skeletal muscle, tests performed on lymphocytes may not be reliable indicators of *RYR1* mutations even in families with such gene defects. These tests also have limited usefulness because there is overlap between values in MHS and normal individuals (Ording *et al.*, 1990).

b) Phosphorus nuclear magnetic resonance spectroscopy has also been tested as a way of distinguishing MHS and normal individuals (Webster,1990; Payen, 1993). Changes in phosphocreatine, inorganic phosphate, ATP, and acidity are measured non-invasively in whole muscles in this test. Muscles are flexed against increasing increments of weight while the arm or leg is placed within a magnet. Concentration of phosphocreatine, ATP, inorganic phosphate and acid production are measured before, during and after exercise. This concept may form the basis for a non-invasive diagnostic test for MH, if a difference in energy utilization can be established between MHS and normal individuals.

c) Two techniques have been used to measure the level of resting Ca^{2+} in the muscle of MH individuals: (1) direct use of implanted Ca^{2+} electrodes, and (2) use of Fura 2, a calcium indicator (personal communication, Dr. Bose) to measure intracellular Ca^{2+} concentration. The intracellular resting Ca^{2+} concentration from biopsies of MH patients was found to be approximately four fold higher than control patients using Ca^{2+} electrodes (Lorpez-Padrino,1993). This imbalance in intracellular Ca^{2+} homeostasis was postulated to be a direct consequence of alternative mechanisms controlling myoplasmic Ca^{2+}. Using Fura 2 as a Ca^{2+} indicator, Iaizzo (1988) was not able to measure any differences in the resting Ca^{2+} concentrations in muscle biopsies from MH (PSS) and normal swine. Therefore, resting Ca^{2+} levels have proven to be controversial. Since these two techniques result in different measurements of resting Ca^{2+}, it is doubtful that this technique can form the basis for an MH diagnostic test.

d) Using a similar experimental protocol to CHCT, contracture induced by ryanodine has been evaluated for its ability to distinguish between MHS and normal individuals. The

results have been consistent with those of other components of the CHCT test (Hopkins, 1991; Lenzen, 1993).

7.0 Porcine Stress Syndrome - An Animal Model for MH

7.1 Description

The widespread use of pigs for experimental surgery in early 1960's and their resulting exposure to halothane and succinylcholine, led to awareness that pigs were susceptible to MH reactions in a similar fashion to human MH (Hall, 1966; Harrison, 1979). Symptoms included high fever, skeletal muscle rigidity, hyperventilation, hypoxia, cyanosis, lactic acidosis and death. In field observations, pigs that were lean and heavily muscled seem to be more susceptible to fatal episodes of shortness of breath, rapid increase in body temperature and skeletal muscle rigidity. Sensitivity to halothane-induced MH and the sudden, stress-induced deaths are both manifestations of the syndrome referred to as porcine stress syndrome or PSS. These reactions are precipitated by various forms of stress, including: weaning, fighting, overeating, exercise, mating, a number of chemical agents or transportation to market (Otsu *et.al.*, 1991). The swine industry also suffers major economic losses due to the development of pale, soft, exudative pork which is the postmortem manifestation of the disease in PSS susceptible animals (Fujii *et al*,1991).

7.2 Genetics

Porcine MH, a presentation of PSS, is inherited in an autosomal recessive fashion. From breed to breed, as well as country to country, the incidence of MH (PSS) in swine varies. An Ontario study estimated that more than 10% of commercial animals are

heterozygous carriers for the syndrome and approximately 1.5% are homozygous for the abnormality (Seeler,1983). Efforts made to eliminate the potentially detrimental gene have not been successful for two reasons: 1) competition between beneficial and deleterious gene effects, and 2) detection. The gene responsible for PSS is associated with leanness and muscle hypertrophy. Both are important market characteristics and hence beneficial. Thus, when breeders were selecting stock with such qualities as large ham conformation and excessive leanness, they were unintentionally selecting for this gene. Until recently, breeders were unable to accurately and economically detect heterozygotes and avoid their use in breeding. As result, the frequency of this gene has stabilized at a high level in the most lean, heavily muscled breads of swine (MacLennan, 1992).

Porcine stress syndrome has been studied extensively as an animal model for experimental investigation of the pathophysiology of human MH, for identification of potential triggering agents, and for the development of therapeutic agents such as Dantrolene.

7.3 Gene Mapping

In early studies of porcine MH, linkage was demonstrated between inheritance of MH and polymorphisms in the gene encoding glucose phosphate isomerase, (*GPI*) which is highly conserved across species (Andersen and Jensen, 1977). Later studies (Archibald and Imlah, 1985), established a linkage group for porcine *HAL* gene (the name for the MH gene giving rise to halothane sensitivity), *GPI* and the gene for 6-phosphogluconate dehydrogenase, (*PGD*), on pig chromosome 6 near the centromere (6p11q21) (Fujii *et al.*, 1991). The *HAL* gene is also known as the *RYR1* gene (Harbitz *et.al.*, 1990).

7.4 Disease Causing Mutation

Evidence for linkage between *RYR1* and porcine MH established *RYR1* as a candidate gene for PSS (Fujii, 1991; Otsu, 1991). Comparison of the *RYR1* cDNA of MH (Pietrain) with normal (Yorkshire) pigs, revealed 18 nucleotide substitutions. However, in the deduced amino acid sequences derived from the *RYR1* cDNAs, only one single amino acid was altered (Fujii, 1991). In the PSS pig, cysteine (Cys) is substituted for arginine (Arg) at position 615 (R615C). This results from a cytosine (C) to thymine (T) transition at *RYR1* position 1843. This nucleotide substitution leads to loss of a *Hin*P1 restriction endonuclease site and to the gain of a *Hgi*A1 site, thus allowing direct mutation detection following amplification by PCR (Fujii,1991; Otsu, 1991). The C1843T base substitution mutation cosegregated with porcine MH in over 450 animals from six breeds of selectively inbred commercially reared pigs with a lod score of 101.75 at a recombination fraction (θ) of 0.00. This mutation is thus strongly implicated as the cause of PSS. The identical mutation appeared in 6 lean, heavily muscled pig breeds which suggests the possibility that this mutation originated in a founder animal. Haplotype and genotype analysis using three markers covering over 100 kb within the *RYR1* gene showed that a specific haplotype is associated with the porcine MH phenotype. This implies that this disease causing mutation did originate in a founder animal and was selected for in breeding stock. The causative nature of the R615C mutation is further supported by biochemical findings of Shomer *et al.*, (1993) in which purified ryanodine receptors from MH pigs, reconstituted into planar lipid bilayers, exhibited longer open times and shorter closed times than did normal calcium release channels. Additional support comes from

the demonstration that the expression of rabbit *RYR1* cDNA containing the Arg615Cys mutation in muscle cells (Otsu *et al.*, 1994) and COS-7 cells (Treves *et al.*, 1994) leads to hypersensitive gating of Ca^{2+} release in these transfected cells.

8.0 *RYR1*: Its Role in Human Malignant Hyperthermia

8.1 The Calcium Release Channel

The Ca^{2+} release channel is characterized by the incorporation of single channels into planar lipid bilayers. Once incorporated, they have been shown to form ligand gated channels. Micromolar concentrations of Ca^{2+} and millimolar (mM) concentrations of ATP or caffeine activate Ca^{2+} release, while mM concentrations of Mg^{2+} inhibite the release of Ca^{2+} through the channels (Endo, 1977; Miyamoto and Racker, 1982; Mori and Tonomura, 1983; Meissner, 1984; Fleischer *et al.*, 1985; Smith *et al.*, 1985; Meissner *et al.*, 1986). Meissner *et al.* (1986) noted that mM concentrations of calmodulin partially inhibited the Ca^{2+} release channel. At first, Dantrolene activates the channel but then closes it (Nelson and Lin, 1993).

It was, however, the Ca^{2+} release channel's high affinity binding to the plant alkaloid, ryanodine that led to its identification and isolation. At nanamolar (nM) concentrations, ryanodine locks the channel in an open state (Smith *et al.*, 1985; 1988). The channel closes at increased concentrations (mM) of ryanodine (Lai *et al.*, 1989). The purified ryanodine receptor exhibits the identical activities of the native channel in planar lipid bilayers, therefore the Ca^{2+} release channel and the ryanodine receptor are the same protein. The Ca^{2+} release channel overall structure has been resolved by the electron

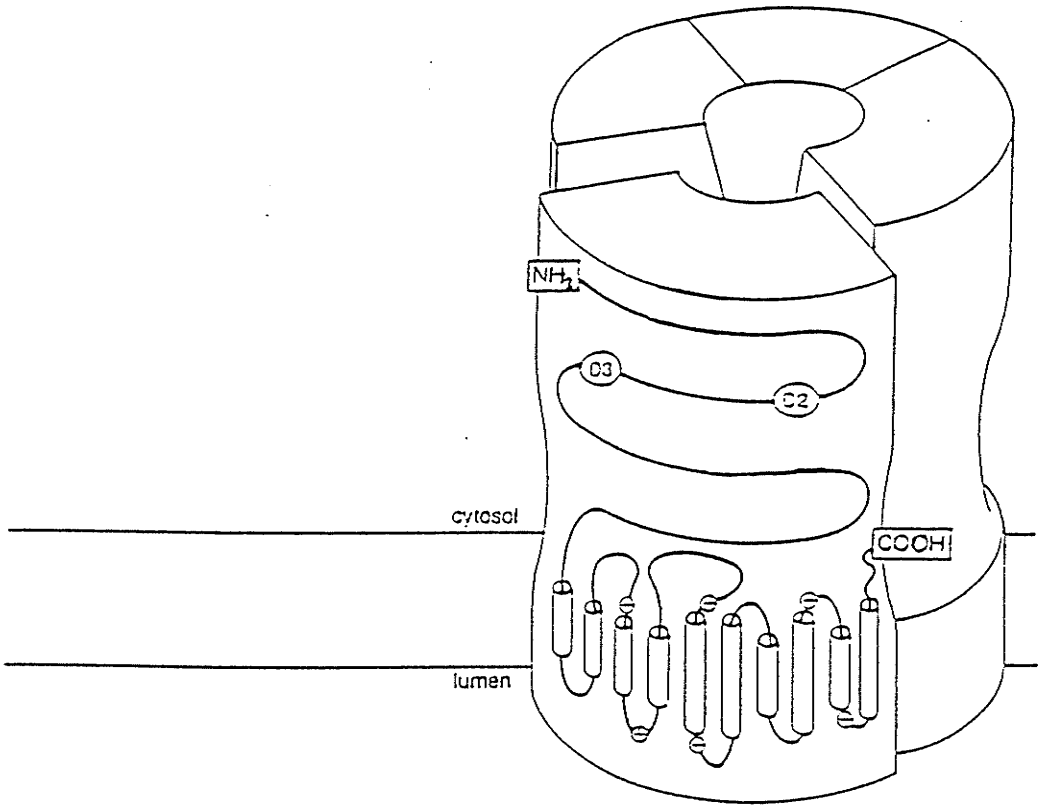


Figure 2:

Schematic model of the ryanodine receptor. Morphological studies suggest that most of the ryanodine receptor is located in the cytoplasmic space between the sarcoplasmic reticulum and the transverse tubular membrane. It is the large N-terminal domain (approximately 4000 amino acids) which extends into the cytoplasm. The remaining fifth of the molecule anchors the molecule in the membrane with the C-terminal of the molecule being located in the cytoplasm. The functional ryanodine receptor is thought to have a tetrameric structure. This figure was adapted from Sorrentino and Volpe (1993).

microscope (Inui *et al.*, 1987; Lai *et al.*, 1989; Wagenknecht *et al.*, 1989; Radermacher *et al.*, 1994). It is a tetramer of 564,000 Da subunits with four fold symmetry. It has a small transmembrane region but the majority of the protein is a huge cytoplasmic domain that projects from the membrane (Lai *et al.*, 1988, Wagenknecht *et al.*, 1989 and Radermacher *et al.*, 1992) (Figure 2).

8.2 Cloning the Ryanodine Receptor from the Sarcoplasmic Reticulum: *RYR1*

The full length cDNAs encoding rabbit (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990), human (Zorzato *et al.*, 1990) and porcine (Fujii *et al.*, 1991) ryanodine receptors have been cloned and sequenced. This was accomplished by first making an affinity purified polyclonal antibody specific for the Ca^{2+} release channel which was used to screen a λgt11 cDNA expression library, constructed from poly (A)+ RNA from rabbit fast-twitch *psoas* muscle (Zorzato *et al.*, 1990). Once the cDNA encoding the rabbit Ca^{2+} release channel was cloned, it was used as a probe to screen a human fetal *psoas* muscle cDNA library in λgt10 to identify human Ca^{2+} release channel cDNA (Zorzato *et al.*, 1990). The cloned human ryanodine receptor cDNA encodes a protein 5032 amino acids long.

Cloning the gene encoding the calcium channel protein has stimulated research on how this protein is situated in the sarcoplasmic reticulum, its structure and possible binding sites for ATP and calmodulin.

There have been two other isoforms of the Ca^{2+} release channel cloned: one from cardiac muscle (*RYR2*) (Nakai *et al.*, 1990; Otsu *et al.*, 1990) and another, *RYR3* (Giannini *et al.*,1992; Hakamata *et al.*, 1992) which appears to be widely expressed

(Sorrentino and Volpe, 1993). The three show approximately 70% homology to one another with some regions which are well conserved in all three isoforms (Sorrentino and Volpe, 1993). There is also overall homology between ryanodine receptors and the inositol 1,4,5-trisphosphate (IP_3) receptors (Takeshima *et al.*, 1989). My thesis will discuss only the first isoform.

8.3 Localization of *RYR1* gene and Linkage to MHS

Porcine MH has been linked to the *HAL* gene, which is a part of the linkage group (*GPI-HAL-PGD*) on pig 6p11q21 (Davie *et al.*, 1988; Fuji *et al.*, 1991). This region of the pig chromosome is part of a syntenic linkage group which has homologies in a variety of species. This directed the original linkage studies in humans to the homologous region on chromosome 19q12-13.2. A genetic study of DNA markers from human chromosome 19q12-13.2 in three large Irish families segregating for malignant hyperthermia showed linkage between markers in the *GPI* region and MHS, more specifically, at the *CYP2A* locus with a lod score of 5.65 (McCarthy, 1990). Subsequent cloning of the human cDNA encoding the ryanodine receptor protein enabled this gene to be mapped using high-resolution somatic cell hybrid analysis. The *RYR1* gene was also localized to 19q13.1, by high-resolution somatic cell hybrid analysis (MacKenzie *et al.*, 1990). These data clearly support the proposal that a defect in *RYR1* underlies both the human and the porcine MHS. This hypothesis was further strengthened when polymorphisms detected by the *RYR1* cDNA were shown to be linked to MHS in nine small Canadian families, with no recombination between the marker and *MHS* (MacLennan *et al.*,1990). Co-segregation of MH with *RYR1* markers, resulting in a lod score of 4.20 at a linkage distance of zero

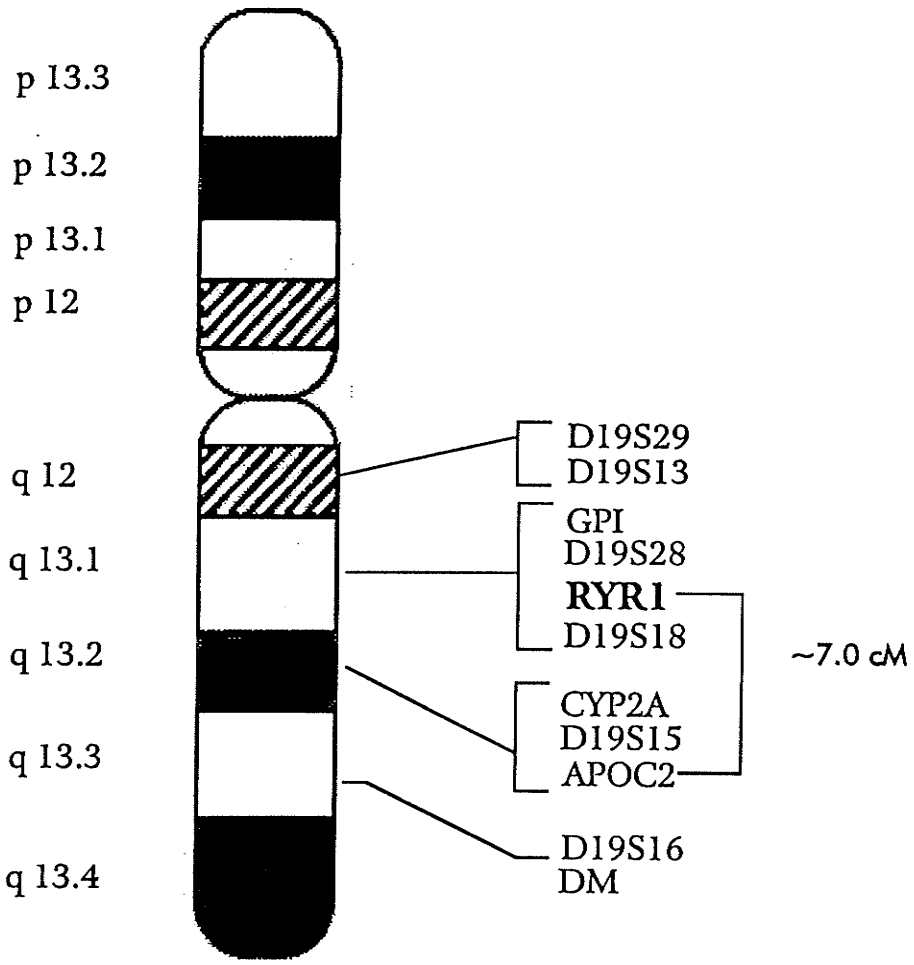


Figure 3:

Linkage map of DNA markers on the long arm of chromosome 19. Showing the chromosomal location of *RYR1* gene. Chromosome 19 is 103 cM. The distance approximate distance between *RYR1* and *APOC2* has been estimated to be 7.0 cM (Genomic Data Base).

centimorgans (cM), suggested possible identity between *RYR1* and MH. Thus, the ryanodine receptor gene was proposed as a candidate gene in MH.

8.4 *RYR1* gene

The *RYR1* gene encoding the Ca^{2+} release channel of human skeletal muscle sarcoplasmic reticulum has been cloned and recently all exon/intron boundaries have been determined (Phillips *et al.*, 1995). The gene has 106 exons, of which two exons are alternatively spliced, giving a coding sequence of 15,117 bp. The exons of the *RYR1* gene vary in size from 15 bp to 813 bp, whereas its introns range in size from 85 bp to roughly 19,000 bp. Sequencing the gene has also revealed that *RYR1* encodes for 5038 amino acids (Phillips *et al.*,1995), five more then originally thought when the gene was first cloned by Zorzato *et al.* (1990). By aligning 16 overlapping genomic phage clones, a cosmid clone and several long PCR products, the length of the *RYR1* gene was determined to be approximately 160 kbp (Phillips *et al.*, 1995). The *RYR1* gene has proven to be a very complicated gene, second only to the human type VII collagen gene (COL7A1) which consist of 118 exons (Christiano, 1994). The *RYR1* gene is much longer than the COL7A1 gene which is only 31 kbp (Christiano,1994) but is still much than the smaller human dystrophin gene which is 2,400 kbp but has only 79 exons (Roberts *et al.*, 1993).

The search for additional MH mutations is hindered by the exceptional size of the cDNA and the gene from which it is derived. Although sequencing the whole cDNA or using other methods of screening for sequence alterations, such as single stranded conformational polymorphism (SSCP) are feasible, they are expensive and laborious. With all exon/intron boundaries now defined, perhaps automated sequencing directly from

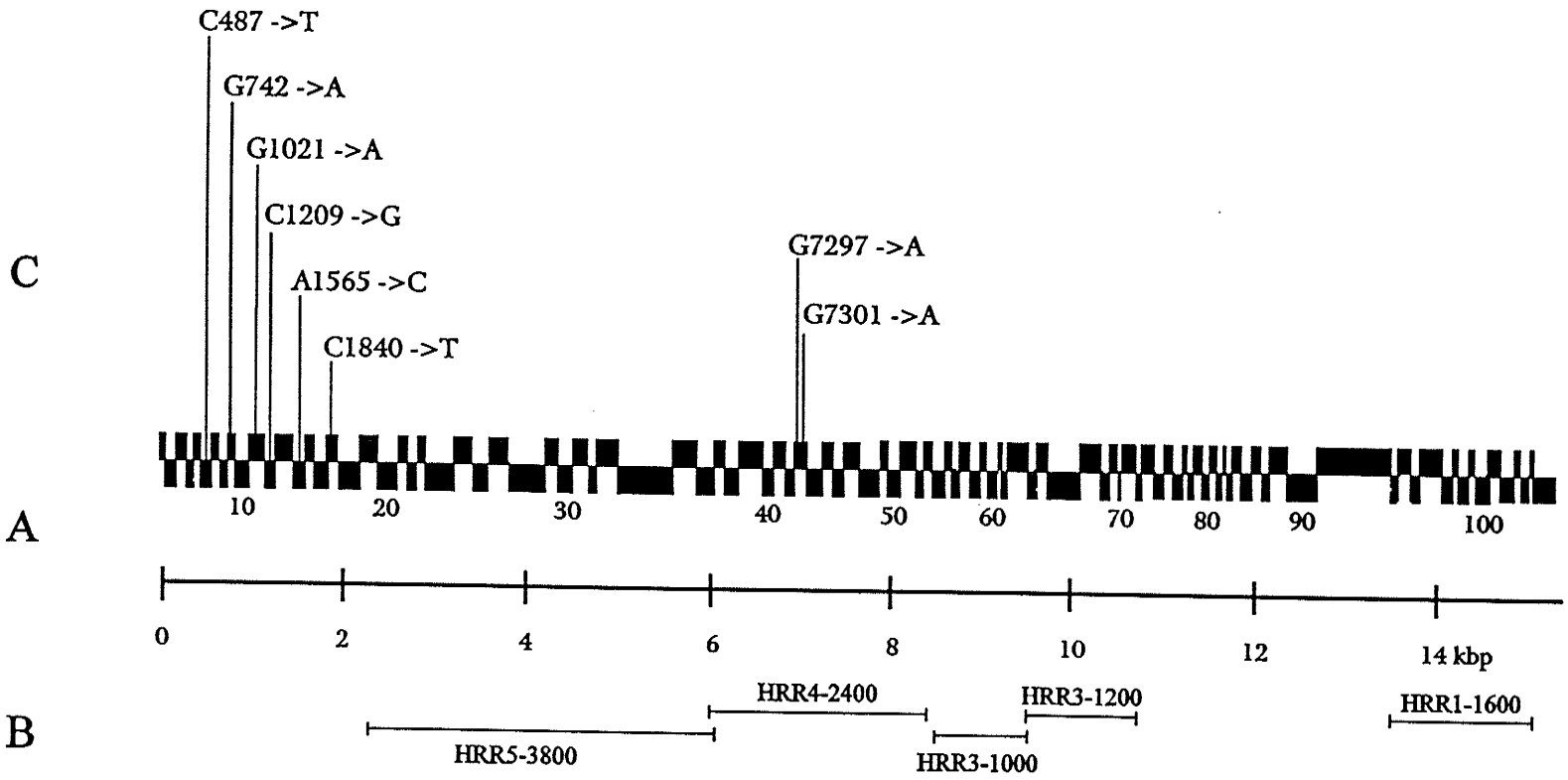


Figure 4:

Schematic diagram of the structure of the human *RYR1* gene. A) The horizontal line represents the 15,393 bp length of the 5' untranslated sequence, the coding sequence plus the stop codon and the 3' untranslated sequence for the human *RYR1* cDNA. The location and relative size of the each exon making up the cDNA are indicated by solid boxes. The largest is exon 91, which is 813 bp, and the smallest is exon 70, which is 15 bp long. B) The location of the five *RYR1* cDNA probes (HRR1-1600, HRR-1000, HRR3-1200, HRR4-2400 and HRR5-3800). C) Exonic locations of the 8 known *RYR1* mutations.

genomic DNA will be possible to search for new *RYR1* mutations.

8.5 *RYR1* and Central Core Disease

Central core disease (CCD) is a rare autosomal dominant, non-progressive myopathy causing hypotonia and proximal muscle weakness during infancy (Quane, 1993a). A typical pattern of mild to moderate weakness of facial and proximal muscles is usually apparent by the first 5-10 years of life (Mulley, 1993). Clinical manifestations of the disease are variable, ranging from normal to severe with a wide variation in muscle involvement. It has been reported that 38% of patients whose muscle demonstrate characteristic "central cores" on muscle biopsy are clinically normal (Zhang, 1993). Diagnosis is made on the basis of the characteristic morphological feature, the presence of amorphous central areas (cores) in type 1 fibers. "Cores" are best demonstrated by oxidative enzyme histochemistry (Dubowitz, 1970). The cores are depleted of mitochondria and therefore appear as negative areas within the normal enzyme activity areas of the surrounding muscle fibre (Quane *et al.*, 1993). Electron microscopic investigation of CCD muscle tissue has demonstrated changes in the contractile apparatus, sarcoplasmic reticulum and t-tubules. However, the biochemical nature and the underlying biochemical defect resulting in the characteristic "cores" are still unknown.

An important feature of the disease is its predisposition to malignant hyperthermia (Denborough, 1973; Eng, 1978; Krivosic-Horber, 1989). This association prompted genetic linkage analysis of CCD using markers from chromosome 19q12-q13.2. These studies have shown that the CCD gene maps to this region of chromosome 19 and, like MHS, is tightly linked to *RYR1* (Quane *et al.*, 1993).

A cumulative lod score of 14.8 for linkage of CCD to *RYR1* at a recombination frequency of 0.0 was obtained (Mulley, 1993; Haan, 1990; Kausch, 1991). Therefore *RYR1* is a candidate locus for CCD because a) malignant hyperthermia is a feature of both MHS and CCD, b) *RYR1* regulates Ca^{+2} release in skeletal muscle, and c) the three loci map to 19q12-q13.2 (Mulley, 1993). It has been suggested that CCD may be another phenotypic manifestation of mutations within *RYR1*.

8.6 Known Mutations in *RYR1*

Eight mutations in the ryanodine receptor (*RYR1*) gene on human chromosome 19q13.1 have been linked to malignant hyperthermia (Table 1). The first human *RYR1* mutation found was the Arg614Cys (R614C) amino acid replacement (Gillard, 1991). This is the corresponding mutation and amino acid replacement which was first found in pigs and implicated in porcine MH. The C1840T transition mutation in *RYR1* results in the cysteine replacing arginine at position 614 of the amino acid sequence (R614C) of the ryanodine receptor protein. The human *RYR1* C1840T substitution in exon 17 eliminates a *Rsa* 1 restriction endonuclease site and provides the basis for screening at-risk individuals (Otsu, 1992). This mutation has since been found in at least 15 MH families worldwide (personal communication with Dr. MacLennan).

In an effort to identify new mutations in the RYR1 gene causing MHS and/or CCD, several laboratories have undertaken systematic mutation screening of the 15.5 kb *RYR1* cDNA in unrelated MHS individuals. Seven additional *RYR1* mutations have been described and shown to segregate in MH families or MH/CCD families: 1) arginine (Arg) for glycine (Gly) at position 248 (G248R) resulting from a G742A transition has been

found in one MH family (Gillard *et al.*, 1992). 2) the single base substitution, C487T, results in the substitution of cysteine (Cys) for arginine (Arg) at position 163 (R163C). This mutation is estimated to be present in 3% of human MHS cases, including one MH/CCD pedigree (Quane *et al.*,1993). 3) a C1209G substitution resulting in the substitution of isoleucine (Ile) with methionine (Met) at position 403 (I403M) (Quane *et al.*,1993). 4) a G7301A transition mutation results in the substitution of arginine (Arg) for histidine (His) at position 2434 (R2423H). This mutation has been found in one MHS/CCD family to date (Zhang, *et al.*, 1993). 5) a novel missense Gly341Arg (G341R) substitution, which is the result of a G1021A mutation, accounts for approximately 10% of Caucasian MHS cases (Quane *et al.*, 1994b). 6) a point mutation A1565C, cosegregating with MHS within an MH family, changes a conserved tyrosine residue (Tyr) at position 522 to a serine residue (Ser) (Y522S) (Quane *et al.*, 1994a). 7) a G7297A transition mutation which results in the replacement of a conserved Gly at position 2433 with Arg (G2433R) in MHS cases. This mutation is believed to account for 4% of MHS cases (Keating *et al.*, 1994; Phillips *et al.*, 1994). None of these seven mutations have been found in the unaffected control population.

The discovery of these *RYR1* mutations in patients with CCD and MHS suggests that this locus is the disease gene for CCD as well as MH. This raises interesting considerations concerning the pathophysiology of these diseases.

Table 1: *RYR1* Mutations Associated with MH or CCD

Amino Acid Substitution	Nucleotide Substitution	Exon Number	Association	Reference
Arg163Cys	T for C487	6	MH, CCD	Quane *et al.*, 1993
Gly248Arg	A for G742	9	MH	Gillard *et al.*, 1992
Gly341Arg	A for G1021	11	MH	Quane *et al.*, 1994b
Ile403Met	G for C1209	12	MH, CCD	Quane *et al.*, 1993
Tyr522Ser	C for A1565	14	MH, CCD	Quane *et al.*, 1994a
Arg614Cys	T for C1840	17	MH	Gillard *et al.*, 1991
Arg615Cys (pig)	T for C1843	17	MH,PSS	Fujii *et al.*,1991
Gly2433Arg	A for G7297	44	MH	Keating *et al.*, 1994 Phillips *et al.*, 1994
Arg2434His	A for G7301	44	MH, CCD	Zhang *et al.*, 1993

Arg - arginine; Cys - cysteine; Gly - glycine; His - histidine; Ile - isoleucine; Met - methionine; Ser - serine; Tyr - tyrosine. T - thymidine; C - cytidine; A - adenosine; G - guanosine. MH - malignant hyperthermia; CCD - central core disease.

9.0 Evidence for Genetic Heterogeneity in Human MH

9.1 Lack of Linkage to *RYR1*

The linkage and mutation data discussed above supports the skeletal muscle *RYR1* gene as the site of the primary defect in some MHS families. Nevertheless, an increasing number of non-chromosome 19q13.1 linked families have been reported (Levitt *et al.*, 1991; Stewart *et al.*, 1991; Deufel *et al.*, 1992b; Fagerlund *et al.*, 1992). In these studies, genetic recombination between MHS segregating in these families and *RYR1* cDNA markers have excluded linkage to *RYR1* on 19q.

MH susceptibility is commonly considered to be a heterogeneous disorder because of the observed clinical variability (Britt and Kalow, 1970; Gronert,1980; McPherson and Taylor, 1982) and its association with several other inherited disorders (Allen, 1993). This perhaps explains why not all MH families can be linked to 19q12-q13.2. It has been estimated that only 30 - 50% of MH families are linked to *RYR1* (Ball and Johnson, 1993).

9.2 Use of CHCT to Determine MH Susceptibility

MH susceptibility is determined by an individual either developing an MH crisis or by CHCT. Approximately 50% of MH families tested using CHCT are linked to *RYR1* (Ball and Johnson, 1993). CHCT has proven to be a relatively accurate method of phenotyping for molecular genetic studies in those families. However 50% of MH families are not linked to *RYR1*. Therefore, it remains crucial to determine if the method of phenotyping is accurate. If even one family member were to be phenotyped incorrectly, it could lead to exclusion of linkage. Accepting the CHCT results as a standard for MH phenotype determination when CHCT has been acknowledged to be less than 100%

accurate (Larach *et al.*, 1992; Larach, 1993; Isaacs and Badenhorst, 1993; Larach *et al.*, 1994; Hopkins *et al.*, 1994) has probably resulted in some of the current confusion in MH genetics (Rosenberg and Fletcher, 1995).

The CHCT measures the contracture of muscle fibers over a background of twitches which demonstrates the viability of the fiber (MacLennan, 1995). A diagnosis of MH susceptibility is established by the development of certain contracture responses to the *in vitro* exposure of specific concentrations of halothane and caffeine (added to separate fibers). The cutoff values used to determine whether a contracture response is abnormal by the North American and European CHCT were chosen to reflect the best statistical analysis possible for the population at large. However, in the analysis of linkage of *RYR1* to MH in a Canadian MH family, MacKenzie *et al.* (1991) reported that altering the cutoff values of the CHCT would permit linkage of an otherwise unlinked MH family to *RYR1*. Therefore, standard cutoff values may be inadequate for analysis of every family (MacLennan, 1995).

The CHCT measurement also includes the interactions between the components of a multifactorial system of contracting muscle fiber which include channels, pumps and contractile proteins. Thus, if the effectiveness of any of the very large number of gene products, none of which may be directly implicated in MH, were altered, this could influence the degree of tension which is used to determine MH status. This becomes evident when one compares the CHCT results of MHS individuals within a family and finds a wide variation in their results (Gillard *et al.*, 1991; Phillips *et al.*, 1994).

9.3 Alternative loci for MH

The search for alternate MH susceptibility loci led investigators to 17q11.2-q24. This area was chosen for examination because it was not represented in previous exclusion maps generated for this disorder (Bender, 1990; McCarthy, 1990; MacLennan, 1990). In addition, both osteogenesis imperfecta and a form of myotonia (Fontaine, 1990; Koch, 1991; Ptacek, 1991) have been mapped to this region of chromosome 17q and both conditions are associated with MH susceptibility. Based on linkage studies in several MH families, a second MHS locus on 17q was proposed (Levitt *et al.*, 1992). The adult muscle sodium channel α-subunit gene (SCN4A) and two subunits of the dihydropyridine receptor, CACNLB1 and CACNLG, which all map to this region, were suggested as three possible candidate loci (Olckers *et al.*, 1992). Subsequent studies of non-chromosome 19-linked European families ruled out linkage of MH to chromosome 17q and to the proposed candidate genes (Sudbrak *et al.*, 1993). Rosenberg and Fletcher (1995) suggested that paramyotonia congenita and other demonstrated defects of the sodium channel α-subunit protein might have been associated with abnormal responses to succinylcholine. The relationship between the sodium channel α-subunit and anesthetic complications in muscle have also been reviewed by Iaizzo and Lehmann-Horn (1995).

Linkage to chromosome 7q was strongly indicated in a single family but the lod score for linkage was less than 3.0 (Iles *et al.*, 1994). The gene encoding the α2/δ subunit of the dihydropyridine receptor (CACNL2A) on chromosome 7q21-22 may be a candidate gene for MH. This gene has not been completely sequenced and no causal mutation has been found, to date.

Several large, non-chromosome 19-linked European MH families have been included in a systematic linkage study using a set of polymorphic microsatellite markers covering the entire human genome (Sudbrak *et al.*, 1995). A single family was linked to chromsome 3q13.1 with a lod score of 3.22. This chromosome 3 locus, to date, provides the best opportunity for identifiying a second causal gene in MH.

10.0 Project Aims

The aims of this project were 1) to identify the genetic basis for MH in sixteen unrelated individuals who had survived an MH crisis or who had had a positive CHCT test, and 2) to compare CHCT testing with DNA analysis in predicting MH susceptibility. None of these individuals had an underlying myopathy or systemic disease. Our hypothesis was that mutations in the *RYR1* gene were responsible for MHS. These individuals were screened using analysis of polymerase chain reaction (PCR) (Saiki *et al*, 1985) amplified DNA for five of the known *RYR1* mutations, namely, G248R, G341R, R614C, G2433R and R2434H. Extended families of five of the 16 individuals were also investigated for linkage to *RYR1* using Southern analysis. In those families where linkage was suspected, single stranded conformational polymorphism analysis (SSCP) was used to search for sequence differences in *RYR1* which may point to potential disease causing mutations.

MATERIALS AND METHODS

11.0 Clinicial Investigation

11.1 General

Sixteen individuals with a well documented MH crisis or a positive CHCT formed the basis for this genetic study. Pedigree information on the extended families of five individuals was obtained (Figures 7, 11, 15 and 19). Each family studied had at least one index case with a documented MH crisis. Following informed consent, peripheral blood was collected for CK testing and DNA extraction from the sixteen individuals and the available members in five extended families. MH status was assigned independently and blindly by Dr. Deepak Bose between 1983-1995 using the caffeine halothane contracture testing (CHCT) of muscle strips obtained by open muscle biopsy on all consenting at-risk family members. All muscles were subjected to routine pathology and histochemistry. All available clinical information on these individuals is found in Table 2.

11.2 Muscle Biopsies and Caffeine Halothane Contracture Testing

As documented earlier, the *in vitro* caffeine halothane contracture test (CHCT) is based on observations that muscle from MHS individuals usually contracts in the presence of smaller amounts of caffeine (Kalow, 1970) or halothane (Ellis, 1973) than muscle from normal individuals. In Manitoba, Dr. Deepak Bose of the Department of Pharmacology, Therapeutics andAnesthesiology, University of Manitoba, runs the major testing center for MH in Western Canada using the following protocol:

Table 2: List of 16 Unrelated MHS Individuals Studied and Their CHCT Results

Name	Year of birth	Sex	Reason for biopsy	Year of biopsy	CHCT Results			
					3%	2%	CSC	CAFF
KS1*	1983	M	MH crisis	1990	5.25 g	3.85 g	1.61mM	-
KS2	1974	M	F/H	1991	0.13 g	0.1 g	2.83 mM	0.3 g
KS3	1957	F	F/H	1991	0.85 g	0.2 g	1.57 mM	0.1 g
KS4	1986	F	MH crisis	1991	0.2 g	0.1 g	1.59 mM	0.1 g
KS6	1979	M	MH crisis	1992	1.9 g	2.6 g	2.58 mM	0.45 g
KS7	1934	M	F/H	1992	3.0 g	0.6 g	1.34 mM	0.3 g
KS8*	1956	M	F/H	1992	6.6 g	4.0 g	1.55 mm	1.65 g
KS9	?	F	F/H	1992	0.55 g	-	2.40 mM	0.0 g
KS10	1980	F	MH crisis	1992	6.3 g	3.5 g	0.84 mM	2.4 g
KS11	1942	M	MH crisis	1992	2.7 g	0.55 g	1.0 mM	1.4 g
KS12	1984	M	MH crisis	1993	1.2 g	0.4 g	3.45 mM	0.2 g
KS14☆	1986	F	MH crisis	1991	2.5 g	1.3 g	1.48 mM	1.1 g
KS15☆	1983	M	F/H	1991	1.0 g	0.5 g	1.78 mM	0.7 g
KS16*	1972	M	MH crisis	1985	muscle condition poor at time of test - no CHCT results			
KS17☆	1965	M	MH crisis	1992	0.8 g	0.0 g	3.20 mM	0.15 g
KS18#	1930	M	F/H	1987	0	-	1.80 mm	1.0 g

Testing criteria are listed in Table 3.
F/H= family history of MH; CSC = concentration of caffeine to produce 1g of tension; CAFF = contraction producted with 2 mM caffeine; # - individual II-3 from MH01 family in which the C1840T mutation is segregating with MHS; * - individuals with the G7297A mutation (KS16 is the proband from MH02 family); ☆ - individuals belong to MH families which have been investigated further, that is, KS14 = II-2 from MH05, KS15 =II-3 from MH04, and KS17 = IV-39 from MH03.

A muscle specimen measuring 3.0 -5.0 cm (length) by 2.0 - 3.0 cm (width) by 1-2 cm (thick) was removed from the quadriceps under local or general anesthetic. The vastus lateralis, rectus femoris or the vastus medialis were recommended sites for a biopsy but the rectus abdominus may also be used (Rosenberg and Reed, 1983). The viable excised muscle was bathed in Krebs-Henseleit solution (118.0 mM NaCl; 4.7 mM KCl; 1.2 mM $MgSO_4 \cdot 7H_2O$; 11.0 mM Glucose; 26.0 mM$NaHCO_3$; 2.5 mM $CaCl_2 \cdot 2H_2O$) while being transported to Dr. Bose's laboratory. The time interval between excision and further processing in the laboratory was between 10 and 30 minutes. This time was not critical provided that the entire test was completed within 5 hours of excision from the body (Britt, 1991). The viable muscle fascicle was secured by clamp or silk suture to a plastic electrode frame amd immersed in Krebs-Henseleit solution (pH 7.4) at 37°C. The other end of the muscle fascicle was attached to a transducer which measures muscle tension, recorded with a polygraph. Stable twitch height and twitch baselines were achieved before drug additions were begun (15 -60 min elapsed time). Caffeine was added directly to the solution bathing the muscle in increments from 0.5 to 32.0 mM. Measurement was made of the contracture achieved 4 min after the addition of caffeine, expressed in grams (g) of tension. The amplitude of contractions induced by 3% halothane was measured in separate muscle strips placed in separate baths. In Manitoba, seven different experimental measurements have been utilized. The criterion for an abnormal test result for each of the seven tests are listed in Table 3.

CHCT criteria have evolved since 1987 with tests 1-4 being introduced post 1987 and test 5 always being part of the study protocol. Tests 6 and 7 were used prior to 1987 (personal communication, Dr. Bose).

Normal muscle required more than 4mM caffeine to give more than 1 g of tension and did not respond as strongly to halothane. Test 4 was the least sensitive and test 2 was the most sensitive. However test 2 was felt to have very poor specificity due to many false positive assignments. Prior to 1991, a muscle biopsy was considered positive (and thus MHS), if any one of the 7 tests was positive. However, in a Manitoba control population, consisting of individuals undergoing hip surgery with a negative family history of MH and therefore assumed not to be MHS, 30% of control muscle biopsies had tested positive for 1 of the 7 tests described above. Test 2 was most often the positive test in controls; potentially due to the relative increased sensitivity to caffeine of the predominant Type I fibres seen histochemically. The percentage of positives decreased to less than 1% in controls, if two positive test results were required for assigning an individual as MHS. The reason for allowing only one positive result for MHS determination despite this high degree of false positives had been the desire to err on the side of caution. The current criteria for scoring muscle biopsies in MHS individuals are still being developed and revised by an international MH Study Group. However, most centers have discontinued the use of test 2 completely. As of 1991, a muscle biopsy in Manitoba was considered to be positive if two of the four post-1987 remaining criteria (test 1,3,4,5) were abnormal. Individuals were then classified as MH susceptible (MHS), or MH normal (MHN) on the basis of the CHCT results. According to North American standards, patients responding

Table 3: Postive Caffeine / Halothane Contracture Testing Criteria

Criteria	Years used
1. ≥ 0.5 g tension with 3% halothane	1987 - present
2. ≥ 1.0 g tension with 1 % halothane and < 0.25 mM caffeine	1987 - 1991
3. ≥ 0.2 g tension with 2 mM caffeine (CAFF)	1987 -present
4 >7% of maximum tension with 2 mM caffeine	1987 -present
5. ≥ 1.0 g tension with 4 mM caffeine (CSC)	1987 - present
6. >200 % potentiation with 2 % halothane	before 1987
7. ≥ 0.2 g tension with 2 % halothane alone	before 1987

to caffeine or halothane, but not both, were included in the MHS category, as C or H responders.

12.0 Molecular Investigation

12.1 DNA isolation

Genomic DNA was isolated from peripheral blood according to the method previously described (Greenberg *et al.*, 1987). In brief, 7.5 ml of whole blood collected in EDTA vacutainer tubes were incubated in 5 volumes of NH_4Cl:Tris at 37°C to lyse the red blood cells. White cells were pelleted by centrifugation at 2000 rpm in a swinging bucket centrifuge (IEC-HN SII from Damon/IEC Division) and the supernatant aspirated and discarded. The pellet was washed in saline solution (0.85% NaCl) and centrifuged again at 2000 rpm. Cells were resuspended in 2 ml of high TE (100 mM Tris, 40 mM EDTA) and lysed immediately in 100 mM Tris; 40 mM EDTA; 0.2% SDS pH8.0; 1 M NaCl. Proteins and insoluble components were removed by two extractions with equal volumes of buffered phenol following which the two phases were separated by centrifugation (IEC-HN SII from Damon/IEC Division) at 2,000 rpm for 5 minutes. Residual proteins and phenol were removed by extraction with equal volumes of chloroform:isoamyl alcohol (24:1) followed by centrifugation. The DNA was precipitated in 1/10 volume of 4 M ammonium acetate and equal volume of isopropanol. The loose DNA pellet was washed once with 70% isopropanol and once with 95% isopropanol to remove excess salts and condense the DNA to a more compact pellet. After drying, the pellet was resuspended in low TE (10 mM Tris; 1 mM EDTA).

12.2 Quantitation of DNA

The quantity and quality of DNA was determined using a Gilson Response Spectrophotometer. Samples were appropriately diluted with low TE and placed in quartz cuvettes and absorbance values at 260 nm and 280 nm were recorded. One A_{260} unit was considered to be equivalent to 50 μg of double-stranded (ds) DNA. An $A_{260/280}$ ratio indicated levels of purity with a value of 1.8 being considered essentially free of contaminating protein in dsDNA preparation.

12.3 Digestion of DNA by Restriction Endonuclease

DNA was digested with various restriction endonucleases (RE) (purchased from Pharmacia, BRL and NBL) according to manufacturer's specifications for the enzyme reaction buffer and temperature optimal for the given RE. Restriction endonucleases were used to cut genomic DNA and plasmid DNA, as well as polymerase chain reaction amplified products.

12.4 Separation of DNA Fragments by Electrophoresis

Electrophoresis of DNA fragments was performed in either agarose or polyacrylamide gels depending on the size of the fragments being separated:

Gel Type	Resolution Range
0.7% agarose	0.8 - 12.0 kb
1.2% agarose	0.4 - 7.0 kb
5.0% polyacrylamide	100 - 500 bp
8.0% polyacrylamide	60 - 400 bp
12% polyacrylamide	50 - 200 bp

(Maniatis, 1992)

13.0 Preparation of DNA Probes

13.1 Description of DNA Probes

Five overlapping *RYR1* cDNA probes were kindly provided by Dr. David MacLennan. These 5 *RYR1* probes encompassed the entire 15 kb *RYR1* cDNA. The cDNA clones of the *RYR1* gene ranged in size from 1000bp to 3800bp and had been cloned into the *Eco*R1 sites of four Bluescript plasmids (see Figure 5 and Table 4).

13.2 Introduction of plasmid DNA into bacteria

13.21 Transformation using calcium chloride

Preparation of competent cells: 250 μ of an overnight saturated culture of DH5α *E. coli.* bacteria were transferred to 25ml of Luria-Bertani (LB) medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 10 g NaCl per liter, pH 7.5) and incubated at 37° C with vigorous agitation for 2 hours (a New Brunswick Scientific gyrotorary shaker, shaking at 200 rpm). The culture was chilled on ice for 10 minutes and the cells pelleted at 4,000 x g for 5 minutes at 4°C (Beckman J2-21). The supernatant was discarded and the pellet resuspended in 1 to 2.5ml of ice cold, sterile $CaCl_2$ buffer (50mM $CaCl_2$ and 10mM Tris-HCl pH8.0) and incubated on ice for 15 minutes. The cells were pelleted and the supernatant discarded. The pellet was resuspended in 1.7 ml of ice cold, sterile $CaCl_2$ buffer and 200 μl aliquots were stored at 4°C for 4 hours to overnight. (Dower *et al.*, 1988)

Transformation of competent bacteria: 1 to 5 ug of plasmid DNA was added to one 200 μl aliquot of competent bacteria with gentle mixing. The mixture was stored on ice for 30 minutes, heat shocked at 42°C for 2 minutes then diluted with 1 ml of LB in a

37°C water bath for 45 minutes. Aliquots of 20 μl, 200 μl and 400 μl were spread onto LB agar plates containing 100 ng/ml ampicillin. Plates were left at room temperature for 20 minutes to allow absorption of the volume. The plates were inverted and incubated in a 37°C incubator for 16 hours. Individual colonies were picked and 2 ml overnight saturation cultures were grown. The plasmid was isolated and digested to determine presence of correct clones.

13.22 Transformation by electroporation

This technique was used to amplify the Bluescript plasmid that contained the 410 bp insert of genomic DNA containing the *RYR1* G248R mutation. This DNA was used as a control for the screen for this mutation (see section 13.244).

Preparation of bacteria cells: (Dower et al, 1988) 2.5 ml of an overnight saturated culture of DH5α *E. coli* cells were used to inoculate 500 ml of LB in a 2 L flask and grown with vigorous agitation (200 rpm) in a New Brunswick Scientific gyrotorary shaker at 37°C until the culture reached a logarithmic growth phase. This corresponded to an OD_{600} reading of 0.5 to 0.6 OD units using a spectrophotometer (Gilson). The culture was chilled in an ice water bath for 10 to 15 minutes. The cells were then transferred to pre-chilled 250 ml centrifuge tubes and centrifuged for 20 minutes at 4,000 x g at 2°C (Beckman J2-21). The supernatant was discarded and the pellet resuspended in 5 ml of ice cold water and diluted with 500 ml of ice cold water. The cells were pelleted and the supernatant poured off immediately. The pellet was resuspended by swirling in the remaining liquid. The cell suspension was mixed well with a second 500

ml volume of ice cold water and pelleted again. The supernatant was again removed. An equal volume of 10% glycerol was added and 50 μl aliquots were dispensed into pre-chilled microfuge tubes and frozen on dry ice (-80°C).

Electroporation of plasmid DNA: The electroporation apparatus (Bio, Richmond, California) was set to 2.5 kV and 25 uf and the pulse controller set to 400 ohms. Routinely, 10-50 pg of pure plasmid DNA were added to a fresh tube on ice. One vial of competent cells was rapidly thawed manually and 25 μl aliquots were added to each vial to be electroporated. The cell suspension was mixed with a pipette tip and transferred to a pre-chilled, sterile (UV irradiated 10 minutes and chilled on ice 5 minutes) 1 mm electroporation cuvette. The outside of the cuvette was wiped free of excess moisture and tapped slightly on the bench-top to settle the cells to the bottom. The cuvette was immediately placed into the sample chamber and the pulse discharged. The cuvette was removed and 1 ml of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgCO_4$, and 20 mM glucose) was immediately used to resuspend the cell suspension. This volume was transferred to a 10 ml culture tube and incubated with agitation (200 rpm) at 37°C in a New Brunswick Scientific gyrotorary shaker for 1 hour. Three aliquots (2 μl, 20 μl and 200 μl) of total volume 200 μl were spread onto LB agar plates supplemented with 100μg/ml of ampicillin. The plates were allowed to dry, inverted and incubated overnight at 37°C. Transformation efficiencies were typically $1x10^7$ to $1x10^9/\mu$g plasmid DNA, which was far more than required.

13.3 Plasmid DNA Amplification and Purification

13.31 Growth conditions

Bacterial strains were grown in LB medium. Media for plates was prepared by adding 14.4 g of agar per liter. Antibiotic addition was dependent on the selectable marker and usually consisted of ampicillin or carbenicillin (final concentration at 40ug/ml).

13.32 Plasmid purification

Plasmid purification was performed as described by Birnboim and Doly (1979). Although 5 ml culture were grown for routine screening, the procedure could be scaled up (linearly) for any culture volume. Overnight cultures were placed on ice for approximately 5 min before centrifugation either at 7000 rpm for 5 min in a Sorvall JA-20 rotor or in a microcentrifuge for 1 min at room temperature. The supernatant was discarded and the pellet resuspended by vortexing (Fisher Scientifc Fisher Vortex Genie 2) in 100 μl of cold Solution I (50 mM glucose, 25 mM Tris-HCL, pH 8.0, 10 mM EDTA, pH 8.0). The cells were incubated for 5 min at room temperature after which 200 μl of freshly prepared SDS solution (1% SDS in 0.2 M NaOH) was added and the samples gently mixed by inversion. Each tube then received 150 μl of 3 M sodium acetate solution (NaAc) (pH 5.2). The slurry was gently mixed, incubated on ice for 15 min then centifuged for 5 min at room temperature to pellet insoluble material. The supernatant was transferred to a fresh tube, subjected to a phenol-chloroform-isoamylalcohol (25:24:1) extraction and centrifuged as above. The DNA in the aqueous phase was ethanol precipitated by the addition of 2 volumes of absolute ethanol at room temperature, followed by a 5 min incubation at room temperature and centrifugation at 7000 rpm for 15 min (apparatus as above). The DNA was rinsed once with 70% ethanol and dried

briefly under vacuum (Savant Speed Vac Concentrator). The resulting pellet was resuspended in 30-40 μl low TE.

13.4 Plasmid insert isolation

The first step in insert isolation involved using the appropriate restriction endonuclease to release the insert from the cloning site. The digest was then run on a mini-agarose gel in order to separate the plasmid and insert based on their difference in size. Two methods to purify the insert were utilized.

13.41 Electroelution

DNA fragments were isolated from agarose gels using the paper:dialysis membrane dam method of Girvitz *et al.* (1980) with minor modification. Longwave UV light from a Mineralight Lamp (Model UVGL-58) was used to localize the insert band in the gel. Using a sharp scalpel, an incision was made in the gel directly in front of the leading edge of the band of interest. This cut was 2 mm wider on either side of the band. A piece of 3MM Whatman filter paper and a piece of single dialysis membrane were cut the width of the slot and slightly deeper than the gel. The paper and the membrane were soaked for 5 min in electrophoresis buffer. Using wide-edged forceps, the walls of the incision were held apart and the 3MM paper backed by the dialysis membrane was inserted. Once the paper was in place, electrophoresis was continued until the band of insert DNA had migrated into the paper. The dialysis membrane trapped the DNA in the paper. The paper and the dialysis membrane were removed from the gel and placed in a 0.5 ml microfuge tube which had been place inside a 1.5 ml tube. The 0.5 ml tube was specially prepared

with a hole punched in the bottom and the lid removed. To the 0.5 ml tube containing the membrane and paper, 100 μl of band elution buffer (0.2 M NaCl, 50 mM Tris pH7.6, 1 mM EDTA and 1% SDS), were added and allowed to incubate for 2 min. The elution buffer and DNA were drawn down into the larger 1.5 ml tube by centrifugation for 15 sec at 3000 rpm while the paper and membrane remained in the top tube. The supernatant was recovered in a new tube. This elution process was repeated three times. The eluates from each spin were pooled, and extracted once with TE-saturated phenol/chloroform (1:1). The aqueous phase was removed and the DNA was precipitated using 2 volumes of cold 100% ethanol and 0.1 volume of NaAc (3 M). The DNA was pelleted by centrifugation at 13,000 x g for 5 min (IEC Micro-MB centrifuge), washed with room temperature 70% ethanol, dried briefly and dissolved in 20-50 μl of low TE.

13.42 Gene clean

The digest was run overnight on a 20 x 20 cm 1% agarose gel to separate the insert and vector. The insert band was identified under low frequency UV light with a Mineralight Lamp (Model UVGL-58). The portion of the gel containing the insert to be purified was then physically removed from the remainder of the gel. The Geneclean Kit (BioCan Scientific Inc) was used to isolate the insert. The agarose slice was then cut into pieces that were approximately 0.4 g and placed into 1.5 ml microfuge tubes. Then 1.0 ml sodium iodine (6 M) was added to the tubes which were placed in a 55°C water bath for 7 min to dissolve the agarose. Once the agarose was dissolved, 12.5 μl of Glassmilk (a suspension of silica matrix in water) was added to each tube and it was vortexed until the contents were in suspension. The suspension was incubated for 5 min at room

temperature and then pelleted by centifugation. The supernatant was discarded and the pellet was washed 3 times with 200 μl of NEW wash solution, being careful to resuspend the pellet between washes. After the third wash, the pellet was resuspended in low TE, and incubated in a 55°C water bath for 3 min. After centrifugation, the supernatant containing the insert DNA was then collected.

14.0 Genomic DNA Manipulation

14.10 Analysis of Genomic DNA Using Southern Hybridization

The localization of specific sequences within genomic DNA was accomplished by the transfer techniques first described by Southern (1975). Genomic DNA samples were digested with various restriction enzymes and the resulting fragments were separated according to size by electrophoresis through an agarose gel. The sized DNA fragments were made single stranded by denaturation and were transferred to a solid support (membrane). The DNA bound to the membrane was hybridized to a radiolabeled DNA probe, and autoradiography was used to locate the position of the bands complementary to the probe. Enzyme/Probe combination used are listed in Table 4.

14.11 Digestion of DNA by Restriction Endonuclease

Typically, digests for Southern blot analysis involved 5-10 μg of genomic DNA in a total volume of 30 μl which contained ddH_2O, 3 μl 10X manfacturer's buffer and 2 μl of appropriate RE. Reactions were boosted approximately 2-3 hrs into their incubation with an additional 2 μl of RE, after which incubation continued overnight.

Table 4: Ryanodine Receptor Probes and Flanking Probes on Chromosome 19

Locus*	Probe Name^	RFLP enzyme	Polymorphic band sizes (kbp)
D19S29	17.4	*Msp*I	3.5/5.5 3.6(c)
D19S13	HW60	*Bgl*II *Taq*I	6.7/15.0 2.6/5.5
D19S28	5B18	*Taq*I	1.7/2.7
RYR1	HRR1-1600	*Ban*I	6.0/13.0
	HRR3-1000	*Hind*III *Pvu*II *Bam*HI	19.0/22.0 6.5/4.4, 1.9 19.0/14.0, 5.0
	HRR3-1200	*Pvu*II	3.9, 1.9/5.8
	HRR4-2400	*Taq*I *Bcl*I *Eco*RV	1.8/1.1, 0.7 3.5(c) 11.5/8.4, 2.9 4.6, 3.0(c) 28.0/2.5 11.0(c)
	HRR5-3800	*Eco*RI *Taq*I *Msp*I	24.0/16.0, 9.0 1.9/1.6, 0.3 1.8/2.2
D19S18	PM6.7	*Msp*I *Eco*RI	1.7/3.0 25.0/40.0
D19S15	JSB6	*Taq*I	4.5/2.7
D19S16	JSB11	*Taq*I *Msp*I	6.5/8.5 1.7/1.6
APOCII	APOCII	*Taq*I	3.5/3.8

Table is adapted from MacLennan *et al* (1990)
* - The order of the flanking probes is as determined by Schonk *et al.*
^ - HRR1 to HRR5 are probes form the human ryanodine receptor cDNA described by Zorzato *et al.*, 1989. Their localization on the cDNA map is as follows: HRR1-1600, bases 13602 to 15243; HRR3-1000, bases 8515 to 9554; HRR3-1200, bases 9549 to 10851; HRR4-2400, bases 6125 to 8493; HRR5-3800 bases 2381-6130 and is illustrated in Figure 4.
(c) - Constant hybridizing bands

The quality of DNA digestion was estimated by electrophoresing 2 μl of a total 30 μl reaction for each sample on a 1% agarose mini-gel containing 1 μg of ethidium bromide (EtBr)/ml added in 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA, final pH approx. 8.5) for 1 hr at 87 volts. A photograph of the mini-gel was used to estimate relative amounts of DNA in each lane. Visual inspection and comparison of the digested DNA samples assured relatively equal loading of DNA on the definitive gel.

14.12 Separation of digested genomic DNA by Electrophoresis

For Southern blot analysis, 1% agarose gels were used to separate digested genomic DNA. Appropriate amounts of agarose were added to 1X TAE and the solution boiled until dissolved. The solution was cooled to 60°C, then gels cast in plastic trays as described in Maniatis *et al.* (1982). The gels were submerged in 1X TAE in the buffer chamber and samples were loaded (1X Orange G used as loading buffer). One lane of the gel was loaded with 200 ng of Lambda DNA double digested with *Hind*III/*Eco*RI or a Lambda ladder which acted as the standard. Typically, 1% agarose gels were run at 87 volts in 7cm x 7cm minigels (approx. running time 30-40 min) or at 54 volts overnight (20 to 22 hours) in 20cm x 25cm large size gels. Following electrophoresis, gels were stained with 0.5 μg/ml EtBr in TAE. Once stained, the gel was placed on the Spectroline ultraviolet transilluminator (Model TR-254 - 254 nm UV) and a Polaroid picture was taken (exposure of 2 to 4 seconds using a red filter and Polaroid type 57 film). The intensities of the fluorescence of the ethidium bromide bound to the DNA was used as a measure of the concentration of DNA in each lane. Furthermore, the intensity of the banding

(representative of repetitive DNA) or smear of the DNA was a measure of the completeness of the digestion.

14.13 Southern Transfer

The transfer of DNA from the agarose gel to Zetaprobe membrane was done according to the specifications of the manufacturer (Bio Rad) and based on the method first described by Southern (1975). Briefly, the gel was first acid depurinated in a 0.25 M HCl solution for 15 minutes which ensured the transfer of high molecular weight DNA. The DNA was denatured in 0.2 N NaOH/ 0.6 M NaCl for 30 minutes. After denaturation, the gel was rinsed with distilled water and transferred to Southern apparatus as outlined in Maniatis *et al.* (1982). The Zetaprobe membrane was equilibrated by soaking in 10X SSC for 15 to 20 minutes. Transfer was set up in the standard way using 10X SSC. DNA was transferred overnight. Then the Zetaprobe membrane was rinsed in 2X SSC for 15 minutes. The membrane was baked for 2 hours at 80°C in a Napco vacuum oven (Model 5831) to fix the DNA to the membrane.

14.14 Hybridization of Probes

a) Synthesis of Radiolabelled DNA Probes

Probes were constructed by the random primer method of Feinberg and Vogelstein (1983). Approximately 50 ng of DNA (as prepared and isolated in section 12.0) were boiled for 2 min in a total volume of 6.25 μl of H_2O and then chilled on ice for 5 min. Then 11.75 μl of reaction buffer (425 mM Hepes, pH 6.6, 10.5 mM $MgCl_2$, 21 mM β-mercaptoethanol, 105 mM Tris-Cl, pH 8.0, 42.5 μM dGTP, 42.5 μM dTTP, 1.7 μg/ml BSA (bovine serum albumin), and 0.02 U/ml $Pd(N)_6$-mer primers), 2.5 μl α^{32}P dCTP,

2.5 μl α^{32}P dATP and 1 μl (10U) Klenow enzyme were added. The cocktail was incubated at 37°C for 50 min and then the reaction was stopped by adding 5 μl of 0.5 M EDTA and 170 μl of ddH_20.

While the probe was incubating at 37°C, a DE81 disc of filter paper was moistened with 100 μl of 0.5 M NaH_2PO_4 and allowed to air dry. After the mixture and ddH_20 was added, 1 μl of the probe was placed on the filter paper which was then placed in a scintillation vial and a scintillation count taken using the Beckman LS 1801 scintillation counter. This count represented the total amount of radioactivity added to the reaction (N_t). The filter paper was rinsed with 100 μl of 0.5 M NaH_2PO_4 and 95% ethanol. The filter was dried and a count obtained again. This count represented the amount of incorporated radioactivity (N_i). The percent incorporation (N_o) was calculated as: the amount of incorporated radioactivity (N_i) multiplied by 100 and then divided by the total amount radioactivity (N_t). This should be 70% or more.

b) Prehybridization and Hybridization

Both the traditional "bag" method and the Robbins Scientific Hybridization Incubator (Model 310) were used for membrane hybridization. The membrane was prehybridized in a prehybridization solution containing 0.5M Na_2HPO_4, 0.5M EDTA pH 8.0, 20% SDS for at least 15 minutes prior to adding the radioactive labelled probe. After this prehybridization step, the solution was discarded and replaced with fresh solution. The denatured radiolabelled probe and 200 μg/ml salmon sperm, acting as blocking DNA, were then added. Hybridization was performed at 65° C for 16 hours in a water bath or with gentle rotation on an orbital rotator (Model 310).

c) Washing conditions, autoradiography and stripping

The membrane was washed in "Church Wash" (0.4M Na_2HPO_4, 20%SDS) for 15 minutes at room temperature and then twice at 60°C for 40 minutes (modified Church and Gilbert, 1984). The damp membrane was placed in a plastic bag or covered with plastic wrap. The membrane was covered with a piece of Kodak-X-OMAT AR5 X-ray film and both were placed in a Kodak X-ray Exposure Holder sandwiched between two Dupont Cronex Lightning Plus intensifying screens. The holder was placed into a black plastic bag and placed at -80°C for an overnight exposure. Films were developed in an automatic film processor (National Imaging Ltd., Winnipeg MB). The blot was stripped for subsequent use by pouring boiling 0.1% SDS/0.1X SSC over the membrane and allowing it to cool to room temperature. The damp blots were stored in a sealed bag at 4°C.

14.2 Polymerase Chain Reaction (PCR)

PCR is a method of selective replication *in vitro* of a selected region of DNA (Saiki, 1988). PCR requires a number of components: a source of DNA (the template), a polymerase that can synthesize the new nucleotide chain, free nucleotides that can be added to the end of a growing nucleotide chain and various cofactors (Mg^{2+}, buffers etc.) PCR is dependent on 2 oligonucleotide primers that are homologous to the DNA at each end of the region to be synthesized. PCR is a cyclic process of denaturation of ds DNA, annealing of the primers to the complementary template and extension of the primers by the polymerase to synthesize replicas of each strand of the original template. Amplification is exponential. PCR technology is used for restriction fragment length

polymorphism (RFLP) analysis, as well as direct and indirect mutation detection.

14.21 Separation of PCR products by Electrophoresis

To prepare 8% mini polyacrylamide gels for elecrophoresis (BioRad mini-gel apparatus) of PCR products, 10 μl of TEMED (Sigma) and 50 μl of 25% ammonium persulfate were added to 5 ml of 8% polyacrylamide stock solution [5.3 ml of 29:1 acrylamide: bisacrylamide (w/w), 2 ml of 10X TBE (900 mM Tris-HCL, 900 mM boric acid, 20 mM EDTA) and 12.7 ml ddH_2O for a total of 20 ml]. The gels (7cm x9cm) were cast in a vertical position and allowed to set for 10 min. The gels were run at 200 volts in 1X TBE buffer for approximately 1 h and then stained in 0.5 μg/ml ethidium bromide in 1X TBE.

Separation of PCR products for SSCP and sequencing is described in Sections 14.23 and 13.43 respectively.

14.24 RFLP Studies Using PCR

PCR was used to amplify a DNA fragment which was then digested with various restriction enyzmes. A list of PCR primers, the enzymes and the resulting band sizes are listed in Table 5.

14.23 Indirect Mutation Analysis: Single Stranded Conformational Polymorphism

In collaboration with Dr. MacLennan at the University of Toronto, we attempted to identify the sequence alterations in *RYR1* genomic DNA that might be associated with MH. We were using a technique called single stranded conformational polymorphism (SSCP) which is an indirect method of mutation detection. SSCP is based on the relationship between the electrophoretic mobility of single-stranded DNA and its folded

Table 5: PCR Restriction Fragment Length Polymorphisms

Amino Acid (silent change)	RE change (recognition site)	Fragment Sizes	Primer Names
Leu197 CTG > CTC	*Alw*NI loss (CAGNNNCTG)	51bp + 39bp / 90bp 37bp common band	1Fi 2Re
Thr 2658 ACG > ACA	*Bsa*JI loss (CCNNGG)	46bp + 86bp / 136bp	3Fe 4Re
Ser2862 AGT > AGC	*Hinp*I gain (GCGC)	205bp / 50bp +153bp	X51Fe X50Re
Leu3229 CTG > CTA	*Sty*l gain (CC(AA or TT)GG)	223bp / 130bp + 89bp	5Fi 6Re

leu - leucine, ser-serine and thr- threonine.

conformation. The fold conformation of ssDNA reflects the DNA sequence of that strand. Therefore, any changes in the nucleotide sequence should, theoretically, cause a shift in the mobility of that analyzed molecule upon electrophoresis in a neutral polyacrylamide gel (Orita, 1989).

PCR-SSCP analysis required the production of a labelled PCR fragment. The PCR product was then subjected to electrophoresis through a neutral polyacrylamide gel using 3 different conditions. Conditions varied in the following ways: 1) the amount of glycerol in the gel; 2) ionic strength of the running buffer; and 3) the temperature in which the gel was run. Briefly, using PCR (Saiki, 1988), 12 exons of the RYR1 gene were amplified, utilizing the forward and reverse primer pair specific to the exons listed in Table 6. PCR products for SSCP analysis were amplified in a reaction volume of 100 μl, with 200 - 500 ng of genomic DNA, 100 ng of each of the appropriate primer pair, 1.25 mM dNTPs, 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatin and 1mM $MgCl_2$. PCR reaction conditions were: denaturation at 94°C for 1 minute, 2 minutes at the annealing temperature specific for the primer pair and extension at 72°C for 3 minutes for 35 cycles.

When necessary, the hot PCR product was cut with a restriction endonuclease which is also unique for the primer pair. Ten μl of PCR product was digested with 1μl of restriction enzyme for 2 hours prior to electrophoresis. Ten μl of the cut hot PCR product was added to 38 μl of the loading buffer (95% deionized formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05% Xylene cyanol). The three running conditions chosen for this screen were:

Table 6: *RYR1* Exons Screened Using Single Stranded Conformational Polymorphism

RYR1 Exon	Primers names	Fragment Size	PCR Annealing Temperature	Restriction Enyzme*
Exon 1	HPR1F and HFL1R	411 bp	53°C	*Sph*I
Exon 2	HFL2F and HFL2R-2	285 bp	53°C	*Ban*II (159,126)
Exon 3	HFL3F and HFL3R	288 bp	53°C	*Ban*II (159,129)
Exon 5	HFL5F anf HFL5R	340 bp	55°C	*Apa*I (214,63)
Exons 6 & 7	HFL6F and HFL7R-2	660 bp	55°C	*Hinf*I (267,232,99^{i},63^{i})
Exon 8 & 9	HFL8F and HFL9R	359 bp	54°C	-
Exon 14	HFL14F and HFL14R	208 bp	55°C	-
Exon 17	HFL17F and HFL17R	334 bp	59°C	-
Exon 18	HFL18F and HFL18R	380 bp	55°C	-
Exon 20	HFL20F and HFL20R	308 bp	55°C	*Sma*I (168,140)
Exon 44	HFX44F and	210 bp	58°C	-

Primer prefixes: HFL - Human Flanking; HPR - Human Promoter; HFX - Human Border; HEX - Human Exon.

* - Large PCR products are cut for better SSCP resolution.

' - Intronic Sequence

1. 5 % glycerol, 0.5X TBE, 6 % acrylamide at roon temperature at 40 watts for 3.5 - 5 hours.
2. 1 % glycerol, 1X TBE, 6 % acrylamide at room temperature at 15 milliamps for 16 - 18 hours.
3. 20 % glycerol, 1X TBE, 6 % acrylamide at 4^0C at 28 watts for 16 - 18 hours.

After electrophoresis, gels were dried using the BioRad 583 gel drier and then exposed to X-ray film.

SSCP results were interpreted by looking for differences between denatured normal and mutant PCR products rather than trying to interpret each band.

14.24 Direct Mutation Analysis

14.241 Detection of the **C1840T** Mutation in *RYR1*

We tested for the presence of the C1840T mutation in human genomic DNA by a combination of PCR and restriction endonuclease digestion. A fragment of 940 bp encompassing the mutation was amplified by PCR (Saiki, 1988) for analysis by *Rsa*I. The alteration of the sequence GTAC1840 to GTAT1840 deletes an *Rsa*I site. The amplification was carried out in 100 μl containing 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatin, 1mM $MgCl_2$, 200-500ng of genomic DNA and 100ng of each of the forward primer (5'-ATCTCTAGATTGCCACATCTTATCCCGATGCGC-3') and reverse (5'-ATCTCTAGAA CCTGTCCAGAGATGCAGTCCATC-3') primer for a total volume of 100 μl. PCR reaction conditions were as follows: denaturation at 94^0C for 1 min, annealing at 53^0C for 2 min, and extension at 72^0C for 3 min for 35 cycles in a Perkin Elmer Thermal Cycler. Digestion of the homozygous normal (N/N) product with *Rsa*I

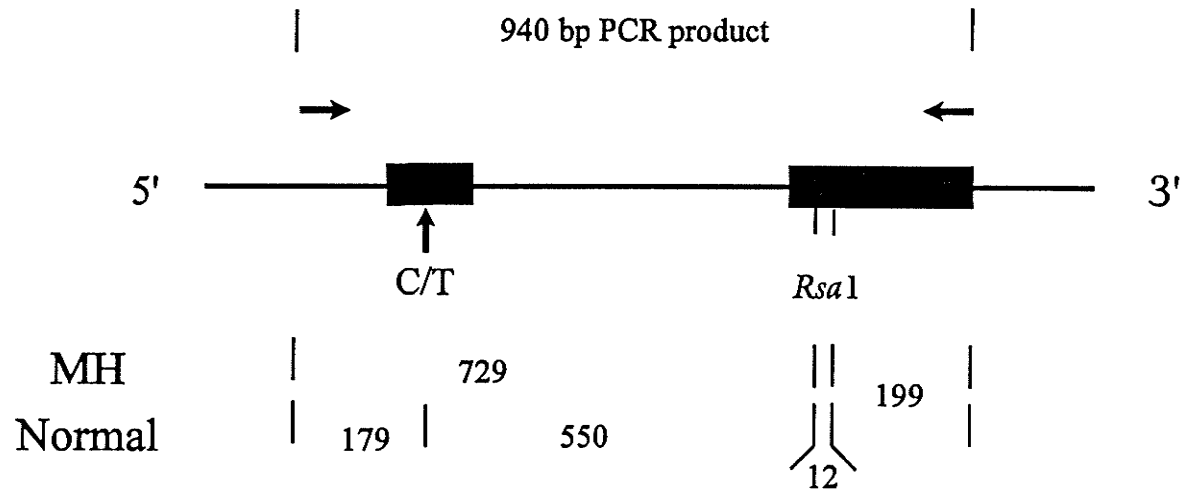


Figure 5:

Detection strategy for the *RYR1* C1840T mutation. The positions of the forward and reverse PCR primers which generate a 940 bp product are indicated by horizontal arrows. The vertical arrow shows the position of the C1840T mutation, eliminating an *Rsa*I site. The position of two constant *Rsa*I sites are also indicated. Diagrammatic representation of the fragment sizes (in base pairs) resulting from the digestion with *Rsa*I in both the MH and normal alleles is indicated. This figure has been adapted from Otsu *et al.* (1992).

generates fragments of 555, 199, 179 and 12 bp, while in a heterozygous state (N/n) fragments of 729, 550, 199,179 and 12 bp are generated. The 940 bp fragment contains a constant *Rsa*I site to serve as an internal control (Otsu,1992).

14.242 Detection of the **G7297A** mutation in *RYR1*

We tested for the presence of the G7297A mutation in human genomic DNA by a combination of PCR and restriction endonuclease digestion. A fragment of 423 bp encompassing the mutation was amplified by PCR (Saiki, 1988) for analysis by *Dde*I digestion. The alteration of the sequence CAGG7297 to CAGA7297 created a *Dde*I site. The amplification was carried out in 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatin, 1mM $MgCl_2$, 200-500ng of genomic DNA and 100ng of each of the forward primer (5'-CTCATCCGGAAGCCTGAGTG-3') and reverse (5'-CTGCAT GAGGCGTTCAAAG-3') primer for a total volume of 100 μl. PCR reaction conditions were: denaturation at 94°C for 1 min, annealing at 53°C for 2 min and extension at 72°C for 3 min for 35 cycles in a Perkin Elmer Thermal Cycler. Digestion of the homozygous normal (N/N) product with *Dde*I generated fragments of 298, 58, 36 and 16 bp, while in a heterozygous state (N/n) fragments of 298, 263, 58, 36, 35 and 16 bp were generated. The 423 bp fragment contained three constant *Dde*I sites to serve as internal controls (Phillips, 1994).

14.243 Detection of the **G7301A** mutation in *RYR1*

As previously discussed, central core disease (CCD) is a morphologically distinct autosomal dominant myopathy with variable clinical features which is closely associated with MH in humans. In a CCD family, a G7301 to A substitution in *RYR1* which results

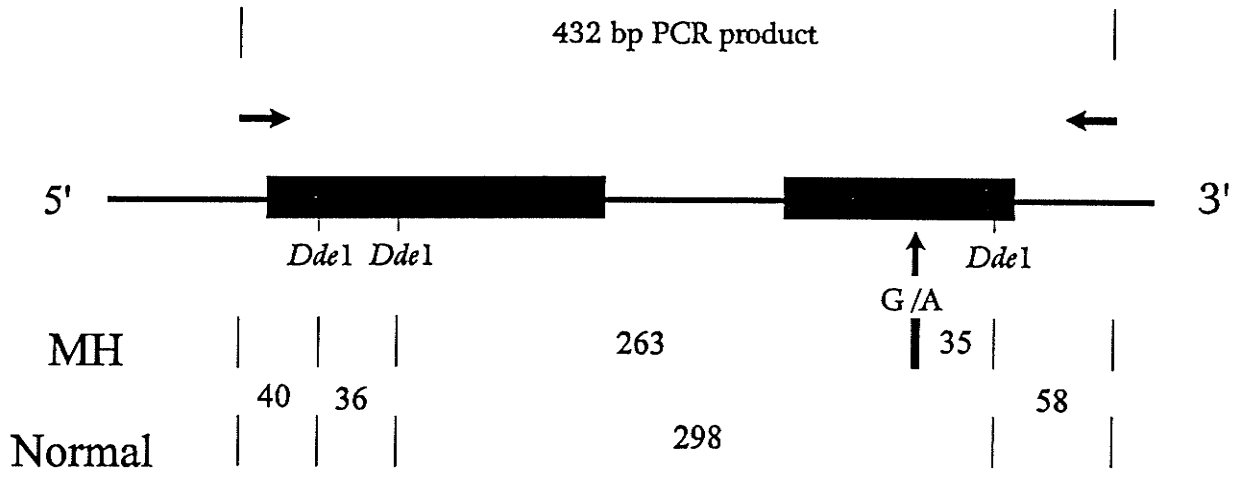


Figure 6:

Detection strategy for the *RYR1* G7297A mutation. The positions of the forward and reverse PCR primers which generate a 432 bp product are indicated by horizontal arrows. The vertical arrow shows the position of the G7297A mutation, creating a new *Dde*I site. Three constant *Dde*I sites are also marked. The fragment sizes (base pairs) resulting from the digestion with *Dde*I in both the MH and normal alleles are also indicated. This figure has been adapted from Phillips *et al.* (1994).

in an Arg2434His mutation has been identified in several members. The family members who were heterozygous for this mutation had been diagnosed with both CCD (using histological staining) and MH (using CHCT). Family members who had had an MH reaction were also heterozygous. In MHN and CCD negative family members, the R2434H mutation was absent (Zhang, 1993). This substitution of A for G7301 mutation abolished an *Hga*I restriction site. Therefore by using PCR to amplify a fragment of *RYR1* encompassing the mutation, followed by restriction endonuclease digestion with *Hga*I, we were able to screen our MH families for this mutation. Briefly, we tested for the presence of the potential alteration (G7301A) in human genomic DNA of our unrelated MHS individuals. A sequence of 210 bp encompassing the mutation was amplified by PCR (Saiki, 1988) for analysis by *Hga*I digestion. Amplification was carried out in a reaction of 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatin, 1mM $MgCl_2$, 200 - 500 ng of genomic DNA, 0.2 mM of each dNTP, 2.5 U of Taq Polymerase and 100 ng of each of the forward primer [5'TTCCCT GCAGCTTTGGTGAGGAACC (+9 bp 5'Xbal1 linker)] and reverse primer [5'GAGTGCC TGCATGAGGCGTTCAAAG (+9 bp 5'Xbal1 linker)] with a total volume of 100 μl. PCR reaction conditions were: denaturation at 94^{0}C for 1 minute, annealing at 58^{0}C for 2 minutes and extension at 72^{0}C for 3 minutes for 35 cycles in a Perkin Elmer Thermal Cycler.

The presence of the G7301 to A mutation was detected by polyacrylamide gel separation of products obtained by digestion with *Hga*I. Digestion of the homozygous normal (N/N) product with *Hga*I generated fragments of 210, 115, 95 bp, while in a heterozygous state (N/n), fragments of 115, 95 bp were generated.

14.244 Detection of the **G742A** mutation in *RYR1*

This Gly248Arg mutation was previously described by Gillard (1992) in one MH family. It was detected during a systematic sequencing of *RYR1* cDNA derived from muscle mRNA belonging to MHS individuals. This G742A transition mutation in *RYR1* did not create or destroy a restriction endonuclease site; therefore a new mutation detection technique needed to be developed.

Using the diagnostic test for Gly248Arg mutation, developed by Zhang *et al* (1993), we screened our MHS families and MHS individuals for this mutation. Zhang *et al* (1993) developed an allele-specific PCR amplification diagnostic test using allele-specific primers with genomic DNA. Two forward primers differing only in the 3' base (mutant A and normal G) were designed to be paired with a common reverse primer. The primer sequences were: FA[5'-GACTTGTCTACTATGAGA*-3']; FG[5'-GACTTGTC TACTATGAGG*-3']; and the R [5'-AAGACCTTTTCTGTCCTC-3']. Therefore, depending on the genomic sequence, the normal allele or mutated allele was generated. Both forward primers generated a fragment of 178 bp. The lack of PCR product using the mutant forward primer was correlated with the absence of the mutation. That is, a PCR product was generated using the forward mutant A primer and common reverse primer if, and only if, the mutation was present. If there was no mutation, there was no PCR product generated. PCR product was generated using the forward normal primer and common reverse primer each time, because at least one allele would have the normal G. A plasmid containing the homozygous mutant allele sequence was used as a PCR control. Amplification was carried out in a reaction of 10 mM Tris pH 8.3, 50 mM KCl, 0.01%

gelatin, 1mM $MgCl_2$, 200 - 500 ng of genomic DNA, 0.2 mM of each dNTP, 2.5 U of Taq Polymerase and 100 ng of each of the either forward primer FA or FG and reverse primer (R) with a total volume of 100 μl. PCR reaction conditions were: denaturation at 94^{0}C for 1 minute, annealing at 53^{0}C for 2 minutes and extension at 72^{0}C for 3 minutes for 35 cycles in a Perkin Elmer Thermal Cycler.

14.245 Detection of the **G1021A** Mutation in *RYR1*

DNA samples were screened for this mutation at Ontario's Provincial Laboratory in Toronto by John Waye. Exon 11 was amplified as a 269 bp fragment using PCR and the following primers: R4R-1-6 [5'-CCTGCTAAACACACAGGCAGAGGA-3',IVS-10] and R4R-1-7 [5'-ACTCAAGCGATTCTCCCACCTCAG-3', IVS-11]. PCR was conducted in a volume of 100 μl containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 15 pmoles of each primer and 2.5 U of Taq Polymerase. PCR was run for 32 cycles consisting of 94^{0}C for 60 seconds and 70^{0}C for 40 seconds in a Perkin Elmer Thermal Cycler.

Following amplification, the PCR products were dot-blotted onto nylon membranes (Bio-Rad) and hybridized to allele-specific oligonucleotide (ASO) probes against the normal [R4R-1-5: 5'-GTGACTCCCCGTACTTGTA-3'] and the mutant [R4R-1-8: 5'-ATCAAGTACAGGGAGTCAC-3'] alleles. Probe labelling and detection were facilitated using the ELC 3'-Oligolabelling and Detection System (Amersham). The ASO probes were 3' end labelled with fluorescein-11 deoxyuridine triphosphate and hybridization was conducted at 50^{0}C for 60 minutes in a solution containing 5X SSC, 0.1% ELC hybridization buffer component, 0.2% SDS, 20 fold dilution of ELC liquid blocking

solution, and 8 ng labelled probe per ml of hybridization solution. Following hybridization, the membranes were washed at 54^{0}C for 15 mintues in 1X SSC/0.1% SDS and the membrane-bound probes were detected using an antifluorescein antibody conjugated to horseradish peroxidase (HRP). HRP enzyme activity was detected by chemiluminescence and brief exposure (1-10 minutes) to X-AR5 X-ray film (Kodak).

14.25 Sex Determination (Lau *et al.*, 1989)

The zinc finger Y (ZFY) is a gene found specifically on the Y chromosome. There is a corresponding homologous gene on the X chromosome designated ZFX. Although the ZFX and ZFY genes are homologous, there are some differences in the sequence that allows us to amplify both X and Y specific sequences of different sizes at the same time. Therefore, a PCR amplification reaction can be used to determine whether a particular DNA sample is from a male or a female. The PCR reaction uses three different primers: 1) ZFC is a constant primer that will bind to both X and Y sequences; 2) ZFY is a Y specific primer that will only bind to the Y sequence. When used in combination with the constant primer (ZFC) for PCR, a 340 bp fragment is amplified; 3) ZFX is a X specific primer that will only bind to the X sequences. When used in combination with the constant primer (ZFC) for PCR, a 488 bp fragment is amplified. Therefore males will have two bands and females have one.

This protocol was used to determine if two DNA sample (one male and one female) had been mixed up in family MH02. Sexing the samples was used to eliminate the possibility of a sample mix up. X and Y specific sequences were amplified using the following primers: ZFC [5'-ATTTGTTCTAAGTCGCCATATTCTCT-'3], ZFY [5'-CAT

CAGCTGAAGCTTGTAGACACACT-3'] and ZFX [5'-AGACACACTACTGAGGCA AAATGTATA-3']. Two reactions were set up for each sample: 1) one with primers necessary to amplify only the Y specific sequence and 2) one with both Y specific and X specific primers. A female and male control were also included. Amplification was carried out in a volume of 100 μl containing 67 mM Tris, 3 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_2$, 10 mMβ-mercaptoethanol, 0.2 mM of each dNTP, 25 pmoles of each primer and 2.5 U of Taq Polymerase. PCR reaction conditions were: an initial denaturation at 94^0C for 7 min followed by 30 cycles consisting of: 94^0C for 30 seconds (denaturation between cycles) and 65^0C for 4 min (annealing and extension of primers). There was a final 10 min extension at 65^0C. PCR products were separated on a mini polyacrylamide gel.

14.3 DNA Sequencing

14.31 Purification of PCR product

The target sequence was amplified by PCR (Saiki, 1988) as described above. The PCR product was then subjected to electrophoresis through a 5% mini-polyacrylamide gel (Bio-Rad apparatus). The gel was stained after running in 0.5 μg/ml EtBr in TBE. The band of interest was visualized under longwave UV light (Mineralight Lamp model 58) and then excised from the gel. The piece of gel containing the band was cut into several small fragments and put into a 15 ml polystyrene tube along with 500 μl of TE buffer. The PCR product was allowed to elute overnight. Further purification involved a series of phenol/chloroform extractions. PCR DNA was precipitated with 1/10 volume 3 M

sodium acetate and 2 volumes of absolute ethanol which was stored at -20°C overnight. After pelleting and washing the DNA with 70% ethanol, the DNA was resuspended in 30 μl of TE or water. 1-2 μl was run on a mini acrylamide gel to estimate amount of DNA recovered.

14.32 DNA sequencing reaction preparation

The Sanger dideoxy chain termination method (Sanger et al, 1977) of sequencing DNA was employed preferentially due to its ease of use and availability of standardized kits. Cycle sequencing permitted direct sequencing of femtomole quantities of ds DNA using PCR technology. Therefore, a dsDNA cycle sequencing kit was used (BRL, Burlington, Ontario). The primer was end labelled in a 5 μl reaction [1 μl 5X kinase buffer (300 mM Tris-HCl pH 7.8, 50 mM $MgCl_2$, 1 M KCl), 1 μl [γ^{32}P]ATP (6000 Ci/mmol), 1 μl (1 u) T4 PNK (T4 polynucleotide kinase) and 2 μl (1 pmol) primer]. The reaction was incubated at 37° C for 30 minutes before termination by heating at 55°C for 5 minutes. The reaction mixture consisted of a 36 μl volume of 4.5 μl of 10X Taq sequencing buffer (300 mM Tris-HCl pH 9.0, 50 mM $MgCl_2$, 300 mM KCl and 0.5% (w/v) W-1), 26 μl of template DNA (approximately 50 fmol) and 0.5 μl (2.5U) Taq DNA polymerase). The reagents were mixed and tubes briefly centrifuged (IEC Micro-MB centrifuge) to collect the volume at the bottom of the tube. This reaction was distributed into 4 tubes containing 2 μl of individual dideoxynucleotide triphosphate (ddNTP) termination mix. Each 10 μl reaction was overlaid with 10 μl of mineral oil, the tube briefly centrifuged and then placed into a Perkin Elmer Thermal Cycler. Temperature cycling was for 95°C for 5 minutes to denature the template, then a quick

cycle of 10 seconds at 95°C and 10 seconds at 65° C. 5 μl of stop buffer (95% v/v formamide, 10mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol) was added to the samples which were then stored at -80°C for no more than 1 week before running on a sequencing gel.

14.33 Sequencing Gels

Denaturing polyacrylamide sequencing gels (50 x 21 cm) were prepared as described in the BioRad literature. Typically, 6% polyacrylamide gels containing 7 M urea were used. Stock solutions of 30% acrylamide (29:1 acrylamide/bisacrylamide) were used in preparing gels. Prior to electrophoresis, gels were pre-run until the temperature of the gel reached 50°C. Electrophoresis buffer was 1X TBE (900 mM Tris-HCL, 900 mM boric acid, 20 mM EDTA). Samples were denatured in loading buffer (95% w/v formamide, 10 mM EDTA pH 8.0, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol) and electrophoresed at 1800-2000 V for 1-3 h. Gel temperature was maintained at 50°C for the entire electrophoresis.

15.0 Data Analysis

15.1 Linkage Analysis

Genetic linkage is defined as the tendency for two or more non-allelic genes or DNA markers to be inherited together more frequently than would be expected by independent assortment, because the two loci are located close to each other on the same chromosome (syntenic) (Risch, 1992). The closer 2 syntenic loci are together, the less

likely their respective alleles will be separated at meiosis. When loci are farther apart, crossing over between homologous chromosomes at meiosis allows for the exchange of alleles resulting in new combinations (Russell, 1996).

The frequency of recombination is defined as the recombination fraction, θ, which can vary from 0.0 to 0.5 and will increase with increasing distance between loci. The amount of recombination that occurs between loci is an indirect measure of the distance in cM between the two loci. The absence of linkage, therefore independent assortment, is indicated when the probability of either a recombinant or a parental chromosome is equal ($\theta=0.5$). Therefore, recombination events along a chromosome that has multiple closely spaced DNA markers can be used to localize a disease gene within a region of the chromosome (Risch, 1992).

Linkage analysis requires defined genetic markers to be available for study and that these markers be readily identifiable and highly polymorphic. (A locus is said to be polymorphic when 2% of the population are heterozygous for the less common allele.) A nucleotide sequence variation between individuals that does not alter the phenotype is referred to as a neutral polymorphism (Levitt, 1992). Throughout the human genome, there are many neutral polymorphisms which create or destroy restriction endonuclease recognition sites, which are useful for linkage analysis. The homologous segments of restriction endonuclease cleaved DNA of differing lengths are known as restriction fragment length polymorphisms or RFLPs. RFLPs can be detected by Southern analysis or by PCR amplification of a specific region of genomic DNA followed by appropriate restriction enzyme digestion. In order to detect linkage between 2 genes, informative

matings are necessary. An informative mating demonstrates clear segregation of the alleles at two loci. One parent must be heterozygous for the marker at the loci under consideration so that segregation analysis will identify recombination between the locus and the disease. When clear segregation cannot be seen, the mating is noninformative (Maynard-Smith et al, 1961).

The measure of linkage is calculated mathematically as a lod score. A lod score is defined as the $\log_{10}$ of the ratio of the probability that the data obtained would have arisen if the loci are linked, to the probability that the data would have arisen from unlinked loci or by chance. The conventional threshold for declaring linkage is a lod score of 3.0, that is a 1000:1 ratio. A lod score between 3.0 and -2.0 is consider inconclusive and more data are required, while a lod score of -2.0 or less excludes linkage (Morton, 1955).

Linkage between MHS and *RYR1* was studied in 5 families by the analysis of a number of such RFLPs within and flanking the *RYR1* locus using Southern blotting and PCR followed by restriction enzyme digestion as previously discussed. Formal lod scores were not calculated because of few scoreable meioses, due in part to the difficulties in phenotypic assignment. (see Discussion)

15.2 Haplotype Construction

RFLP data was analyzed and then used to construct an individual's haplotype. A haplotype is a combination of very closely linked polymorphic markers that tend to be transmitted as a unit to the next generation. Haplotypes are transmitted, in a Mendelian fashion, one from each parent (Lewin, 1990) . MHS was assigned to specific haplotypes

within a family and the presence or absence of recombination events were noted by inspection of the data.

15.3 Mutation Segregation Studies

In families where an *RYR1* mutation was found, all available family members were screened for that mutation. It was then determined whether the mutation cosegregated with MHS, as assigned by CHCT. The numbers were too small to perform a formal χ^2 analysis.

RESULTS

16.0 General Results

We studied sixteen unrelated individuals with a well documented MH crisis or a positive CHCT. These sixteen individuals were screened for five known MH associated *RYR1* mutations: Gly248Arg, Gly341Arg, Arg614Cys, Gly2433Arg and Arg2434His using PCR protocols designed for their direct detection. Relevant demographic information pertaining to these individuals is found in Table 2. Within this patient population, we found one individual who was heterozygous for the Arg614Cys mutation (KS18) and three individuals who were heterozygous for the Gly2433Arg mutation (KS1, KS8 and KS16). We have been able to study other family members of 2 of the 4 families with an identified *RYR1* mutation. We have also studied the families 3 of the remaining 12 individuals in whom no *RYR1* mutation has been identified to date, using RFLP analysis and SSCP.

17.0 The Arg614Cys mutation:

The diagnosis of MH susceptibility was established in the proband (III-2) from this large Manitoba Mennonite family when she died at the age of 45 years of an MH crisis following a general anesthetic (Figure 7). She was admitted for a left oophorectomy at her local hospital in 1979. There was no previous history of any adverse anesthetic reactions. She was anesthetized with succinylcholine, thiopental, nitrous oxide and halothane. Toward the end of her two hour laparotomy, she was noted to be hypotensive, hyperthermic (40.5°C), hypertonic and mottled. Her urine was noted to be red. She

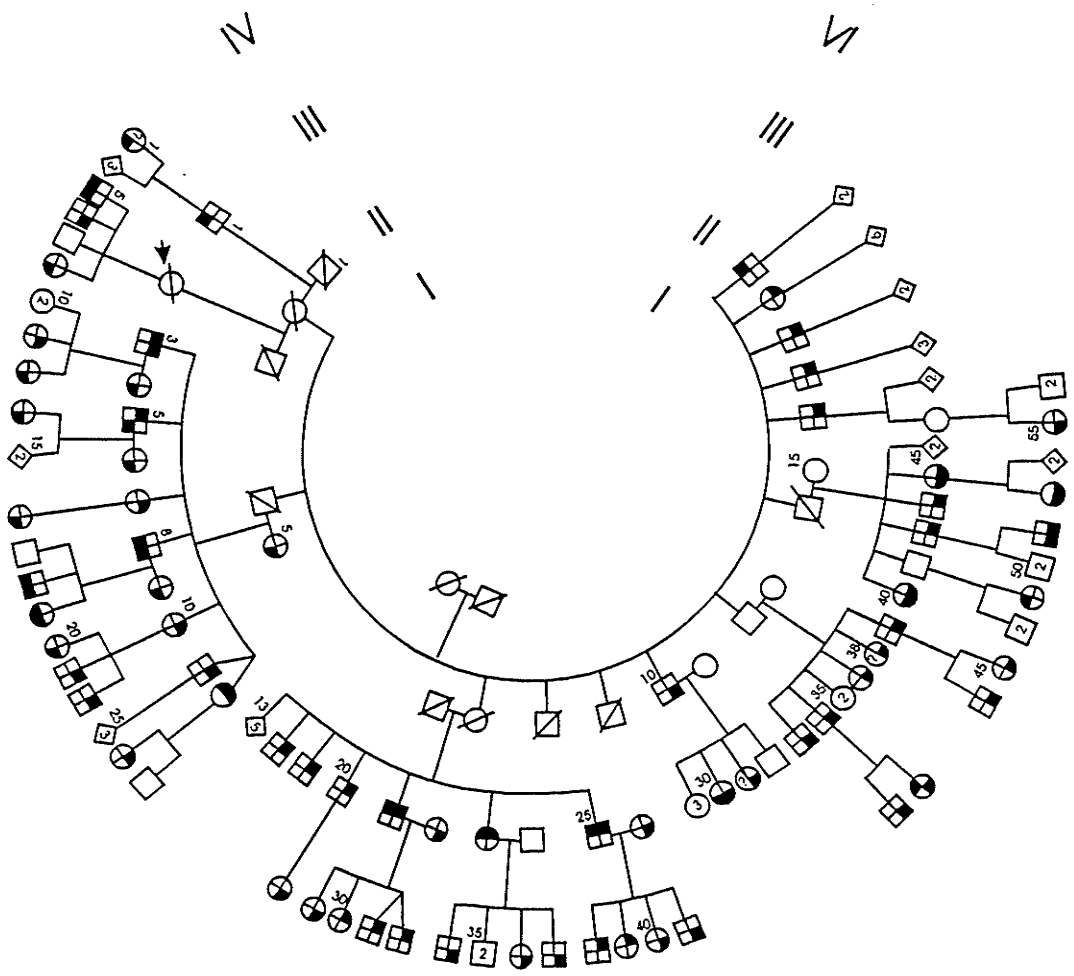


Figure 7:

Partial pedigree of a large Manitoba Mennonite MH kindred (MH01). Two individual: (↗) in this family have had a documented MH crisis. The proband, III-2, died from a MH crisis. MH status as determined by CHCT and the presence of the C1840T mutation is indicated with symbols: ◔ = MHN by CHCT; ◕ = MHS by CHCT; ⓘ = MH status by CHCT is unknown; ◔ = C1840T mutation present; ◔ = C1840T mutation absent; ◯ = not studied.

Table 7: Malignant Hyperthermia Status Based on CHCT and DNA Results for Members of the MH01 Family

ID #	Date of Biopsy	Twitch Quality	2% Hal	3% Hal	CSC	CAFF	MH Status	
							CHCT[c]	DNA
III-3	22/5/87	Good	0 g	-	*1.8 mM	*1 g	MHS	+
III-5	9/12/91	Good	*0.5 g	*3.9 g	*2.1 mM	*0.8 g	MHS	-
III-8	12/5/89	Poor	0 g	*3.6 g	4.18 mM	0.1 g	Unknown	-
	4/6/92	Good	0 g	0.35 g	4.49 mM	0 g	MHN	-
III-12	24/8/93	Good	*0.5 g	*1.4 g	*1.46mM	*1.4 g	MHS	+
III-21	20/2/90	Good	*3.8 g	*7.6 g	*1.53mM	*1.1 g	MHS	+
III-23	7/12/90	Good	*3.2 g	*8.6 g	*1.71mM	*0.9 g	MHS	+
III-25	21/1/86	Good	0 g	-	*3.27mM	*0.6 g	MHS	+
III-30	14/12/92	Good	0 g	0.4 g	4.95 mM	0 g	MHN	-
III-31	12/ 5/92	Poor	0 g	0 g	*2.72mM	0 g	Unknown	-
III-38	5/5/83	Poor	0 g	-	*2.98mM	*0.65 g	Unknown	-
III-40	28/8/92	Good	0 g	0 g	5.25 mM	0 g	MHN	-
III-44	11/5/93	Good	0 g	0 g	4.22 mM	0 g	MHN	-
IV-1	4/6/86	Poor	0 g	-	-	0 g	Unknown	-
IV-5†	8/12/87		-	0.3 g	16 mM	-	MHN	-
IV-18	21/3/90	Good	0 g	0.3 g	7.9 mM	0.1 g	MHN	-
IV-19	6/12/89	Good	0 g	0 g	4.4 mM	0 g	MHN	-
IV-38	No	-	-	-	-	-	Crisis	+
IV-43	7/4/89	Good	-	0.75 g	3.0 mM	0.65 g	MHS	-
IV-51	26/8/86	Good	0.15 g	0 g	16 mM	-	MHN	-
IV-52	13/5/86	Good	0 g	-	8.14 mM	0 g	MHN	-

Testing criteria are listed in Table 3
CSC = Concentration of caffeine to produce 1 g of tension
CAFF = Contraction produced with 2 mM caffeine
Unknown = Status could not be assessed because of poor muscle quality
* Abnormal test result
† done in Calgary
+ C1840T mutation present
- C1840T mutation absent

developed disseminated intravascular coagulation, renal failure, and cardiogenic shock. She never regained consciousness and died one day post-operatively. Subsequently, a second individual (IV-38) was identified as having survived an MH crisis. This 3 year, 10 month old boy developed generalized muscle rigidity and cyanosis after administration of succinylcholine, nitrous oxide, and halothane for a right inguinal hernia repair. Myoglobinuria was documented and his CK level rose to 18,000 U/L the following day. He recovered uneventfully following supportive management.

Approximately 126 individuals in this family are known to be at 50% or 25% risk for MHS. Standardized open biopsy of the vastus lateralis muscle was performed in 21 at-risk individuals during the period 1986 to present. One individual, III-8, had 2 biopsies. These muscle biopsy specimens were studied using standard histochemistry and CHCT protocols of the North American Group and Registry (after 1987). Family members were classified as MH susceptible (MHS), or MH normal (MHN) on the basis of the CHCT (Table 7).

Early investigation of the inheritance of MH susceptibility in this family involved linkage analysis of MHS to cDNA markers from *RYR1* on chromosome 19q12-q13.22. A list of the enzyme/probe combinations found in Table 4 were used. One haplotype segregated with MHS suggesting *RYR1* as a candidate gene. Therefore, we embarked on a search of the *RYR1* gene for a disease causing mutation using SSCP to screen for single nucleotide changes in the *RYR1* gene of the MHS patient, III-3. Thirteen *RYR1* exons were screened (exons 1-3, 5-9, 14, 17, 18, 20 and 44). An aberrant SSCP pattern was detected in Exon 17 of the *RYR1* gene (Figure 8). Direct sequencing of the amplified

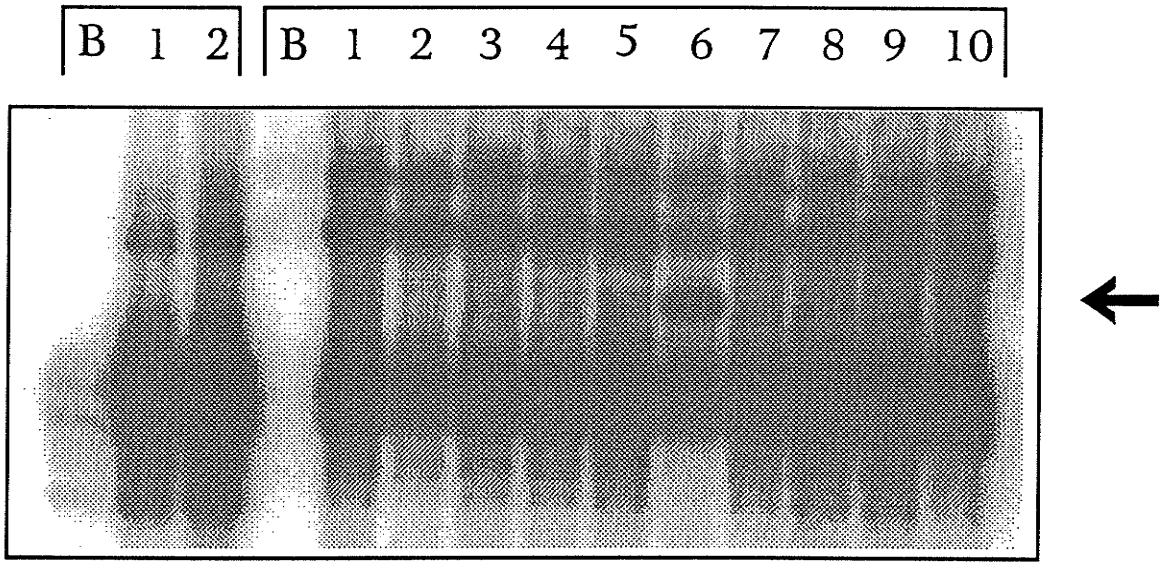


Figure 8:

Detection of the C1840 to T mutation by SSCP analysis. The 334 bp PCR amplified product, containing exon 17 of the *RYR1* gene, was digested with *Pst*I to improve its resolution on the SSCP gel. Samples 1-10, from unrelated MHS probands, were separated on a sequencing size non denaturing gel which contained 10% glycerol and was run at 15 mA at room temperature overnight (16 hours). The arrow indicates a variant SSCP banding pattern in lane 6 which is associated with the C1840T mutation.

fragment from patient III-3 showed the presence of the single base substitution C1840T (Figure 9). The C1840T substitution resulted in the replacement of an Arg 615 to Cys. This mutation caused the loss of a *Rsa*1 site. Therefore screening for this mutation involved PCR amplification of Exon 17 of *RYR1* gene followed by *Rsa*1 digestion (Figure 10). This comparison formed the basis for the manuscript which has been recently published in the journal *ANESTHESIOLOGY* (Appendix A). DNA samples from 68 family members, including 16 who had undergone muscle biopsies and one who had a documented crisis, but not biopsied, were screened for the C1840T mutation. Twenty-two family members were found to be heterozygous for this mutation. Of these, 5 individuals had a prior positive CHCT and one had an MH crisis. DNA analysis revealed that 46 family members were homozygous for the normal allele. Of these, 9 had been classified as MHN and 2 as MHS on the basis of CHCT results. Therefore the correlation between DNA results and CHCT assignments was not absolute and several possible explanations were considered (see discussion).

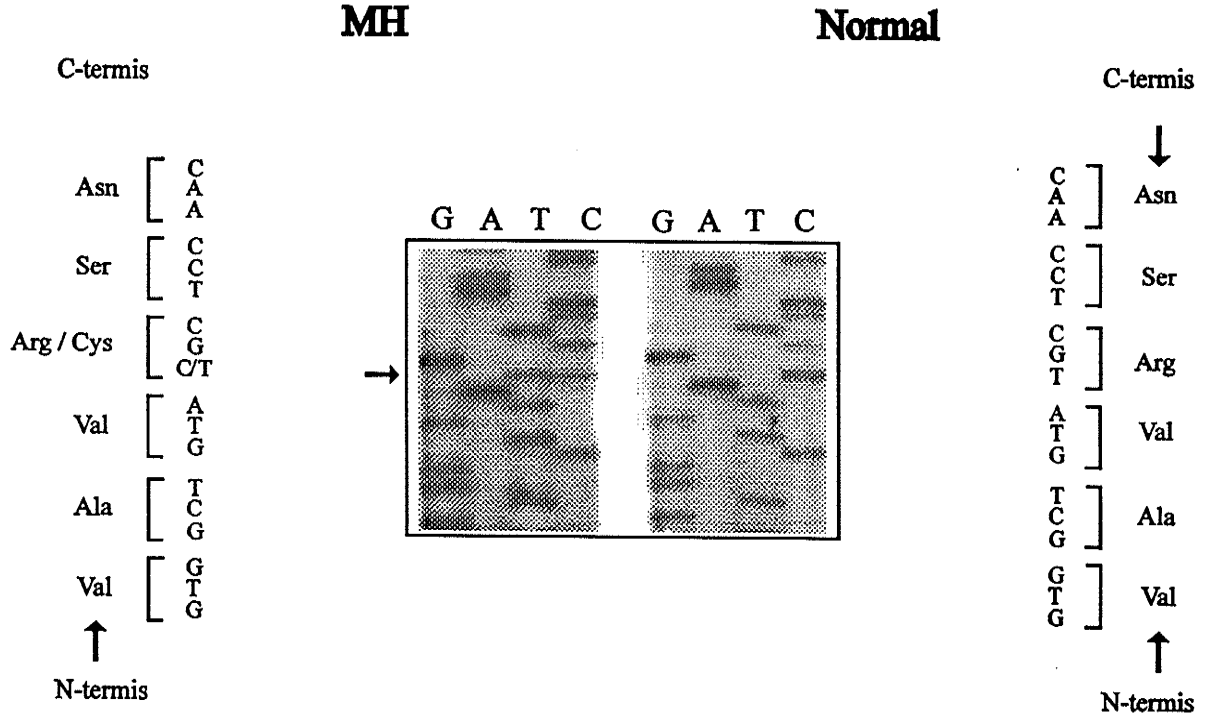


Figure 9:

Direct sequencing of PCR amplified exon 17 of the *RYR1* containing the C1840T mutation. The substitution of T for C1840 in the coding strand is indicated by an arrow. The normal panel contains DNA from a normal individual and MH panel contains DNA from the individual III-3 (MH01).

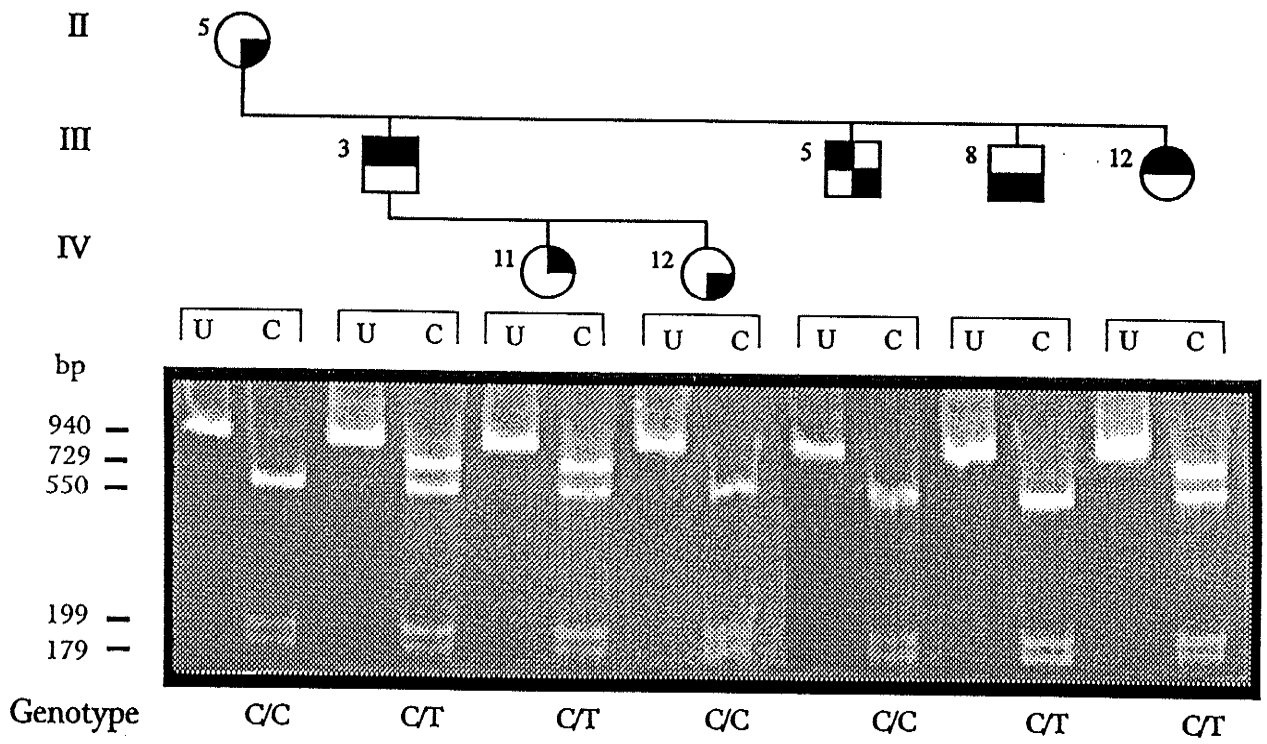


Figure 10:

*Rsa*I restriction endonuclease analysis of the C1840 to T mutation from a grouping within the large pedigree, MH01, described in Figure 7. *Rsa*I digestion of the amplified 940 bp PCR product yields a 729, 550 ,199 and 179 bp fragments for DNA containing the C1840T mutation. Each individual genotype is indicated by a C-for the normal allele and T-for the mutant allele. Pedigree symbols are as in Figure 7.

18.0 The Gly2433Arg mutation:

18.1 The diagnosis of MHS was established in the proband (IV-7) from this family when he survived an MH crisis (Figure 11). Individual IV-7 had been admitted in October of 1984 for an elective bilateral lengthening of his Achilles tendon. During induction of anesthesia with halothane and succinylcholine, he became hyperthermic to 39^{0}C, tachycardic, and developed a metabolic acidosis. The patient was given dantrolene, the anesthetic was discontinued and diuresis was induced with mannitol. The patient recovered uneventfully. His CK rose to 184,200 U/L post operatively and he developed myoglobinuria within 48 hours. His father, and subsequently his paternal grandfather, underwent muscle biopsies for CHCT testing. Both had positive CHCT and therefore were determined to be MHS. All of the proband's grandpaternal relatives were therefore considered at risk for MH and were encouraged to have further testing. CHCT results for family members is found in Table 8.

The early investigation of this MH family involved linkage analysis between the gene for MHS and several cDNA markers within and surrounding the *RYR1* gene. Using the information obtained with each of the enzyme probe combinations (Table 4), we were able to construct haplotypes in order to follow allele segregation in this family. Figure 12 illustrates the inheritance of the specific haplotypes in this family. II-7 was determined to be MHS by CHCT testing done in 1987. However, MH susceptibility appeared to be segregating with his orginally assumed affected wife's (II-8) "M1" haplotype in this family. The maternal "M1" haplotype appeared to be segregating with the MHS in II-8's MHS offspring, III-6 and III-10, while her MHN child, II-4, had inherited the alternate

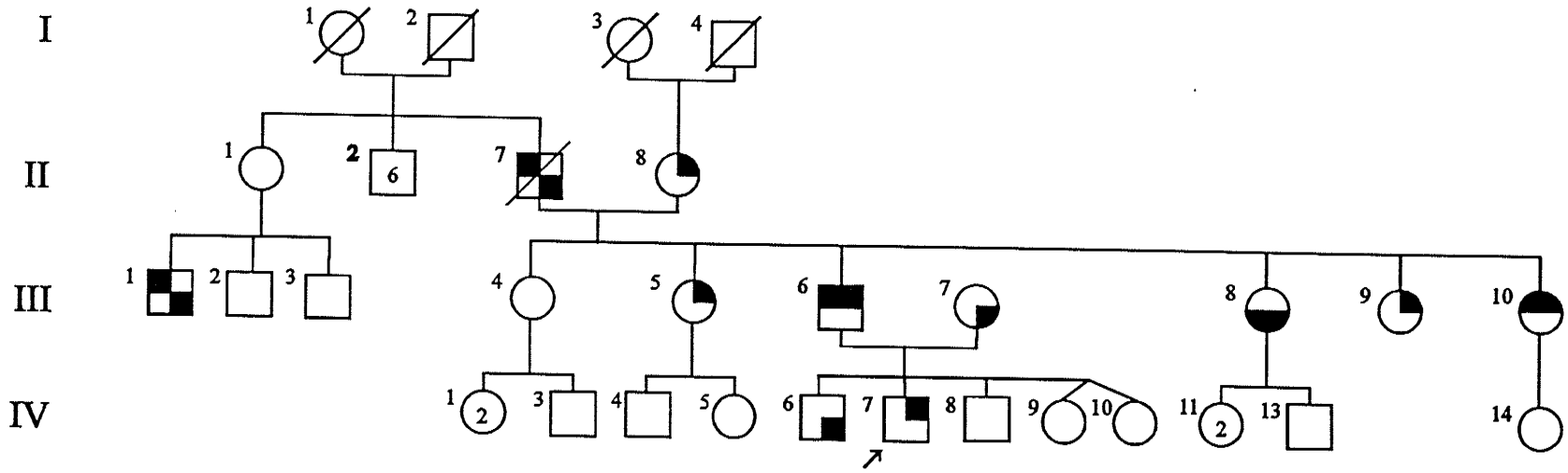


Figure 11:

Partial pedigree of a Manitoba MH kindred. Proband IV-7 (↗) survived a documented MH crisis. MH status as determined by CHCT and the presence of the G7297A mutation is indicated by the symbols: ◔ = MHN by CHCT; ◐ = MHS by CHCT; ⓘ = MH status by CHCT is unknown; ◔ = G7297A mutation present; ◔ = G7297A mutation absent; ◯ = not studied.

Table 8: Malignant Hyperthermia Status Based on CHCT and DNA Results for Members of the MH02 Family

ID #	Date of Biopsy	Twitch Quality	2 % Hal (g)	3 % Hal (g)	CSC (mM)	CAFF (g)	MH Status	
							CHCT	DNA
II-7†	8/5/87	Good	0.2*	-	6.67	-	MHS	-
III-1	28/2/92	Good	0.8*	2.7*	1.90*	-	MHS	-
III-6	13/3/87	Good	0.0	-	1.25*	-	MHS	+
III-8	25/11/87	Good	-	-	6.04	-	MHN	-
III-10	29/1/88	Good	-	0.8*	1.57*	1.2*	MHS	+
IV-7	4/10/85	muscle had been treated with dantrolene prior to testing - no CHCT results					MH crisis	+

Testing criteria are listed in Table 3.
CSC = concentration of caffeine to produce 1 g of tension; CAFF = contraction produced with 2 mM caffeine; + = G7297A mutation present; - = G7297A mutation absent; * = abnormal result.
† = Additional positive result - 216 % potentiation.

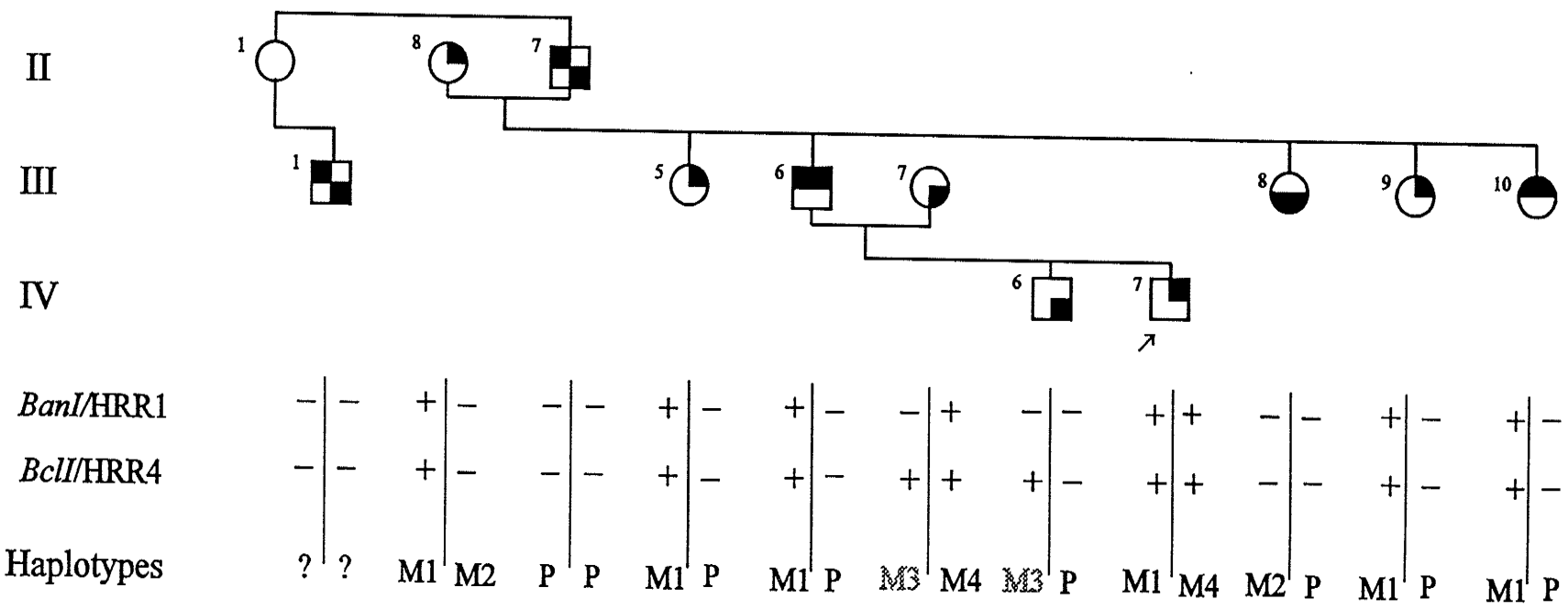


Figure 12:

Analysis of segregation of alleles of markers HRR1 and HRR4 (which are polymorphisms from within the *RYR1* locus) in the MH02 family with predicted chromosome haplotypes shown under each individual. Alleles are as described (MacLennan *et al.*, 1990) and are presented as haplotypes "-" indicates absence of restriction site and "+" indicates presence. Pedigree symbols are as in Figure 11.

"M2" haplotype. III-6 also passed this "M1" haplotype on to his son (IV-7), who had the MH crisis.

This family was not included in the SSCP screen of *RYR1* exons because it seemed, after the original RFLP studies, assuming the paternal grandfather (II-7) was MHS, that MH susceptibility could not be due to an *RYR1* mutation or a mutation linked to *RYR1*. However, individual III-2 was included in the general screen for five of known mutations in *RYR1*. It was discovered that in fact IV-7 has the Gly2433Arg mutation in exon 44 of the *RYR1* gene. Sequencing this exon confirmed a substitution of A for G7297 in III-2 (Figure 13). This mutation caused the loss of a *Dde*1 site. Therefore screening for this mutation involved PCR amplification of Exon 43 and 44 of *RYR1* gene followed by *Dde*1 digestion.

We used this assay to screen all available members of this family for the Gly2433Arg mutation (Figure 14). This mutation was found in the proband and also in III-6 and III-10, who were MHS by CHCT. It was also found in III-5 and III-9 who had no previous history of anesthetic reactions and have not had CHCT testing. This mutation was absent from III-8 who was determined to be MHN by CHCT. It was also found in individual II-8 who had not been subjected to CHCT but carried the *RYR1* haplotype "M1" which seemed to be segregating with MHS in this family. This mutation was absent from II-7 who had a positive CHCT. This would be explained if samples II-7 and II-8 had been mislabelled during DNA extraction. However, when new DNA samples were obtained, the results were still the same. Sexing was also performed on the samples and II-8 had no PCR product when primers for Chromosme Y, while II-7 did. A muscle

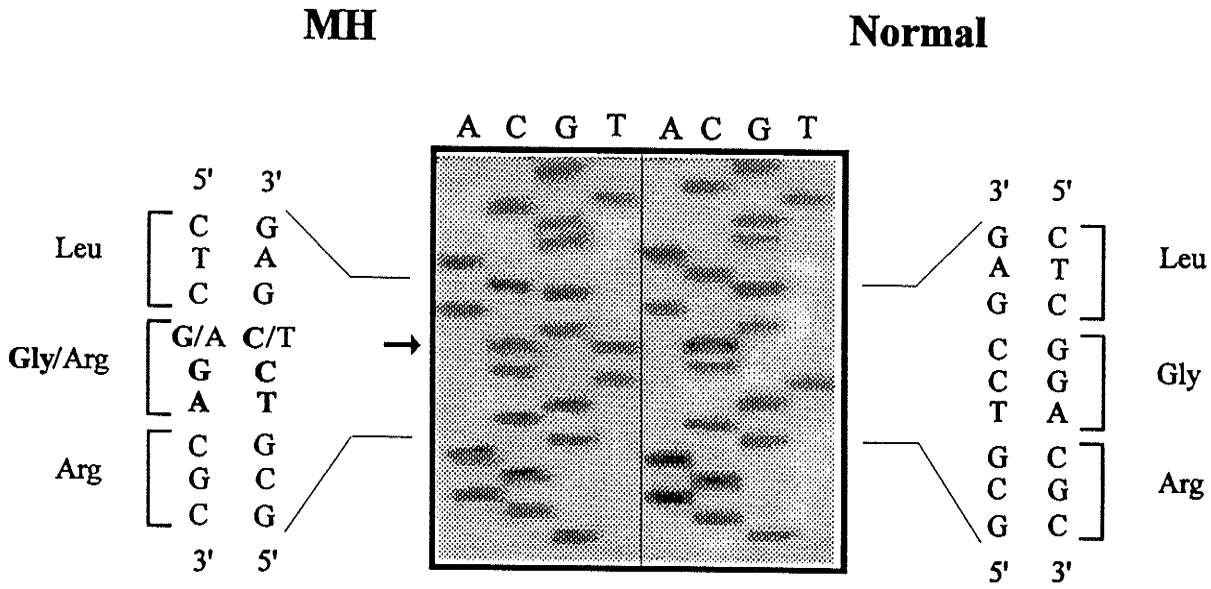


Figure 13:

Direct sequencing of PCR amplified exon 44 of the *RYR1* containing the G7297A mutation. The substitution of A for G7297 in the coding strand is indicated by an arrow. The normal panel contains DNA from a normal individual and MH panel contains DNA from the individual IV-7 (MH02) who has had a documented MH crisis. The G7297 to A mutation results in the glycine to arginine amino acid substitution.

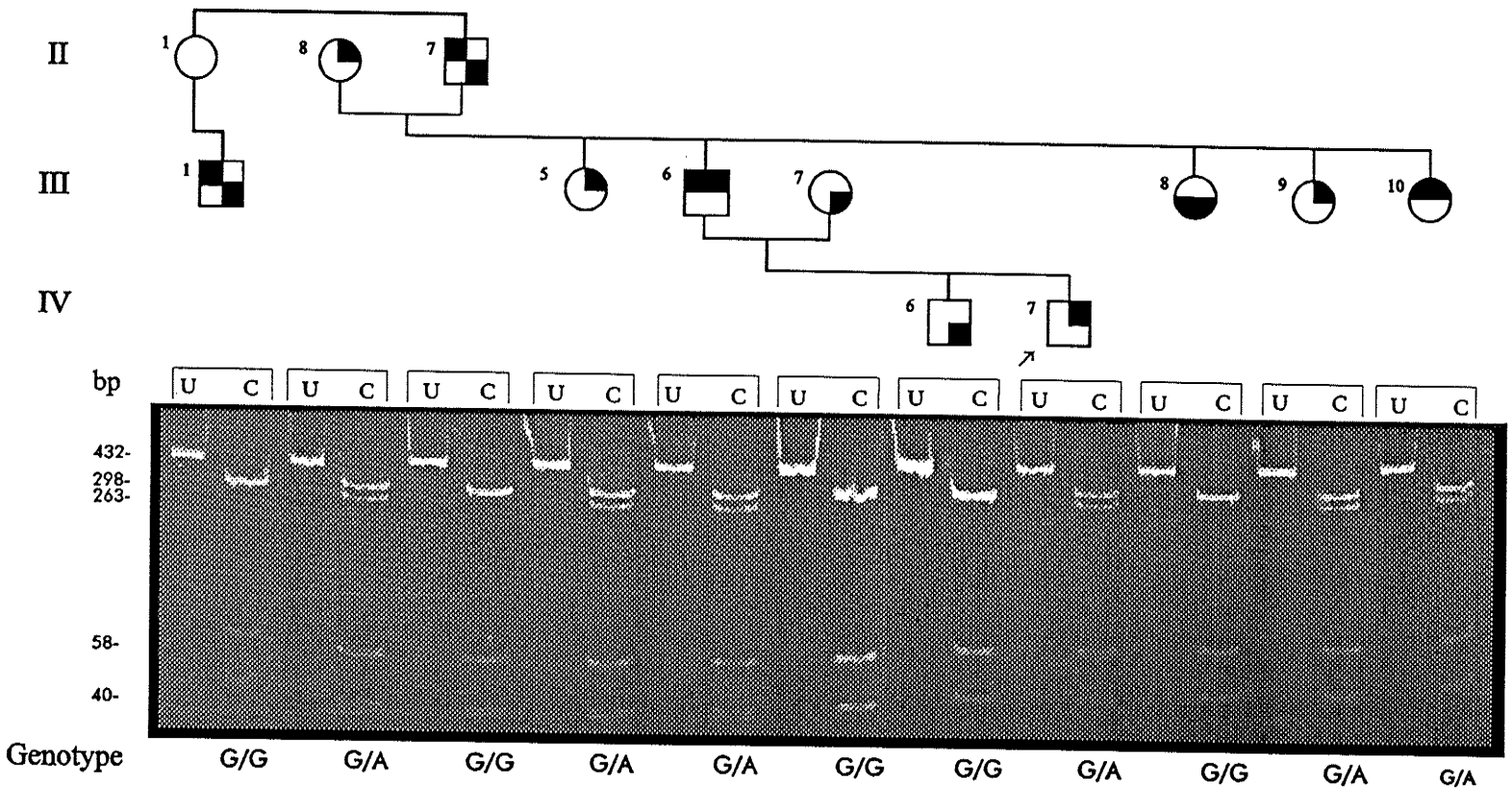


Figure 14:

*Dde*I restriction endonuclease analysis of the G7297 to A mutation from a grouping within the pedigree, MH02, described in Figure 11. *Dde*I digestion of the amplified 432 bp PCR product yields a 298, 263, 58 and 40 bp fragments for DNA containing the G7297A mutation. Each individual genotype is indicated by a G- for normal allele and A- for the mutant allele. Pedigree symbols are as in Figure 11.

biopsy and CHCT MH assignment had been requested for individual II-8 but as of yet no surgery has been scheduled.

18.2 We have identified a second individual from our study population who had this Gly2433Arg mutation using the *Dde*1 digestion of PCR amplified exon 44 of *RYR1*. This individual's (KS1) DNA from exon 45 of *RYR1* was sequenced and the presence of a G7297A nucleotide substitution was confirmed. This individual survived an MH crisis after receiving a general anesthetic in November of 1989 for a colonoscopy. He was anesthetized using fentanyl, halothane and succinylcholine. He was monitored with a blood pressure monitor, electrocardiography and pulse oximeter. He showed generalized muscular rigidity and a rapid increase in temperature almost immediately after induction of anesthesia. His post operative serum CK was 32,296 U/L. Anesthesia was discontinued and the anesthetic machine changed. The patient was then hyperventilated with 100% oxygen and was given Dantrolene, as well as active cooling and fluid loading. He recovered without further complications. In March of 1990 he had a muscle biopsy. Histology and histochemistry of the muscle was normal. CHCT results were positive, leading to the diagnosis of MHS. His mother was also biopsied in 1990 and her CHCT results also indicated MHS. She had one general anesthetic in 1978 without complication. A sample of her DNA was also tested for the presence of the Gly2433Arg mutation. She also carried this mutation.

18.3 A third individual was identified as having the Gly2433Arg mutation by utilizing

the *Dde*1 digestion of PCR amplified exon 44 of *RYR1*. This individual's (KS8) DNA from exon 45 of *RYR1* was sequenced and the presence of a G7297A nucleotide substitution was confirmed. The individual underwent a muscle biopsy for CHCT testing because his mother survived an MH crisis in 1984 during a dental procedure. His materal uncle also had a severe general anesthetic complication in 1971. This individual has had one general anesthetic and one other anesthetic without complication. His CHCT results were strongly positive Table 2. He is an only child but has three children, who will be encouraged to have a blood sample drawn for DNA testing and have a muscle biopsy.

19.0 Families Without Any of the Five *RYR1* Mutations:

19.1 The diagnosis of MHS was established in the proband (IV-39) from this family after a well documented MH crisis (Figure 15). He was admitted in December of 1985 for a laparotomy. There was no previous history of any adverse anesthetic reaction. Soon after the procedure began the patient's temperature rose rapidly and peaked at 38.7°C. This increase in body temperature was coupled with a rise in his pCO_2, potassium, blood pressure and heart rate. The patient was treated with dantrolene and within 24 hours his temperature and vital signs were normal.

Approximately 34 individuals in this family were known to be at 50% or 25% risk for MHS. Standardized open biopsy of the vastus lateralis muscle was performed in 17 at-risk individuals during the period 1986 to present (Table 9). These muscle biopsy specimens were studied using standard histochemistry and CHCT protocols of the North American Group and Registry (ref). Fourteen family members were classified as MHS,

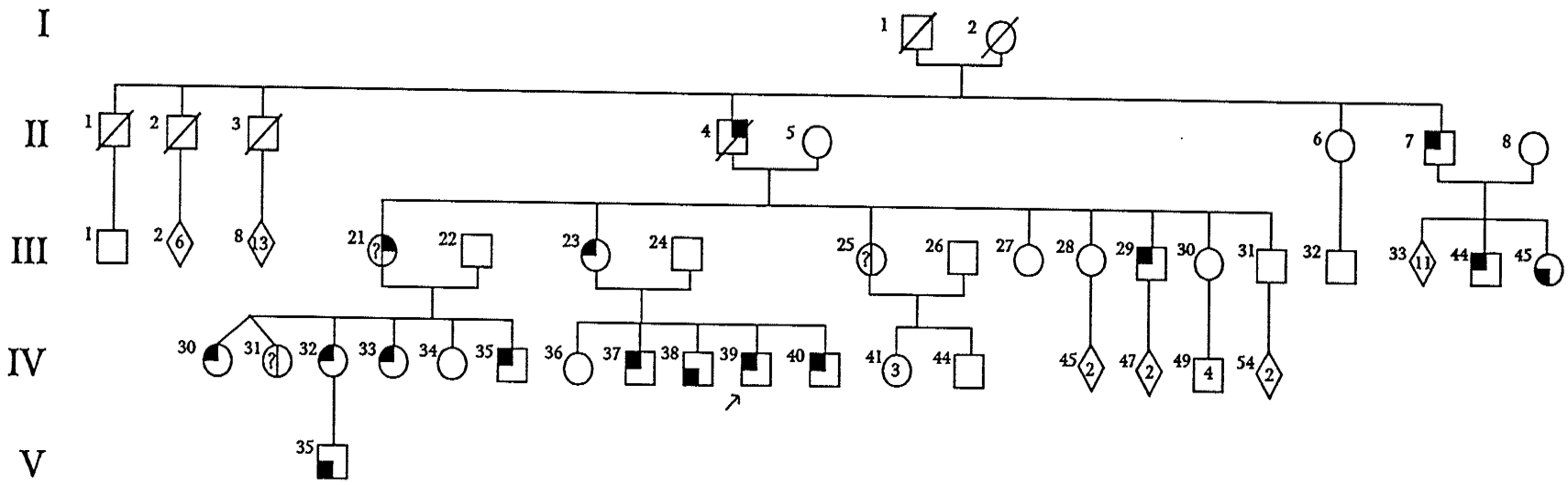


Figure 15:

Partial pedigree of the MH family (MH03). The proband is this family is IV-3 (↗) who has survived a MH crisis. The MH status of family members as determined by CHCT or pedigree is indicated by: ◒ = MHN by CHCT; ◓ = MHS by CHCT; (?) = CHCT has been performed but MH status is still unknown; ◔ = MHS as determined by pedigree information; ◯ = not studied.

Table 9: Malignant Hyperthermia Status as Determined by CHCT for Members of the MH03 Family

ID #	Date of Biopsy	Twitch Quality	2 % Hal (g)	3 % Hal (g)	CSC (mM)	CAFF (g)	MH Status
II-7	9/5/89	Good	-	1.5*	1.47*	1.5*	MHS
III-21	20/11/86	Poor	-	-	-	-	unknown
III-23		Good	-	0.6*	3.23*	0.0	MHS
III-25	6/3/92	Poor	-	-	-	-	unknown
III-29	21/1/92	Good	0.6	3.5*	3.06*	0.5*	MHS
III-44	12/4/88	Good	-	0.5*	3.47*	0.05	MHS
III-45	9/4/90	Good	-	-0.5	10.06	0.0	MHN
IV-30	17/12/91	Good	0.4	1.0*	2.97*	0.1	MHS
IV-3	2/10/87	Poor	-	-	-	-	unknown
IV-32	11/3/88	Good	-	0.0	4.05	0.25*	MHS
IV-33	25/9/87	Good	0.3	-	-	-	MHS
IV-35	10/1/92	Good	1.8	3.2*	1.66*	0.7*	MHS
IV-37	30/7/91	Good	0.25	0.7*	3.26 *	0.2*	MHS
IV-38	2/8/91	Good	0	0	4.15	0.1	MHN
IV-39	6/7/92	Good	0	0.8*	3.02*	0.1	MH crisis
IV-40	26/7/91	Good	0.7	3.0*	1.32*	0.4*	MHS
V-35	22/7/88	Good	-	0	4.75	0.0	MHN

Testing criteria are listed in Table 3.
CSC = concentration of caffeine to produce 1g of tension; CAFF = contraction produced with 2mM caffeine; Unknown = MH status could not be assessed because of poor muscle sample quality at the time of the test; * = abnormal results.

or MHN on the basis of the CHCT (Table 9). The MH status of three family members who had undergone CHCT remains unknown because of insufficient muscle sample or poor muscle quality (no twitch) at the time of the test.

Investigation of this MH family involved linkage analysis between the gene for MHS and the *RYR1* gene. A battery of restriction enzyme digests and DNA probe combinations were utilized until the maximum number of meioses could be analyzed. The RFLP markers were then used to construct haplotypes. Haplotype data for this family are shown in Figures 16 -18.

In Figure 16 , individual II-4's inferred haplotype, P, appears to be segregating with MHS in his 2 MHS children, as determined by CHCT. The P haplotype is also inherited by III-21 who is the mother of MHS children (by CHCT) and therefore by pedigree inspection is considered MHS. Individual II-7 may also carry this P haplotype, which has been inherited by his MHS son. Individual III-45, who is MHN by CHCT, has inherited the alternative haplotype, M1, from her father. This same P haplotype is also inherited by IV-39, the proband, who has had a MH crisis and a positive CHCT, as shown in Figure 17. Individual III-25's other 2 MHS (by CHCT) sons have also inherited the P haplotype, while her MHN son (by CHCT) has inherited the alternate haplotype, M2. In Figure 18, MHS (by CHCT) individuals, IV-30 and IV-32, have inherited the same P haplotype from their mother III-21, who is assumed MHS because she has MHS (by CHCT) children. Individual V-36 does not inherited his mother's P haplotype but rather the alternate, P2, haplotype. However, the P haplotype is not inherited by all of III-21's MHS (by CHCT) children as would be expected if MHS was linked to *RYR1* in this

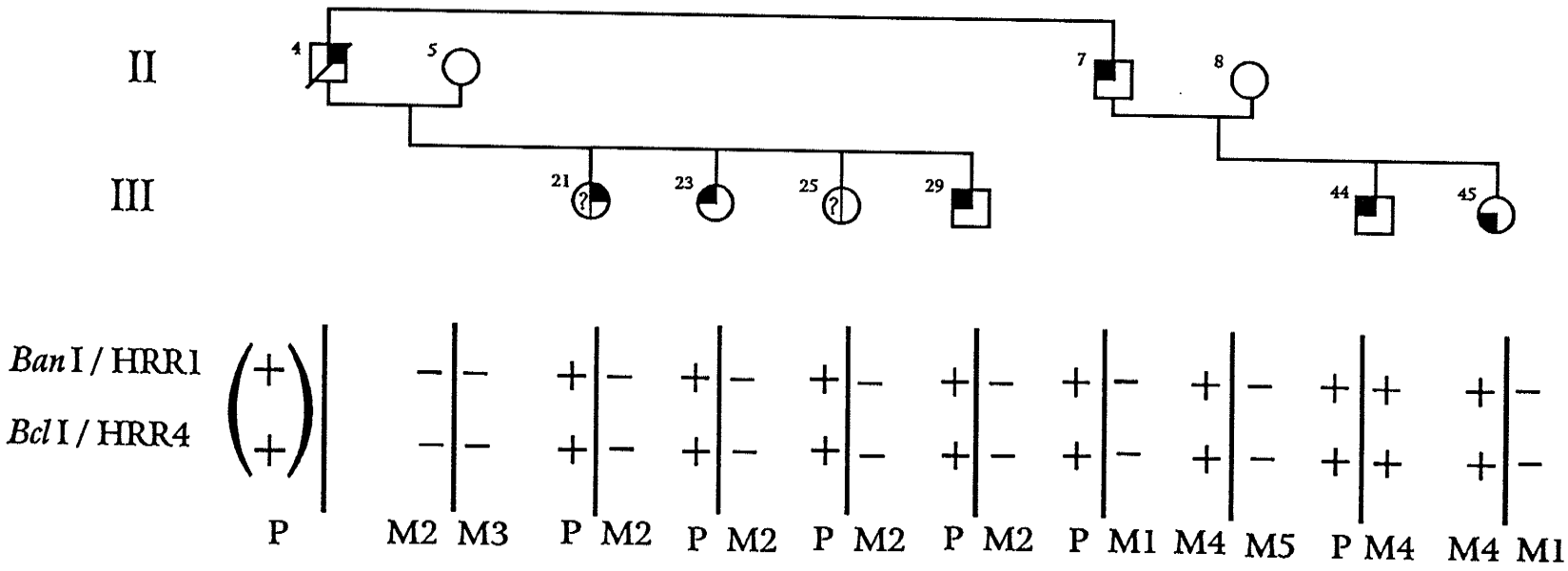


Figure 16:

Analysis of segregation of alleles of markers HRR1 and HRR4 in a branch of the MH03 family with predicted chromosome haplotypes shown under each individual. Alleles are as described (MacLennan *et al.*, 1990) and are presented as haplotypes "-" indicates absence of restriction site and "+" indicates presence.

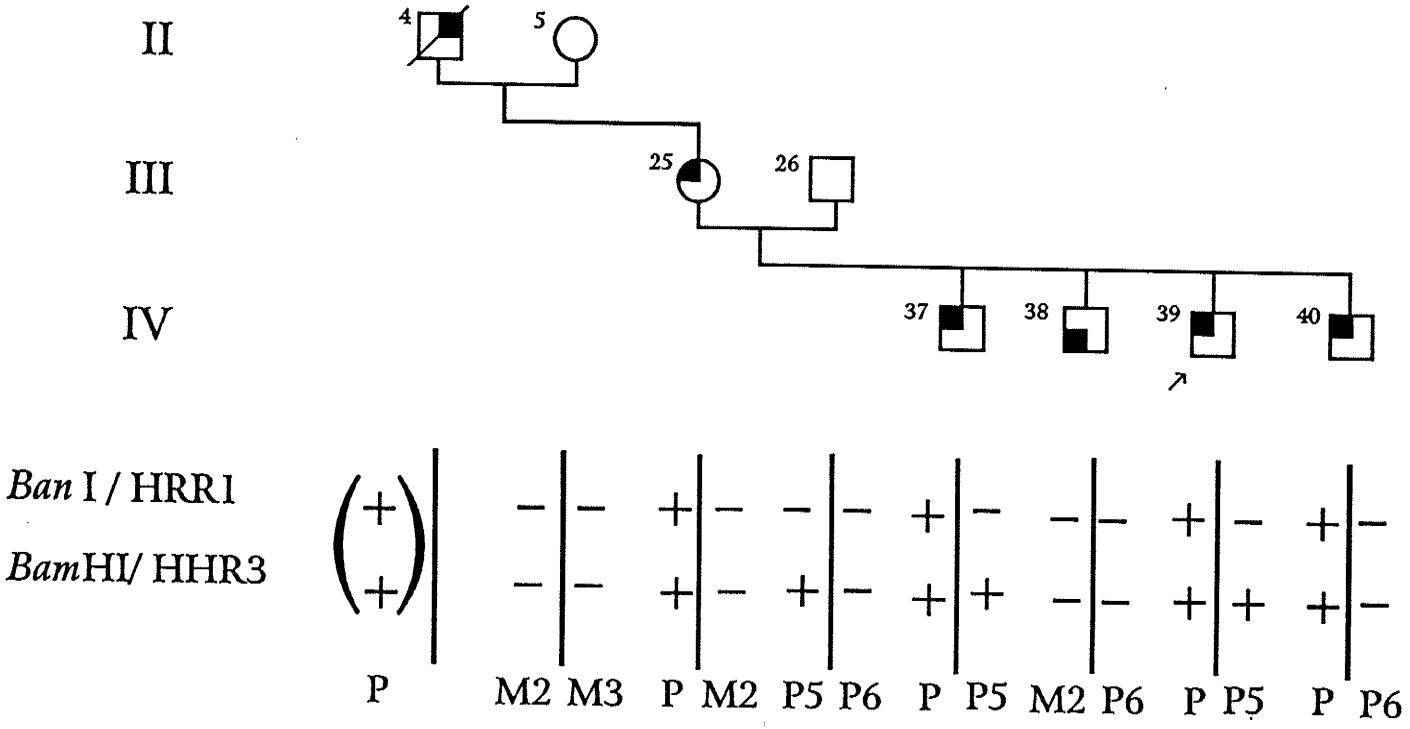


Figure 17:

Analysis of segregation of alleles of markers HRR1 and HRR3 in a branch of the MH03 family with predicted chromosome haplotypes shown under each individual. The haplotype in brackets was inferred. Alleles are as described (MacLennan *et al.*, 1990) and are presented as haplotypes "-" indicates absence of restriction site and "+" indicates presence.

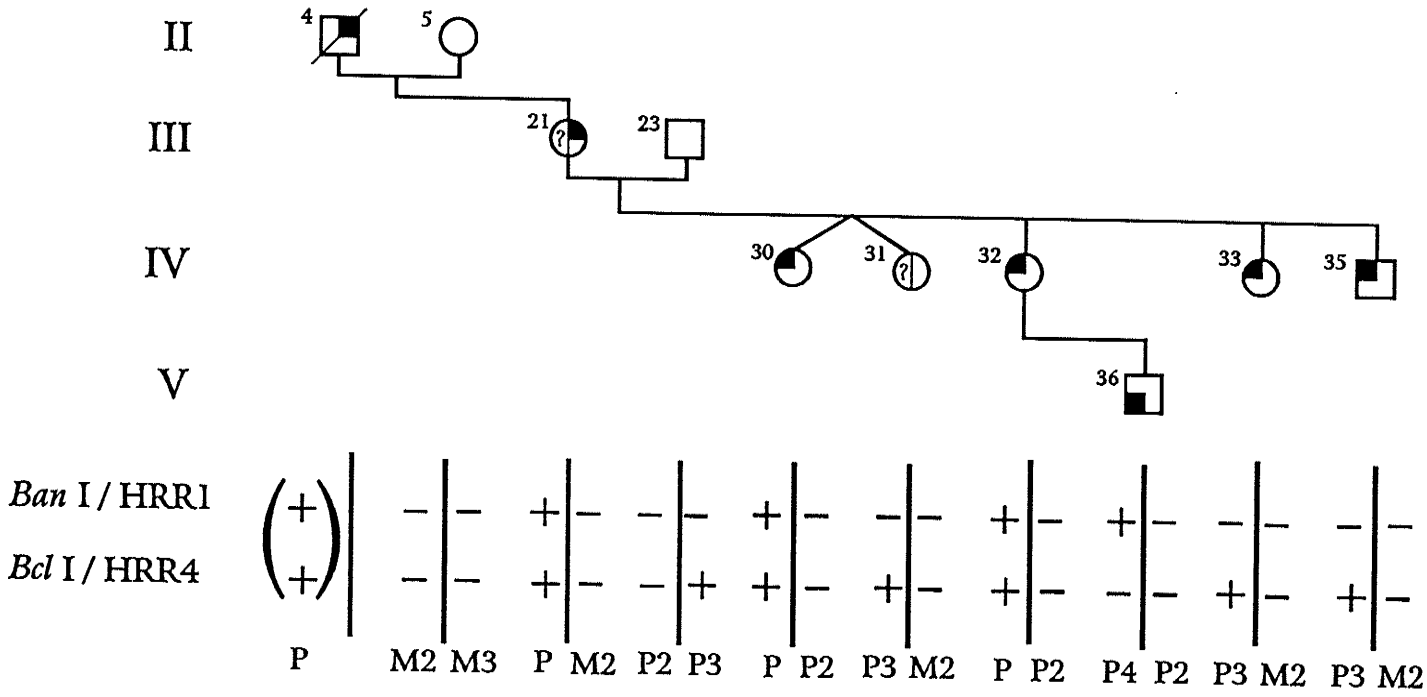


Figure 18:

Analysis of segregation of alleles of markers HRR1 and HRR4 in a third branch of the MH03 family with predicted chromosome haplotypes shown under each individual. The haplotype in brackets was inferred. Alleles are as described (MacLennan *et al.*, 1990) and are presented as haplotypes "-" indicates absence of restriction site and "+" indicates presence.

family. MHS individuals IV-33 and IV-35 are recombinants having inherited the alternate, M2, haplotype from their mother.

Individual IV-6 was included in the search of *RYR1* gene for a disease causing mutation via SSCP. Single nucleotide changes were screened for in the *RYR1* gene of unrelated MHS patients. To date, thirteen of the greater than 100 exons of *RYR1* have been screened including exons 1-3, 5-9, 14, 17, 18, 20 and 44. There was an SSCP shift noted in exon 44 of *RYR1* in this individual. Both the Gly2433Arg and Arg2434His mutations were excluded by sequencing exon 44. However, a C7257T base substitution, which results in a neutral His to His amino acid replacement, was found when exon 44 was sequenced. SSCP analysis was attempted with other members of his immediate family. The shift appeared to represent this His to His neutral polymorphism which was segregating in this family. This shift pattern was difficult to visualize on the original autoradiograph and was not reproducible. Therefore it was not determined whether the polymorphism was segregating with MHS. Individual IV-6 was also included in the screen for the mutations in the *RYR1* gene at positions 248, 341, 1840, 2433 and 2434. None of these mutations were found in this individual.

19.2 The diagnosis of MHS was established in the proband (I-1) from this family (Figure 19) after she survived an MH crisis in Guatemala. In April of 1986, the proband underwent a tubal ligation one hour and 15 minutes post-partum. She was anesthetized with Rophynol, succinylcholine and halothane. There was slight mandibular rigidity and intubation was difficult but the procedure continued. Two hours later when the procedure

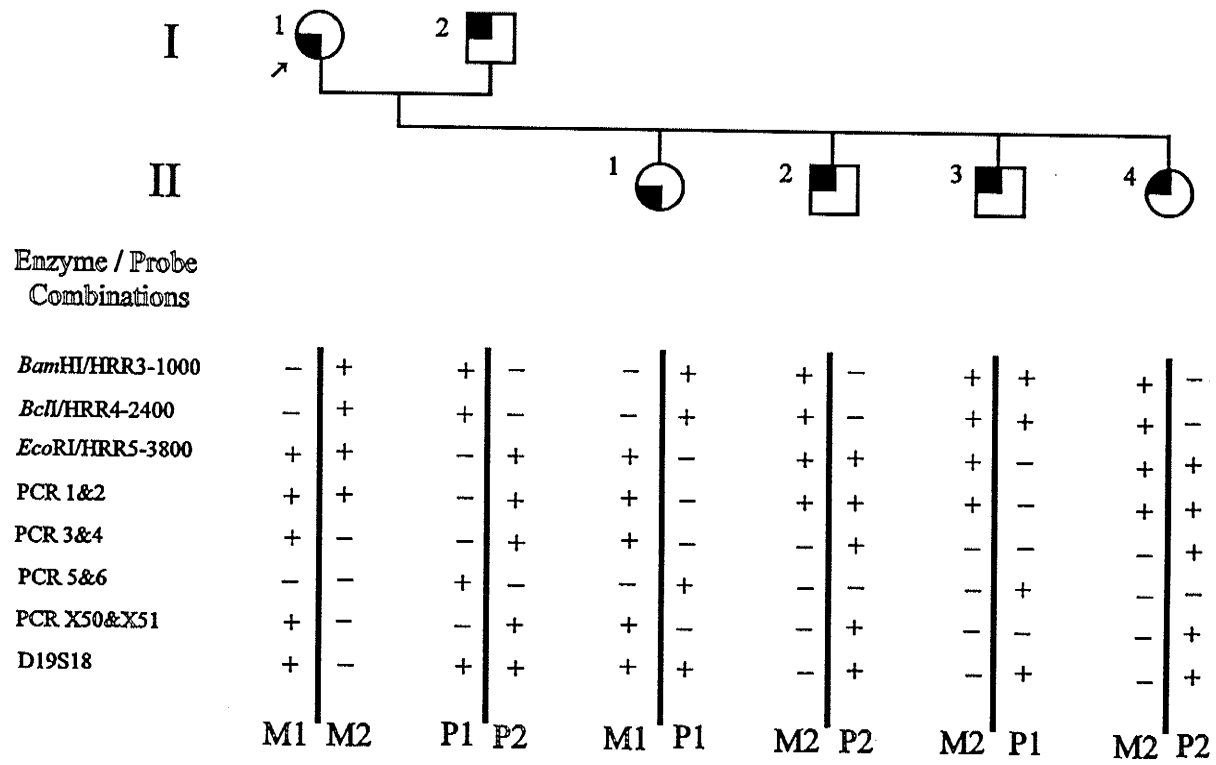


Figure 19:

Analysis of segregation of alleles of markers HRR1 and HRR4 in a MH04 family with predicted chromosome haplotypes shown under each individual. Alleles are as described (MacLennan *et al.*, 1990) and are presented as haplotypes "-" indicates absence of restriction site and "+" indicates presence. Pedigree symbols are as follows: ◔ = MHN by CHCT; ◔ = MHS by CHCT. The proband I-1 (↗) survived a MH crisis.

Table 10: Malignant Hyperthermia Status as Determined by CHCT Results for Members from the MH05 Family

ID#	Date of Biopsy	Twitch Quality	2% HAL	3% HAL	CSC	CAFF	MH Status
I-1†	12/4/91	Good	0.0 g	0.0 g	4.42 mM	0.0 g	MHN
	24/2/92	Good	0.0 g	0.0 g	4.07 mM	0.0 g	MHN
I-2	24/2/92	Good	1.1 g*	2.35 *g	3.02 mM*	0.1 g	MHS
II-1	12/11/91	Good	0.0 g	0.3 g	3.89 mM*	0.0 g	MHN
II-2	15/11/91	Good	0.5 g*	1.0 g8	1.78 mM*	0.7 g*	MHS
II-3	15/11/91	Good	1.1 g*	1.5 g*	2.80 mM*	0.3 g*	MHS
II-4	12/11/91	Good	0.6 g*	0.5 g*	2.96 mM*	0.0 g	MHS

Testing criteria are listed in Table 3.
† = This individual had a MH crisis; CSC = concentration of caffeine to produce 1g of tension; CAFF = contraction produced with 2 mM caffeine; * = abnormal test.

was completed, the patient experienced accentuated muscle rigidity, tachycardia and had "hot skin". Her temperature peaked at 39.5°C. The patient was treated with Dantrolene and active cooling and recovered uneventfully. Her serum CK 24 hours post surgery was 307 U/L. Since returning to Canada, she and her husband and their 4 children have all undergone muscle biopsies followed by CHCT to determine their MH status. The CHCT result for I-1 indicated an MH negative response on two separate occasions a year apart. Three of her four children were determined to be MHS and one was assigned MHN. The proband's husband also had a muscle biopsy and his CHCT results indicated that he was MHS (Table 10).

This family was investigated for linkage of MHS to *RYR1* gene using cDNA markers within and surrounding the *RYR1* region of chromosome with both the traditional Southern analysis and PCR RFLPs. Haplotypes were constructed utilizing all the marker information. As shown in Figure 19 MHS appears to be segregating with the "M2" haplotype derived from the proband I-1 despite the fact that she has had two negative CHCT tests.

The proband from this family and her son, II-3, were included in the SSCP screening for 13 *RYR1* exons. No shifts in mobility for either individual was noted. II-3 was also included in the screen for known *RYR1* mutations 248, 341, 1840, 2433, and 2434. None of these mutations were present.

19.3 The diagnosis of MHS was established in the proband, II-2, from this family by her clinical response to anesthesia. At three years of age, this child suffered an MH crisis

while undergoing oral surgery. She was anesthetized with nitrous oxide, halothane and succinylcholine. She developed severe masseter spasm and generalized muscular rigidity, metabolic acidosis and myoglobinuria. The anesthetic was discontinued and Dantrolene was administered. She was also hyperventilated with 100% oxygen and placed on a cooling blanket. Her post-operation serum CK peaked at 74,800 U/L. She was biospied in 1991 and CHCT results indicated that her muscle fibers responded abnormally to both caffeine and halothane leading to the assignment of MHS. Both her father I-2 and then her half-sister II-1 underwent a muscle biopsy/CHCT and were determined to be MHS.

Linkage studies in this family revealed that both II-2 and her half-sister, II-1, inherited the same paternal "P1" haplotype. Therefore, linkage to *RYR1* on chromosome 19q is suggested and these individuals were included in the SSCP screen of *RYR1* exons. No SSCP shift had been discovered in the thirteen *RYR1* exons screened thus far. Individual II-2 was also screened for the known MH associated *RYR1* mutations 248, 341, 1840, 2433, and 2434. None of these mutations were found.

DISCUSSION

We have studied 16 individuals who have survived an MH crisis or have a positive CHCT result following a muscle biopsy. Using the molecular genetic technique of PCR followed by restriction enzyme digestion we have been able to screen these individuals for five previously identified *RYR1* mutations, namely G248R, G341R, R614C, G2433R and R2434H. We have found two of these *RYR1* mutations, R614C (Arg614Cys) and G2433R (Gly2433Arg) in unrelated individuals.

The C1840T mutation resulting in the R614C (Arg614Cys) mutation was identified in a large Manitoba Mennonite family. Many family members were investigated for the presence of this C1840T mutation and there proved to be a very high but not absolute correlation between the presence of the R614C mutation and MHS as determined by CHCT. In addition, 3 unrelated individuals with the G7297A *RYR1* mutation resulting in the G2433R (Gly2433Arg) amino acid substitution were found but further family studies could be pursued in only one of these families. The two extended families with the different *RYR1* mutations have provided us with the opportunity to compare CHCT and DNA-based diagnoses of MH susceptibility.

In the study of the large MH family in which the C1840T mutation resulting in the R614C amino acid substitution is segregating, 19 members of the family had been subjected to the CHCT. After careful analysis of CHCT data, obtained over a period of several years, it was concluded that 4 of the 19 tests were, in fact, inconclusive, since the twitch quality of the biopsied muscle was noted to be poor at the time of assay. Data from these 4 tests could not be used in the final comparative analysis between CHCT and DNA-based assignments.

One of these patients had originally been diagnosed as MHS. On rebiopsy, this patient responded as MHN and was included in the study as MHN (Table 7). Of the 16 CHCT results remaining, 2 were discordant with the DNA based diagnosis. Three hypotheses may account for these results: (a) the Arg614Cys mutation is not linked to MH in this family; (b) a second MH mutation is segregating in the family, giving rise to positive CHCT results for the two individuals who do not carry the Arg614Cys mutation; or (c) the positive CHCT results for individuals III-5 and IV-43, who do not carry the Arg614Cys mutation, are false positive results.

Although our data for CHCT and DNA-based MH diagnoses are not absolutely concordant, there is a strong correlation between the two tests. This strong correlation for linkage of MH to chromosome 19q13.1, without complete concordance, might suggest that the C1840T mutation is tightly linked to a true MH allele, but is separated from it by recombination in some individuals. In one segment of the family where we found discordance, we determined the haplotype for several hundred kilobases around the C1840T mutation and found no recombinants within this branch of the family (Figure 20). This finding would be more supportive of the view that the C1840T mutation is a causal mutation rather than the view that it is near to a causal mutation but does not, itself, play a causal role.

We cannot rule out that a second MH allele is segregating in individuals III-5 and IV-43, but this seems unlikely, since these individuals are found in two different branches within the large pedigree and there is no evidence of segregation of a putative second MH allele in either their siblings or their offspring.

There is strong evidence that the C1840T mutation causes MH in both swine and

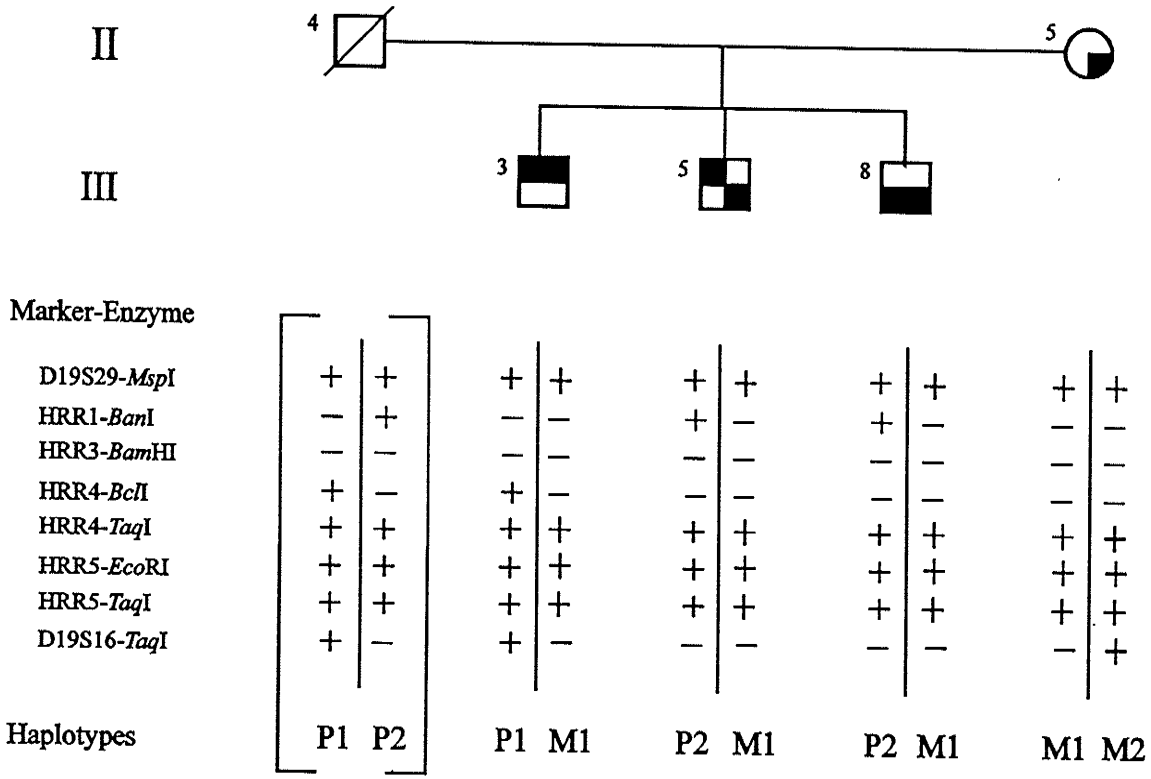


Figure 20:

Haplotypes of a nuclear MH family (MH01) showing the inheritance of the same low risk paternal haplotype P2 by individuals III-5 and III-8, and inheritance of the high risk paternal haplotype P1 by their MHS sib III-3. Alleles as described (MacLennan *et al.*, 1990) and are presented as haplotypes "-" indicates absence of restriction site and "+" indicates presence. Maternal haplotypes are M1 and M2. Paternal haplotypes P1 and P2 have been inferred and are bracketed. Pedigree symbols are as in Figure 7.

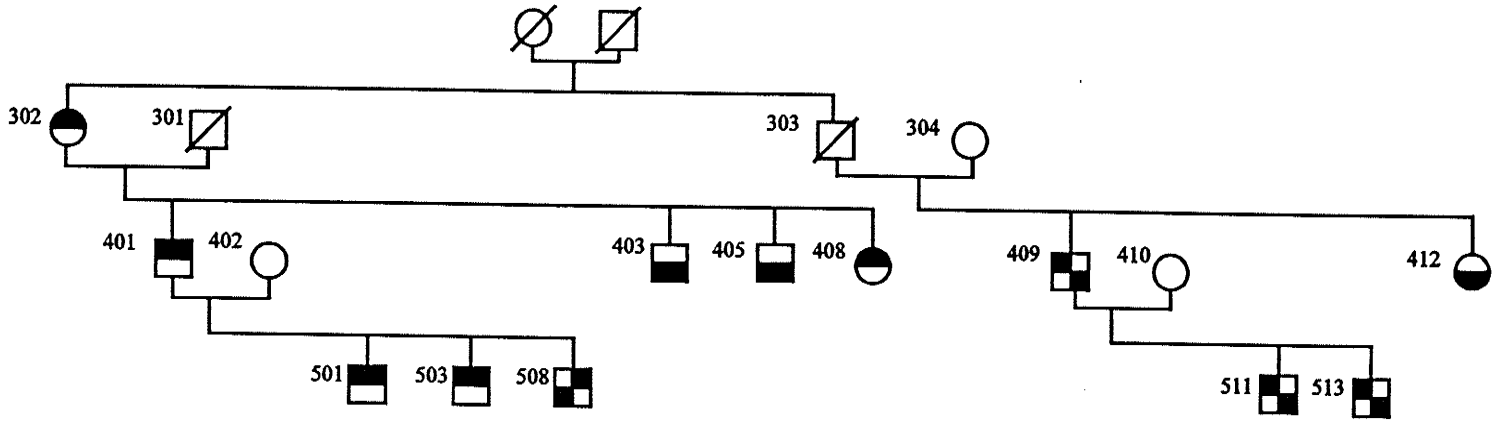
human. Specifically, a lod score of 102 at $\theta = 0.00$ favouring linkage with MH in swine (Otsu *et al.*,1991) combined with the association of the mutation with MH across a species barrier, in humans (Gillard *et al.*, 1991; Hogan *et al.*, 1992) provides strong genetic evidence. Furthermore, Shomer *et al.* (1993) studied purified ryanodine receptors from both MHS and normal pigs by reconstituting them in planar lipid bilayers. It was shown that ryanodine receptors from MHS pigs remained open longer and had shorter closed times than those of normal pigs. When rabbit *RYR1* cDNA containing the Arg614Cys mutation was expressed in muscle cells (Otsu *et al.*, 1994) and in COS-7 cells (Treves *et al.*, 1994), hypersensitive gating of the Ca^{2+} release was also observed in these transfected cells. Biochemical findings such as these add additional support that the Arg614Cys mutation in *RYR1* causes MH in swine and humans.

If we were to conclude that the Arg614Cys mutation is not causative of MH in this family, we would have to discount all of the evidence for the causal nature of the mutation, including the fact that this mutation has been shown to segregate with MH in two other MH families (Gillard *et al.*, 1991; Hogan *et al.*, 1992). In addition, we would have to define the CHCT as being 100% accurate, which would not reflect the results of studies on the accuracy of this test (Larach *et al.*, 1993; Larach *et al.*, 1992a; Larach *et al.*, 1992b). Larach and her colleagues at The North American MH Registry continue to evaluate North American CHCT results with the goal of standardizing CHCT procedures and establishing diagnostic cutpoints (Larach *et al.*, 1993; Larach *et al.*, 1992a; Larach *et al.*, 1992b). In their studies, current diagnostic cutpoints can achieve sensitivities approaching 100%, but with specificities approaching only 80% (M. Larach, personal communication). The European MH group have

not published error rates for their CHCT protocol, but members of their group have acknowledged that the test is not 100% accurate (Hopkins *et al.*, 1994). False negative test results have been reported for the European CHCT protocol (Isaacs *et al.*, 1993).

In light of our own experiences, in particular, the finding that rebiopsy can lead to a reversal of a test outcome, and in the face of clear evidence that the CHCT is not 100% sensitive, we are unable to consider the CHCT as 100% accurate. Accordingly, we are unable to accept alternative (a), that the Arg614Cys mutation is not linked to MH in this family. We are able, however, to accept alternative (c), that the CHCT gave rise to 2 false positives in our study of 16 CHCT results. Family members have been told the results of their DNA tests but were counselled that their genotype data must still be interpreted cautiously,particularly if their DNA test result was negative, at least until we more fully understand the basis for false positive CHCT results.

Our study can be compared with a study by Deufel *et al.* (1995) of a very complex MH family. In this family, two Arg614Cys mutations were found on two different haplotypes in one branch of the MH family (Figure 21). MH susceptibility also segregated in another branch of the family in whom no Arg614Cys mutation was present. CHCT results for MH susceptibility segregated with the presence or absence of the Arg614Cys mutations in 7 of the 8 individuals tested in the left branch of the family, including one who was homozygous for the mutation. Individual 508, however, was negative in the CHCT, but was heterozygous for the mutation. In order to achieve concordance in this branch of the family, Deufel *et al.* (1995) would have had to accept that individual 508 was diagnosed as a false negative by the CHCT. In the right branch of the family, the Arg614Cys mutation was absent, but an attempt



D19S75	3	2	1	2	7	2	1	2	-	-	1	7	2	2	2	2	1	3	2	2	1	2	2	4	1	2	2	4	1	2					
D19S191	6	5	-	-	3	5	1	5	-	-	1	3	5	5	5	5	1	6	10	3	10	10	11	8	10	11	10	8	10	10					
RYR1-MS	2	4	2	4	4	4	2	4	2	4	2	4	4	4	4	4	2	2	4	4	4	4	4	4	4	4	4	4	4	4					
RYR1-C1840T	T	C	T	C	C	C	T	C	T	C	T	C	C	C	C	C	T	T	C	C	C	C	C	C	C	C	C	C	C	C					
D19S190	1	2	2	2	2	2	2	2	2	2	2	2	3	2	3	2	2	1	2	2	1	2	3	2	1	3	2	2	1	2					
D19S47	6	2	-	-	2	2	6	2	6	2	6	2	2	2	2	2	6	6	2	2	2	2	6	6	2	6	2	6	2	2					

Figure 21:

Pedigree MH011 with MHS adaptation of Figure 1 from Deufel *et al.*, 1995. MHS as determined by CHCT and presence of the C1840T mutation is indicated by the symbols as follows: ◒ = MHN by CHCT; ◓ = MHS by CHCT; ◔ = C1840T mutation present; ◒ = C1840T mutation absent; ◯ = not studied.

was made to correlate CHCT results with chromosome 19q13.1 haplotypes. In order to achieve concordance in this branch of the family, one of the four tests carried out would have to be assumed to be a false positive and one a false negative. This would not be unreasonable if one accepts that the CHCT is not 100% accurate. An understanding of the inheritance of MH in this branch of the family will require further study.

In both our study and the study of Deufel *et al.* (1995), a high correlation (14/16 in our study; 7/8 in Deufel's study), but complete concordance was not found between CHCT and DNA-based diagnoses for MH in families in which the Arg614Cys mutation was segregating. In our opinion, the CHCT is not 100% accurate and these results can be reconciled by the reasonable assumption that the CHCT can yield both false positive and false negative results. Deufel *et al.* (1995) however, suggested that their results questioned both the causal nature of the Arg614Cys mutation and the role of *RYR1* in MH. An equally valid conclusion could be that CHCT has an inherent inaccuracy resulting in misassignments of MH phenotype. (Serfas *et al.*, 1996).

We have found a G7297A mutation in three of the 16 individuals screened for this mutation using PCR followed by enzyme digestion. This was confirmed by sequencing exon 44 of *RYR1*. This mutation was first described by Phillips *et. al.* and then by Keating *et. al.* in 1995. It is believed to be responsible for 5% of MH cases. This G7297A mutation, which results in a substitution of Arg for Gly at position 2433 in the ryanodine receptor protein, is considered a strong candidate for causal MH mutation for many reasons: a) this mutation is found beside a (Arg2434 to His) mutation which segregates with CCD and MH, b) this mutation involves a gain or a loss of Arg residue which has also been observed in five of the

seven potential MH mutations identified to date, c) it has been published that this base substitution is absent from 256 normal chromosomes (Phillips, 1995). Despite the evidence which points to the causal nature of this mutation, we found discordance between the DNA diagnostic test for this mutation and the CHCT results which were used to determine MH status.

There can be two reasons for the discrepancy: 1) inaccuracy in the determination of the MH status using CHCT as discussed previously, or 2) there may be a second MH mutation yet undefined in *RYR1* or another MHS gene. In our family, the presence of the G7297A mutation did not correspond to the CHCT determined MH status in two of the six family member who had had muscle biopsies. The first of the individuals is the paternal grandfather of the index patient. His CHCT results Table 8 indicate that he is MHS but the G7297A mutation is not present. His positive CHCT results were based on his muscle responses to 2% halothane with 0.2 g of tension which is, although considered positive, very close to the cut-off point. His muscle gave a negative response to caffeine because 6.67 mM of caffeine was required to give 1 g of tension. In the European IVCT (*in vitro* contracture test) an individual must respond positively to both caffeine and halothane in order to be consider MHS. Responding to only halothane or caffeine, but not both, is considered an MH-equivocal (MHE) result. It has been previously illustrated that individuals who are MHE can inherit either normal or mutant allele in the case of the Gly 341 Arg mutation (Quane *et al.*, 1994) Further investigation into this family has identified the G7297A in the paternal grandmother who was previously assumed to be MH negative since her husband had a positive CHCT. Early RFLP haplotype analysis, as illustrated in Figure 12, suggested that

MH was inherited from the paternal grandmother. When these results were first discovered, it was believed that a sample mix-up between the grandparents had occurred, so new samples were requested. These new samples however gave the same RFLP results. When it was discovered that the grandfather did not carry the G7297A mutation, all grandpaternal samples were sexed to exclude sample mix-up. Sexing of the samples revealed that no sample mix up had occurred. Our study is not the first in which lack of concordance between this *RYR1* mutation and MH that have been noted. The Gly2433Arg mutation in *RYR1* was detected in eight MH families (Quane *et al.*, 1995; Phillips *et al.*, 1995) but was concordant with MH in only six. In one small family (Phillips *et al.*, 1995), two brothers were diagnosed as MHS by the CHCT. One gave exceptionally strong test results and carried the Gly2433Arg mutation. The other was well within the positive category, but did not carry the Gly2433Arg mutation. In the absence of further information, it is reasonable to suggest that the individual with the strong CHCT result carried two MH mutations, while his brother carried only the one that was not detected in assays for the Gly2433Arg mutation. In the other discordant family (Phillips *et al.*, 1995), inheritance patterns and haplotype analysis did not support a second MH mutation. As in our present family, it was logical to invoke false positive CHCT results as the basis for discordance. In our study, the paternal grandfather was believed to be a false positive. His nephew, who was determined to be MHS via CHCT, does not carry the Gly2433Arg mutation either. He, too, might be considered a false positive, or MHS may be the result of an as yet unknown mutation in *RYR1* or another MHS gene.

Three other MH families were studied by RFLP analysis using DNA markers within and surrounding the *RYR1*. Linkage was difficult to prove in these families for a number of

reasons. In the first family (MH03), MH status was undetermined or inconclusive in many key individuals. However, when those individuals whose MH status was determined by CHCT were investigated with RFLP analysis, recombination between MHS and *RYR1* was seen to have occurred in two individuals IV-33 and IV-35. These individuals may have been diagnosed incorrectly by CHCT and therefore may be false positives. Linkage between MHS and *RYR1* would then be indicated.

In the second family (MH04), a very interesting observation has resulted in the inability to link *RYR1* to MHS in this family. The proband in this family had a MH crisis in Guatemala in 1986. The diagnosis of an MH crisis was , in fact, made by an anesthetist who is a relative of Dr. Beverly Britt, a well known anesthetist and a MH expert from Toronto. When she returned to Canada in 1991, she underwent CHCT testing and her result was normal. All of her children then had muscle biopsies for CHCT to determine their MH status(Table 9). Three of her children (2 sons and 1 daughter) were determined to be MHS, while the other daughter was determined to be MHN. The proband was retested on a separate occasion many months after her initial test but once again was determined to be MHN. Her unrelated nonconsanguineous husband underwent a muscle biopsy for the purpose of MH assignment by CHCT and he was found to be MHS. Although the CHCT results seem puzzling, RFLP haplotypes were carried out on this small family. Based on the assumption that our proband, in fact, had an MH crisis and that she and her husband were misdiagnosed by CHCT (i.e. I-1- false negative and I-2-false positive), her M2 haplotype is inherited by all three of her MHS children, while her MHN daughter inherits her M1 haplotype. Linkage between MHS and *RYR1* would then be possible in this family.

However, if our proband did not have an MH crisis and her CHCT assignment is in fact correct, the MHS of the children would have to have been inherited from their MHS (by CHCT) father. Haplotype analysis revealed that not all the MHS children inherited the same paternal haplotype. Two MHS children inherited P2, whereas the paternal haplotype P1 was inherited by a MHS and the MHN child. In this case, II-3, who is discordant, would be considered a false positive if MHS is indeed linked to *RYR1* in this family. Thus, the third possibility may be that MHS is not linked to *RYR1* in this family, supporting the concept of genetic heterogeneity for MHS.

The third family (MH05) studied is very small, consisting only of a father and his two daughters. In our RFLP haplotype studies, there was no recombination between MHS and the *RYR1* marker. However, because of the size of this family, linkage could not be definitely concluded.

In order to perform linkage analysis between a disease locus and DNA markers, one must be sure of the diagnosis of the disease. In MH, the disease phenotype is not visible on visual examination but requires the interpretation of whether the muscle fibre taken from the individual responds within a normal or abnormal range to caffeine or halothane (CHCT) or an individual must have had a documented MH crisis. As previously mentioned, it has been documented that CHCT is not 100% accurate. Therefore, it is possible that some individuals could be been misdiagnosed. This may be the cause of some the confusion in the molecular studies of MH. One incorrect phenotypic assignment may negate linkage and be the reason why up to 50% of MH families cannot be linked to *RYR1*.

Despite the fact that linkage was not absolutely concluded for our three families, one

member from each were included in the SSCP screening of *RYR1* exons and no motility shifts indicating potential mutations were found.

It has been estimated that only 30 to 50% of MH families are linked to the *RYR1* gene (Ball *et al.*, 1993). Therefore, researchers have been looking for other MH loci. Alternative loci for MH have been described on chromosomes 17q (Levitt *et al.*, 1992; Olkers *et al.*, 1992), 7q (Iles *et al.*, 1994) and 3q13.1 (Sudbrak *et al.*, 1995). Of these 3 potential additional MH loci, the locus on chromosome 3 is currently the best candidate for a second MH locus (Sudbrak *et al.*, 1995). Assignment of alternate loci using the CHCT, however, has its own potential for error. An understanding of the limits of accuracy of the CHCT, in studies of the linkage of MH to *RYR*1 and alternate loci, will be an important factor in all future research on the genetic basis of MH.

CONCLUSION AND SUMMARY

Sixteen individuals were screened for 5 of the 7 known mutations in *RYR1*. In four individuals of the sixteen screened, an *RYR1* mutation was identified. One proband was found to carry the R614C (Arg614Cys) mutation while three others carried the G2433R (Gly2433Arg) mutation. The extended family of the proband with the R614C mutation and the extended family of one of the three probands with G2433R mutation were investigated further to determine if the DNA-based results correlated with the CHCT assignments. An absolute correlation could not be made in either. Discordance could be explained by three possible hypotheses: 1) the *RYR1* mutation is not linked to MH; 2) a second MH mutation is segregating in the family or ; 3) there are errors in the CHCT assignments.

There is very strong genetic and biochemical evidence that the C1840T (R614C) mutation causes MH in swine and humans. This mutation lies within one of the two predicted regulatory domains at the NH_2-terminal end of the ryanodine receptor protein (Chen, *et al*, 1993). The causal nature of the G7297A (G2433R) mutation is supported by the presence of the Arg2434His mutation which is linked to both MH and CCD within a family (Zhang, *et al.*, 1993). These two mutations occurring side by side at the COOH-terminus appear to fall within a regulatory domain which may control the Ca^{2+} release channels (Chen, *et al*, 1993; Zorzato, *et al.*, 1990). In the family with the R614C mutation, the possibility of a second MH mutation segregating in the pedigree would be unlikely since the discordant individuals are not closely related. A second mutation can not be completely discounted in the second pedigree where G2433R is segregating. However, in both families MH status was determined by CHCT testing. Since CHCT is not 100% accurate, we propose

that the observed discordance between DNA test results and CHCT assignment in our two families results from four false positive diagnoses by the CHCT, two in each family. Until the basis for false positive CHCT results is more fully understood, we would recommend that members of families in which an *RYR1* mutation has been identified, be informed of their results of their DNA tests, but counselled that their genotype data must be interpreted cautiously.

It has been estimated that approximately 30 to 50% of MH families are linked to the *RYR1* gene (Ball and Johnson, 1993). Although linkage describes a probability, positive linkage to a candidate gene requires complete concordance. Thus discordance for even one member of a family implies lack of linkage. Linkage analysis has been successful in identifying many chromosome 19 linked families. This fact strongly argues that in many cases, CHCT is an accurate method of phenotyping for molecular genetics. Inaccuracy in CHCT MH assignments of some of the family members may be the reason why it has been difficult to determine, with certainty, if two of the three additional families investigated here (by linkage analysis) are linked to *RYR1*. MHS in these families could also be due to a mutation in other MHS loci. Alternative loci for MH have been described on chromosome 17q (Levitt, *et al.*, 1992; Olckers, *et al.*, 1992), 7q (Iles, *et al.*, 1994) and 3q13.1(Sudbrak, *et al.*, 1995). Given CHCT limitations, identification of alternate loci will also be problematic. An important factor in all future research into the genetic basis of MH will be appreciation of the limited accuracy of CHCT when studying linkage of MH to both *RYR1* and other loci.

Currently anesthesiologists tend to treat all blood relatives of a MH crisis survivor as MHS, regardless of their CHCT MH assignment, electing to use non-triggering agents to

anesthetize these "at-risk" individuals. Their caution in utilizing even the results from the "gold standard", CHCT, used to assign MH status makes it unlikely that clinical practice decisions will be made solely on the basis of DNA assignment of MH susceptibility. Perhaps in the future, as our knowledge broadens, this anesthetic management policy will change to incorporate DNA test results in MH patient care.

QUESTIONS FOR THE FUTURE

Caffeine halothane contracture testing is currently the "gold standard" for determining MHS. Yet is clear that both false positive and false negative MH assignments may result from CHCT testing. How should we approach the problem in the future given the discrepancies in linkage analysis and phenotypic assignment by CHCT? Should research into identifying *RYR1* and other locus mutations leading to MHS be pursued? If so, could anesthetic management of a patient belonging to a family with a known *RYR1* mutation such as R614C be based exclusively on the presence or absence of a point mutation? Perhaps succinylcholine, the main MH crisis triggering agent, should be eliminated from the general population undergoing elective anesthesia and only be used in emergency situations. These are only some of the very complex issues that will require further study and input from numerous disciplines.

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Appendix A:

Comparison of the Segregation of the *RYR1* C1840T Mutation with Segregation of the Caffeine/Halothane Contracture Test Results for Malignant Hyperthermia Susceptibility in a Large Manitoba Mennonite Family

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Comparison of the Segregation of the RYR1 *C1840T Mutation with Segregation of the Caffeine/Halothane Contracture Test Results for Malignant Hyperthermia Susceptibility in a Large Manitoba Mennonite Family*

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Background: **Malignant hyperthermia (MH) is an important cause of anesthesia-induced death. Malignant hyperthermia susceptibility is diagnosed using the *in vitro* caffeine/halothane contracture test (CHCT) in fresh muscle biopsy specimens. The CHCT test is highly invasive, expensive, and lacks 100% specificity. Genetic and biochemical evidence provide strong support for the view that the substitution of cysteine for arginine 614 (Arg614Cys) in the human ryanodine receptor gene is one of several mutations that are likely to cause human MH. DNA testing was compared with CHCT as a means of predicting MH susceptibility in a large MH family in which the Arg614Cys mutation was detected.**

Methods: **A comparison of CHCT and DNA-based diagnosis was conducted in a large Manitoba Mennonite MH kindred identified by an index patient who died at age 45 yr of an MH crisis after general anesthesia. The presence of the Arg614Cys mutation was detected through a combination of polymerase chain reaction and restriction endonuclease digestion. Blood samples for DNA analysis were obtained from 68 family members, including 19 who had undergone muscle biopsies and 1 who had a documented crisis but did not undergo biopsy. Family members were classified as MH-susceptible or MH-normal on the basis of the CHCT.**

Results: **Twenty-two persons were found to be heterozygous for the Arg614Cys mutation. Five of these persons had prior positive CHCT results and one had an MH crisis but did not undergo biopsy. On DNA testing, 44 persons were found to be homozygous for the normal allele. Of these, ten had been classified as MH-normal and five as MH-susceptible on the basis of the CHCT. On reevaluation of the data obtained in our earlier CHCT diagnoses, we found that the condition of the muscle was poor, with no twitch, for three of five individuals homozygous for the normal allele but originally classified as MH-susceptible and for one who was homozygous for the normal allele and originally classified as MH-normal. Caffeine/halothane contracture test results for these four persons were considered invalid. The twitch response was good for the two remaining persons who were homozygous for the normal allele but classified as MH-susceptible, because contracture was observed with appropriately low levels of both caffeine and halothane.**

Conclusions: **An absolute correlation between DNA test results and CHCT assignment could not be made in this kindred. Possible explanations for discordance are that the Arg614Cys mutation is not linked to MH, that a second MH mutation is**

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segregating in the family, or that there are errors in the CHCT. Because there is strong evidence supporting the causal nature of the Arg614Cys mutation, the discordant persons are not closely related within the pedigree as they would be if a second MH mutation were segregating, and the CHCT is not 100% accurate, we propose that the observed discordance between DNA test results and CHCT assignment in this kindred results from two false-positive diagnoses by the CHCT. (Key words: Anesthetics, volatile: halothane. Caffeine/halothane contracture test. Calcium release channel (ryanodine receptor). Malignant hyperthermia susceptibility testing. Mutation analysis. Neuromuscular blocking agent: succinylcholine. *RYR1* C1840T mutation testing.)

MALIGNANT hyperthermia (MH) is an inherited human skeletal muscle disorder and is one of the main causes of anesthesia-induced death.[1,2] Commonly used halogenated anesthetics, such as halothane, and the depolarizing neuromuscular blocking agent, succinylcholine, can trigger MH crises in MH-susceptible (MHS) persons.

A principal objective of MH research has been to identify MHS individuals before administration of anesthetics so that alternative, safe anesthetics and nondepolarizing muscle relaxants can be used. Malignant hyperthermia susceptibility is currently diagnosed using the *in vitro* caffeine halothane/contracture test (CHCT) on fresh muscle biopsies. The basis for this test is that contracture of skeletal muscle strips from MHS persons are more sensitive to caffeine[3] or halothane[4] than fibers from normal persons. In the two decades since the CHCT was first developed, recommended standards for a positive CHCT have evolved in both North America[5] and Europe.[6]

The CHCT has proven to be a valuable clinical test.[7] When it is carefully executed and appropriate cutoff points are used, the test achieves 92–95% sensitivity,[8,9] defined by Larach[7] as the percentage of positive test results in the diseased population and calculated from the formula: 100 × [true-positives/(true-positives + false-negative)], and 53–75% specificity, defined by Larach[7] as the percentage of negative test results in the absence of disease and calculated from the formula: 100 × [true-negative/(true-negatives + false-positives)]. Because failure to detect MHS persons can result in a serious or fatal outcome, sensitivity approaching 100% is more important for clinical diagnosis than specificity.[7] In spite of its value as a clinical test, the lack of 100% specificity in the CHCT reduces its value as a predictor of phenotypic carriers of the genetic abnormality, MH. The CHCT is invasive and expensive and therefore is not a practical screen for all patients before general anesthesia. Thus, there is a need for a reliable, inexpensive, and noninvasive test for MH susceptibility.[2]

A primary MH defect has been proposed to involve abnormal gating of the calcium release channel (ryanodine receptor) of human and porcine skeletal muscle sarcoplasmic reticulum.[10–16] Genetic studies also support *RYR1*, the gene encoding the skeletal muscle isoform of the ryanodine receptor, as a causal gene for MH in humans[17–26] and porcine stress syndrome in pigs.[27–28] In the MHS pig, the substitution of T for C at position 1843 in *RYR1*, resulting in the substitution of cysteine for arginine 615 in the ryanodine receptor, was the only amino acid difference detected in a comparison with a normal animal.[27] This mutation cosegregated with MHS in more than 450 animals from 6 breeds of selectively inbred pigs with a lod score of 101.75 at a recombination fraction $\theta = 0.00$.[28] This strongly implicated it as the causal mutation for porcine MH. The corresponding human C1840T mutation (Arg614Cys) has been linked to MH in unrelated families.[19,20] The mutation, located in exon 17 of *RYR1*, eliminates a *Rsa*I restriction endonuclease site, providing the basis for diagnosis of at-risk individuals.[19]

Linkage of MH to *RYR1* has been possible in only 30–50% of all cases studied[29] and, in one case, lack of linkage of the Arg614Cys mutation to MH was reported in a complex MH family.[30] There are at least three possible reasons why the Arg614Cys mutation or other *RYR1* mutations may not segregate with MH in all cases. First, there may be no linkage. Second, more than one MH allele may be segregating in the family. Third, there may be linkage, but inaccurate phenotypic assessment may prevent the demonstration of linkage.

In a screen of our own series of 15 unrelated patients from our Manitoba probands with an MH crisis or positive CHCT, one person was heterozygous for the Arg614Cys mutation. This person belongs to a very large pedigree of Mennonite descent. In this study, we have compared the inheritance of the Arg614Cys mutation with inheritance of the MHS or MH-normal (MHN) phenotype, as defined by CHCT.

Methods

Patients and Caffeine/Halothane Contracture Testing

The index patient (III-2) in this large Manitoba family of Mennonite descent died at the age of 45 yr of an MH

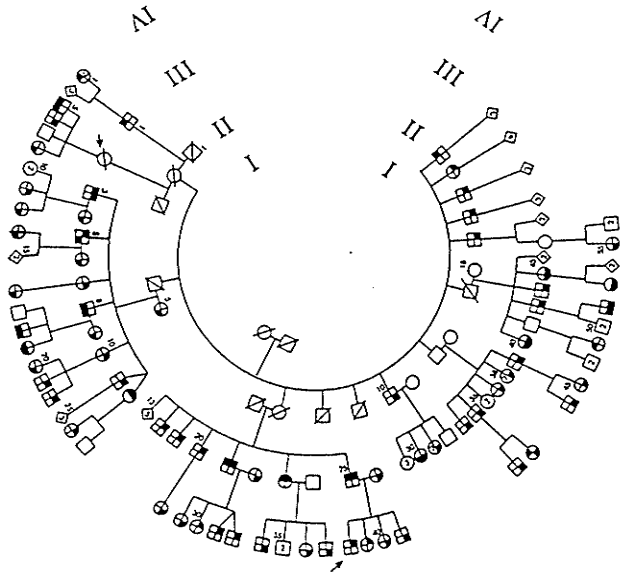

Fig. 1. Partial pedigree of large Manitoba kindred with 2 persons (↗) with documented malignant hyperthermia crisis. (⊕) = malignant hyperthermia normal by CHCT; (⊖) = malignant hyperthermia susceptible by CHCT; (⊘) = malignant hyperthermia status by CHCT unknown; (⊖) = C1840T mutation present; (⊖) = C1840T mutation absent; (○) = not studied. Numbers inside symbols refer to number of persons. Numbers to the upper left of symbol refer to pedigree position in each generation.

crisis after administration of a general anesthetic (fig. 1). She was admitted to the hospital for a left oophorectomy in 1979. There was no previous history of adverse anesthetic reactions. She was anesthetized with thiopental, nitrous oxide, succinylcholine, and halothane. Toward the end of her 2-h laparotomy, she was noted to be hypotensive, hyperthermic (40.5°C), and hypertonic. Her skin was mottled and her urine was red. She developed disseminated intravascular coagulation, renal failure, and cardiogenic shock. She never regained consciousness and died 1 day postoperatively. Subsequently, a second person (IV-38) was identified as having survived an MH crisis. This 3-yr, 10-month-old boy developed generalized muscle rigidity and cyanosis after administration of succinylcholine, nitrous oxide, and halothane for a right inguinal hernia repair. Myoglobinuria was documented and his creatine kinase level increased to 18,000 U/L the next day. He recovered uneventfully after supportive management.

Approximately 126 persons in this family are known to be at a 50% or 25% risk for MHS. Standardized open biopsy of the vastus lateralis muscle was performed in 21 at-risk persons during the period 1986 to present. One person, III-8, had 2 biopsies. These muscle biopsy specimens were studied using standard histochemistry and CHCT protocols of the North American Group and Registry.[5] Caffeine/halothane testing criteria have changed during the past 9 yr and those criteria used in Manitoba during that time are listed in table 1. Family members are classified as MHS, or MHN on the basis of results of the CHCT. In accordance with North American Standards, patients responding to caffeine or halothane, but not both, are included in the MHS category, as C or H responders.

Mutation Analysis

Blood samples for DNA extraction were obtained from 68 family members, including 19 of the 21 persons who had undergone muscle biopsies and one who had a documented crisis. Genomic DNA was isolated from whole blood as described previously.[31] The presence of the C1840T mutation in human genomic DNA was detected through a combination of polymerase chain reaction and restriction endonuclease digestion as described.[32]

Results

The pedigree of our MH family is presented in figure 1. Since subject III-2, who died after a documented MH crisis, was maternally related to subject IV-38, who also experienced an MH crisis, the maternal relatives of III-2 were all presumed to be at risk for MH. Subject III-3 was the first in this kindred to be identified as heterozygous for the *Rsa*I polymorphism. Direct DNA sequencing confirmed that the loss of the *Rsa*I site was the result of a C1840 to T transition (data not shown). In all, 22 persons were found to be heterozygous for the C1840T mutation. Of these, five (III-3, III-12, III-21, III-23, and III-25) had prior positive CHCT results and one (IV-38) had an MH crisis.

Table 1. Positive Caffeine/Halothane Contracture Testing Criteria

Criteria	Years Used
≥0.2 g tension with 2% halothane alone	Before 1987
≥0.5 g tension with 3% halothane	1987–present
≥1.0 g tension with 4 mM caffeine (CSC)	1987–present
≥0.2 g tension with 2 mM caffeine (CAFF)	1987–present

Table 2. Malignant Hyperthermia Status Based on CHCT and DNA Tests

ID No.	Date of Biopsy	Twitch Quality	2% Halothane (g)	3% Halothane (g)	CSC (mM)	CAFF (g)	MH Status	
							CHCT	DNA
III-3	22/5/87	Good	0	—	1.8*	1*	MHS	+
III-5	9/12/91	Good	0.5*	3.9*	2.1*	0.8*	MHS	−
III-8	12/5/89	Poor	0	3.6*	4.18	0.1	Unknown	−
	4/6/92	Good	0	0.35	4.49	0	MHN	−
III-12	24/8/93	Good	0.5*	1.4*	1.46*	1.4*	MHS	+
III-21	20/2/90	Good	3.8*	7.6*	1.53*	1.1	MHS	+
III-23	7/12/90	Good	3.2*	8.6*	1.71*	0.9*	MHS	+
III-25	21/1/86	Good	0	—	3.27*	0.6*	MHS	+
III-30	14/12/92	Good	0	0.4	4.95	0	MHN	−
III-31	12/5/92	Poor	0	0	2.72*	0	Unknown	+
III-38	5/5/83	Poor	0	—	2.98*	0.65*	unknown	+
					2.58*	0.75*		
III-40	28/8/92	Good	0	0	5.25	0	MHN	−
III-44	11/5/93	Good	0	0	4.22	0	MHN	−
IV-1	4/6/86	Poor	0	—	—	0	Unknown	−
IV-5†	8/12/87		—	0.3 (4%)	16	—	MHN	−
IV-18	21/3/90	Good	0	0.3	7.9	0.1	MHN	−
IV-19	6/12/89	Good	0	0	4.4	0	MHN	−
IV-38	No biopys	—	—	—	—	—	Crisis	+
IV-43	7/4/89	Good	—	0.75	3.0	0.65	MHS	−
IV-51	26/8/86	Good	0.15	0	16	—	MHN	−
IV-52	13/5/86	Good	0	—	8.14	0	MHN	−

CSC = concentration of caffeine to produce 1 g of tension; CAFF = contraction produced with 2 mM caffeine; Unknown = status could not be assessed because of poor muscle quality; + = C1840T mutation present; − = C1840T mutation absent.

* Abnormal test result.

† Done in Calgary.

Forty-four subjects were found, on DNA testing, to be homozygous for the normal allele. Of these, 14 had undergone muscle biopsies and CHCT (table 2), resulting in the initial classification of 9 (III-30, III-40, III-44, IV-1, IV-5, IV-18, IV-19, IV-51, IV-52) as MHN and 5 (III-5, III-8, III-31, III-38, IV-43) as MHS. Reevaluation of all of our CHCT data, including the discrepancies, however revealed that all muscle strips from the biopsies of discordant subjects III-8, III-31 and III-38, as well as the biopsy for concordant subject IV-1, were poor, with no twitch. Accordingly, these CHCT results are considered invalid and the MH status of these persons is considered to be unknown. Subject III-8, however, underwent a repeat biopsy in 1992. The condition of his muscle strips was excellent and his CHCT assignment was clearly MHN, in agreement with his normal DNA test (table 2). The reassignments of III-31, III-38, and IV-1 from MHS to unknown and of III-8 from MHS to MHN, are reflected in figure 1 and table 2.

The two other CHCT-positive, DNA-negative subjects (III-5 and IV-43) remain problematic. Subject III-5, analyzed in 1991, generated a strong contracture response of 3.9 g tension with 3% halothane, greater than 1 g tension with less than 4 mM caffeine and greater than 0.2 g tension with 2 mM caffeine, resulting in his classification as MHS. Subject IV-43, biopsied in 1989, generated a contracture response of 0.75 g tension with 3% halothane, 0.65 g tension with 2 mM caffeine, and greater than 1 g tension with less than 4 mM caffeine, resulting in her classification as MHS.

We have determined the haplotypes for siblings III-3, III-5, III-8, and their mother, and deduced the haplotype of their father, using *RYR1* intragenic and flanking markers. The data presented in figure 2 show that subject III-3, who is MHS by both DNA and CHCT, inherited a deduced haplotype, p1, from his presumed MHS father, subject II-4, whereas MHN subject III-8, who is MHN by both DNA and CHCT, inherited the deduced normal p2 haplotype. Subject III-5 is MHS by CHCT, but inherited the p2 haplotype, including the absence of the C1840T mutation, like his normal brother. Thus, the possibility that the C1840T mutation is not a causal mutation, but is only tightly linked to

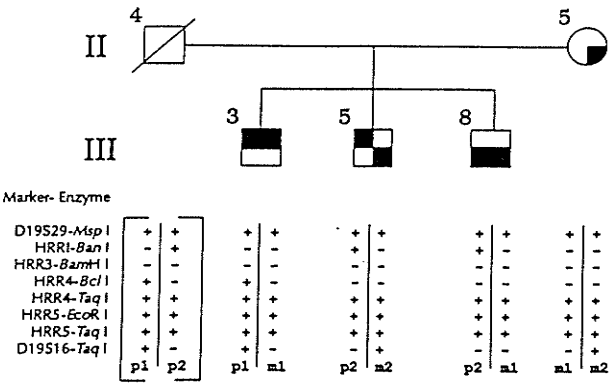

Fig. 2. Haplotypes of a partial nuclear family showing inheritance of the same low risk paternal haplotype p2 by subjects III-5 and III-8, and inheritance of the high risk paternal haplotype P1 by their MHS sib III-3. Alleles are as described[16] and are presented as haplotypes. "−" indicates absence of restriction site; "+" indicates presence. Maternal haplotypes are m1 and m2. Paternal haplotypes p1 and p2 have been inferred and are bracketed. Pedigree symbols are as in figure 1.

an unknown MH allele, located on chromosome 19q13.1 and lost by recombination in subject III-8, is unlikely, because subjects III-5 and III-8 have the same haplotypes, extending over several hundred kilobases.

Discussion

In this study of a large MH family in which the C1840T mutation in *RYR1* is segregating, we have had the opportunity to compare CHCT and DNA-based diagnoses of MH susceptibility. The CHCT and the DNA-based diagnoses were initially discrepant in 5 of 19 members of the family who had been subjected to CHCT. Careful analysis of our CHCT data, obtained during a period of several years, led us to the conclusion that four of our tests were, in fact, inconclusive, because the muscle quality was poor at the time of assay. We had the opportunity to rebiopsy one of those patients who had originally been diagnosed as MHS. On rebiopsy, the patient responded as MHN and was included in the study as MHN (table 2). Of the 16 CHCT results remaining, 2 were discordant with the DNA-based diagnosis. These results are consistent with at least three hypotheses: (1) that the Arg614Cys mutation is not linked to MH in this family; (2) that a second MH mutation is segregating in the family, giving rise to positive CHCT results for two persons who do not carry the Arg614Cys mutation; and (3) that the positive CHCT results for subjects III-5 and IV-43, who did not carry the Arg614Cys mutation, are false-positive results.

Although our data for CHCT- and DNA-based MH diagnoses are not concordant, there is a strong correlation between the two tests ($P < 0.05$). This strong correlation for linkage of MH to chromosome 19q13.1, without complete concordance, might suggest that the C1840T mutation is tightly linked to a true MH allele, but is separated from it by recombination in some persons. In one segment of the family where we found discordance, we determined the haplotype for several hundred kilobases around the C1840T mutation and found no recombinants within this family grouping (table 2). This finding would be more supportive of the view that the C1840T mutation is a causal mutation than the view that it is near to a causal mutation but does not, itself, play a causal role.

While we cannot rule out that a second MH allele is segregating in subjects III-5 and IV-43, this seems unlikely, because these persons are found in two different groupings within the large pedigree and there is no evidence of segregation of a putative second MH allele in either their siblings or their offspring.

There is strong evidence that the C1840T mutation is causal of MH in both swine and human MH. Specifically, a lod score of 102 at $\theta = 0.00$ favoring linkage with MH in swine,[28] combined with the association of the mutation with MH across a species barrier, in humans,[19,20] provides strong genetic evidence. This is further supported by the biochemical findings of Shomer *et al.*[16] in which purified ryanodine receptors from MHS pigs, reconstituted into planar lipid bilayers, exhibited longer open times and shorter closed times than did normal calcium release channels. It is also supported by the demonstration that expression of rabbit *RYR1* cDNA containing the Arg614Cys mutation in muscle cells[14] and COS-7 cells[15] leads to hypersensitive gating of Ca^{2+} release in these transfected cells.

If we were to conclude that the Arg614Cys mutation were not causative of MH in this family, we would have to discount all of the evidence for the causal nature of the mutation, including the finding that this mutation has been shown to segregate with MH in two other MH families.[19,20] In addition, we would have to define the CHCT as being 100% accurate. Such a definition would be completely out of line with studies of the accuracy of this test.[7–9] Larach and colleagues at The North American Malignant Hyperthermia Registry continue to evaluate North American CHCT results with the goal

of standardizing CHCT procedures and establishing diagnostic cutpoints.[7–9] In their studies, current diagnostic cutpoints can achieve sensitivities approaching 100%, but specificities approaching only 80% (M. Larach, personal communication). The European Malignant Hyperthermia group have not published error rates for their CHCT protocol, but members of their group have acknowledged that the test is not 100% accurate.[33] False-negative test results have been reported for the European CHCT protocol.[34]

In light of our own experiences, in particular, the finding that rebiopsy can lead to a reversal of a test outcome, and in the face of clear evidence that the CHCT is not 100% sensitive, we are unable to define the CHCT as 100% accurate. Accordingly, we are unable to accept the alternative that the Arg614Cys mutation is not linked to MH in this family. We are, however, able to accept the alternative that the CHCT is giving rise to 2 false-positive results in our study of 16 CHCT results. Family members were told the results of their DNA tests but were counseled that their genotype data must be interpreted cautiously, at least until we more fully understand the basis for false-positive CHCT results.

Our study can be compared with one by Deufel *et al.*[30] of a very complex MH family. In this family, two Arg614Cys mutations were found on two different haplotypes in one branch of the MH family. Malignant hyperthermia susceptibility also segregated in another branch of the family in which no Arg614Cys mutation was present. Caffeine/halothane contracture test results for MH susceptibility segregated with the presence or absence of the Arg614Cys mutations in seven of the eight persons tested in the left branch of the family, including one who was homozygous for the mutation. Subject 508, however, was negative in the CHCT, but heterozygous for the mutation. To achieve concordance in this branch of the family, Deufel *et al.*[30] would have had to accept that subject 508 was diagnosed as a false-negative by the CHCT. In the right branch of the family, the Arg614Cys mutation was absent, but an attempt was made to correlate CHCT results with chromosome 19q13.1 haplotypes. To achieve concordance in this branch of the family, one false-positive and one false-negative CHCT result, out of four tests carried out, would have had to be invoked. This would not be unreasonable if one accepts that the CHCT is not 100% accurate. An understanding of the inheritance of MH in this branch of the family will require further study.

In both our study and the study of Deufel *et al.*,[30] a high correlation (14 of 16 in our study; 7 of 8 in Deufel's study), but not concordance was found between CHCT- and DNA-based diagnoses for MH in families in which the Arg614Cys mutation was segregating. In our view, the CHCT is not 100% accurate and these results can be brought into concordance by the reasonable assumption that the CHCT can yield both false-positive and false-negative results. Deufel *et al.*,[30] however, suggested that their results threw into question both the causal nature of the Arg614Cys mutation and the role of *RYR1* in MH.

This study is not the first in which lack of concordance between *RYR1* mutations and MH have been noted. The Gly2433Arg mutation in *RYR1* was detected in eight MH families,[22,26] but was concordant with MH in only six. In one small family,[26] two brothers were diagnosed as MHS by the CHCT. One had exceptionally strong test results and carried the Gly2433Arg mutation. The other was well within the positive category, but did not carry the Gly2433Arg mutation. In the absence of further information, it is reasonable to suggest that the person with the strong CHCT result carried two MH mutations, while his brother carried only the one that was not detected in assays for the Arg2433 mutation. In the other discordant family,[26] inheritance patterns and haplotype analysis did not support a second MH mutation. As in the family currently being studied, it was most logical to invoke both false-positive and false-negative CHCT results as the basis for discordance.

It has been estimated that only 30–50% of MH families are linked to the *RYR1* gene.[29] Although linkage describes a probability, positive linkage requires concordance. Thus, discordance for even one member of a large family can lead to lack of linkage. That linkage analysis has been successful in identifying so many chromosome 19 linked families argues strongly that, in many cases, the CHCT is an accurate method of phenotyping for molecular genetic studies. Alternative loci for MH have been described on chromosomes 17q,[35,36] 7q,[37] and 3q13.1.[38] Linkage to chromosome 17q has not been confirmed.[39] Linkage to chromosome 7 was strongly suspected in a single family but the lod score for linkage was less than 3[37] and a causal gene and a causal mutation have yet to be found. Linkage to chromosome 3 with a lod score over 3 has been reported, making this locus the best candidate for a second MH locus.[38] Assignment of alternate loci using the CHCT, however, has its own potential for error. An

understanding of the limits of accuracy of the CHCT, in studies of the linkage of MH to *RYR1* and alternate loci, will be important to all future research on the genetic basis of MH.

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To whom it may concern:

Kimberly Serfas has permission from the authors of Serfas *et al.*, undersigned here, to include the article entitled "Comparison of the Segregation of the *RYR1* C1840T Mutation with Segregation of the Caffeine/Halothane Contracture Test Results for Malignant Hyperthermia Susceptibility in a Large Manitoba Mennonite Family" which was published in February 1996 issue *Anesthesiology* as an appendix in her Master's thesis.

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