MOLECULAR GENETIC ANALYSIS OF MALIGNANT HYPERTHERMIA

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The candidate confirms that the work submitted is his own and that appropriate credit has been given were reference has been made to the work of others.

Abstract

Malignant hyperthermia (MH) is the most common cause of unexpected death due to general anaesthesia using common volatile anaesthetic agents. It is an autosomal dominant pharmacogenetic abnormality of skeletal muscle with a high degree of genetic and clinical variability. It is associated with elevated calcium ion levels in skeletal muscle cells. Apparently it is caused by defect in a calcium release channel, encoded by the *RYR1* gene which is located on chromosome 19q.

Studies show only around 50% of MH families are linked to the *RYR1* gene so that heterogeneity for this abnormality is suggested. Further studies proved more genes or a candidate regions such as chromosome 7q, 3q are involved in malignant hyperthermia susceptibility.

In this search for more understanding of the malignant hyperthermia disorder and for identifying the gene or genes which may be causative in MH, some UK MH families for the *RYR1* gene and other candidate regions were studied. Furthermore based on reports of the correlation between MH and mutations in the *RYR1* gene in 19-linked families the screening of UK MH families for three mutations from eight putative causative mutations in the *RYR1* gene was carried out.

The ultimate goal of this MH project has been to identify individuals susceptible to MH in advance of anaesthesia. Genetic analysis by direct mutation testing or linkage analysis potentially offers an alternative non-invasive and accurate test for diagnosis of MH susceptibility. However once the linkage

relationships are firmly established, they provide a sensitive, non-invasive technique for the presymptomatic diagnosis of MH.

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List of abbreviations

APS	Ammonium persulphate
bp	base -pair
CCD	Central Core Disease
CK	Creatine phosphokinase
cM	centi Morgan
CRC	Calcium release channel
DH	Dynamic halothane
DHPR	Dihydropyridine receptor
dNTPs	deoxynucleotide triphosphates
dATP	2′deoxyadenosine triphosphate
dCTP	2′deoxycytosine triphosphate
dGTP	2′deoxyguanosine triphosphate
dTTP	2′deoxythymidine triphosphate
EMHG	European Malignant Hyperthermia Group
EMHGGS	European Malignant Hyperthermia Group Genetics Section
EtBr	Ethidium bromide
IP_3	Inositol 1,4,5-trisphophate
IVCT	In Vitro Contracture Test
kb	kilo base
LIPE	Hormone-sensitive lipase gene
Low A	reduced concentration of Adenosine triphosphate
MH	Malignant Hyperthermia

MHS	Malignant hyperthermia susceptible
MHE	Malignant hyperthermia equivocal
MHN	Malignant hyperthermia normal
min	minutes
mM	milliMolar
nt	nucleotide
PCR	polymerase chain reaction
PSS	porcine stress syndrome
RF	Recombination fraction
RYR1	Ryanodine receptor (skeletal muscle)
s	second
SC	Static caffeine
SC	Static halothane
SCN4A	Adult skeletal muscle sodium channel
SDS	Sodium dodecyl sulphate
SR	Sarcoplasmic reticulum
STR	Short tandem repeat
TE	Tris-EDTA (10mM Tris-HCl, 1mM EDTA, pH 8)
UV	Ultra violet
VNTRs	Variable number of tandem repeats

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Chapter one

Introduction

Malignant Hyperthermia, MH, is perhaps the commonest cause of unexpected death in patients due to general anaesthesia using common volatile anaesthetic agents (Ellis and Halsall 1980). Malignant hyperthermia is an autosomal dominant pharmacogenetic abnormality and life threatening disorder of skeletal muscle with a high degree of genetic and clinical variability (Denborough *et al* 1962; Britt *et al* 1969). It is an inherited condition that occurs in humans and some species of domestic animals, such as dog, horse and swine (Gronert 1980; Britt 1987). It can be triggered by a variety of factors, such as stress, anaesthetic drugs and skeletal muscle relaxants (Wingard 1974).

1.1 History of malignant hyperthermia (MH)

Malignant hyperthermia was reported for the first time in humans by Denborough and Lovell in Australia (1960). They reported an accelerated metabolism during anaesthesia, in a 21-year-old man, who survived after an episode of extreme hyperpyrexia. Subsequent investigation revealed that he had lost 10 relatives since 1922 as a result of anaesthesia and in three of these cases where records were available high body temperature was associated with the death. For this anaesthesia they used volatile anaesthetic agents, halothane and nitrous oxide. The family history of the young man suggested this condition had been

transmitted as an autosomal dominant with reduced penetrance (Denborough *et al* 1962).

Further case reports were published and muscle rigidity, hyperthermia, tachycardia and other symptoms of malignant hyperthermia as a result of general anaesthesia were described (Saidman *et al* 1964; Thut and Davenport 1966; Lavoie 1966; Davies and Graves 1966). In subsequent MH studies treatment schemes and hypotheses of its aetiology, its relationship to other metabolic and muscle diseases such as central core disease (CCD) and neuroleptic malignant syndrome have been described. These raised the possibility that in previous years some of the sudden deaths which had occurred during or immediately after anaesthesia associated with high fever might have been caused by malignant hyperthermia susceptibility. The word “malignant” before hyperthermia or hyperpyrexia was used for the first time by Gordon in his description of the disorder (Gordon 1966).

MH is not a disease with unique symptoms, a unique hereditary pattern and a singular aetiology. It does not necessarily occur during the first anaesthesia (Britt *et al* 1969). Halsall *et al* (1979) reported one patient who had had 13 previous general anaesthetics without any apparent problem before experiencing an MH crisis and subsequently was identified as MH susceptible. Therefore the occurrence of an MH episode is dependent on the background susceptibility of subjects and other possible environmental factors, and for this reason MH susceptibility is considered to exhibit autosomal dominance with reduced penetrance. During dental and other minor surgery using anaesthetic vapour for a brief exposure time, MH is usually not a complication. Unfortunately compared

with most muscular disorders MH does not have any phenotypic key features and the phenotype remains cryptic until a susceptible individual is exposed to triggering anaesthetic agents. It is generally believed that a defect in calcium regulation of muscle contraction and metabolism is responsible for this abnormality (Britt and Kalow 1970b; Mickelson *et al* 1986; Carrier *et al* 1991; Nelson 1991; Iaizzo *et al* 1988) although the precise mechanism is not known.

1.2 An animal model of MH

A condition similar to MH in some features, called porcine stress syndrome (PSS) has been recognised in some races of pigs including the Dutch, Landrace, Poland China, and Pietrain breeds. This syndrome in predisposed pigs is induced by stress such as separation, shipping, weaning, fighting, coitus, slaughter, and heat in addition to exposure to volatile anaesthetics. Porcine MH or PSS presents with shortness of breath, associated with rapid increase in body temperature, metabolic acidosis, muscle rigidity, and death (Gronert 1980; Mabry *et al* 1981). PSS in addition to death can have serious economic consequences in the pork industry. It causes hypermetabolism in skeletal muscle which results in the deterioration of the muscle so that the carcass yields an unsuitable quality of pork (Briskey 1964). This meat has little commercial value and it is described as pale, soft and exudative pork (PSEP).

In attempts to further understand the syndrome the relations between PSS as a disorder of muscle and rigidity during suxamethonium-halothane anaesthesia (Hall *et al* 1966) and nitrous oxide-halothane anaesthesia (Harrison *et al* 1968) in

susceptible pigs have been investigated. Discoveries on the clinical, metabolic and biochemical changes in susceptible pigs with MH have demonstrated its importance as an animal model for the human phenotype. It was ideal for experiments on pathophysiology and identification of susceptible individuals which could not be carried out in humans (Berman *et al* 1970).

The finding of the role of altered skeletal muscle sarcoplasmic reticulum, SR ryanodine receptor in susceptible pigs compared with controls and the implication of a biochemical abnormality of the SR calcium release channel as the cause of MH was an important step towards understanding the aetiology of malignant hyperthermia susceptibility or PSS (Mickelson *et al* 1986; 1988; Eravasti *et al* 1991). This is supported by molecular genetic evidence where a mutation in the gene encoding the skeletal muscle ryanodine receptor in susceptible pigs was reported. The susceptibility to PSS in pigs, unlike MH in humans, is an autosomal recessive trait and transmitted by a single gene, *Hal* (Andresen and Jensen 1977; Reik *et al* 1983) which is located on chromosome 6 (Hartbitz *et al* 1990).

1.3 MH signs and symptoms

A human MH crisis is indicated by a spectrum of symptoms, which are categorised in eight groups ranging from the classic cases described as fulminant, with frequency less than 10% (Rosenberg 1988), to those with mild or unusual presentation (Ellis *et al* 1990; Halsall and Ellis 1993). Although a typical physical appearance of MH susceptible individuals is not proven, anecdotal evidence exists

to suggest that MH susceptible (MHS) individuals tend to be well built, muscular and also may have a greater muscle bulk and power than normal (Britt 1974; Ellis 1980). The MH diversity of different clinical presentations of MH may reflect differences in anaesthetic techniques used in different countries and centres. Indeed MH is associated with a variable combination of different characteristics. Conclusions about specific signs are difficult to determine and as a syndrome the clinical and biochemical symptoms can be categorised as below.

1.3.1 Clinical aspects:

Following administration of an inhalational agent with or without suxamethonium (succinylcholine) the clinical characteristics of MH may appear in susceptible individuals and early recognition of the symptoms is vital to successful management of the patient. The classic presentation begins after a few minutes with an increase in heart rate (tachycardia) and arterial CO_2, respiratory acidosis and tachypnoea and are the earliest signs of MH. Muscle rigidity, especially masseter muscle spasm or jaw rigidity, have been described as the most common clinical signs of MH with an incidence of 50 - 64%, but the appearance of masseter muscle spasm is not correlated with MH in every patient. MH has also been described in anaesthesia in association with muscle diseases, such as myotonia congenita (Ellis and Halsall 1984). Muscle rigidity can occur without hyperthermia and also hyperthermia can happen without rigidity in 20% of MH cases (Kalow 1970; Rosenberg and Fletcher 1994). The MH symptoms may occur at any time during anaesthesia and even afterwards in the recovery period.

In an MH episode an increase in body temperature of 2-6°C per hour up to a temperature of 43°C is not uncommon (Kalow 1970; Keaney and Ellis 1971; Gronert 1980). Body temperature may begin to rise a few minutes after the initiation of the earliest signs of MH or may be delayed for a longer time and may even only be observed post operatively in the recovery room. Generally it is a relatively late sign of the syndrome if it occurs at all (Rosenberg and Fletcher 1994). The rise in body temperature reflects an imbalance between the heat production and heat loss. This results from skeletal muscle hypermetabolism which is related to the high concentration of Ca^{2+} in the myoplasm (Nelson *et al* 1983; Mickelson *et al* 1988). This phenomenon gives rise to an increase in cardiac output and consequently to tachycardia.

1.3.2 Biochemical signs

The loss of intracellular calcium balance which is initiated by triggering agents in susceptible individuals and the attempts of muscle cells to restore normal levels of Ca^{2+} ions leads to the consumption of large amounts of ATP to provide enough energy for sarcoplasmic reticulum Ca^{2+} pumps. This rapid ATP consumption in re-establishing the resting Ca^{2+} balance requires an extreme increase of muscle metabolism to five or six times the basal level. This excessively high metabolic rate, described as a metabolic storm, results in malignant hyperthermia symptoms, increase in body temperature, acidosis, tachycardia, increased oxygen consumption and muscle rigidity. Depletion of ATP can induce muscle membrane permeability to potassium ions, and results in hyperkalaemia. This elevated serum potassium level may contribute to the production of cardiac

arrhythmia including asystole (Hopkins and Ellis 1995; Rosenberg 1988). In addition, increasing muscle permeability causes increased serum levels of creatine phosphokinase and other muscle enzymes like lactate dehydrogenase (LDH) and aspartate amino transferase (Rutberg *et al* 1983). The induced derangement after an acute MH episode will continue by breakdown of muscle fibres and the increase in myoglobin excretion causes damage in renal tubules.

1.4 Triggering agents

As mentioned before, PSS can be induced in susceptible pigs by different factors but in humans MH is triggered by volatile anaesthetic drugs, and possibly the depolarising muscle relaxant, suxamethonium. Some workers also consider that a few cases may be induced by injection of some intravenous anaesthetics such as propofol, opioids and barbiturates although it is a controversial matter (Strazis and Fox 1993; Dripps *et al* 1988). Fruen *et al* (1995) reported that propofol does not activate ryanodine receptor Ca^{2+} channels of normal or MH susceptible pigs. Wingard (1974, 1981) argued that stress could also precipitate MH in humans but supportive evidence is lacking. It is well known that inhalation anaesthetic drugs including halothane, diethyl ether, methoxyflurane, enflurane, isoflurane, desflurane, sevoflurane, and chloroform (Ellis and Halsall; 1980; Ellis 1992; Hopkins and Ellis 1995; Rosenberg and Fletcher 1994) and some depolarising muscle relaxants like suxamethonium (Hall *et al* 1966) may act as triggering agents in malignant hyperthermia induction.

For safe anaesthesia, nitrous oxide, local anaesthetic, intravenous anaesthetics, sedatives and non-depolarising relaxants do not trigger MH (Halsall and Ellis 1993; Rosenberg and Fletcher 1994).

1.5 Frequency and incidence of MH

Apart from the spectrum of expression of the clinical features of MH there seems to be great variation from individual to individual, and even in the same individual at different times to susceptibility and response to triggering agents in predisposed individuals (Halsall *et al* 1979; Ellis *et al* 1986). Thus uneventful anaesthesia with exposure to triggering agents does not exclude the possibility of malignant hyperthermia susceptibility. This makes the exact determination of the prevalence of malignant hyperthermia susceptibility difficult. Currently the frequency of MH crises is estimated to be between 1 in 40 000 and 1 in 50 000 general anaesthetics in the North American and European populations (Kalow 1970; Ellis and Halsall 1980; Ellis *et al* 1986; Halsall and Ellis 1993; Strazis and Fox 1993). It is suggested that the frequency of MH susceptibility is 1 in 5000 in the general population of the UK (West 1996). The discrepancy between frequencies may arise from the following factors : lack of a unique practical anaesthetic method in different centres; brief exposure time (duration of anaesthesia which may be insufficient time to induce MH); type of triggering agents used, for example, in more than 90% of operations, the standard triggering agents are used (Campling *et al* 1993). In addition, susceptibility and the use of non-volatile anaesthetic drugs are involved in expression of the malignant hyperthermia phenotype (King *et al* 1972; Ellis 1980). Halsall *et al* (1979)

supposed the probability of MH developing in susceptible patients is 44% on each occasion of exposure to triggering agents.

In general anaesthesia the estimated frequency of MH in children and young adults is higher than for other age groups and so it is supposed that the MH phenotype may be affected by age. Studies show the age range of the majority of index cases is between 3-19 years (McPherson and Taylor 1982; Halsall and Ellis 1993). This higher incidence of MH crises in childhood may also be influenced by many factors, which may reflect a reduced penetrance of MHS with increasing age illustrated in fig 1.1 (redrawn from Halsall and Ellis 1993).

Despite MH being an autosomal dominant trait it appears that more males than females suffer from the clinical reaction. This may be a reflection of different life styles, for example the higher number of males than females who present with trauma, which is one of the most common reasons for anaesthesia between the ages of 10-30 years (Halsall *et al* 1979; McPherson and Taylor 1982) although the death rates for both sexes being the same and IVCT reveals approximately equal numbers of both sexes and being susceptible to MH. As illustrated in fig 1.1 the frequency of individuals experiencing MH is reduced in the over 30 year age groups for both sexes. This may be caused by reduction in sensitivity to anaesthetic drugs with advancing age (Kalow 1970). In conclusion, the susceptibility to MH, although not a sex-linked disease, may be a sex influenced trait. Other environmental factors may also influence the onset of an MH episode, such as stress, premedication trauma and exercise (Wingard 1974; Ellis and Halsall 1980; Halsall *et al* 1979; Strazis and Fox 1993; Britt and Kalow 1970a). MH susceptibility is reported in all

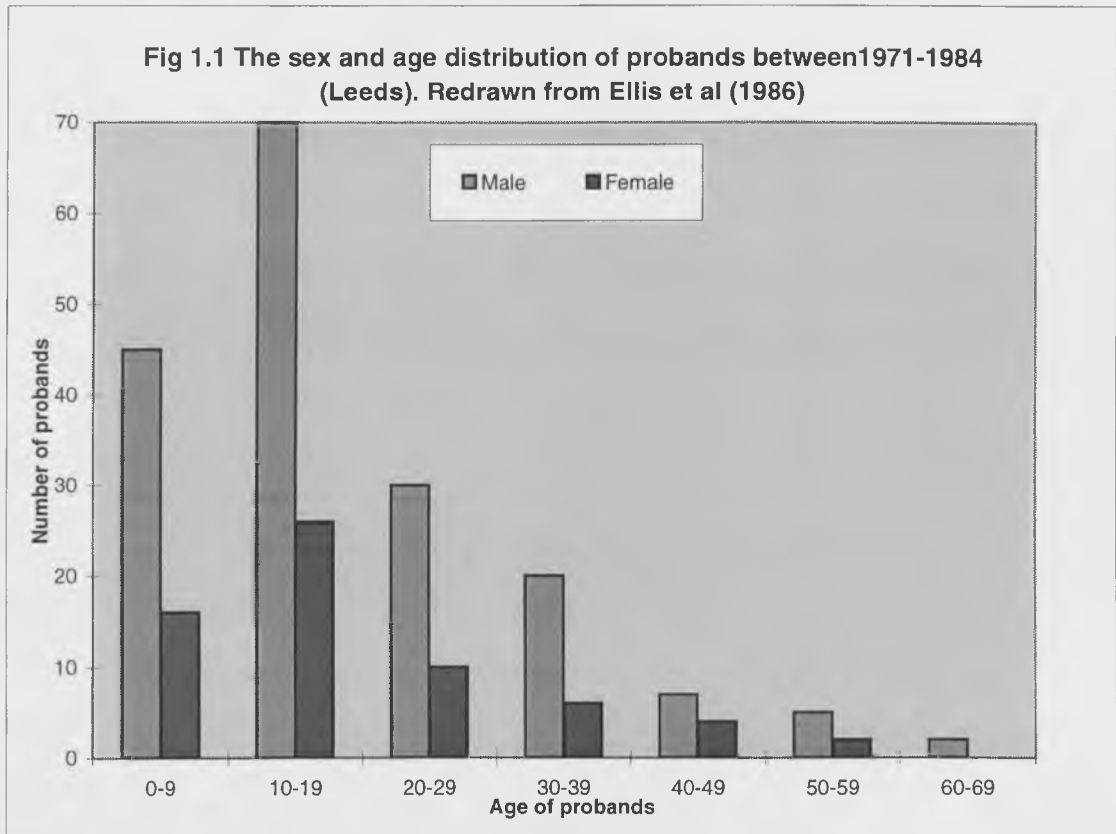


Fig 1.1 MH crises predominate in the 10-19 year age group and are considerably more prevalent in males than females. The frequency of MH crises decrease with increasing age. Possible reasons for these observations are discussed in section 1.5.

racial groups but is more common in the Caucasian population (McPherson and Taylor 1982) and is particularly high in the Japanese population.

1.6 Treatment

The frequency of mortality as a result of an MH episode was reported at 70% in 1970 (Halsall and Ellis 1993) and at present, although it has not been eliminated, it is less than 10% (Strazis and Fox 1993). In reducing mortality and successful treatment of susceptible individuals, the early recognition of an impending MH crisis is very important. With regard to malignant hyperthermia as a life threatening disorder and the lack of a generally applicable pre-anaesthetic screening method for MH susceptibility, the knowledge of anaesthetists of the early symptoms of an MH episode, and rapid response by terminating the anaesthetic process, is critical. In this procedure the elimination of the volatile drugs from the body, enhanced by hyperventilation, and intravenous dantrolene therapy are the main methods of treatment. Dantrolene is believed to stop the release of calcium from the sarcoplasmic reticulum by blocking the calcium release channel, controlling high temperature and muscle rigidity regardless of causes (Gronert *et al* 1988). It is very important in the successful treatment of an MH crisis and has reduced the MH caused death rate in anaesthesia (Ellis *et al* 1974; Kolb *et al* 1982). If treatment is delayed, the development of muscle rigidity and restriction of the blood supply to muscle cells causes pathological development and leads to the crucial stage which becomes irreversible. In addition to cessation of surgery and dantrolene therapy in recovery after an MH episode, hyperventilation (95% of oxygen, 5% CO_2) and active cooling with an electric cooling blanket or iced water,

and intravenous infusion with ice-cold fluids (mannitol) is necessary (Halsall and Ellis 1993; Rosenberg and Fletcher 1994).

Patients who develop the symptoms of MH may die within minutes from ventricular fibrillation, within hours from pulmonary oedema or coagulopathy, or within days from neurological damage or obstructive renal failure if treatment is not initiated immediately. After successful treatment, they will be ill for several days from heavy loss of potassium, acidosis, unstable body temperature and severe muscle pain (Ellis 1980; MacLennan 1992) and will require intensive care therapy. Longer term symptoms such as muscle pain may continue for several weeks (Ellis and Halsall 1980).

In conclusion, the following steps are vital in preventing an MH episode from becoming irreversible. At first the administration of triggering agents, must be stopped and then injection of dantrolene sodium (1-10mg / per kg of body weight) administered intravenously. The next step is active body cooling, at this step one may cool the body sufficiently to inhibit the MH response at the cellular level, and finally in the recovery period , frequent observation of the body temperature, biochemical signs and attention is necessary.

1.7 Identification of susceptible individuals

Many methods have been described in the literature for MH diagnosis but only two methods are currently used, and these are the creatine phosphokinase activity (CK) measurement and the In Vitro Contracture Test (IVCT).

1.7.1 The measurement of CK

Serum creatine phosphokinase (CK) activity is increased in many MH-susceptible individuals but in others it is normal (Isaacs and Barlow 1970; Gronert 1980). Efforts to use concentration of CK as an indicator in MH susceptibility prediction have been made (Issacs and Barlow 1970) but with insufficient sensitivity and specificity to be used as a screening test in malignant hyperthermia diagnosis. The increase of CK in several diseases which are not related to MH, and the variability of CK levels in MHS individuals (Ellis *et al* 1975) and also CK increase after exercise (Ellis *et al* 1972) renders it of little value as a screening test (Ellis 1992). As an initial screening test, a high level of CK in some susceptible families to MH can be used in making a decision on priority of individuals for investigation by muscle biopsy.

In conclusion, it is supposed that the estimate of serum CK appears unreliable and so it cannot be used in susceptible patients as a predictive test, although the patients with high values of CK, 10 000-20 000 units per litre (whereas the normal range is 200-220 units) after anaesthesia have more chance of susceptibility to MH especially when it is 20 000 units or more per litre. In some susceptible families, high resting levels of CK may correlate with malignant hyperthermia susceptibility (Kalow 1970; King *et al* 1973; Ellis *et al* 1972; 1975; Ørding 1988; Rosenberg 1988).

1.7.2 In Vitro Contracture Test (IVCT)

The limitation of using the biochemical tests and also the lack of phenotypic symptoms to predict malignant hyperthermia susceptibility before general anaesthesia makes the *in vitro* contracture test IVCT a "gold standard" test in malignant hyperthermia diagnosis. The IVCT has been widely accepted by most European testing centres. It remains the most reliable and acceptable method in identifying MH susceptibility although it is time-consuming, expensive and invasive and for these reasons is not appropriate as a population-screening test. In addition, viability and type of muscle used, and some known myopathies, also influence the IVCT results. An IVCT protocol was established by Kalow *et al*, who reported that muscle from malignant hyperthermia susceptible individuals is more sensitive to caffeine (Kalow *et al* 1970; Kalow *et al* 1977). In the following year the sensitivity of muscle to halothane was described (Ellis *et al* 1971; Ellis *et al* 1972; Ellis and Harriman 1973). Nowadays two main protocols for IVCT are used in MH investigation Units. These are the European protocol used by all members of the European Malignant Hyperthermia Group (EMHG 1984; 1985) and the North American protocol (Larach 1989).

The North American method, Caffeine Halothane Contracture Test (CHCT) differs in a few aspects from the European test. Ørding and Bendixen (1992) have reported an agreement of 78%-88% between results from the two protocols. The North American method is not performed by EMHG because it produces both false positive and negative results (MacKenzie *et al* 1991; Ellis 1992; Isaacs and Badenhorst 1993; Larach 1993; Wedel and Nelson 1994). The CHCT is carried

out according to the protocol supported by the North American Malignant Hyperthermia Registry (NAMHR). This group was formed to establish uniformity in testing among laboratories from Canada and United States (Larach 1989). In this method two tests are required. These are the exposure of muscle strips to 3% (v/v) halothane alone and exposure to incremental caffeine concentrations (CSC) alone. In addition to these tests, there are two optional tests. These are exposure of muscle strips to a combination of both 1% halothane and incremental caffeine concentrations, and the other one is using 2% halothane alone. The abnormal contracture responses or MH susceptibilities identified as :

A positive response is defined by a contracture of more than 0.2 - 0.7g (dependent upon the individual laboratory) on exposure to 3% halothane for 10 minutes.

Abnormal contracture response to 2mM caffeine or caffeine specific contracture (CSC) at <4mM and induction of 0.2 g tension or more or the development of 1g contracture after exposure to a concentration of 1mM or less caffeine in presence of 1% halothane (Larach 1989; Melton *et al* 1989).

In this test protocol a few points need consideration. Firstly, the range of values for abnormality response is determined by each laboratory. This means that the threshold for deciding diagnosis of MHS and MHN is variable and this could underlie the false positive and negative results from individual centres. Secondly, while the combined test may increase sensitivity in some cases, it may result in decreased specificity (Wedel and Nelson 1994). Thirdly in the North American protocol, contracture response to halothane alone, caffeine alone or the combined

challenge is indicative of MH susceptibility. At the recent NAMHR 5th MH Biopsy Standards Conference Meeting acceptance of the MHE group defined by the European protocol as an intermediate between MHS and MHN was recommended (Iaizzo and Lehmann-Horn 1995). This step if accepted may help in reaching an agreement on an international protocol.

In Europe the *in vitro* contracture test is carried out according to the updated European standardised protocol which was adopted by the European Malignant Hyperthermia Group or EMHG in 1984 and updated in 1985 (EMHG 1984; 1985; Ellis 1984; Ellis *et al* 1986; Ellis 1992). In this test, muscle biopsy specimens measuring 3cm, 0.4cm, and 0.2cm are taken from the motor point of the *vastus internus* muscle under general or regional anaesthesia. The fascicles are immediately placed in oxygenated (95% O_2 and 5% CO_2) modified Krebs solution (NaCl 118.1mM, KH_2Po_4 1.2mM, KCl 3.4mM, $MgSo_4$, 0.8mM, $NaHCO_3$ 25mM, glucose 11.1mM, $CaCl_2$ 2.5mM and pH 7.4) at room temperature prior to contracture testing. The time from biopsy to the completion of all tests should not exceed 5 hours. The bundles of living muscle at 37°C in a tissue bath are exposed to the different doses of triggering agents, caffeine (0.5mM, 1mM, 2mM, 3mM, 4mM and 32mM) and halothane (0.5%, 1%, and 2%) to produce a sustained increase of at least 0.2g in baseline tension.

From the results of IVCT, the patients may be classified into three diagnostic groups, MHS, MHN, and MHE, based on the responses to contracture inducing agents. MHS indicates that the individual is susceptible to malignant hyperthermia, with abnormal responses to both caffeine (2mM or less) and

halothane(2% or less) in the muscle contracture test (fig 1.2). MHN or normal individuals have no abnormal response to either halothane (2% or less) or caffeine (2mM or less) (fig 1.3). MH equivocal or MHE individuals show a positive response to caffeine (MHE_c) or halothane (MHE_h) but not both. The MHE category allows maximum specificity to be assigned to the MHS and MHN phenotypes. For clinical purposes MHE individuals are advised that they are sensitive to halothane (EMHG 1984; 1985) but for investigative purposes they are regarded as equivocal. In addition to halothane and caffeine in contracture tests, use of ryanodine may be useful in distinguishing between the MHN and MHE classes (Wappler *et al* 1994; 1996). The ryanodine test appears to be more sensitive than the caffeine test in distinguishing between MHS and MHN (Halsall and Ellis 1993) and may become an integral part of the IVCT protocol. When a suspected clinical reaction is observed, all probands except those under 10 years old, or patients who have died, should be tested for MH susceptibility. In these situations the diagnosis of MH status may be obtained indirectly by testing both the parents.

In an interesting review of the reliability of the IVCT (based on European protocol), 65 subjects who survived a clinical episode of fulminant reaction of MH have been investigated with the IVCT. Of these, 89% (58) have been designated susceptible and ~11% equivocal (4 MHE_h and 3 MHE_c) with no MHN (P. M. Hopkins, pers. comm.).

Although IVCT is at present the only accurate and reliable method for MH diagnosis it is not an ideal test for the following reasons. A sizeable muscle biopsy

a) Halothane concentrations : → 0.5% 1% 2%

↓ ↓ ↓

1gr

b) Caffeine concentrations :

3mM 2mM 1.5mM 1mM 0.5mM

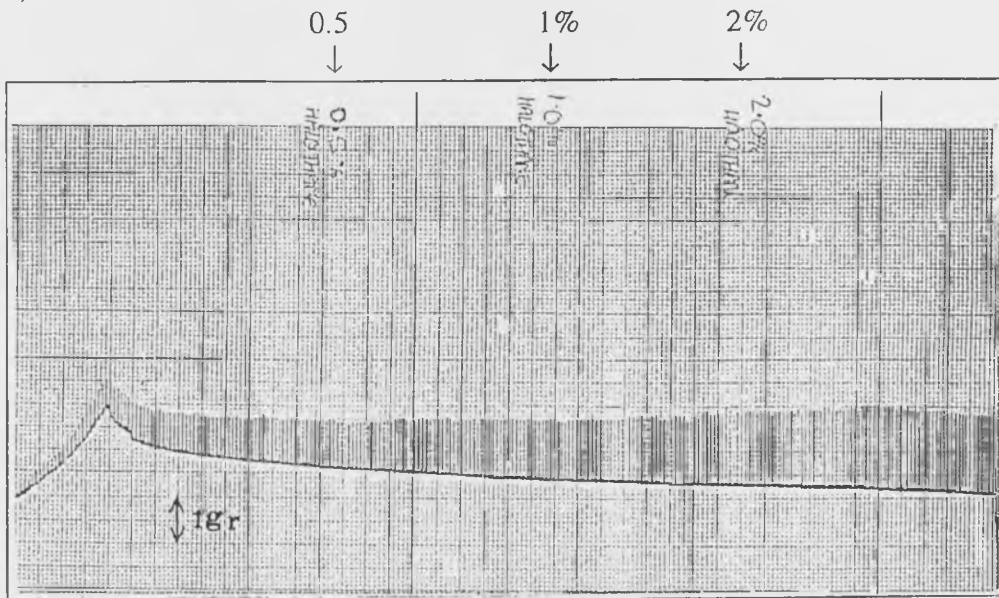
↓ ↓ ↓ ↓ ↓

1gr

Fig 1.2 The effect of different concentrations of a) halothane (0.5, 1, 2 and 3%) and b) caffeine (0.5, 1, 1.5, 2, 3, 4 and 32 mM) on muscle samples from individual no. 2134, who shows positive contracture to halothane and caffeine at 0.5% and 0.5mM respectively and is therefore diagnosed as MHS.

a) Halothane concentrations :

0.5 ↓ 1% ↓ 2% ↓



b) Caffeine concentrations

1.5mM ↓ 2mM ↓ 3mM ↓ 4mM ↓ 32mM ↓

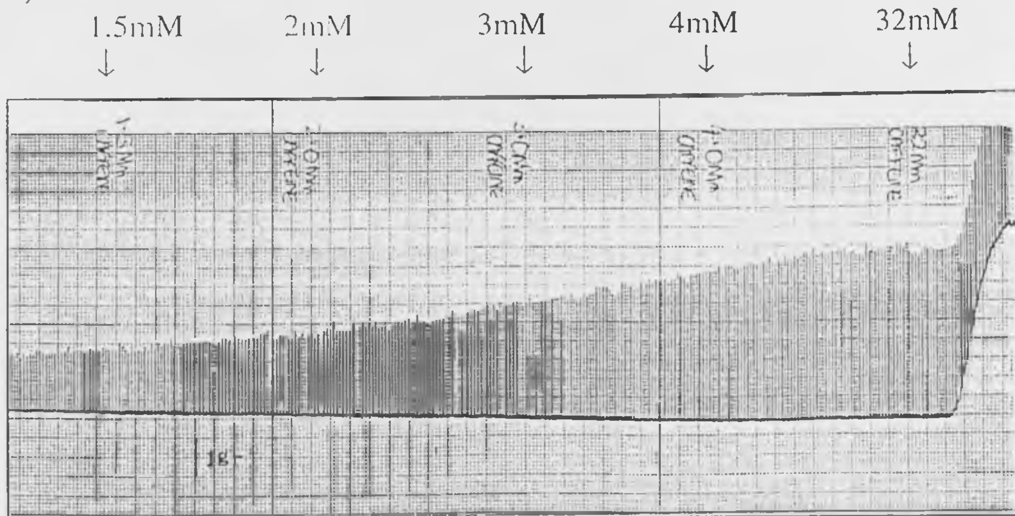


Fig 1.3 The effects of a) halothane (0.5, 1 and 2%) and b) caffeine (0.5mM to 32mM) on muscle samples from individual 1879 who shows no contracture to halothane and caffeine and is therefore diagnosed as MHN.

is required which has to be taken using anaesthetic and involves hospitalisation of the patient. This protocol is an expensive test that needs at least two nights stay in hospital, and a special laboratory and expert staff. It is possible that the variability and fibre types of muscle (type I and II) used influence the test results because the muscle bundles are a mixture of slow (type I) and fast (type II) muscle fibres (Gallant and Goetti 1989; Sudo 1995). IVCT is not absolutely specific, for example, false positive results have been obtained for patients with other known myopathies.

Although it is claimed that the fibre composition has an influence on halothane and caffeine contractures in cat, rat, and swine muscles (Brownell and Szabo 1982; Nelson and Schochet 1982), further studies indicate that the composition does not influence the discrimination between MHS responses in contracture test results in human biopsies (Heiman-Patterson *et al* 1987). Of course the halothane sensitivities for a given muscle fibre type (I or II) are different between MHS and MHN patients, but both types of MHS fibres had a significantly lower threshold compared with normal fibres (Adnet *et al* 1994). In conclusion the effects of fibre types on response to halothane or caffeine is a controversial subject and more experiments are necessary. The report of false negative results in young children (Issacs and Bandenhorst 1993; Hopkins and Ellis 1995) is another disadvantage of IVCT. Because of these problems, muscle biopsy testing of young children is seldom performed; not only it is undesirable to take the samples but also because the immature muscle frequently gives erratic responses in this test (Ellis *et al* 1986).

1.8 MH and other diseases

Most patients who have been identified as MH susceptible do not show any other disease related to it. However in some cases the association between MH and a second disease is reported (Denborough *et al* 1962; Steers *et al* 1970; Ellis *et al* 1971; Harriman *et al* 1973), for example the association of MH with neuromuscular disorders has been recognised since 1970 (Isaacs and Barlow 1970). Some diseases which have been associated with MH are as follows :

Central core disease (CCD) in muscle fibres was first described by Shy and Magee (1956) in five members from a family pedigree with three generations. The association of this apparently rare myopathy with MH was reported by Denborough *et al* (1973). It is a slow or non-progressive morphological myopathy which affects the normal development of muscle fibres in affected individuals from birth. CCD appears in patients with quite variable clinical features, ranging from being asymptomatic to severely disabled as result of muscle weakness. CCD is inherited as an autosomal incomplete dominant genetic trait, and apparently more than one genetic factor is involved in manifestation of disease (Engel *et al* 1961; Denborough *et al* 1973) although in a study in nine CCD families no heterogeneity was reported (Schwemmle *et al* 1993). The CCD diagnosis depends upon the demonstration of poorly staining "cores" in a significant proportion of type I Skeletal muscle fibres (Denborough *et al* 1973). Significant pathological changes in sarcoplasmic reticulum and t-tubules and reduction in the number of triads in CCD families was reported by Hayashi *et al* (1989). Due to the wide clinical

variation it is not clear whether this reflects a high mutation rate, low penetrance or superficial diagnosis. In subsequent studies the abnormal contraction with IVCT in a CCD patient was reported by Brownell (1988). Islander *et al* (1995) suggested that the members of families with CCD could be at risk of being malignant hyperthermia susceptible. Co-segregation of MH with CCD has been described in patients using linkage studies with chromosome 19q markers (Kausch *et al* 1991) and based on this correlation it has been proposed that these two abnormalities are allelic (Kausch *et al* 1991). With the strong association between the *RYR1* gene and surrounding markers on 19q12-q13.2, the *RYR1* gene is a likely candidate for CCD (Mulley *et al* 1991; Schwemmle *et al* 1993).

Neuroleptic malignant syndrome (NMS) is another rare disease which mimics some features of MH like muscle rigidity, high CK, increased body temperature and acidosis due to metabolic stimulation (Caroff *et al* 1987; Heiman-Patterson 1993). Triggering agents in NMS are neuroleptic drugs but not anaesthetic agents. This abnormality responds to dantrolene therapy although it is differentiated from MH by the IVCT (Ellis and Heffron 1985; Adnet *et al* 1980) and the time course of the onset of the syndrome which is hours to days in contrast with MH in which it is minutes (Rosenberg 1988; Renwick *et al* 1992). However, while the clinical manifestations of the two syndromes are similar, their underlying aetiology is different (Keck *et al* 1995).

Myotonia congenita (myotonia may be defined as a delayed relaxation of muscles) is a rare dominant trait characterised by muscle spasm after suxamethonium administration (King *et al* 1972; Heiman-Patterson *et al* 1988;

Ellis 1981). Saidman *et al* (1964) described a patient with myotonia congenita who developed malignant hyperthermia after anaesthesia. In the treatment of these MH-like events, dantrolene may be a useful drug due to its effect on the excitation-contraction coupling mechanism (Ellis 1981). This abnormality is reported in a few cases of MH patients with positive IVCT (Heiman-Patterson 1988). Usually it is associated with muscle hypertrophy and its histological appearance also has similarities with the hypertrophied muscle fibres in MHS individuals, and greater variation of fibre size than normal (Ellis 1981).

Several other abnormalities have been reported with MH. **King syndrome** is a slowly progressive myopathy with short stature. The usual manifestation of this abnormality are fever and tachycardia and elevated CK levels. It begins in childhood and patients with King syndrome should be considered as MH susceptible. This abnormality has been reported in four unrelated boys (King and Denborough 1973) and in a girl by McPherson and Taylor (1981). In a few cases it has been reported in association with malignant hyperthermia.

Osteogenesis imperfecta: an association between MH and this abnormality has been suggested. Patients suffering from this abnormality may display increasing temperature during general anaesthesia although this alteration may have a different basis (Rampton *et al* 1984). In a survey of seven patients with osteogenesis imperfecta only one of them was MH susceptible using a North American protocol IVCT (Rosenberg 1988).

Sudden infant death syndrome (SIDS) is a disorder with probably multiple aetiology reported in a few families associated with malignant hyperthermia susceptibility (Denborough *et al* 1982). Further studies have not confirmed the association between MH and SIDS (Ellis *et al* 1988).

Duchenne Muscular Dystrophy (DMD), is inherited as an X-linked recessive muscular dystrophy and is the commonest of the muscular dystrophies. During anaesthesia with halothane and or suxamethonium, tachycardia, hyperthermia, acidosis, hyperkalaemia, myoglobinuria and high level of CK have been reported (Ellis 1981; Ellis and Heffron 1985; Wang and Stanley 1986; Brownell 1988). The reactions, if detected early in their course, appear to be reversed by discontinuing the volatile drugs. Children with DMD may develop tachycardia and cardiac arrest post-operatively due to muscular weakness (Ellis and Heffron 1985). In some cases muscle biopsies from patients showed contracture on exposure to caffeine (Brownell *et al* 1983) and halothane (Rosenberg and Heiman-Patterson 1983). **Becker Muscular Dystrophy** (BMD) another type of muscular dystrophy with a few differences such as milder effects and later age of onset. Patients usually survive until middle life. DMD and BMD have both been shown to be due to defects of the gene coding for a sarcolemmal protein, dystrophin. This protein in very low levels protects the fibres (especially in skeletal muscle cells) from damage due to repeated contraction. This protein is present in an altered form in BMD patients but in nearly all DMD patients is absent. Almost all DMD patients have deletions in the dystrophin gene which give rise to alterations in the reading frame and change the coding sequence downstream

of the mutation. These events consequently cause the DMD disease. But in comparison deletions which do not alter the reading frame induce BMD. There have been no recent reports of anaesthesia problems in BMD but muscle from a BMD patient has been shown to have abnormal IVCT response to halothane (Heiman-Patterson *et al* 1988).

Hyperkalaemic periodic paralysis (HyperPP) is caused by an autosomal dominant mutation in the α-subunit gene of adult skeletal muscle sodium channel, *SCN4A* which is located on chromosome 17q (Fontaine *et al* 1990). The mutations in this subunit may be causing masseter muscle rigidity and whole-body rigidity (symptoms of MH) using succinylcholine (Vita *et al* 1995). Some individuals with hyperPP show positive contracture using IVCT (Lehmann-Horn and Iaizzo 1990). An attack of hyperPP may be induced by different factors such as cold, fasting, sleep, infection and anaesthesia and may be treated by high sodium, low potassium, high carbohydrate diet, intravenous glucose and insulin (Ellis 1981; Hopkins and Ellis 1995). **Hypokalaemic periodic paralysis** (HypoPP) has been observed in a patient with MH-like reactions and a positive response to halothane using IVCT and may be due to deregulation of Ca^{2+} (Lehmann-Horn and Iaizzo 1990; Lambert *et al* 1994). This abnormality is associated with a reduction in serum potassium concentration and is inherited as an autosomal dominant abnormality with full penetrance in males and incomplete penetrance in females. The locus for hypoPP is reported to be located on chromosome 1 at q31-32 (Fontaine *et al* 1994). An attack of hypoPP can be induced by anaesthesia and other factors such as heavy exercise, stress, or by eating a heavy meal especially if it contains a high proportion

of carbohydrate. It can be treated with combined low sodium and high potassium therapy (Hopkins and Ellis 1995).

These abnormalities are clinically and genetically distinguishable from each other. Indeed the disorders mentioned reported to coexist with MH are infrequent and do not represent a typical presentation of MH. On the other hand the positive IVCT results in other myopathies than MH are not specific and may be based on pathophysiological mechanisms different from MH (Heytens *et al* 1992). In conclusion the patient with either myopathy or related conditions such as hernia, strabismus and orthopaedic abnormalities must be considered to be at a greater risk than the general population, or on the other hand they may be predisposed to MH if exposed to the appropriate triggers (Ellis *et al* 1972; McPherson and Taylor 1981). However these associations may be coincidental and are independently inherited.

1.9 Aetiology of MH

A genetic abnormality which leads to MH causes a primary lesion in the skeletal muscles. This is because muscle rigidity is one of the first macroscopic signs of an impending MH episode, and also the muscle relaxant dantrolene blocks development of the syndrome, and in addition many susceptible individuals and most pigs have elevated muscle-specific serum creatine phosphokinase activity (Hall *et al* 1976; Isaacs and Barlow 1970; Harrison1971; Gronert *et al* 1976; Heffron and Mitchell 1975). In an attempt to find the possible primary abnormality in MH the relation between it and the sympathetic nervous system was studied. In a well-designed study, Gronert *et al* (1977) showed that the changes in the

metabolic rate in susceptible pigs are unrelated to abnormalities of the central or sympathetic nervous system (Artru and Gronert 1980; Gronert *et al* 1988). Finally these indications of a myogenic aetiology have given rise to a very large number of studies of the biochemical basis of MH using muscle from susceptible pigs and human muscle biopsies.

1.9.1 The role of Ca^{2+}

The main cause of malignant hyperthermia induction is an alteration in mechanisms that control and maintain Ca^{2+} homeostasis and its low cytoplasmic concentration in skeletal muscle (Lopez *et al* 1985; Heffron 1988). Lopez *et al* (1988) in a study measured free Ca^{2+} during and after an MH episode, and also, using dantrolene, proved that the concentration of Ca^{2+} increased in swine MH skeletal muscle fibres and reduced after dantrolene administration. Calcium release from the sarcoplasmic reticulum SR, the main store of Ca^{2+} in skeletal muscle is induced by membrane depolarisation. Three membranes, the sarcolemma, the sarcoplasmic reticulum membrane and the mitochondrial membrane are involved in intracellular calcium in muscle. These membranes through their constituent proteins act together in controlling calcium movements during the coupled processes of excitation and contraction coupling.

Furthermore proteins like the dihydropyridine receptor function co-ordinately to regulate the transduction of depolarisation signals from the sarcolemma to the intracellular calcium store and to effect the release of calcium into the sarcoplasm. Mutation in any one of the genes encoding these proteins

could conceivably lead to the abnormality in Ca^{2+} regulation found in MH muscle (Lopez *et al* 1988; Iaizzo *et al* 1988). Increased free Ca^{2+} in the myoplasm could result from increased release of Ca^{2+} from the SR, increased release of Ca^{2+} from mitochondria, or an increased entry of Ca^{2+} through the sarcolemma from the extracellular fluid. Although uptake of Ca^{2+} from the sarcoplasm by the SR by calcium pumps is important in calcium regulation, in surveys of calcium pumps in MH susceptibility, experiments in halothane positive and negative pigs indicated no causative role for the calcium pump which is responsible for calcium re-uptake against a concentration gradient (Louis *et al* 1992).

Iaizzo *et al* (1989) reported that the development in MHS muscle contracture is not related to alterations in the surface membrane caused by anaesthetic agents (Iaizzo and Lehmann-Horn 1989). Furthermore depolarisation is not required for contracture development in porcine MHS fibres (Gallant 1988). They indicated that Ca^{2+} significantly increases during a clinical episode with no change in the resting membrane potential Vm (Lopez *et al* 1986; 1988). The lack of detectable change in Vm during the increase in intracellular calcium concentration in an MH episode demonstrates that this increase is not membrane-potential dependent and therefore depolarisation does not appear to be related to induction of the syndrome in pigs.

In a well designed study using pig cells, halothane did not alter the resting membrane potentials of MHS or MHN surface membranes but it alters the electrical properties action potential (raise, peak and fall) of the muscle fibres. These effects were more pronounced in MHS skeletal muscle fibres but were not

related to the occurrence of a contracture (Iaizzo *et al* 1989). Based on many aetiological studies of MH, attention has focused on the SR as the probable site of the lesion. This is because the increasing Ca^{2+} concentration is fundamental to induction of the MH crisis and SR is the main store of Ca^{2+} for release and re-uptake. Therefore it is likely that any defect in SR could give rise to an MH episode (Denborough *et al* 1973; Gronert *et al* 1979).

1.9.2 Sarcoplasmic reticulum and RYR1

The sarcoplasmic reticulum is the main store of intracellular calcium, 80% of the Ca^{2+} in skeletal muscle, and therefore plays the main role in calcium homeostasis in muscle (Artru and Gronert 1980). Transfer of the neural stimulation and depolarisation of the sarcolemma, the action potential, via the t-tubule to the terminal cistern of the SR and consequent release of calcium initiates the excitation-contraction coupling. The sequence of the different events leading to the contraction of the cell in response to the nerve influx is called excitation-contraction coupling.

Two processes are involved in excitation-contraction coupling, first depolarisation of the motor end plate occurs in response to acetylcholine released from the nerve ending, and second, this causes a transient membrane permeability resulting in an influx of sodium and efflux of potassium. As a result, during excitation-contraction coupling, transmission of the electric potential from the sarcolemma propagates in muscle to the t-tubules to the SR. This process is believed to occur at triad junctions where the t-tubules and SR membranes are

close together and gives rise to a massive calcium release into the myoplasm of muscle fibre. This step is believed to result from a conformational change in the dihydropyridine receptor and direct physical coupling between two calcium channels (for explanation of the E-C coupling the two following hypotheses are ruled out as the main mechanisms, Ca^{2+} induced Ca^{2+} release which causes direct activation of the RYR channel by extracellular calcium ions via the t-tubule, and the role of second messenger for E-C coupling in skeletal muscle fibres) (Marty *et al* 1994; Ronjat 1995). The next step causes actin and myosin filaments to interact and produce the contracture (fig 1.4). A defect of the SR could cause impaired re-uptake of calcium from or uncontrolled release of calcium into the aqueous sarcoplasm. A number of independent physiological studies comparing halothane-positive with halothane-negative pigs of different breeds have detected an abnormality in calcium release from SR as the primary cause of MH (Nelson *et al* 1983; Ohnishi *et al* 1983; Mickelson *et al* 1986; O'Brien 1986). Alteration in the properties of the MHS calcium channel ryanodine receptor protein due to the halothane sensitivity gene (*Hal* locus), results in a modified SR calcium release channel activity and elevated rate of Ca^{2+} release from vesicles of the SR in MHS individuals.

The ryanodine receptor, the sarcoplasmic reticulum calcium-sensitive calcium release channel protein, has been identified as the key protein involved in the regulation of SR Ca^{2+} release. Its function is regulated by Ca^{2+}, ATP, Mg^{2+}, inositol 1, 4 and 5-trisphosphate (IP3) and calmodulin. Thus an abnormality in the ryanodine receptor protein or these factors might alter pharmacological sensitivities and gating properties of the SR Ca^{2+} release channel. RYR or ryanodine receptor

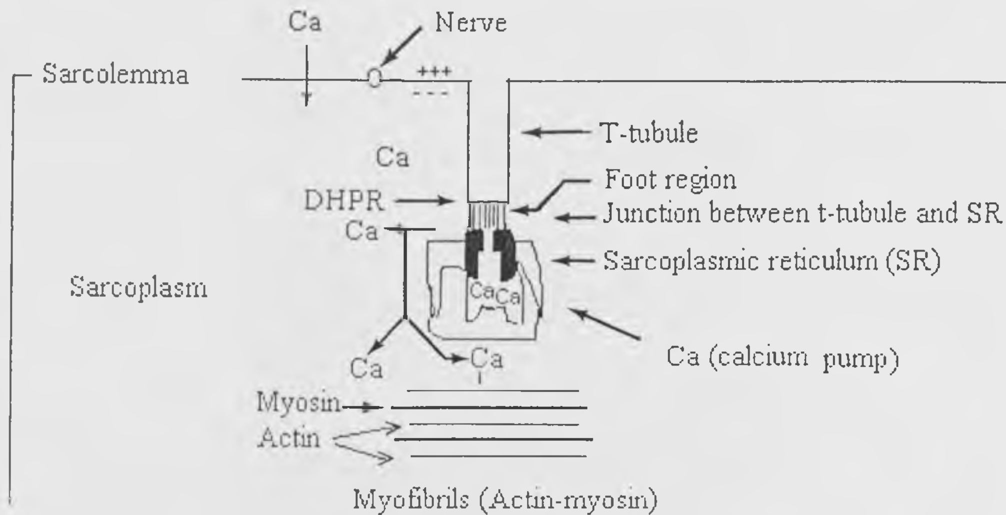


Fig 1.4 A proposed relation between Ca^{2+} ions and different factors which are involved in excitation-contraction coupling.

The process initiates when the nerve impulse reaches the sarcolemma causing depolarisation of the membrane. The generalised depolarisation of the sarcolemma activates the voltage-gated calcium channels of the t-tubules. These events activate the ryanodine receptor at the sarcoplasmic reticulum and calcium is released (different mechanisms such as calcium-induced calcium release are involved in calcium release, section 1.9.2) into the myoplasm. The increase in myoplasmic calcium concentration causes the myofibrils in the muscle cell to contract. The abnormality in the factors which are involved in excitation-contraction coupling (such as DHPR or dihydropyridine receptor in the t-tubule and ryanodine receptor or RYR in the sarcoplasmic reticulum) may give rise to malignant hyperthermia (adapted from MacLennan 1992).

binds and is blocked by ryanodine, a plant alkaloid (used as an insecticide). This is a homotetrameric complex, made from identical subunits, located in the SR membrane projecting into the myoplasm at the junction of the SR and T-tubules. Finding out more about the skeletal muscle ryanodine receptor (RYR) was achieved by Lai *et al* (1988). They purified the channel protein from rabbit SR and reconstituted it as a functional unit in a lipid bilayer. It consists of two domains, a junctional foot which spans the 10-20nm gap between the sarcoplasmic reticulum terminal cisternae and transverse tubule membranes, and transmembrane domain in the SR membrane. RYR has been purified from skeletal muscle as a 30S protein complex composed of four identical polypeptides of about 5000 amino acids.

It has been suggested that in mammals at least three distinct *RYR* genes exit and they show differential tissue expression, *RYR1* is present in skeletal muscle. *RYR2* is expressed in cardiac muscle and brain but not in skeletal muscle. Therefore this gene is not considered to be a potential candidate in MH because the model of potential-induced calcium release in skeletal muscle is not compatible with *RYR2* defects (Ogawa 1994). The *RYR3* gene appears to be expressed in a wide range of tissues including skeletal muscle (Giannini *et al* 1992) and has been proposed as a possible candidate gene in malignant hyperthermia susceptibility.

Comparison of sarcoplasmic reticulum samples from halothane sensitive and insensitive pigs suggests that RYR binds ryanodine with higher affinity in MH-susceptible than normal SR (Lai *et al* 1988; Mickelson *et al* 1988). It appears to bind when the calcium release channel is in the open configuration and blocks the channels open (Mickelson *et al* 1989). Furthermore comparison of homozygous

halothane sensitive and halothane insensitive pigs with heterozygous pigs showed that the SR isolated from the heterozygous pigs has Ca^{2+} release properties intermediate between those of halothane positive and halothane negative animals (Gallant *et al* 1989). As a result heterozygous pigs do not exhibit a typical halothane positive response to triggering agents. One explanation for this observation is that there is a mixed population of RYR1 channels in the heterozygous pigs with 0, 1, 2, 3 or all 4 of the subunits being of the mutant type. Therefore there may be adequate normal channels to avoid ryanodine blocking. Ca^{2+} flux through a heterotetrameric channel with less than four affected subunits may not be altered sufficiently to precipitate an MH crisis. Indeed the Ca^{2+} release channels in heterozygous pigs can be assembled from mutant and normal subunits that combine to form heterotetrameric channels (Shomer *et al* 1995). The SR from pigs heterozygous for the halothane-sensitivity gene, however, demonstrates intermediate values for the rate of calcium release and affinity for tritiated ryanodine. Thus alteration in SR Ca^{2+} release and ryanodine binding in pigs comparing only one copy of the halothane-sensitivity gene demonstrates a distinct intermediate phenotype and indicates a truly heterozygous state (Mickelson *et al* 1989).

These studies using SR vesicles and muscle bundles indicate that both MHS and normal SR Ca^{2+} release channel genes are expressed in the muscle of heterozygous pigs (Gallant *et al* 1989). However, SR from heterozygous pigs has more affinity for ryanodine, a lower threshold for contraction (Gallant and Jordan 1996), and intermediate properties in Ca^{2+} release compared with SR from normal pigs.

In conclusion these intermediate responses to triggering agents are further proof that the ryanodine receptor is the main cause of gene in halothane sensitivity in pigs.

1.9.3 T-tubule and DHPR

In skeletal muscle the 1,4 dihydropyridine receptor (DHPR) acts as the voltage sensor and calcium channel located on the transverse tubule (t-tubule) membrane which is a part of the sarcolemma. The t-tubules are the internal tubular membrane systems which are orientated transversely with respect to the fibre axis (Caspar Ruegg 1988). They function as an L-type voltage-dependent calcium channel and transducer of membrane depolarisation of the interior of the muscle fibre, and so fulfil a critical role in excitation-contraction coupling (E-C) and in sarcoplasmic calcium regulation. The DHPR functions primarily as a voltage sensor in E-C coupling and exhibits voltage-gated Ca^{2+} channel activity although the entry of extracellular Ca^{2+} is not required for skeletal muscle contracture (Catteral and Striessing 1992). Moreover it is suggested that the DHPR complex is a binding site for dantrolene sodium which acts to block calcium release in malignant hyperthermia therapy. The DHPR is comprised of a hetero-pentameric protein involved in excitation-contraction coupling. The purified DHPRs are composed of five polypeptide subunits, α_1, α_2, β, δ and γ.

1.9.4 Mitochondria

There is no report of a significant causative role of mitochondrial defects in malignant hyperthermia induction but they may be involved in the progress of the

syndrome once initiated. Many of the changes observed in mitochondria have also been seen in other myopathies and are probably non-specific manifestations of the disease process (Gronert and Heffron 1979). Furthermore Britt *et al* (1973) reported no difference between normal and MH mitochondria in regard to respiratory function, and later Gronert and Heffron (1979) stated that mitochondrial respiration is not greatly impaired in MH, if at all.

1.9.5 Miscellaneous

It is considered that the skeletal muscle CRC (calcium release channel) is not strongly voltage gated, and cannot be activated directly by membrane potential changes in the transverse tubule or SR membranes. Therefore the action potential at the t-tubule / SR junction for calcium release activation from SR needs a chemical or physiological signal. Among the factors being investigated to account for calcium release and MH induction, low molecular weight compounds including Ca^{2+} and inositol 1,4,5-trisphosphate have been considered as second messengers in this process (Fletcher *et al* 1995a) and intermediates which affect Ca^{2+} release.

1.9.5.1 Inositol trisphosphate

1,4,5-inositol trisphosphate, IP3, is an intracellular second messenger which acts in calcium release from intracellular stores of a variety of cell systems, including the sarcoplasmic reticulum of skeletal muscle (Berridge and Irvine 1989) and then it serves as the chemical messenger of excitation-contraction coupling. It is a product of the hydrolysis of phosphatidylinositol 4,5-bisphosphate and causes Ca^{2+} release from non-mitochondrial intracellular stores, sarcoplasmic reticulum

(Henzi and MacDermott 1992). In addition release of calcium from sarcoplasmic reticulum suggests IP3 may cause opening of dihydropyridine sensitive calcium channels in animal models (Vilven and Coronado 1988). The IP3 receptor is located in the sarcoplasmic reticulum membrane and shows some homology with the ryanodine receptor.

The increase of IP3 concentration in MHS samples is demonstrated in human and pig skeletal muscle, and thus the pathophysiological role of inositol trisphosphate in MH development is considerable especially as it is reported that halothane inhibits the hydrolysis of IP3 in swine (Foster *et al* 1989; Scholz *et al* 1991; Scholz *et al* 1993; 1995). In attempts to identify MHS susceptible individuals, using IP3 as an indicator and measurement of IP3 and IP3 5-phosphatase activity, there were no significant results (Foster 1993; Scholz *et al* 1995).

1.9.5.2 Calcium pump

As mentioned before (section 1.9.1), changes in the Ca^{2+}-ATPase are not believed to play a primary role in the pathogenesis of MH in pigs. This suggestion was supported in humans (Everts *et al* 1992; McSweeney and Heffron 1990).

1.9.5.3 Calmodulin

The function of calmodulin as a biological regulator and Ca^{2+} receptor is linked to the level of free Ca^{2+} in the cell and there is evidence that it may be involved in the control of the movements of cellular Ca^{2+}. It is formed from a

single polypeptide with four high affinity Ca^{2+} binding sites. In different studies it has been shown that the calcium release is independent of calmodulin inhibition (Holland *et al* 1992; Marjanen *et al* 1984). Although finding the relationship between this protein and MH susceptibility was reported, this is not probably the site of halothane action on sarcoplasmic reticulum (O'Sullivan and Heffron 1995).

1.9.5.4 Phospholipase A2

This enzyme has been suggested to be involved in MH induction. The increased sarcoplasmic Ca^{2+} may activate membrane-associated phospholipases (Heffron 1988). Consequently increased activity of phospholipases may result in increased membrane permeability resulting in fatty acid liberation. The enhanced activity of phospholipase A2 can cause increased Ca^{2+} release from mitochondria which causes increased Ca^{2+} release from the sarcoplasmic reticulum. This procedure is called calcium induced Ca^{2+} release (Ellis and Heffron 1985).

1.10 The genetics of MH

1.10.1 The genetics of MH

The mode of MH inheritance as an autosomal dominant has been confirmed by pedigree analysis and results of family screening using the *in vitro* contracture test (Ellis *et al* 1986). The chance of transmission of the susceptibility allele by an affected individual to each offspring based on Mendelian principles is 50%. The malignant hyperthermia abnormality in susceptible individuals appears with reduced penetrance and variable expressivity (Britt *et al* 1969). In this section

possible causative genes and their mutations which may be involved in MH susceptibility are described.

1.10.2 Genetic mapping of RYR1 in pigs and humans

The general clinical and biochemical characteristics of MH syndrome in humans and pigs are similar (Berman *et al* 1970; Britt and Kalow 1970b), and so it is possible that a similar gene or genes may be responsible for MH in humans and pigs. Genetic mapping in the pig showed that the *Hal* locus, the causative gene in PSS or halothane sensitivity, is tightly linked to the phosphohexose isomerase locus, *phi,* also known as glucose phosphate isomerase, *gpi* (Andresen and Jensen 1977). These loci were observed to be linked with the S and H blood group loci and glucose phosphate dehydrogenase, *pgd.* The gene order was deduced to be *S-(phi-Hal) - H - po2 - pgd.* This linkage group was located near the centromere of porcine chromosome 6, at p12-q21 (Archibald and Imlah 1985; Harbitz *et al* 1990). In humans *gpi* has been mapped to the q12-13.2 of region of chromosome 19q and this region shows homology with the centromere region of pig chromosome 6 (Davies *et al* 1988). Previous studies by Mickelson *et al* had indicated that the skeletal muscle SR calcium release channel in halothane sensitive pigs is abnormal and this calcium channel is the product of the *Hal* gene (Mickelson *et al* 1988; 1989). At this time MacKenzie *et al* (1990) reported that the human skeletal muscle ryanodine receptor is located on chromosome 19 in the region 19cen-q13.3.

Linkage studies in humans between MHS and markers that span 19q12-13.2 in three large Irish families indicated co-segregation of the MHS gene and the related polymorphic markers surrounding the *RYR1* gene from the *GPI* gene region

(McCarthy *et al* 1990). In this region two genes were suggested as candidates for MH susceptibility. These were *RYR1* and the hormone-sensitive lipase gene (MacLennan *et al* 1990; Levitt *et al* 1990). Identification of *RYR1* as the candidate gene was demonstrated by the observation of tight linkage between the calcium release channel and the *hal* locus in pigs (Harbitz *et al* 1990, Otsu *et al* 1991). MacLennan *et al* (1990) reported that the polymorphic markers for the *RYR1* gene co-segregate with human MH susceptibility. Confirmation of MHS linkage to 19q13 has been obtained by two groups in Germany and Britain (Deufel *et al* 1992; Ball *et al* 1993). Co-segregation of MHS and *RYR1* in human families (Healy *et al* 1991; McCarthy *et al* 1990; Ball *et al* 1991; Couch *et al* 1991b; Stewart *et al* 1991) combine with physiological data indicated that the *RYR1* gene is a highly likely candidate for the MH susceptibility, and basic defects in the *RYR1* gene underlie the MH episode (Davies 1990). In addition close linkage was demonstrated in pigs between the halothane sensitivity locus and polymorphic microsatellites flanking the porcine calcium release channel gene (Bolt *et al* 1991).

1.10.2.1 Mutations in the RYR1 gene

Linkage between the *RYR1* gene and MH made it imperative to initiate a search for sequence differences between MH and normal individuals. For the first time in a comparison of the *RYR1* cDNA sequence of MH (Pietrain) and normal (Yorkshire) pigs a single amino acid change was reported (Fujii *et al* 1991). Indeed the large size of the skeletal muscle ryanodine receptor gene with more than 204kb makes it probable that many different mutations could occur to give rise to the MHS phenotype.

The *RYR* cDNA is over 15000 base pairs long, arranged in 105 exons, two of which show alternative splicing, and encodes an RYR1 protein with 5035 amino acids (Zorzato *et al* 1990; Phillips and MacLennan 1995).

In a study of the sequence of the *RYR1* gene, Fujii *et al* (1991) found 18 single nucleotide differences between halothane positive and halothane negative pigs but no evidence for small insertions, deletions or internal stop codons. Only one of these sequence variants, the C1843T resulted in an amino acid change, substitution of Arg^{615} by Cys and was considered to be a possible causative mutation. This mutation was investigated in blood samples from more than 450 animals (halothane positive, homozygote pigs and halothane negative) from six breeds of pigs, and it can account for all cases of MH in swine (Fujii *et al* 1991; Otsu *et al* 1991; 1994). In a survey of human MHS, Gillard *et al* (1991) observed conversion of C1840 to T (Arg^{614} to Cys) in a single MH family of thirty five families investigated. As a result it was proposed as a causative gene for malignant hyperthermia susceptibility. To date eight potentially pathological mutations and further polymorphisms in the *RYR1* gene have been reported in MHS and CCD individuals. In a well designed study, Gillard *et al* (1992) reported twenty-one polymorphic sequence variants in the *RYR1* gene although most were normal variants of the *RYR1* gene sequence observed in the general population. Fourteen of the polymorphic sequences resulted in loss or gain of a restriction endonuclease site. The other four of these changes caused amino acid substitution in which only one of these, Gly^{248} to Arg, segregated with malignant hyperthermia susceptibility in a single family. In addition in this potentially causative gene seven more amino

acid changes have been reported in MHS and CCD families. These are listed in table 1.1. These mutations are clustered in two regions, between exons 6 and 18 and around exon 45 (Phillips and MacLennan 1995). Despite a few families reported to show co-segregation of MH susceptibility with the pig mutation C1840T (Hogan *et al* 1992; Moroni *et al* 1995) it was not found in 100 unrelated MHS individuals from the UK (Hall-Curran *et al* 1993). In addition it has been reported in MHN individuals diagnosed by the European IVCT protocol as normal that the C1840 to T mutation is observed (Hartung 1994.). The C487T base substitution is another mutation which is reported in the *RYR1* gene in two MHS and one CCD families (Quane *et al* 1993), the point mutation resulting in the amino acid substitution Arg^{163} to Cys. The C742 to A mutation was first described by Gillard *et al* (1992). Co-segregation of C742 to A with MH susceptibility appeared only in a single family and was proposed as a pathological mutation. This mutation has not been detected in European MHS families (data of EMHG, May 1996). The G1021A mutation reported by Quane *et al* (1994a) causes malignant hyperthermia susceptibility. This is the most common mutation observed so far in the European population and accounts for 7-10% of MHS individuals (Heytens *et al* 1995). Amino acid substitution, Ile^{403} to Met resulting from nucleotide substitution, C1209G was reported by Quane *et al* (1993) in single CCD family. Beside these mutations Quane *et al* (1994b) reported another mutation in the *RYR1* gene at A1565 which causes a transversion to C in a single MHS/CCD family. Zhang *et al* (1993) reported the G7301T point mutation in the skeletal muscle ryanodine receptor gene. This base exchange cause amino acid conversion of Arg^{2434} to His. It was found in only one CCD family. There are no further

reports detecting this mutation in MHS or CCD families. In an effort to identify new mutations the G7297 to A base substitution is reported by Keating *et al* (1994) in three CCD families. This point mutation causes conversion of Gly^{2433} to Arg and together with the Arg^{2434} to His mutation may indicate a second cluster of MHS/CCD mutations in the *RYR1* gene. In conclusion, taken together, the linkage and mutation data seem to confirm that molecular lesions in the *RYR1* gene may give rise to MH in humans in at least some families, and may be supposed that one mutation is not sufficient in MH induction in some cases or on the other hand a combination of more than one mutation, or involvement of a mutation and other factors is not impossible.

1.10.2.2 RYR1 gene and other disorders

The *RYR1* gene in addition to being a candidate for malignant hyperthermia is involved in other diseases as a possible candidate e.g. CCD and myotonic dystrophy. Myotonic dystrophy is an autosomal dominant disorder for which the causative gene has been localised on chromosome 19q13.2-13.3 (Harley *et al* 1991; Brunner *et al* 1989). The *RYR1* gene has been excluded as a candidate gene for myotonic dystrophy based on recombination between the two loci (MacKenzie *et al* 1990).

However strong co-segregation of *RYR1* and CCD with no recombination and a lod score of 11.8 have confirmed linkage between these two genes and the

Table 1.1 ***RYR1* gene mutations.** The eight putative *RYR1* gene mutations found in MH susceptible and CCD patients are listed in following table. As it is shown some mutations are very rare and observed in single cases and also all mutations are common between MHS and CCD families.

Base change at cDNA	**Amino Acid change**	**Status**	**Frequency**	**References**
C^{487} to **T**	Arg163 to Cys	MHS and CCD	3-5% 1%	Quane *et al* (1993) Fletcher *et al* (1995b)
G^{742} to **A**	Gly248 to Arg	MHS	single family	Gillard *et al* (1992)
G^{1021} to **A**	Gly 341 to Arg	MHS	~10%	Quane *et al* (1994a)
C^{1209} to **G**	Ile403 to Met	CCD	single family	Quane *et al* (1993)
A^{1565} to **C**	Tyr522 to Ser	CCD / MHS	single family	Quane *et al* (1994b)
C^{1840} to **T**	Arg614 to Cys	MHS	~5% 2%	Gillard *et al* (1991) Fletcher *et al* (1995b)
G^{7297} to **A**	Gly2433 to Arg	MHS / CCD MHS	3-5% 4%	Keating *et al* (1994) Philips *et al* (1994)
G^{7301} to **A**	Arg2434 to His	CCD	single family	Zhang *et al* (1993)

disease (Mulley *et al* 1993). These studies and the fact that patients with CCD are apparently prone to develop malignant hyperthermia may indicate that MHS and CCD are causally related or allelic.

1.10.3 Heterogeneity in MH

The term "genetic heterogeneity" is used when the same phenotype is caused by defects in different genes such that at least two genes would be involved in the same phenotype. Following the mapping of MHS to 19q12-13.2, further studies revealed lack of linkage between MHS and polymorphic genetic markers either within the *RYR1* gene or very close to it in many families (Ball *et al* 1992, Deufel *et al* 1992; Levitt *et al* 1992). In European MH pedigrees from the UK, Germany, Belgium and Scandinavia heterogeneity is considered to account for up to 50% of families investigated (Ball *et al* 1993; Deufel *et al* 1992; Fagerlund *et al* 1992). These important findings confirm that *RYR1*, or a gene in the region of q12-13.2, is not the only possible MHS causative gene. Therefore MH exhibits both allelic heterogeneity (different predisposing mutations in the same gene) and locus heterogeneity (mutation in different genes).

There are many other candidate genes which encode proteins involved in Ca^{2+} regulating levels and membrane stability by regulating fatty acid metabolism or IP3 levels in skeletal muscle. In the following section putative genes which are supposed to be involved in MH susceptibility will be detailed. The findings based on linkage between MH susceptibility and other genes, DHPR subunits and SCN4A are considerable.

1.10.4 Identification of further MHS loci

Since all cases of malignant hyperthermia are not genetically linked to defects in the skeletal muscle sarcoplasmic reticulum calcium release channel gene then identifying a gene or genes related to MH susceptibility in non 19-linked families is important. The search for other possible defective candidate genes has followed two approaches, firstly by the investigation of genes which encode the proteins involved in Ca^{2+} level and IP3 level regulation. This approach has been successful in finding interesting candidates genes or regions such as on chromosome 7q11.23-q21.1 (Iles *et al* 1994). Secondly a genome search is being undertaken to investigate the linkage relationships of MH with polymorphic markers distributed throughout the entire human genome.

1.10.4.1 Genomic search approach

Such a project has been undertaken by the European Malignant Hyperthermia Group firstly in collaboration with the Généthon Institute near Paris and more recently in collaboration with the Berlin-Buch Genome Centre. To permit genomic searching, large IVCT tested non 19q linked families (with more than one recombinant in each family) are required. It is important that they have an unambiguous MH crisis in at least one index case, exhibit a clear pattern of autosomal dominant inheritance and are sufficiently large to generate a lod score of more than +3.

For this collaborative work, eleven pedigrees (Généthon, 7 families and Berlin-Buch, 4 families) have been included in a systematic linkage study using

polymorphic microsatellites markers covering the entire human genome. To date one new MHS locus (*MHS4*) located on chromosome 3q13.1 with a lod score of over +3 has been identified by this study (Sudbrak *et al* 1995). This linkage has only been found in a single German family.

1.10.5 Candidate genes

1.10.5.1 RYR2 and RYR3 genes

In addition to the *RYR1* gene, two further ryanodine receptor genes have been identified, *RYR2* and *RYR3* (Sorrentino and Volpe 1993). These three distinct genes express different isoforms in specific locations. The *RYR3* gene appears to be widely expressed in several tissues including spleen, lung, kidney, skeletal muscle (with a level of 100-fold lower than those of *RYR1* and *RYR*2 in skeletal and cardiac muscles respectively), stomach, and some smooth muscles (Ogawa 1994). The gene for has *RYR*3 recently been reported to be localised to chromosome 15 in the region 15q14-q15 (Sorrentino *et al* 1993). Linkage studies between *RYR3* and MHS did not detect any evidence for an MH locus in this region in three non-19 linked German MHS families (cited from West 1996).

1.10.5.2 The DHPR genes

A possible second causative candidate for MH susceptibility on chromosome 17q11.2-q24 was reported by Levitt *et al* in 1992 and indicated a locus *MHS2*. In this region there are two subunit genes, β and γ (*CACNLB1* and *CACNLG*) the subunits of 1 ,4-dihydropyridine receptor (DHPR), and in addition the locus encoding another plausible candidate adult skeletal muscle sodium

channel (SCN4A)(Olkers *et al* 1992). There is only one report in which the α_2/δ subunit of the DHPR probably is involved in malignant hyperthermia susceptibility (Iles *et al* 1994).

1.10.5 2.1 α_1-subunit

The α_1 subunit of the dihydropyridine receptor is a particularly interesting candidate for MHS because it functions as a voltage sensor for excitation-contraction coupling, and depolarising muscle relaxants such as suxamethonium can trigger an MH crisis via this subunit. It may interact with *RYR1* in triggering calcium release from the sarcoplasmic reticulum. It is a tissue specific gene and three kinds of polypeptide are reported in brain, cardiac muscle and skeletal muscle. The genes encoding the brain (*CACNL1A2*) and cardiac isoforms (*CACNL1A1*) of DHPR α_1-subunits have been localised on human chromosomes 3 and 12p13 respectively (Seino *et al* 1992; Powers *et al* 1992). The skeletal muscle α_1-subunit (*CACNL1A3*) is located on chromosome 1q31-q32 (Gregg *et al* 1993a). This subunit comprises a 175 kDa peptide which contains the binding-site for Ca^{2+} channel antagonists, 1,4 dihydropyridine, phenylalkylamines, and benzothiazepines (Takahashi 1987; Catterall *et al* 1988). The α_1-subunit is the central component of the DHPR complex, forms the ion pore structure, functions as a voltage sensor (Miller 1992) and when expressed in mammalian cells functions as a calcium channel (Perez-Reyes *et al* 1989; Mikami *et al* 1989). The α_1-subunit is necessary for both slow calcium currents and voltage sensing in excitation-contraction coupling. Investigation of the α_1-subunit of human skeletal muscle DHPR reveals

that it is comprised of five segments, I, II, III, IV and linker. Sequencing the cDNA segments reveals that the α_1 subunit has a cytoplasmic II-III loop, a transmembrane segment (IS3), and an IS3-IS4 linking peptide (IS3/IS4-IS4). These are the main highly specialised components which are critical in E-C coupling (Nakai *et al* 1994, Zhang *et al* 1994; O'Brien *et al* 1995). Any abnormality in either of these regions might alter the pharmacological sensitivities and gating properties of the SR Ca^{2+} release channel. This study showed no difference in molecular structure of these peptides between MHS and MHN patients which are not linked to chromosome 19q. This study with others studies may exclude the α_1-subunit as a causative gene in malignant hyperthermia susceptibility. In a survey, linkage between MH and the locus encoding the α1-subunit, *CACNL1A3* on chromosome 1 in the region of 1q31-q32 has been excluded in nine European MH families in which there was also no evidence for linkage to other MHS loci including the 19q and 17q regions (Sudbrak *et al* 1993) although tight linkage between this gene and HypoPP has been reported (Elbaz *et al* 1995).

1.10.5.2.2 β and γ-Subunits

A second possible candidate locus for MH susceptibility (*MHS2*) was reported by Levitt *et al* (1992) on chromosome 17q11.2-q24. Three possible candidate genes are located in this area. They are the adult skeletal muscle sodium channel (SCN4A) and subunits of β_1 and γ of the skeletal muscle dihydropyridine receptor.

The DHPR β-subunit gene (*CACNLB1*) is localised on chromosome 17q11.2-q24 (Gregg *et al* 1993b), in the region which is proposed as a candidate for MHS2. This gene has been excluded as a second candidate for malignant hyperthermia susceptibility (Sudbrak *et al* 1993) for reason which are described in section 1.10.5.3.

The DHPR γ-subunit (CACNLG) is one the five polypeptides that make up the dihydropyridine receptor. In humans the *CACNLG* locus (γ gene) is tightly linked to the growth hormone gene at chromosome 17q23. This region also contains the adult skeletal muscle sodium channel gene (*SCN4A*). This gene is 12.5 kb in length and consists of four exons encoding a 222 amino acid transmembrane protein which is expressed only in skeletal muscle (Powers *et al* 1993). In a mutation screening study in the *CACNLG,* three mutations were found (Lynch *et al* 1995). Two of them were rare variants with no amino acid alteration and the other one although it caused a Gly to Ser change was also detected in normal samples and so is not pathological. In addition the γ-subunit as a candidate for malignant hyperthermia susceptibility was excluded by Iles *et al* (1993). These studies demonstrated no MH causative mutation in the γ-subunit gene of DHPR.

1.10.5.2.3 α_2/δ-subunit

The gene encoding the α_2/δ subunit (*CACNLA2*) of the DHPR is located on chromosome 7q21-q22 (Powers *et al* 1994). These two subunits result from the cleavage of a single precursor protein and are linked together by disulphide bonds. They may be involved in the regulation of calcium current density and together

with β-subunit pronouncedly increase the voltage-sensitivity of channel activation and inactivation. Evidence for linkage between markers flanking this gene and malignant hyperthermia susceptibility was observed in one large three-generation European family (Iles *et al* 1994). Subsequent studies have not repeated this observation in 30 non-19, non-17 linked MHS families studied by the European Malignant Hyperthermia Group (West 1996).

1.10.5.3 Adult skeletal muscle sodium channel (SCN4A)

In searching for further MHS genes a region on chromosome 17q has been implicated. This region showed linkage with MH susceptibility in five small North American and South African pedigrees. The adult skeletal muscle sodium channel gene is located in this region and Olckers *et al* (1992) proposed it as a candidate gene for MH susceptibility. Three point mutations in this gene have been identified in patients with myotonia fluctuans, two additional mutations cause other unique forms of myotonia and another 11 mutations have been identified within the same gene to cause hyperkalaemic periodic paralysis or paramyotonia congenita. It is confirmed that mutation in this candidate gene is associated with suxamethonium-induced masseter muscle rigidity (MMR), whole body rigidity and an abnormal IVCT (Vita *et al* 1995). But it is generally considered that there is no supporting evidence for the role of *SCN4A* in malignant hyperthermia susceptibility and the report on linkage of malignant hyperthermia to the markers in the region 17q11.2-q24 is probably due to misdiagnosis of HyperPP as MHS (Iaizzo and Lehmann-Horn 1995).

1.10.5.4 The hormone-sensitive lipase

The hormone sensitive lipase gene (*LIPE*) is another candidate gene which may be involved in malignant hyperthermia (Levitt *et al* 1990). This hypothesis is based on the abnormal metabolism of free fatty acids in MHS skeletal muscle. These fatty acids originate from triglycerides in skeletal muscle (Fletcher *et al* 1989). In MHS samples high levels of free fatty acid (two and half times the normal levels) are detected in human muscle homogenates (Cheah 1983; Fletcher *et al* 1986) and similar results have been obtained using isolated mitochondria from pigs. This increase in the concentration of free fatty acids can enhance the release of Ca^{2+} induced Ca^{2+}-release from sarcoplasmic reticulum by lowering the threshold and causing a reduction in the Ca^{2+} uptake by the sarcoplasmic reticulum (Fletcher *et al* 1990). In addition there is excessive heat production as a result of the uncoupled oxidative metabolism. The role of hormone-sensitive lipase in free fatty acid mobilisation from stored triglycerides is crucial. It is regulated by hormonal and neuronal factors (Holm *et al* 1988). Hormone-sensitive lipase (LIPE) is activated by catecholamines and inactivated by insulin. If this inactivation did not occur in MHS individuals then the resulting increase in free fatty acids would cause disruption of muscle metabolism (Levitt *et al* 1990).

During a hyperthermic crisis catecholamines are elevated which stimulates LIPE and this results in free fatty acid production (Levitt *et al* 1990). The LIPE gene has been mapped on chromosome 19q13.1-13.2 (Holm *et al* 1988; Levitt *et al* 1995). Based on this background the *LIPE* gene has been proposed as a candidate

gene in MH families which are linked to chromosome 19q (Levitt *et al* 1990; 1995; Liu *et al* 1993).

1.10.6. Genetic diagnosis of malignant hyperthermia

A genetic method for use in diagnosis should establish a procedure for screening patients or persons who are at risk of malignant hyperthermia susceptibility. If a genetic method is proved, it may be possible to provide a cheap, rapid, accurate and non-invasive screening test for those who are at risk. On the other hand the useful indication should be to find affected families in which susceptibility is associated with the MH gene. A possible way for finding MH patients probably can be carried out with a non invasive, accurate and rapid DNA test. These are based on using the potential DNA tests in MH diagnosis is by finding mutations in the *RYR1* gene and other candidate loci. But before mutation analysis can be performed, linkage analysis is necessary to determine linkage of MH in each family. Of course linkage analysis in small families is less reliable for determining linkage between markers and malignant hyperthermia susceptibility. But compared with small families, the risk in large families is lower and once the allele of a marker which is associated with MHS is identified by linkage analysis, it can be used directly in diagnosis of the genetic disorder in individual members of affected families. But it should be remembered that linkage analysis is not absolutely reliable in diagnosis of MH cases and it probably will not be without false diagnoses.

1.11 Aims of this project :

The main goal of this project has been to establish a genetic procedure to identify individuals susceptible to malignant hyperthermia. If a genetic method is found, it may be possible to provide a cheap, rapid, accurate and non-invasive screening test for those who are at risk of malignant hyperthermia susceptibility.

Two approaches have been adopted. Firstly genetic linkage analysis has been used to identifying the chromosome (haplotype) and region on the chromosome that carries the defective gene in a particular family. Secondly, mutation analysis has been performed on one candidate gene.

Chapter two

Human gene mapping

In gene mapping the location of a disease locus can be detected by using physical methods such as in situ hybridisation and somatic cell hybrids and linkage analysis.

2.1 Physical methods

The somatic cell hybrid method (Szybalski and Szybalska 1962; D'Eustachio and Ruddle 1983) is based on the fusion of cultured cells and formation of heterokaryons from different species (human and rodents, usually mouse and hamster). The development of methods to hybridise human with mouse cells allowed the assignment of many genes to specific chromosomes and the construction of a human linkage map by Ephrussi and Weiss (1969). In situ hybridisation or FISH (fluorescent in situ hybridisation) is another technique in physical mapping and based on using a DNA probe which is labelled by incorporation of modified nucleotides (such as biotinylated nucleotides) or reporter molecules. Following hybridisation of the probe to metaphase chromosomes, after denaturation, the chromosome preparation is incubated in solution containing fluorescently labelled affinity molecules which bind to the reporter on the hybridised probe. The results of FISH can be observed by fluorescent microscopy.

Mapping of genes using associated chromosomal aberrations such as deletions, translocations or inversions especially in human cancers is another

technique in physical methods. Some chromosomal aberrations affect more than a single gene and so cause a complex phenotype syndrome to appear. Sometimes a specific disease is regularly associated with a specific aberration (deletion in Duchenne muscular dystrophy disease or some syndromes like Cri du chat which is caused by deletion in chromosome 5p). This method was involved in the initial identification of the gene responsible for FAP (familial adenomatous polyposis) gene mapping in which a possible tumour suppressor gene is defective. In tumour cells the high incidence of particular aberrations such as deletions and translocation is not unusual. Finding the correlation between specific disorders and related genes may contribute to gene mapping.

Gene mapping became a practical possibility using linkage analysis based on the segregation of alleles from many co-dominant polymorphic loci which can be analysed at the DNA level. This is based on segregation of two loci or more, using RFLPs (Restriction Fragment Length Polymorphisms) (Botstein *et al* 1980) and hypervariable markers (VNTRs). The genetic map distance between two genes is estimated by the number of cross over events on a single chromosome strand (chromatid) between two loci during meiosis as a proportion of the number of opportunities for observing these events. Here gene mapping based on linkage analysis is generally described.

2.2 Linkage analysis

2.2.1 Introduction

Two distinct ways for finding the genetic locus which gives rise to a genetic disorder can be supposed. The first one is based on previous knowledge of biochemistry and physiology of the disease. For example the haemoglobin and phenylalanine hydroxylase loci are candidate loci for haemoglobinopathies and most cases of phenylketonuria (PKU) respectively (a few percent of PKU cases are due to mutation in the dihydrobiopterin synthetase or dihydropteridine reductase gene).

The second approach does not require any knowledge of the biochemical nature of the trait or the nature of alterations in DNA responsible for the trait and isolation of a specific gene is not required. This method is dependent on genetic linkage. It uses many genetic markers at known locations on all the chromosomes to examine them in a collection of families with the condition. This process is known as genomic search. Finding a marker that co-segregates with the condition when statistically it is significant can identify the probable location of the disease gene close to the marker locus. Further searching within the candidate region is necessary to identify the related gene or genes which may be expressed in the relevant tissues and finding probable mutations in a particular gene which are associated with the condition. This process is known as positional cloning. This approach has been successfully applied in diseases where their causation previously was not clear, like cystic fibrosis (Tsui *et al* 1985; White *et al* 1985).

Genetic linkage analysis is a method that allows the localisation of genes in the absence of any information about the biochemical defects that cause such disorders. It has been particularly useful for the mapping of disease gene loci. Linkage analysis is a technique that uses polymorphic markers to find the chromosomal location of a disease causing gene. The method relies on availability of markers (proteins or DNA) which detect polymorphisms. The existence of polymorphisms in linkage analysis is very important and it means when two or more alleles are at significant frequency in the population where the rarer allele has a frequency greater than 1%. It is based on the investigation of the segregation patterns of the alleles of a large number of polymorphic DNA markers distributed throughout the entire genome with the disease phenotype in affected families. Indeed using the linkage analysis approaches to human disorders was started with the successful identification of linkage between the loci for colour blindness and haemophilia on the X chromosome (Bell and Haldane 1937). The technique relies on the meiosis events in diploid germ cells. Before explanation of gene segregation during meiosis the brief description below is not without benefit.

2.2.2 Principle of linkage analysis

In 1903 Sutton and Boveri independently proposed the chromosome theory of hereditary and according to their theory the chromosomes carry the hereditary factors or genes (as cited in Goodenough and Levine 1974). As it was clear that the genes are located in a linear fashion on the chromosomes (Morgan, Bridges and Sturtevant 1913, as cited in Vogel and Motulsky 1979) then it was logical that the genes on the same chromosome will be transmitted together. But

cytogenetically it is known that during meiosis certain chromosome segments are exchanged between homologous chromosomes (Bateson, Punnett, Saunders 1908, as cited in Vogel and Motulsky 1979). Based on Mendel's law the chance of assortment and transmission of genes of paternal and maternal origin should be equal. The two terms, "*coupling*" (genes are localised in a doubly heterozygous parent on the same chromosome AB/ab) and "*repulsion*" (when they are localised on homologous chromosomes Ab/aB) were introduced. But for these two aspects Morgan and his colleagues used the "*linkage*" term and they supposed that these are two features of the same phenomenon observed in the fruit fly, *Drosophila melanogaster*.

These events (coupling and repulsion) of genes assortment are based on gametic cell division or meiosis. Meiosis comprises two divisions, meiosis I and II which via different processes produce haploid cells (fig 2.1). The main difference between these two division is the distribution of gametic materials (chromosomes) to daughter cells with similar copies of DNA. The main events in meiosis I from the point of view of linkage occur in prophase with formation of bivalents (homologous pairs of maternal and paternal chromosomes come together) and synapsis. The conformation of the synaptonemal complex between homologous chromosomes permits recombination (crossing-over) between bivalents. Crossing over was first proposed by Morgan (1910, as cited in Vogel and Motulsky 1979) to explain the formation of recombinant combinations of genes that were shown to be linked by genetic data. It is caused by breaking in sister chromatids (after replication each parental chromosome consist of two chromatids which are called sister chromatids, fig 2.1) at chiasmata, these are physical connection points

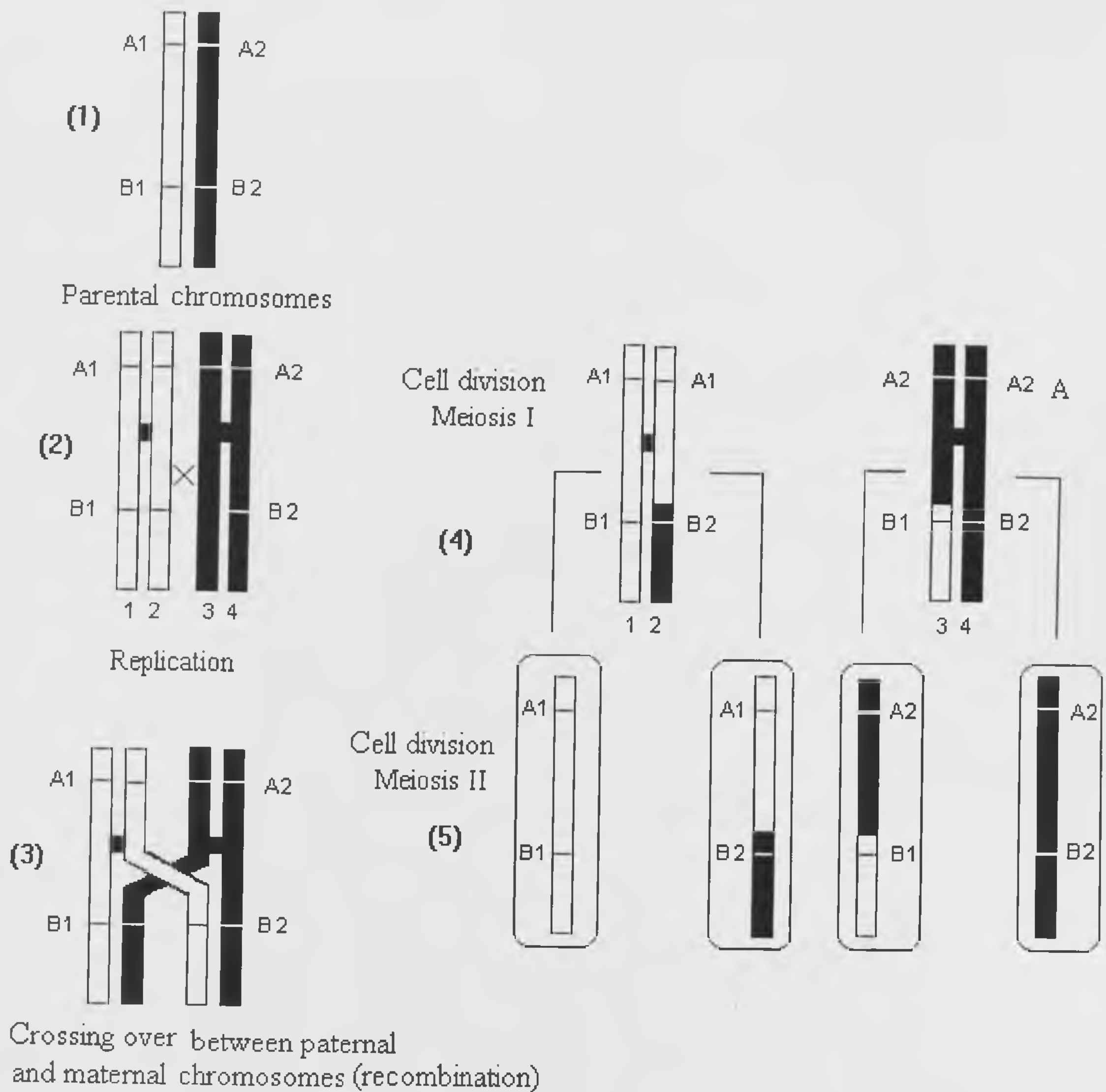


Fig 2.1 Segregation and recombination between alleles at loci A and B during meiosis and gametogenesis.

After replication of parental chromosomes the chromosomes come close together during prophase of meiosis I and via chiasma formation and crossing over between chromatids may yield new combinations of chromosomes. Cell division in meiosis I results in two diploid cells with duplicated recombinant chromosomes. Cell division in meiosis II results in four haploid cells with unique recombinant chromosomes. Depending on the distance between loci the frequency of formation of recombinants (A1-B2 and A2-B1) or non-recombinants (A1-B1 and A2-B2) haplotypes will vary.

between chromatids from different parents that are believed to be the cytogenetical sites of recombination and were first reported in amphibians. This results in exchanging the chromatid fragments in a reciprocal fashion. In addition double cross overs can occur between two, three, and four chromatids in a bivalent. Direct cytogenetical evidence that homologous chromosomes exchange parts during crossing over was first obtained in 1931 by Stern in *Drosophila melanogaster* and Creighton and McClintock in maize (as cited in Vogel and Motulsky 1979). In each chiasma two of the four chromatids in the bivalent are involved. The location of crossing over is not random and it is supposed that there are preferential sites due to the existence of interference and recombinational hotpoints.

During meiosis the alleles of loci on different homologous chromosomes undergo independent assortment (Mendel's laws), i.e. they will be inherited together with a probability of 0.5, whereas loci close together on the same chromosome tend to be inherited together with probability greater than 0.5.

Linkage between two loci is measured by the recombination fraction (RF or θ), which estimates the distance separating the two loci. The recombination fraction is defined as the proportion of recombinant offspring. It is used to estimate the distance between two or more loci in constructing genetic maps (Ott 1991). The recombination fraction can be expressed as:

$$RF = \frac{\text{total number of recombinants}}{\text{total number of recombinants and non recombinants}}$$

Since the recombination fraction is an estimate of an absolute value of the genetic distance it is important to know the error involved in its estimation. The standard error (SE) of a recombination fraction is expressed by below equation where the n>30 :

$$SE = RF \pm \sqrt{\frac{\text{recombination fraction} \times \text{non-recombination}}{\text{total number of offspring (n)}}}$$

The percentage RF is presented in centi-Morgan (cM) as an estimate of the genetic distance or map distance between two loci for short distances (< 10 cM). This distance physically for example, 1% recombination, corresponds to a genetic map of 1 cM or 1 map unit. This correlation is not accurate for larger values of recombination fraction (Haldane 1919). Then for longer distances the linear relationship between recombination fraction and genetic distance breaks down mainly because of multiple cross-overs occurring (between two loci) and interference, a phenomenon which reduces the chance of a cross-over event occurring in the close vicinity of another cross over. Mapping functions have been proposed for relating recombination fractions (θ) with genetic map distances. In human genetic linkage analysis the mapping function which is commonly used is the Kosambi function but other map functions have been proposed where the estimation of genetic map distances show differences between these methods. For example, in Haldane's map function θ =0.27% translated to 39 cM but in Kosambi's mapping function this θ value is equal to 30 cM (cited from Terwilliger and Ott 1994).

RF indicates the likelihood that two linked loci will be separated by a cross over during meiosis. On the other hand RF is the probability that a gamete produced by a parent is a recombinant. The two, three or more unlinked alleles on the same chromosome are equally transmitted to the same or to different gametes duration meiosis. Figure 2.1 shows a simple method of gene segregation in one tetrad, one pair of replicated homologous chromosome in gametogenesis of germ cells. Recombination events that occur at random positions between paired homologous chromosomes at meiosis make it more likely that loci located close together rather than more distant loci, are inherited together (fig 2.1). For instance we can assume the alleles of A1 and B1 (from A and B loci) are closely linked together with a high chance of segregation to the same gamete. So during meiosis the probability for co-segregation of A1-B1 alleles to one gamete will be greater than the A1-B2 combination or haplotype. The haplotype is a set of different genes or markers received by an individual from one parent on one chromosome. Then the A1-B1 combination will appear more frequently amongst the offspring but if not, the combination of A1-B2 or A2-B1 will be possible.

On the other hand, in the segregation of unlinked alleles the production of new recombinant or non-parental gametes is 50% but this rate will be altered for linked genes or markers and close genes will appear together more frequently than 50%. In this method of genetic linkage analysis, two genetic markers are said to be linked if they segregate together in pedigrees more often than by random chance.

If an allele of a DNA marker is linked to a disease gene they will tend to co-segregate during meiosis, owing to the fact that meiotic recombination occurs

infrequently between closely linked loci. If a specific marker is close to a disease gene they co-segregate together; if not the frequency of recombination between two markers can indicate their distance. Thus simple segregation of alleles can be used as a background in linkage analysing three.

2.2.3 Markers used for linkage investigations:

Using markers in human genetics research and diagnostics may be referred to the 1910-1960 period during which the blood groups and were helpful. In the period up to 1960's gene mapping studies had been carried out using blood group and phenotypic markers. After that HLA (human leukocyte antigen) tissue types in 1970 were used although these were limited to a specific chromosome. The use of RFLPs (restriction fragment length polymorphisms) in 1979 (Solomon and Bodmer 1979) as a tool in genetic analysis was an important step in the development of linkage markers (cited from Botstein *et al* 1980). These markers were found to be associated with disease and may be of value in diagnosis and gene mapping (Solomon and Bodmer 1979). Subsequently Botstein *et al* (1980) proposed RFLPs as the solution to the marker problem for genetic analysis in humans. These markers, although first discovered in yeast strains, but rapidly introduced in human as markers in haemoglobinopathies (Maniatis *et al* 1978; Kan and Dozy 1978). DNA VNTRs (variable number of tandem repeats) or minisatellites in 1985 (Jeffreys *et al* 1985; Nakamura *et al* 1987) and DNA STRs (short tandem repeats) or microsatellites in 1989 (Weber and May 1989) were introduced as markers in molecular genetic with higher numbers of alleles in comparison with RFLPs.

A marker's usefulness for linkage analysis depends on the number of alleles and their gene frequencies which determine its degree of polymorphism. The degree of polymorphism may be measured using two parameters, the heterozygosity and polymorphism information content or PIC value. These measurements depended on two factors, the number of alleles and allele frequency. Botstein *et al* (1980) introduced the PIC value for a genetic marker in place of heterozygosity for using in linkage analysis. Heterozygosity simply estimates the frequency of heterozygotes for a genetic marker in a population but the PIC value estimates the frequency of informative matings for that marker. For example in an A1A2×A1A2 mating half of the progeny are heterozygous (A1A2) and so they are not informative for linkage analysis because they could have inherited the A1 allele from either parent. But using markers having more than two alleles the chance of informative meioses will increase as heterozygous matings of the type A1A2×A3A4 will occur which are fully informative. Markers with multiple alleles show higher heterozygosity and PIC comparison with RFLPs. The RFLPs or restriction site polymorphisms have usually two alleles with low PIC and a maximum heterozygosity of 0.5 if the their alleles have equal frequency. But the introduction of VNTRs provided markers with multiple alleles and therefore most people are heterozygous for different alleles and so their PIC value can be very high. Values of PIC from a marker with several common alleles (VNTRs) can reach to 0.99 in comparison with the maximum PIC value of 0.375 for an RFLP marker with only two alleles. The PIC and heterozygosity will increase where the alleles have equal population frequencies. This can be described for two alleles of

an RFLP marker with frequencies of 0.5 and 0.5, or 0.46 and 0.56, or 0.03 and 0.97 where the PIC values of the first two loci are more than third one.

Using PCR as a technique in DNA amplification of microsatellites using small amounts of DNA (about 100ng compared with 10µg for RFLPs) of was another advantage of STRs. With concern to the last two groups of markers (minisatellites and microsatellites) these will be described as follows.

The human genome contains repeated DNA sequence families which are composed of tandem and individual unit repeats. These segments are generally non-coding sequences interspersed in all chromosomes and are more likely to be present in introns or between genes. These tandem repeats include satellite DNA, minisatellites and microsatellites. Satellite DNA's ("satellite" means the highly repetitive non-transcribed DNA) are long segments found in the centromere area of some chromosomes and are not suitable for linkage analysis. Minisatellites are long arrays of tandem repeats and are not common in all chromosomes; the majority of these are found in or near the centromere and telomeres. Microsatellites are found in large numbers of very simple repeats, and are highly informative sequences.

After using PCR in DNA amplification in 1985, discovery of these repeats in 1989 was an important step in molecular biological techniques and caused rapid development in molecular genetics. These short repeats are found in the genomes of most eukaryotes except yeast. These sequences, compared with minisatellites or variable number tandem repeats (VNTRs), are more common and contain short motif in repetition. These are small tandem repeats which are simple in sequence

(1-4bp) and interspersed throughout the genome (e.g. *WUT1.9*, chapter three). The number of copies of the repeat units at any chromosome position usually varies from person to person (or chromosome to chromosome) so different lengths of these repeat sequences are present in the population. These segments are usually non-coding DNA with specific genetic map locations and so these loci are being used increasingly in gene mapping and genetic diagnosis (Weber and May 1989; Hearne *et al* 1992). They are used for amplifying the region containing the sequence of interest by using the polymerase chain reaction. The length of the repeat units or alleles found on the pair of chromosomes in each individual may then be resolved by gel electrophoresis.

In linkage analysis the DNA markers used are generally either sequence variations giving rise to restriction fragment length polymorphism (RFLPs) or polymorphic short tandem repeats (STRs) commonly CA dinucleotide repeats. RFLPs normally have two detectable alleles, one of which contains the specific restriction enzyme site while the other one lacks this site. These markers due to poor informativeness usually are of limited value in genetic analysis

In linkage analysis a useful microsatellite or marker should be typable using an easily available source of DNA such as epithelial cells in mouth washes, or white blood cells in venous blood samples and as well as it must be inherited in a simple codominant pattern. It must reveal a polymorphism, that is the existence of two or more alleles at significance frequency in the population under investigation. The usefulness of the marker depends on its informativeness. A marker is informative when it is possible to distinguish the two alleles passed to offspring

(figs 2.2 and 2.3 show phase known and unknown in two and three generation pedigrees respectively). The alleles of a marker are not of value when their frequencies are close to zero or one; the locus is then not very useful since then there is a good chance that both parents in a family will be homozygous for the same allele. Then a more polymorphic allele is more suitable for linkage analysis. It should have a known chromosomal location and preferably sub-chromosomal location. Finding suitable markers in genome mapping is an important step which is stated from early this century and usually many attempts were carried out to guess the total length of haploid genome and the number of markers (qualified markers) which are needed for this purpose. Following this idea and based on the estimation of total length of the haploid genome which is supposed to be 3300 cM (Renwick 1969) it was estimated that 200-300 markers spaced at 10 cM intervals are needed for the entire mapping of the human genome 3×10^9 bp (Solomon and Bodmer 1979). But Botstein *et al* (1980) proposed that 150 markers would be needed for entire genome mapping with 20 cM intervals. The map of markers for human genetic linkage is rapidly improving. The first DNA polymorphism map to cover the genome was published in 1987 by Donis-Keller *et al* and had an average spacing between markers of 10-15 cM. However recently a comprehensive genetic map of the human genome was published by the Généthon group (Dib *et al* 1996). This map consists of 5 264 short tandem $(AC/GT)_n$ repeat polymorphisms. The map spans a sex-average genetic distance of 3 699 cM and the average interval size is 1.6 cM which identify 2 335 resolved map location.

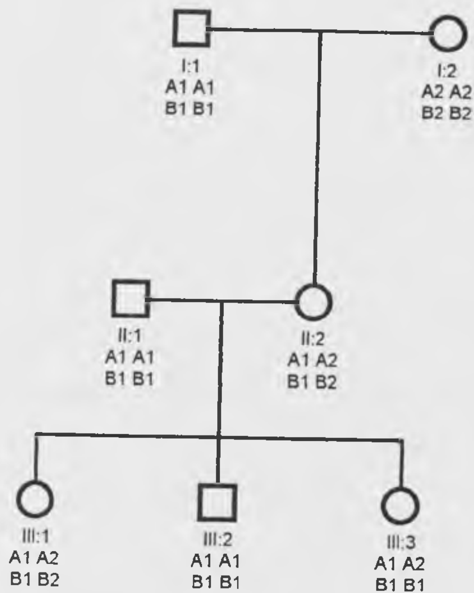


Fig 2.2 Segregation of alleles in a phase known three generation pedigree.

Segregation of alleles *A1* and *A2* at locus A, *B1* and *B2* at locus B in a three generation family. In this family phase in the doubly heterozygous parent, II.2 can be determined from the grandparents, as II.2 must have received *A1* and *B1* alleles from his father, I.1 and *A2* and *B2* alleles from his mother, I.2 and therefore the phase is defined with A1B1 in " coupling " or on the paternally derived chromosome.

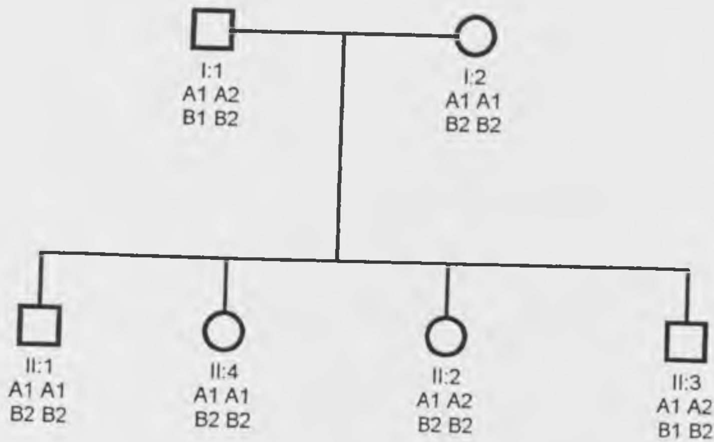


Fig 2.3 Segregation of alleles in a phase unknown two generation family at loci A and B.

In this two generation family the phase of the doubly heterozygous parent, I.1, is unknown. It is equally likely that I.1 carries *A1* and *B1* on the same chromosome as *A1* and *B2.* Therefore it is not possible to define which of his offspring are recombinant.

2.2.4 Computation of lod scores

To assess genetic linkage in humans, especially when the three-generation pedigrees are not available then it is not possible to count recombinants so a direct estimation of recombination fraction is not possible. For this reason an indirect statistical approach is used which is based on maximum likelihood estimations. This method was introduced by Morton (1955) and it can combine the linkage data from phase known and unknown sources in lod scores calculation. Then in following this method is described.

A likelihood ratio, L is estimated by computing the ratio of the likelihood of the two loci being linked at a defined genetic recombination fraction (θ) against likelihood of the two loci being unlinked ($\theta = 0.5$) or on the other hand

$L(\theta)= L(\theta)/L(0.5)$ or,

$$L = \frac{\text{likelihood of linkage at a given value of } \theta}{\text{likelihood that two loci are not linked } (\theta = 0.5)}$$

In linkage analysis this " L " ratio is called *odds ratio* or *odds for linkage*. The introduction of the lod score method made the analysis of human linkage data practical (Morton 1955). In many two generation families there is no information on the phase of the double heterozygote. Using lod scores has the advantage that information from phase known and phase unknown linkage data can be combined. Evidence for linkage is expressed statistically as a maximum likelihood expression as a logarithm to base ten of the odds on linkage (lod) and indicated by "Z".

$$Z = \log_{10} \frac{\text{Likelihood of linkage at a given value of } \theta}{\text{Likelihood that two loci are unlinked } (\theta = 0.5)}$$

or $\quad Z(\theta) = \text{Log}_{10}L(\theta) = \log_{10}[L(\theta)/L(0.5)]$

Lod scores are used in confirming or refuting the linkage between markers and assignment into regions or candidate gene. One example of a lod score calculation for a three generation pedigree showing segregation of an autosomal dominant disease and an assumed locus as illustrated in fig 2.4 is carried out as following:

In the second generation, in individual II.1 who is doubly heterozygous the phase is known and alleles 3 (from the test locus) and D (from the disease locus) are transmitted from his father, I.1, and alleles 2 and N (normal allele of disease locus) from his mother individual I.2.

In the third generation, the alleles of 3 and D are transmitted together to individuals, III.1, III.3, III.5, III.8 and III.10 as parental combinations of alleles or non recombinants. However individual III.6 has inherited alleles 2 and D from her father and so is a recombinant type. The lod scores could be calculated as following:

Chance of individual II.1 transmitting allele 3 to III.1 (III.1 and eight more individuals of third generation are non-recombinant) is ½ and

chance for transmitting of alleles 3 and D if are linked is $½\times(1-\theta)$ (the $1-\theta$ = non-recombination fraction) and if are unlinked is $½\times½$

then likelihood of observing the haplotype of III.1 is $½\times(1-\theta)/½\times½$.

The likelihood of observing individuals III.2 to III.5 and III.7 to III.10 are identical to III.1. In individual III.6, who is recombinant chance of linkage

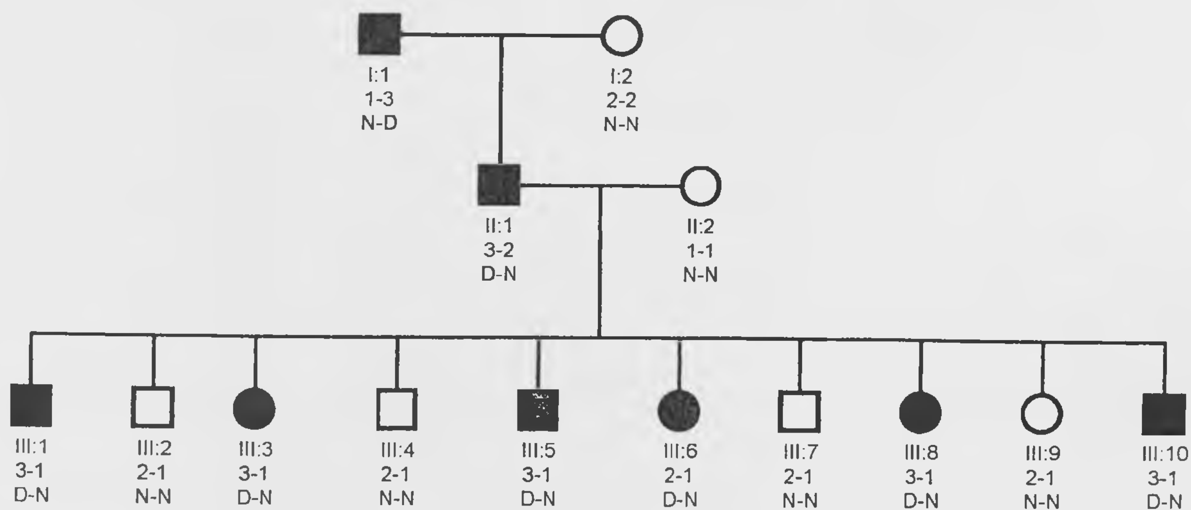


Fig 2.4 One sample of a three-generation family used in lod score calculation.

Based on the marker segregation the lod score calculation is carried out at three different value of recombination fraction or $\theta = 0.05$ and $\theta = 0.1$ (see text).

between alleles 2 and D is $\frac{1}{2}\times(\theta)$ and if they are unlinked is $\frac{1}{2}\times\frac{1}{2}$ and likelihood is equal to $\frac{1}{2}\times(\theta)/\frac{1}{2}\times\frac{1}{2}$. The likelihood for whole family is equal to

$L(\theta) = L(III.1)^9\times L(III.6)^1 = [(1-\theta)/\frac{1}{2}]^9\times[(\theta)/\frac{1}{2}]^1$ and so is equal to

$L(\theta) = [2(1-\theta)]^9\times(2\theta)^1$ and lod score is equal to: $Z = \log_{10} L(\theta)$ $= \log_{10} [2(1-\theta)]^9\times(2\theta)^1$.

For this family the calculation of lod scores with two hypotheses, (a) when the RF (θ) is 0.05 and second (b) when the RF is 0.1 is followed as below:

for (a) $L = [2(1-0.05)]^9\times2(0.5)^1 = 32.268$

$Z = \log_{10} L(\theta)=+1.508$

(b) for the second situation when $\theta = 0.1$ the chance for linkage is 90% and for recombination is 10%. Then as (a) $L = [2(1-0.1)]^9\times2(0.1)^1 = 39.671\times$ and $\log_{10}$ for L which is indicated by "Z" is equal to +1.598 which is the maximum lod score.

A lod score of zero means the assumptions of linkage or non-linkage are equally likely [$\log_{10}(1) = 0$]. This level of statistical proof is derived from a Bayesian calculation which assumes a prior probability of linkage of any two loci on the human genome to be 1 in 50. Lod scores are usually calculated for recombination fractions of 0.00, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50. The lod scores at recombination fraction of 0.00 and 0.01 show very close linkage and up to 0.40 represent loose linkage.

2.2.5 Practical considerations

In linkage analysis the size of pedigree is important as well as the number of generations. For instance in a two generation family segregation between the disease gene and marker may be difficult to interpret because in the phase unknown double heterozygote it is not clear which two alleles were received as a haplotype from one parent. This problem probably will be removed in the third generation. In fig 2.3 in the first generation it is not clear which markers are syntenic in I.1, A1B1 or A1B2 but in the second generation in fig 2.2 the phase will be known and the identification of recombinant and non-recombinant offspring is unambiguous. Finally despite the importance of human genetic analysis in clinical applications and preventive diagnosis it is faced by some problems. The long generation times, small family size, limitation in crosses and non-paternity can be problems.

2.2.6 Two point analysis

In two point linkage analysis, pedigree data are used to check whether two loci are genetically linked, to estimate the recombination fraction between the two loci and to evaluate the support for this linkage. In this manner evidence for linkage is considered significant when the lod score is +3 or more and a lod score -2 or less is taken as evidence against linkage at that recombination fraction. The lod score can be calculated for any value of the recombination fraction or " θ " between 0.00 (complete linkage) and 0.5 (no linkage, 50% recombination).

In this project linkage analysis between hypervariable microsatellite markers and disease locus for MH susceptibility was done by using the LINKAGE

computer programme (version 5.1) (Terwilliger and Ott 1994). The LINKAGE programme estimates the likelihood of linkage between gene disease and makers.

There are a few programmes for linkage analysis and lod score calculation. Two of these are LIPED (for LIkelihood in PEDigrees)and LINKAGE. Both programs calculate the likelihood of linkage from family pedigree data given the various parameters such as recombination fraction and gene frequencies. The LIPED programme is designed for pairwise analysis loci (two point analysis), while the LINKAGE programmes are not limited in the number of loci that may jointly be analysed. LIPED is written in FORTRAN but the LINKAGE programme is in the Pascal language. The major difference between these two programs is in definition of penetrance, while LIPED uses penetrance in a general sense the LINKAGE program use the penetrance as the probability of being affected given a genotype (based on affection status locus) and inducing liability or penetrance class. The LINKAGE package consists of several analysis programmes such as MLINK, ILINK and LINKMAP which calculate two point or multipoint likelihoods in human pedigrees. MLINK calculates lod scores for given set parameter values such as a set of recombination fractions in interlocus intervals. ILINK estimates recombination fractions, but LINKMAP program assumes a fixed map of markers and calculates likelihoods for a new locus at various points in each interval along the known map. ILINK is a program for two point analysis and only estimates the maximum likelihoods. In linkage analysis based on the pedigree drawing the MLINK option (using Cyrillic package version 2.0 which is used for pedigree drawing and published by Cherwell Scientific publishing Ltd 1996)

automatically creates two files, PRE files for pedigree and DAT files for linkage analysis (Ott 1991).

2.2.7 Multipoint analysis

The next step in linkage analysis is multipoint analysis, using two, three or more markers and disease status. In multipoint analysis the closely linked markers which may be informative in different individuals in the pedigree are analysed then a more definitive lod score may be obtained. In two point linkage analysis when an individual may be informative for one marker but others in the same pedigree may be uninformative or homozygous and vice versa for other markers, pairwise lod scores cannot combine information from more than two loci. This situation can affect the results in two point analysis but in multipoint analysis this disadvantage does not arise. The other advantage of this analysis method is that it is useful in establishing the order of a certain set of linked loci on a chromosome.

2.3 Genetic diagnosis of diseases

In genetic diagnosis for an inherited disease (here MH) it is necessary to follow the following steps :

2.3.1 Linkage studies

Finding marker(s) on known chromosome which are closely linked to disease locus and study of association of these markers with disease phenotype in family members. In this step using pedigrees for following the transmission of disease and linkage analysis method is a prerequisite. Having distinguishable and

informative alleles in progenies and a clear mode of inheritance in marker selection are important. In the following pedigrees the examples for informative and uninformative markers are presented. This method is fully described in section 2.2.

2.3.2 Identification of the candidate gene

The next step is identifying the region in which the causative gene is located, using flanking markers and results of linkage analysis may give rise to the determination of a possible abnormal gene. Once a linkage region has been identified it has to be searched for candidate genes either by screening candidates indicated by other studies for example in physiology, biochemistry, pharmacology etc. or by positional cloning strategies.

2.3.3 Identification of mutations associated with the disease

When the gene is recognised, sequencing and screening for causative mutation(s) and following the mutation(s) in susceptible and normal individuals will be necessary (some polymorphism in genes which are normal does not mean a mutation and these base changes need not result in alteration of gene function). The search for existence and co-segregation of causative mutation in people who have suffered the specific disease and lack of this mutation are essential at this step.

2.3.4 Determination of the causative nature of a mutation

After these last steps and making enough results the next important step is introducing the mutant candidate gene into a line of normal animals and then analysing the results from the transgenic line.

2.4 Malignant hyperthermia and genetic methods

In genetic analysis of malignant hyperthermia two methods, the DNA-based test for MH diagnostic and linkage programme for linkage analysis were used as briefly described as below.

2.4.1 Using the linkage programme

For two point linkage analysis, lod scores were calculated using the MLINK option of the LINKAGE program (Version 5.1) on a personal computer (DX4 486 100MZ). Linkage contains different ability to calculate recombination fractions, likelihood, lod scores with two alleles or more in two point and multipoint analysis in two generation or three generation. The MLINK program which is used in this study has the ability to calculate the lod score and risk analysis with two or more loci. This programme in two point analysis calculates the likelihood of the data on two alternative assumptions, when two loci are linked at a given recombination fraction, and when two loci are unlinked. But in multipoint analysis using a few loci and the disease locus together with different locations for the MH locus between the marker loci. Pedigrees were created using the Cyrillic programme,

version 2.0. Penetrance for MH susceptibility was taken to be 0.98 allowing for 2% false positive rate in IVCT, and it was taken as 0.02 (a false negative rate or frequency of phenocopies) for all individuals typed as MHN. All untested individuals were given the disease status unknown and the frequency of MH allele for the disease gene in population assumed to be 0.0001. MHE (malignant hyperthermia equivocal) individuals were assigned unknown status (i.e., they do not contribute to the lod score in used pedigrees). These are the recommendations of the European Malignant Hyperthermia Group; Genetics Section and agreed at their meeting in July 1991.

Finally the principle drawback with this method is that due to genetic heterogeneity in MH, only large pedigrees with many members already diagnosed by the IVCT are able to be analysed in this way.

2.4.2 Mutation analysis

In human to date eight mutations in *RYR1* gene are reported in MH and CCD patients. Probably these are related and causative to some degree in CCD and MH susceptibility but no mutation for other candidates are reported. Although it is predictable using DNA-based test for MH diagnosis in human (as in pig) but now there is no evidence to supporting this idea as the relation between *RYR1* causative mutations and MH susceptibility is ambiguous.

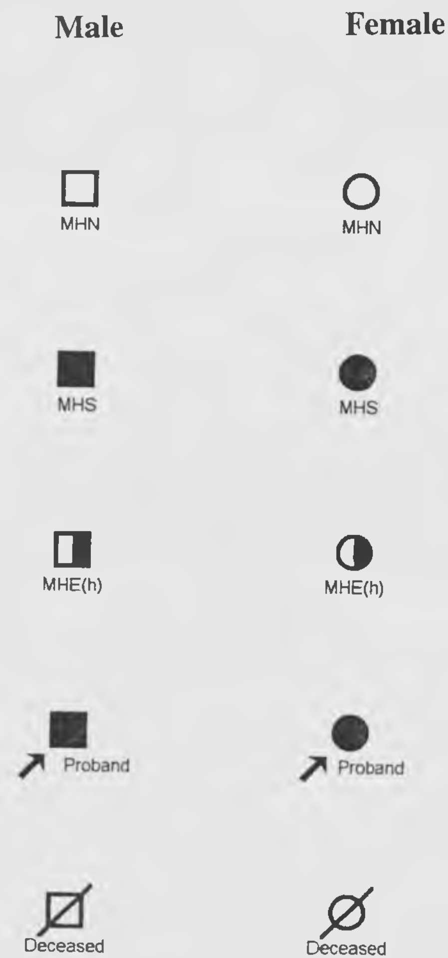
Chapter three

Materials and methods

3.1 Patients and families

Patients came from well characterised families ascertained through the UK Malignant Hyperthermia Investigation Unit at Leeds except family AN2. All probands (except very young probands, under ten years old or deceased probands), and their at risk relatives were investigated by MH Unit using the IVCT. Prior to 1984, the static halothane, dynamic halothane and caffeine tests were also used. However each of these tests was not used for every patient and sometimes additional tests such as a dynamic caffeine test was used. Patients were classified as MH positive or MH negative. Since 1984 all patients have been tested using the European IVCT protocol (EMHG 1984, 1985). On the basis of IVCT patients were classified as MHS, MHN and MHE. MHS patients were those whose biopsies responded to exposure to caffeine, 2mM (or less) and halothane, 2% (or less) by generating a contracture (0.2gr). MHN patients were those whose biopsies responded to neither caffeine nor halothane at the stated concentrations. MHE patients were those whose biopsies responded to either caffeine or halothane (see section 1.7.2).

In a few cases, patients characterised as MHE on the basis of IVCT were known to have experienced a hyperpyrexic response to anaesthesia. These patients were nonetheless considered MHE for the study of linkage analysis (see 2.4.1). This allowed general uniformity with European practice.



SH : Static halothane test

SC : Static caffeine test

DH : Dynamic halothane test

Fig 3.1 Key to Figures 3.2 to 3.9 and description of symbols used in pedigrees. Black symbols showing 0.2g contracture (positive responses or MHS) using specific concentrations of halothane and caffeine. Half-blacked symbols show 0.2g tension using specified concentration of halothane, described $MHE_{(h)}$ and blank symbols are normal or have no response using threshold concentrations (MHN).

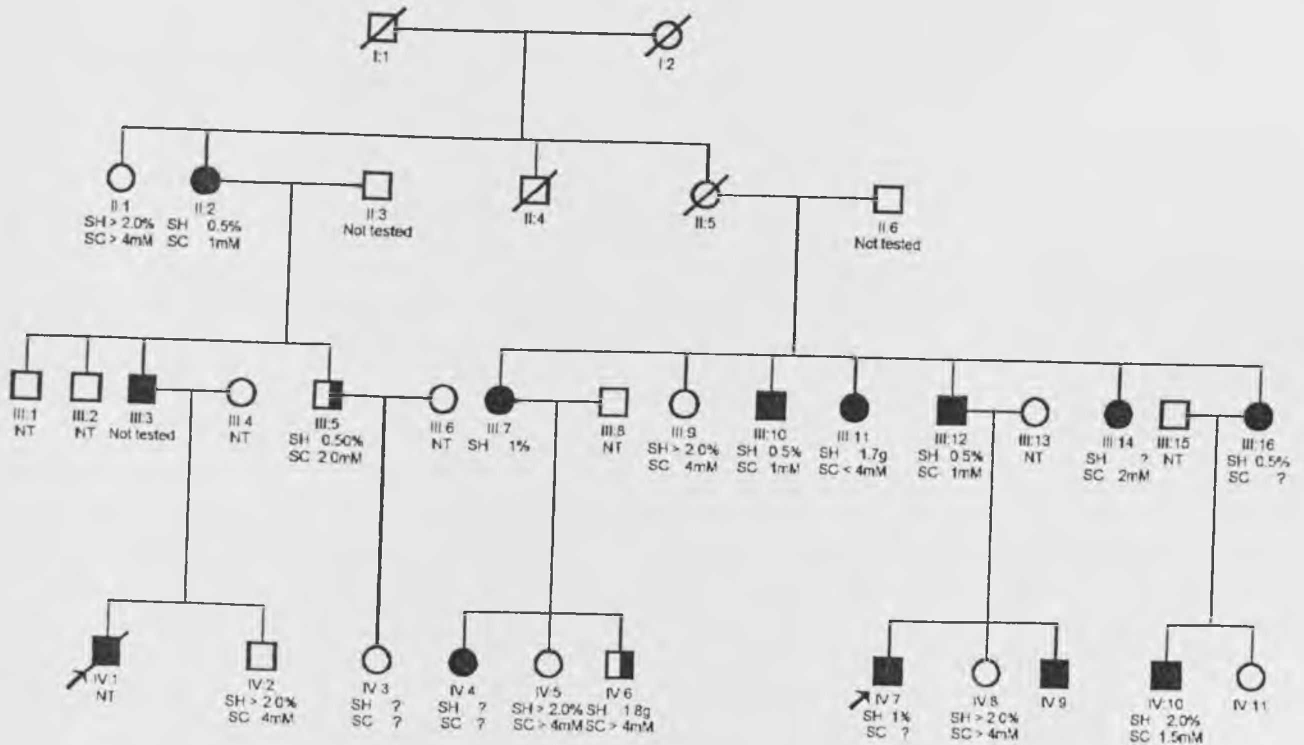


Fig 3.2 Pedigree of family LMH03 showing results of IVCT diagnosis.

This four-generation pedigree, LMH03 composed of 18 individuals which are tested by the IVCT. Two of them are MHE and show positive contracture to halothane only. The first proband IV.7 is a nine year old male. He developed severe masseter spasm during anaesthesia with succinylcholine. Post-operatively he developed marked myoglobinuria with a serum creatine kinase level of 2850 units per litre. His paternal grand mother had died unexpectedly during anaesthesia for a hysterectomy when aged 46. The second proband of this family IV.1 is a great nephew of the above grand mother. He developed an increasing heart rate after one hour of anaesthesia followed by arrhythmia and asystole. He had muscle rigidity and a temperature of 40C prior to death when aged 14. Previous linkage analyses with markers from chromosome 19q, 17q, 7q, 3q, and 1q have excluded linkage with these candidate gene regions (Dr J. Curran, pers. comm.).

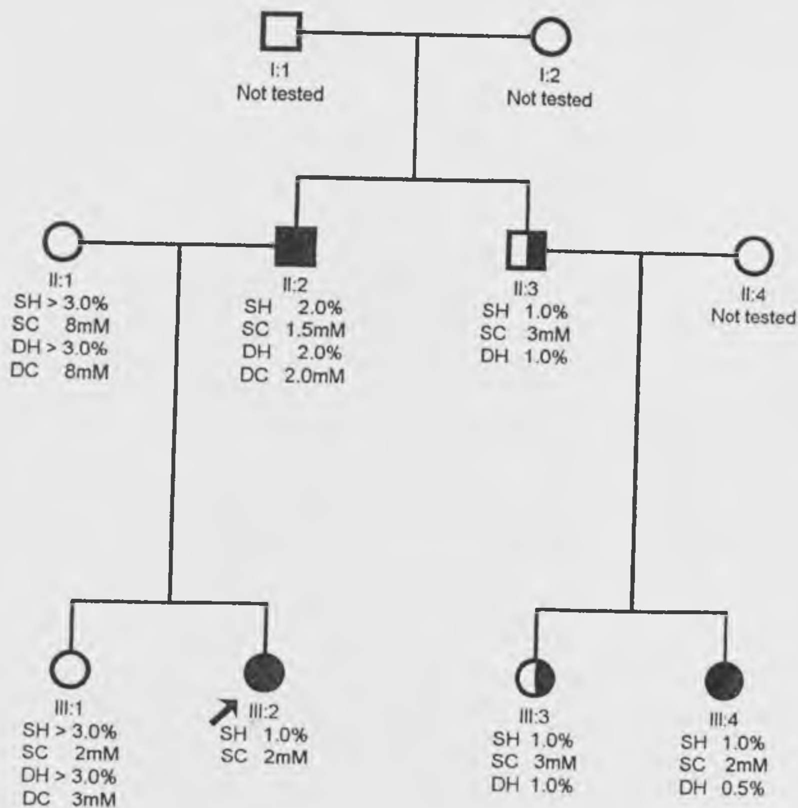


Fig 3.3 Pedigree of family LMH07 showing results of IVCT diagnosis. This family comprises three MHS, two MHN, two $MHE_{(h)}$ and three individuals who are not tested by IVCT. The proband, III.2 is a young female who expressed clinical MH reaction during anaesthesia using suxamethonium. Her sister and her mother are normal and it appears that the disease allele is inherited from her father who is MHS. The proband's uncle (II.3) and cousin (III.3) are both $MHE_{(h)}$. It would appear that the $MHE_{(h)}$ status of the uncle represents a susceptibility diagnosis because he has an MHS daughter, III.4. This family is excluded from chromosome 19q linkage (Dr J. Curran pers. comm.).

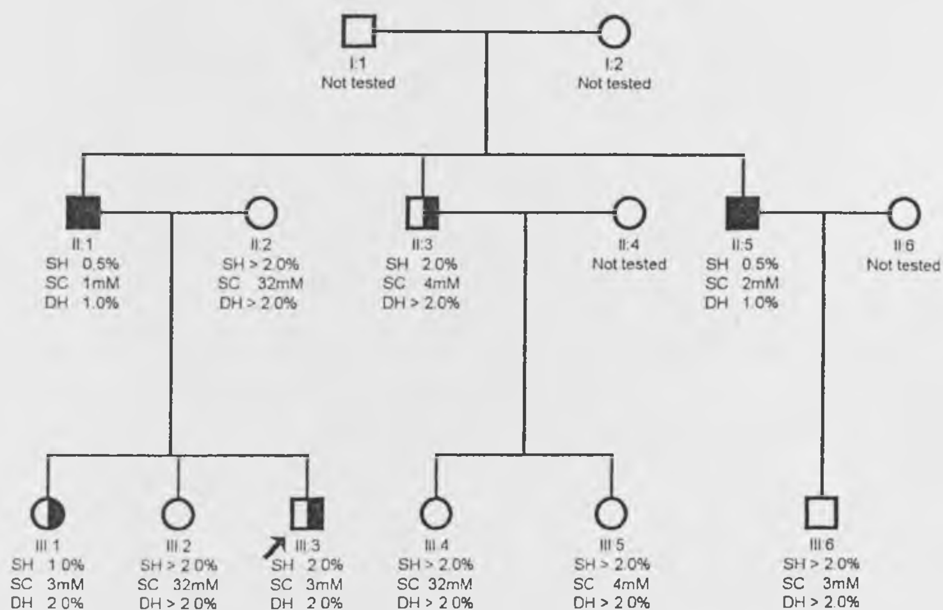


Fig 3.4 Pedigree of family LMH12 showing results of IVCT diagnosis. The LMH12 family includes 10 individuals tested. Three are $MHE_{(h)}$, two MHS and four MHN. The proband of this family is MHE using halothane test. He showed clinical reactions (such as hyperpyrexia) triggered by halothane. This family with one MHS recombinant is excluded from chromosome 19q markers using linkage analysis (Dr J. Curran per. comm.).

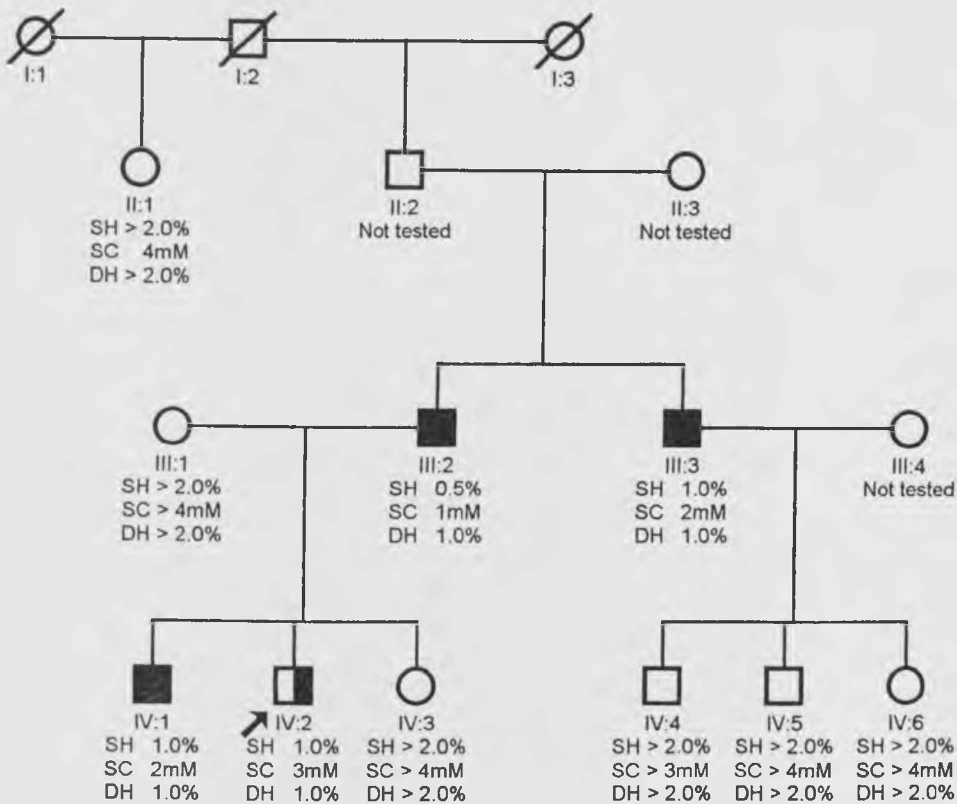


Fig. 3.5 Pedigree of family LMH15 showing results of IVCT diagnosis. This family is composed of three MHS, one $MHE_{(h)}$ and seven MHN individuals. The proband of this family IV.2 is $MHE_{(h)}$ by IVCT despite the clinical reaction during anaesthesia. In a dental extraction he presented the muscle spasm and raised CK with halothane and suxamethonium. His father and his brother are MHS although his sister is normal. His uncle, III.3 is MHS but his three children are MHN.

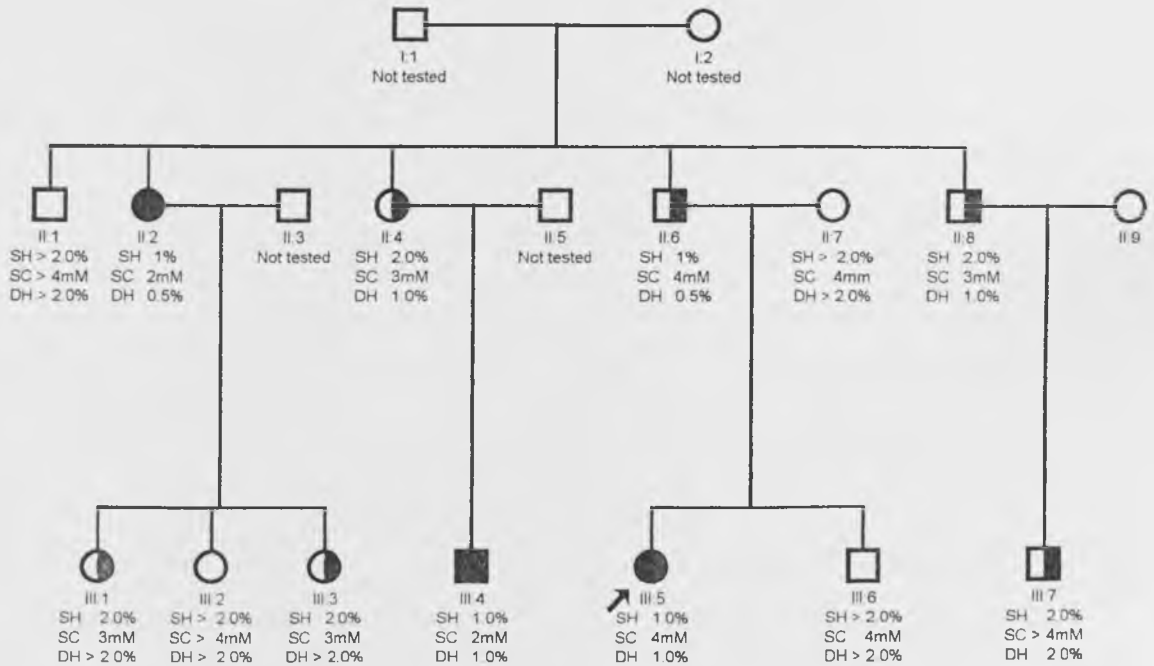


Fig. 3.6 Pedigree of family LMH16 showing results of IVCT diagnosis. III.5 is the proband. She developed muscle spasm and jaw rigidity after induction of anaesthesia with suxamethonium for tonsillectomy. She proved to be MHS by IVCT testing but her father has no positive response to caffeine but halothane induced positive contracture using her muscle biopsy. Thirteen people have been tested by IVCT in this pedigree with three MHS and 6 $MHE_{(h)}$ individuals identified.

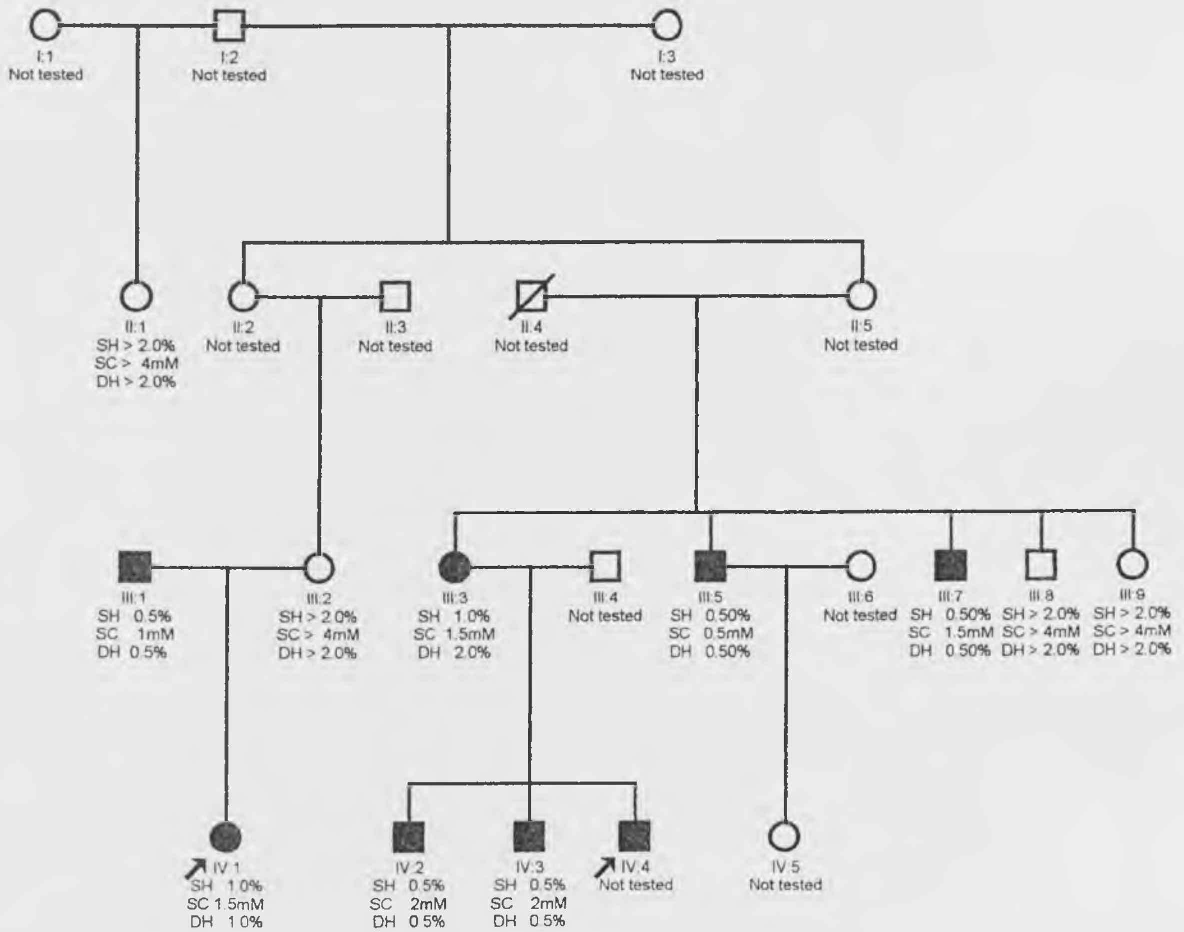


Fig. 3.7 Pedigree of family LMH17 showing results of IVCT diagnosis. The first proband in family LMH17 is IV.4 who is not tested but his mother with two his brothers are MH susceptible by IVCT testing. During anaesthesia he developed tachycardia and increased in body temperature after isoflurane, thiopentone and fentanyl. The second proband of this family, IV.1 was anaesthetised for a broken nose when she was 12 years old. During general anaesthesia using halothane and suxamethonium she developed masseter muscle spasm and raised CPK, 26 000 unit per litre and myoglobinurea. When she was old enough, her MHS status was established using *in vitro* contracture testing. Apparently the second proband is affected by disease gene through her father who is not a relative of this family.

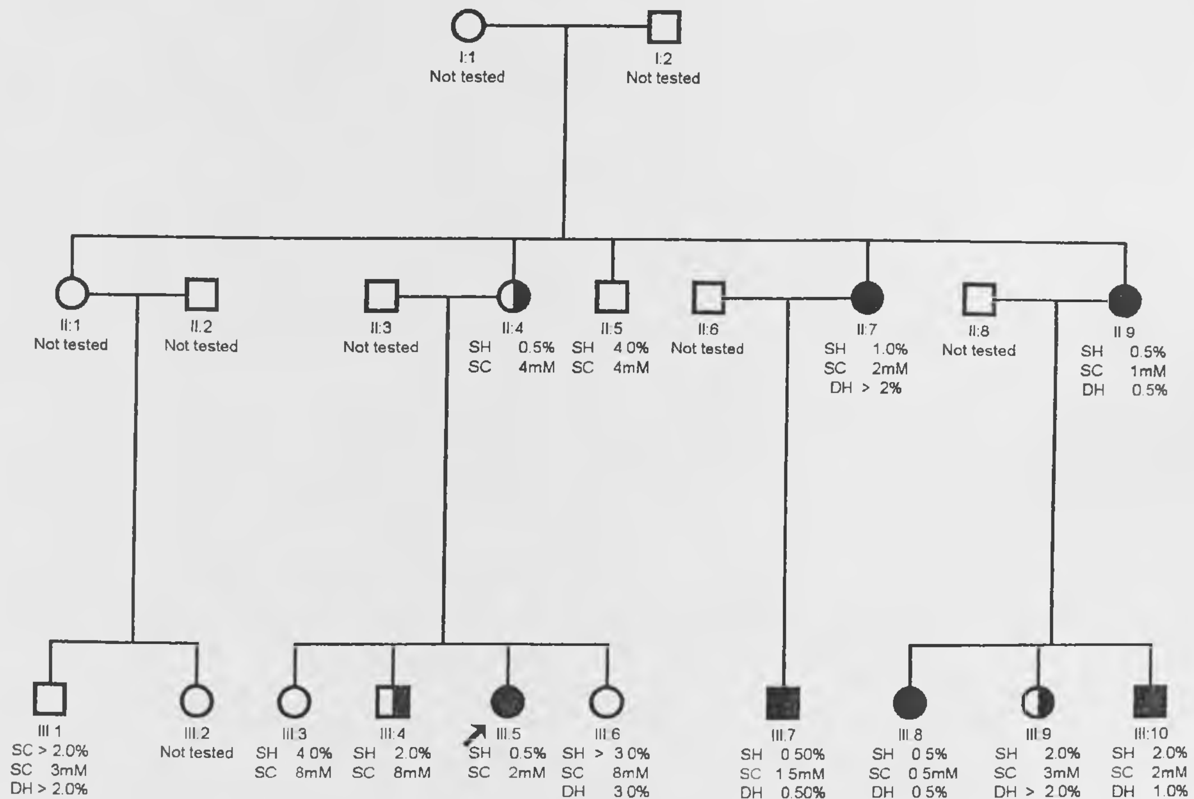


Fig. 3.8. Pedigree of family LMH18 showing results of IVCT diagnosis. The proband III.5 was recognised during anaesthesia for squint because she developed rigidity with suxamethonium. After administration of drug her temperature increased from 37 to 38.6°C in 20 minutes and CK increased to 8 700 units post operatively. The proband's mother is $MHE_{(h)}$ with a strong response to halothane in IVCT but not to caffeine. Thirteen members of this family were screened by IVCT with 7MHS, 2 $MHE_{(h)}$, 4MHN individuals and five people not tested.

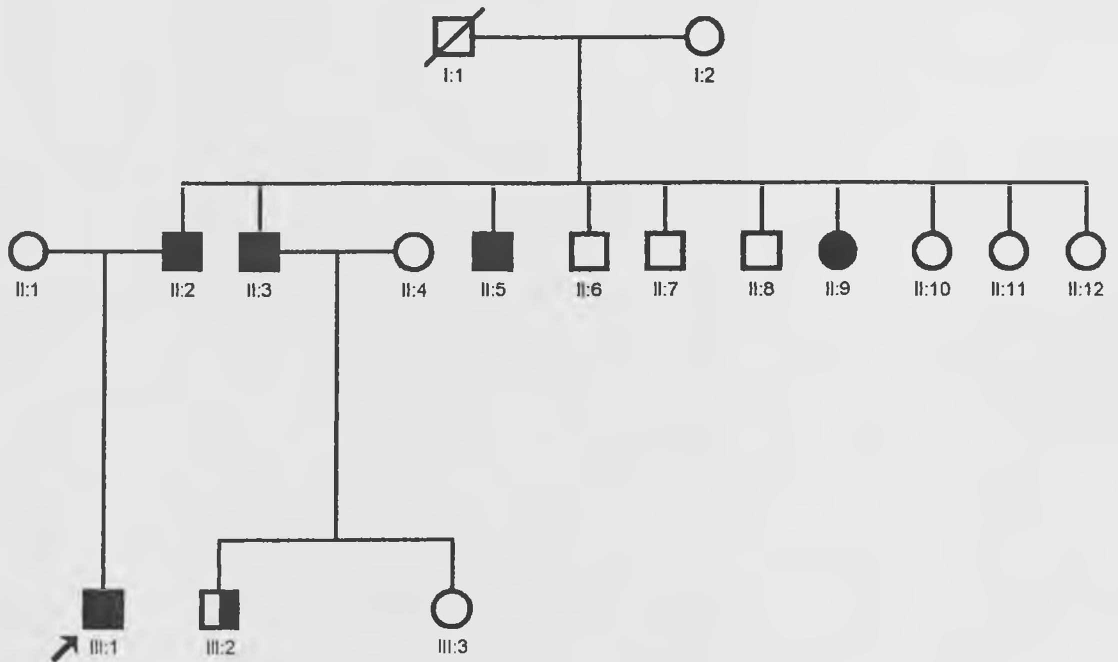


Fig 3.9 Pedigree of family AN2.
The IVCT for this family has been carried out according to the European protocol at Antwerp, Belgium. This family is a three-generation family with 15 individuals of whom fourteen have been tested by the IVCT. This family as LMH03 has been excluded from 19q, 7q, 3q, 1q candidate gene regions and investigated using markers from chromosome 2p.

For this study families were selected for linkage analysis on the basis of size and clear MHS/MHN results and for mutation analysis all families were investigated. In figures 3.1 to 3.9 the pedigrees of MH susceptible families investigated for linkage are shown with their IVCT results for all individuals tested.

3.1.1 CCD families

Details of seven MH/CCD families using IVCT are described below (table 3.1). Three pedigrees of CCD families are shown in Figures of 3.10 and 3.11

3.1.2 Families and samples used in mutation screening

242 families have been investigated for three *RYR1* mutations, C487T, G1021A and C1840T. The IVCT results of these families are listed in table 3.2.

3.2 Methods

3.2.1 Preparation of DNA samples

Genomic DNA was extracted from whole blood samples according to salting out procedures for fresh blood and a phenol-chloroform extraction method for frozen blood samples.

3.2.1.1 Blood samples

Blood samples were collected by venepuncture. 10ml of blood was collected into 15ml polypropylene tubes contained anticoagulant. DNAs were either extracted within 12 hours of blood collection or samples were frozen at -80°C.

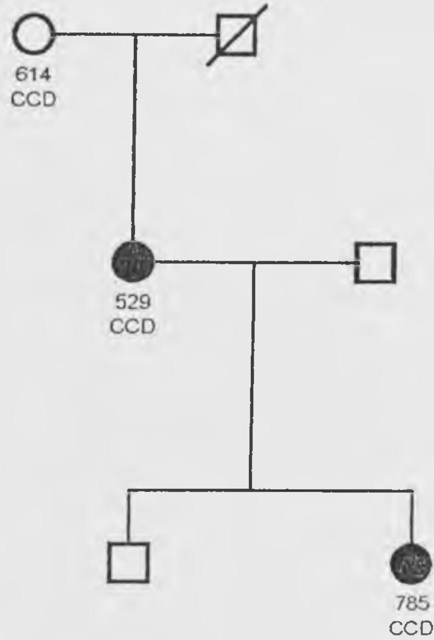


Fig 3.10 Pedigree of CCD family used in mutation screening. This family is very small and has not been entered in linkage analysis. The numbered individuals have been tested using IVCT and vice versa. The individuals with black and blank symbols are MHS and MHN respectively. The individuals with no number have not been tested.

Fig 3.11 Two samples of CCD families. These families are entered in mutation analysis for C487T and G1021A mutations. The results show no mutation in these two families. The individuals with black and blank symbols are MHS and MHN respectively.

Table 3.1. The IVCT results of seven CCD families used in mutation screening. S and D, symbols are used for static and dynamic halothane respectively. The quantities of halothane and caffeine are the amount required to induce 0.2g of muscle tension.

Family	Number	Halothane	Caffeine	Status	Relation-ship
Bar-Lan	2379	0.5%(S) / 0.5%(D)	1mM	MH+ve/CCD	Proband
	2499	>2%(S) />2%(D)	32mM	MH-ve	Sister
Gib-Mir	529	2%(S)	<8mM	MH+ve/CCD	Proband
	614	4%(S)	16mM	MH-ve/CCD	Son
	1976	>2%(S) / >2%(D)	>4mM	MH-ve	Daughter
Jon-Nor	3076	2%(S)	2mM	MHS/CCD	Proband
Mas-Boo	2954	>2%(S) / >2%(D)	4mM	MHN/CCD	Proband
Pay-Nor	2176	0.5%(S) / 0.5%(D)	0.5Mm	MHS/CCD	Proband
	2449	>2%(S) / >2%(D)	4mM	MHN	Sister
	2451	0.5%(S) / 0.5%(D)	1mM	MHS/CCD	Father
Sel-Dar	2586	0.5%(S) / 0.5%(D)	0.5mM	MHS/CCD	Proband
	2936	0.5%(S) / 1%(D)	1.5mM	MHS/CCD?	Father
	2937	0.5%(S) / 0.5%(D)	0.5mM	MHS/CCD?	Sister
Smi-Tam	742	2%	4mM	MHE/CCD?	Relative
	1776	0.5%(S) / 0.5%(D)	4mM	MHS/CCD	Proband
	1843	0.5%(S) / 0.5%(D)	2mM	MHS	Daughter
	1844	>2%(S) / >2%(D)	32mM	MHN	Daughter

Table 3.2. IVCT results of individuals used in mutation screening.

The star (*) shows heterozygoute individuals for G1021 to A mutation.

Pedigree identification	Identification number of individuals	Threshold contraction (0.2g)		MH status
		halothane %	caffeine mM	
AIN-BON	2136	1	0.5	MHS
AKH-OXF	3174	>2	>2	MHN
ALC-BUR	3161 3162	>2 >2	4 3	MHN MHN
ALL-STA	2796	>2	4	MHN
AMO-SUR	2723	1	1.5	MHS
BAM-MIL	2654	>2	4	MHN
BAN-LIN	3150	2	2	MHS
BAR-BAR	1567 1606 1633 1751 3159	1 >2 0.5 0.5 1	1.5 >4 2 2 1.5	MHS MHN MHS MHS MHS
BAR-LAN	2379 2499	0.5 >2	1 32	MHS MHN
BAW-SON	2658	>2	3	MHS
BEA-DOR	2866	>2	4	MHN
BEA-EXE	1502 1533	1 >2	3 >4	MHE MHN
BEA-WIL	2646	>2	4	MHN
BIR-MID	1569 1614 2338	2.0 >2 >2	3 32 3	MHE MHN MHN
BLA-AEV	2698	1	1.5	MHS
BLA-ARM	2748 3033 3170	0.5 1 2	2 2 3	MHS MHS $MHE_{(h)}$
BLA-DER	2631	1	1.5	MHS
BLA-DEV	2801 2811	>2 >2	4 3	MHN MHN
BLA-DON	2781	>2	4	MHN
BLE-MID	2142	>2	4	MHN

continued.....

Table 3.2 continued

BOO-SUN	2694	1	1.5	MHS
BOU-BIR	2686	>2	32	MHN
BOW-CLN	2975	2	2	MHS
BOW-OWL	3192	1	2	MHS
BRA-TIN	2789	>2	3	MHN
BRA-DER	2831	>2	4	MHN
BRE-NDR	2692	>2	4	MHN
BRI-BAN	2684	>2	4	MHN
BRO-DER	2722 3038	>2 1	4 2	MHN MHS
BRO-GLA	2687	>2	3	MHN
BRO-MID	2805	>2	4	MHN
BRO-NOR	2771	>2	3	MHN
BUL-LON	2855 2794	>2 >2	4 4	MHN MHN
BUR-BUG	2749	>2	32	MHN
BRO-PON	3194	>2	32	MHN
BUR-LYN	2778	>2	32	MHN
BUT-WAR	3090	2	2	MHS
CAN-CHI	3156	1	2	MHS
CAR-LLA	2860 3075	2 1	1.5 2	MHS MHS
CAT-TEN	3094	1	1.5	MHS
CHE-LON	2754	>2	32	MHN
CLA-PAI	2264	2	1	MHS
COL-IOW	2262	>2	32	MHN
COU-LIN	2758	>2	32	MHN
CRA-SKE	3173	>2	32	MHN
CRE-BUC	2850	>2	32	MHN
CRO-HCD	2714	>2	4	MHN
CUM-ABE	2879	2	2	MHS *
DAV-NOT	2851	>2	4	MHN
DAV-SWA	2753	>2	4	MHN
DIC-EDI	2810 2847	2 >2	2 32	MHS MHN
DON-CRO	2200	>2	4	MHN
DOR-SHE	2929	>2	32	MHN

continued….

Table 3.2 continued

ECT-PRE	2713	>2	4	MHN
ELL-PLY	2691	1	2	MHS
FAI-BOS	2247	>2	3	MHN
FEA-BUC	2804	2	2	MHS
FID-LEE	2924	0.5	0.5	MHS
FIS-PRE	2650 2705 2706 2728 2894 3191	0.5 1 >2 1 2 >2	1 1 4 1.5 1.5 4	MHS MHS MHN MHS MHS MHN
FOR-ESS	2148 2149 2884	2 >2 0.5	2 3 0.5	MHS MHN MHS
FOX-BLA	713 1227 1298 2337 2685	0.5 2 2 0.5 >2	2 3 2 0.5 3	MHS MHE MHS MHS MHN
GAM-DON	2637	1	1.5	MHS
GAM-HAN	2701 2978 3099	0.5 1 0.5	1.5 2 1	MHS MHS MHS
GIB-HUD	529 614	2 4	-VE 16	MHS MHN
GIB-MIR	1976	>2	32	MHN
GIL-LEI	3068	1	2	MHS
GRA-IOW	2786	>2	4	MHN
GRA-STO	2680	>2	32	MHN
HAK-BLA	2842	>2	>3	MHN
HAL-CRO	2710	1	1.5	MHS
HAL-SUN	2667 3176	2 >2	2 4	MHS MHN
HAM-LEE	2735	>2	4	MHN
HAN-DER	2699	>2	3	MHN
HAR-BRA	3177	>2	32	MHN
HAR-CAR	2841	>2	32	MHN
HAR-NOT	2769	>2	32	MHN
HAY-WIG	2761	>2	32	MHN
HEY-WIG	2800	>2	32	MHN
HIN-BUR	2651	2	2	MHS

continued….

Table 3.2 continued

HIN-SOT	2832	0.5	1.5	MHS
HOP-DUN	2776	1	1.5	MHS
	2861	2	32	MHN
HOW-SHR	2834	>2	4	MHN
HUN-LOW	3178	>2	>3	MHN
HYD-DUR	2822	1	1.5	MHS
INK-BRI	2147	1	1.5	MHS
	2196	1	1.5	MHS
	2251	>2	32	MHN
	2627	>2	4	MHN
IRV-KIR	3163	>2	4	MHN
JAC-SOM	2157	0.5	1.5	MHS
JEF-BIR	742	2	4	MHS
JON-CAR	2632	>2	32	MHN
	2633	>2	32	MHN
	3139	2	2	MHS
JON-DOR	2835	>2	32	MHN
JON-GLA	2648	>2	32	MHN
	2767	>2	4	MHN
JON-NOR	3076	2	2	MHS
JON-WIG	2137	>2	4	MHN
KEL-POR	2568	0.5	1	MHS *
	2709	>2	3	MHN
KIN-BRI	2774	1	1.5	MHS
KIN-CDR	2682	>2	32	MHN
KYN-BAS	2673	0.5	1	MHS *
	2729	2	2	MHS
	2807	2	3	MHE_h
	2808	>2	32	MHN
	2809	>2	4	MHN
	2907			
LAN-BIR	2561	0.5	1.5	MHS
LAN-SHE	2813	>2	32	MHN
LAT-COK	2688	>2	4	MHN
LEA-SUS	2134	0.5	0.5	MHS *
	2236			MHS *
	2235	1	1.5	MHS *
LEE-NOR	2144	1	3	MHE_h
LEG-TOR	2732	>2	3	MHN
	3083	1	2	MHS
	3179	2	2	MHN
	3181	>2	32	MHN
IEW-EAS	2693	>2	32	MHN

continued.....

Table 3.2 continued

LEW-MIL	2840	>2	>2	MHN
LIN-PIN	2606 2629 2708 2900	0.5 0.5 0.5 0.5	0.5 0.5 1 1.5	MHS MHS MHS MHS
LIN-SOT	2918	0.5	1.5	MHS
LIT-BAT	2675	>2	32	MHN
LOA-BRI	3188	1	2	MHS
LOO-HAL	2661	>2	4	MHN
LYN-LON	2736	2	1.5	MHS
MAC-GUI	2976 3160	0.5 >2	1.5 4	MHS MHN
MAI-LON	2765	2	2	MHS
MAR-LLA	2133	>2	4	MHN
MAS-BOO	2954	>2	4	MHN
MCB-GLA	2166 3166	0.5 >2	1.5 2	MHS MHS
MCL-SCO	2836 3167 3169	2 >2 1	1.5 4 2	MHS MHN MHS
MCM-ANT	2674 2854	>2 1	3 2	MHN MHS
MCM-GLA	2168	1	1.5	MHS
MCM-UTT	2730	>2	4	MHN
MEA-LEE	3096	1	1.5	MHS
MIL-BOU	2268	>2	4	MHN
MIN-BIR	3091	1	2	MHS
MIT-DUR	2945	2	2	MHS
MOB-CAT	2138	2	32	MHE_h
MUB-GLA	2669	1	0.5	MHS
MUL-GLA	2843 2844	>2 0.5	32 1	MHN MHS
MUR-NEW	2161	0.5	1	MHS *
NAR-WAT	2712 3072	1 0.5	1.5 1.5	MHS MHS
NAU-FIF	2552 2696 2697	0.5 1 >2	0.5 2 32	MHS MHS MHN
NEW-BRI	2657	>2	3	MHN
NEW-WAR	2136	>2	32	MHN
ODO-CHE	2659	>2	4	MHN
OHA-STO	2703	>2	4	MHN

continued.....

Table 3.2 continued

ORW-BLA	2830	>2	32	MHN
PAL-LEE	2638	>2	3	MHN
	2750	>2	4	MHN
	2784	>2	4	MHN
PAL-PET	2653	>2	4	MHN
PAR-BUE	2754	>2	32	MHN
PAR-ORE	2165	>2	32	MHN
PAT-LIN	2644	>2	32	MHN
PAY-NOR	2176	0.5	0.5	MHS
	2449	>2	4	MHN
	2451	0.5	1	MHS
PER-LEI	3057	0.5	1.5	MHS
PIN-LEE	2681	>2	3	MHN
PIN-NLE	2864	>2	4	MHN
POP-BAR	1437	>2	4	MHS
	1826	2	3	MHE_h
	1852	1	2	MHS
	1879	>2	>4	MHN
PRE-DUN	2770	>2	32	MHN
	2791	>2	4	MHN
PRI-BAN	2825	>2	4	MHN
QUA-MAN	2949	>2	32	MHN
	2950	1	2	MHS *
	2988	>2	1.5	MHS *
RIC-DEW	2654	>2	4	MHN
RIC-MDI	3064	1	1.5	MHS
RIL-STO	2883	1	1.5	MHS
	3016	1	2	MHS
ROB-BIR	900	>3	8	MHN
	954	2	1.5	MHS
ROO-RED	2782	>2	4	MHN
ROW-HAN	2865	>2	32	MHN
	3029	>2	4	MHN
ROW-MAN	2816	0.5	0.5	MHS
ROW-PON	2757	>2	32	MHN
ROW-STO	2162	1	2	MHS
	2180	0.5	1	MHS
	2536	0.5	1	MHS
	3104	0.5	1.5	MHS
RUD-CAR	2550	0.5	1.5	MHS
RYA-LEE	2888	1	1.5	MHS
SAB-DON	2727	>2	4	MHN

continued.....

Table 3.2 continued

SAW-GLA	2772	>2	32	MHN
SEL-DAR	2586	0.5	0.5	MHS
	2936	0.5	1.5	MHS
	2937	0.5	0.5	MHS
SHA-CAM	2780	>2	32	MHN
SHB-DON	2785	>2	32	MHN
SHO-MIR	2150	0.5	1	MHS
	2641	>2	3	MHEh
	2662	>2	3	MHN
SIM-MAC	2768	>2	3	MHN
SIM-SUS	3051	0.5	1	MHS
SLA-ROT	2733	>2	3	MHN
SMI-BOL	2649	>2	32	MHN
	2790	>2	32	MHN
SMI-GLA	2756	0.5	0.5	MHS
	2826	1	1	MHS
SMI-TAM	1776	0.5	2	MHS
	1843	0.5	2	MHS
	1844	>2	32	MHN
SPO-GRI	2799	>2	4	MHN
SPO-GRU	2140	>2	4	MHN
SQE-SHE	2652	>2	4	MHN
STA-BAT	3175	>2	32	MHN
STA-DER	2742	>2	3	MHN
STE-GEW	2634	1	2	MHS
	2635	>2	3	MHN
	2636	>2	4	MHN
STR-ESS	2556	0.5	0.5	MHS
STR-HAR	2738	2	2	MHS
SUS-CLE	2678	2	2	MHS
SUT-BAS	2766	>2	4	MHN
	3143	1	2	MHS
	3013	1	2	MHS
SUT-SAU	2664	1	1	MHS
TAY-LEI	2737	>2	>2	MHN
TER-LIN	2990	1	1	MHS
TEW-DON	2715	1	1.5	MHS
	2824	2	3	MHEh
	2891	1	2	MHS
	2915	2	2	MHS
	3077	1	2	MHS
THD-BRA	2679	>2	4	MHN
TIM-FYL	2852	>2	4	MHN

continued......

Table 3.2 continued

TIM-ROT	2163	0.5	1.5	MHS
TIN-NEW	2630 2642	>2 >2	3 4	MHN MHN
TOO-WOO	2145	>2	4	MHN
TOT-COR	2702	>2	32	MHN
UNS-HUD	2856 2858	0.5 >2	0.5 3	MHS MHN
VER-SOM	2270 2704	0.5 >2	1.5 3	MHS MHN
VET-POR	2716	>2	4	MHN
WAL-PRE	2153 2175	0.5 >2	1.5 32	MHS MHN
WAR-LAN	2583 2731 2814	2 1 >2	1.5 2 >2	MHS MHS MHN
WAR-NOR	2504	2	2	MHS
WEA-HAR	2798	>2	32	MHN
WEB-WOB	2717	>2	3	MHN
WEB-WOK	2643	>2	4	MHN
WEL-BEV	2828	>2	32	MHN
WHI-AUS	3109	0.5	1	MHS
WHI-DUD	2655	2	2	MHS
WHI-HAR	2139	>2	32	MHN
WIL-BLA	2740	>2	32	MHN
WIL-GLA	2660 2720	>2 >2	32 4	MHN MHN
WIL-HUN	2829 3164	1 1	2 2	MHS MHS
WIL-WHI	2151 2739	1 1	1 2	MHS MHS
WOO-BRA	2683 2792 2793 2862	0.5 0.5 >2 0.5	0.5 0.5 32 1.5	MHS MHS MHN MHS
WRO-SEL	3102	0.5	1.5	MHS

3.2.1.2 DNA extraction from fresh blood

Fresh venous blood samples (10 ml) were collected in 15ml polypropylene heparinised or EDTA tubes. After spinning at 900g for 5 minutes, the buffy layer containing white cells was removed with a pipette and placed in a 30ml polypropylene tube containing 20ml lysis buffer (0.86% ammonium chloride) and incubated for 15 minutes at 37°C. After centrifugation at 900g for 15 min the pellet was washed twice with lysis buffer and re-suspended in 5 ml STE (100mM sodium chloride, 1M Tris-HCl pH 7.8 and 500mM EDTA) with 1% SDS and 50μl proteinase k (10mg/ml, Boehringer Mannheim). The mixture was incubated overnight at 37°C. After incubation 2.5ml 7.5M ammonium acetate was added and the mixture placed on ice for 40 minutes. Samples were centrifuged for 30 minutes at 1300g and then the supernatant transferred to a fresh 15ml tube. Two volumes of cold ethanol (4°C) were added and mixed by inverting the tubes several times until the DNA precipitated. The precipitated DNA strands were hooked out using a bent Pasteur pipette, and placed in 1.5ml microcentrifuge tubes containing an appropriate volume, usually 100-500μl, of 1XTE (1M Tris-HCl pH 7.6, 500mM EDTA) depending on the yield of extracted DNA. Finally before using or storage of samples they were rotated at room temperature for at least 2 hours to dissolve the DNA.

3.2.1.3 DNA extraction from frozen blood

When the blood samples were more than 12 hours old or had been frozen the phenol-chloroform method was used. The phenol extraction removes the

proteins and RNA, the chloroform removes any traces of phenol which may inhibit later reactions, of course phenol can cause severe chemical burns on skin and will damage clothes. 10ml blood samples in either heparin or EDTA were mixed with 10ml of lysis buffer (0.86% ammonium chloride) and incubated at 37°C for 30 minutes. After spinning at 1300g for 30 minutes pellets were resuspended in 1% SDS and 50µl proteinase k (10mg/ml) samples were incubated at 37°C overnight. An equal volume of equilibrated phenol was added to the DNA sample mixture (in 30ml polypropylene tube) and after shaking well but gently and centrifuging (1300g) for 5 minutes the upper aqueous layer was removed to a 13ml polypropylene tube. This extraction step was repeated with phenol and then using chloroform instead of phenol. After the final chloroform extraction the upper layer was transferred to a 30ml tube and two volumes of ethanol were added and mixed by inverting the tubes several times until the DNA precipitated. The precipitated DNA strands were hooked out using a bent Pasteur pipette and placed in 1.5 ml microcentrifuge tubes containing usually 100µl to 500µl of 1X TE depending on the yield of extraction. Then before using or storage of samples they were rotated at room temperature for at least two hours to dissolve the DNA.

3.2.1.4 Quantitation of DNA

For quantitation of extracted DNAs and to confirm that the extraction has been successful, from both methods the DNA samples were run on 0.8% agarose gels.

3.2.2 Gel electrophoresis

3.2.2.1 Agarose gel electrophoresis

Gels, dependent on the size of DNA samples, were prepared with different concentrations of agarose (Boehringer Mannheim GmbH) and electrophoresis was carried out as described in Sambrook *et al* (1989). For different percentage (n% = n gram agarose/100ml gel volume) of normal agarose gels, the appropriate weight agarose, with 1X TBE (Tris base, boric acid, 500mM EDTA) were used. The mixture placed in a conical flask in a microwave oven and heated on full power for about 2 min until the agarose had completely dissolved then 5μl of 10mg/ml ethidium bromide was added. The mixture then was poured into trays with a well former in place and left to set. Gels were set in different sizes of trays (dependent on the size and number of samples, tray 10×8.5 cm or 15×20 and tanks 18.5×10 cm or 35×17 cm) and running of samples after mixing with loading buffer (0.5mM xylene cyanol, 1mM bromophenol blue, 20% Ficoll 400) was performed in horizontal electrophoresis tanks (Bio-RAD mini sub, DNA cell). For gel running a Bio-RAD (model 250/2.5) power supply with appropriate voltage (depend to the size of DNA fragments between 70-100v) and time (50-80 min) was used. Then DNA bands were visualised in UV light and photographed with photographic equipment (UVP inc) using Upp-110HA (Sony) film.

3.2.2.2 Electrophoresis of extracted DNA

The extracted DNA samples were run on 0.8% agarose gel which was prepared (1XTBE, ethidium bromide (Sigma) 0.5μg/ml with care because of

mutagenicity), and after gel setting the samples (5μl) were run at 80 volts for 40-60 minutes (fig 3.12).

3.2.2.3 Acrylamide gels

A 19:1 mix of acrylamide : bisacrylamide was used (Fison) to make 100ml of 6% gel containing 1XTBE, 7M urea, (to keep the samples denatured), 1ml 10% freshly prepared ammonium persulphate (1g / 10ml water) and 40μl TEMED (Sigma) and swirling gently to mix. Gels were prepared as described in Sambrook *et al* (1989) with some slight modifications. Glass plates (short : 33×39 cm and long 33×42cm) were washed with soapy water, rinsed with distilled water, and then one plate siliconised on its inner surface with siliconising agent (Sigmacoat, Sigma). This plate was then rinsed with 95% ethanol. Two 0.4mm spacers were placed along the long edges of one plate and the second plate placed on top and the plates sealed together with tape. The gel mixture was prepared and immediately using the barrel of a 60ml syringe was poured carefully into the gel mould (~ 60ml). The flat edge of a sharkstooth comb (Gibco/BRL) was used to make a straight edge at the top of the gel and then butterfly clips applied around this area to ensure that the comb did not leak.

After gel polymerisation the comb was inverted to make the sample loading spaces. Then gel was placed in a sequencing gel rig (model S2, Gibco/BRL) and connected to a power supply (3000xi, Bio-RAD) and 1XTBE was used in both the upper and lower buffer chambers. The gel was pre-run before sample loading for 30 min. at 60W until the gel reached approximately 50°C. Prior to loading the

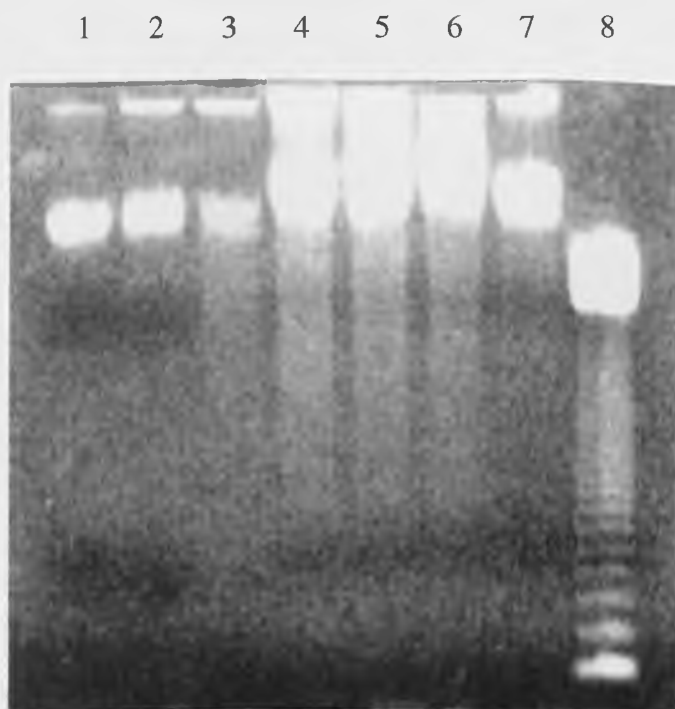


Fig 3.12 DNA extraction.
Some samples of extracted DNA are shown in lanes 1 to 7. Lane 8 shows a 123bp DNA ladder. The molecular weight of the genomic DNA samples can be greater than the last band(4200bp).

samples an equal volume of loading buffer was added to the PCR product and it was denatured for 3min at 94°C and then 2-5 μl was loaded into each lane. The gel was run at a constant power of 60W for an appropriate time dependent upon the sample size. After running the gels were fixed in 10% methanol (for dehydration and to remove any unbound urea which could cause fuzzy bands) and transferred with care from the glass plate to a sheet of 3M chromatography paper (Whatman). The gel was covered with Saran wrap and placed into a gel dryer (model 58, Bio-RAD) under vacuum at 80°C for 30-60min before exposing the sensitive films (blue sensitive x-ray film GRI Ltd) for 2-72 hours depending upon the level of radioactive incorporation determined using a Geiger monitor). Finally after exposure the film processing was carried out using developer (Ilfospeed, Ilford) and fixer solutions (Hypam fixer, Ilford).

3.2.3 PCR methods

3.2.3.1 Labelled PCR

Amplification of all microsatellite repeats of chromosome 19 were carried out in a thermocycler (Perkin-Elmer Cetus & Biometra trio-thermoblock) in a total volume of 20 μl, reaction buffer, containing 1.5-2mM $MgCl_2$ 0.25 mM dNTPs (dCTP, dGTP, dTTP and dATP Promega / Pharmacia), 50ng genomic template DNA, 1-2μCi [α -^{32} p]dATP (10mCi \ ml (3000 Ci / mmol), Amersham Life Science), 0.25U Taq DNA polymerase (5 Unit/ 1μl, Promega), 0.5mM each primer (Genetic Research) and 1μl containing 50ng genomic DNA was added. The reactions were covered with a drop of light mineral oil (Sigma). The samples were

denatured at 94°C for 5 minutes. Then amplification was carried out for 30 cycles at certain conditions (tables 3.3 to 3.7). After a final extension step at 5 min at 72°C an equal volume of loading buffer (2X formamide loading buffer : 950µl deionised formamide, 200µl 5XTBE, 0.1mg bromophenol blue and 0.1mg xylene cyanol) was added. The samples might be stored frozen or heated to 95°C for 3min and then placed on ice. Alleles were separated using 3-5µl each sample on 6% polyacrylamide gels as described in section 3.2.2.3. This used method of gel running was the same for all microsatellites except *WUT1*.9 which was amplified non-radioactively and resolved on low melting agarose gels (2% Metaphor agarose or 2.5% Nusieve in 1XTBE) instead of polyacrylamide gels.

3.2.3.2 PCR for mutation detection

The PCR condition for amplification of candidate region in mutation screening using mentioned primers (table 3.8) were carried out in 25µl volumes contain 10mM Tris-HCl, 1.5mM $MgCl_2$, 200µM dNTP, 0.5µM each primer, 50-100ng of genomic DNA, 0.25-0.5 U *Taq* polymerase (Promega). Then the reactions were covered with a drop of mineral oil. The reactions were processed 5 minutes for initial denaturation at 94°C and 30 cycles (94°C, 1 min, 58°C 1 min, and 72°C 1 min). The products had a final extension stage at 72°C for 10 minutes.

3.3 Primers

Primers were obtained from different suppliers (Pharmacia and Oswel) and used for two purposes, microsatellite amplification for linkage analysis and

amplification of *RYR1* gene regions for mutation screening. Some primers for amplification of chromosome 19q, 7q, 3q and 1q regions in linkage analysis are intragenic and the others are intergenic. The second group of primers are intragenic sequences used for mutation detection. These mutations are C487T, G1021A and C1840T. All primer sequences are shown in tables and are written in the 5′ to 3′ direction and primers for different chromosomes are described separately. These primers with sequences, location, size and PCR conditions are summarised in tables and figs in sections 3.4.1 and 3.5.1 for linkage analysis and mutation detection respectively.

3.4 Linkage analysis

3.4.1 Primers used for linkage analysis

3.4.1.1 Primers for linkage analysis of chromosome 19q

Seven pairs of primers (forward and reverse primers) were used to amplify *D19S49, D19S191, D19S220, WUT1.9, RYR1, D19S47* and *D19S178* for chromosome 19q linkage analysis. Figure 3.13 shows the location of these markers on chromosome 19q. This figure is adapted from Ashworth *et al* (1995) Levitte *et al* (1995), Weber *et al* (1993), Weissenbach *et al* (1992), Matise *et al* (1994). Details of these primers are listed in table 3.3. Three samples of chromosome 19q markers, *RYR1*, *D19S178* and *WUT1.9* are shown in figures 3.14, 3.15 and 3.16.

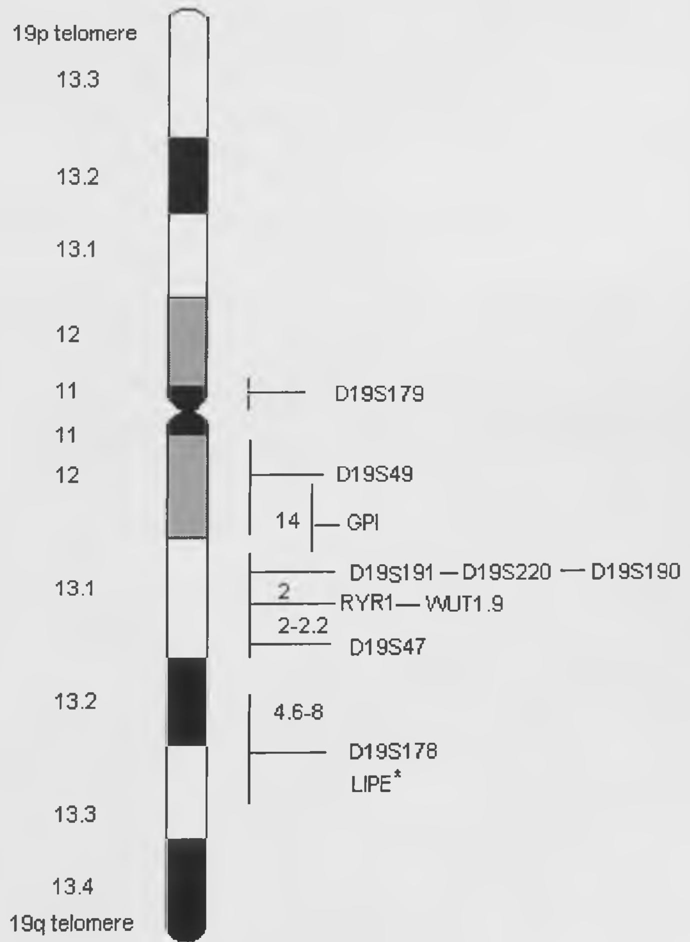


Fig 3.13 Chromosome 19q microsatellites used in linkage analysis.

The localisation of the eight markers used and their genetic distances are illustrated in this figure. The *GPI* and *LIPE* genes were not entered in linkage analysis but are illustrated because these loci have been related to malignant hyperthermia susceptibility.

Locus	Location	Primer sequences	PCR conditions	Product size (bp)	Number of alleles	References
D19S49	19q12.1	For ACTCATGAAGGTGACAGTTC Rev GTGTTGTTGACCTATTGCAT	94°C 30s, 57°C 90s, 28X	106-122	5	Weber *et al* (1990)
D19S191	19q13.1	For AGTAAAGAGGTTGAATTAATGACC Rev TGCCAGCGAAGCTATCTGG	94°C 45s, 45°C 90s, 30X	103-125	11	Iles *et al* (1992)
D19S220	19q13.1	For ATGTTCAGAAAGGCCATGTCATTTG Rev TCCCTAACGGATACACAGCAACAC	94°C 45s, 58°C 90s, 30X	265-283	10	Weissenbach *et al* (1989)
*WUT1.9**	19q13.1	For AGTCTTGAAGTTATCTGTCTG Rev TGGATGGACAGAGCGAGGC	94°C 45s, 56°C 45s, 72°C 60s, 30X**	211-398	10	Wolz *et al* (1996)
RYR1	19q13.1	For GCAATGGCATAATCTCAGCT Rev GCATCACGGTCTGCAATTCA	94°C 60s, 54°C 40s, 30X	221-223	3	Couch *et al* (1991)
D19S47	19q13.1	For GATGTCTCCTTGGTAAGTTA Rev AATACCTAGGAAGGGGAGGG	94°C 30s, 57°C 90s, 28x	88-106	8	Weber & May (1989)
D19S178	19q13.2	For CACAACACTGTTCATTTGTC Rev TTTCAGTAGAATTTCAGGCC	94°C 45C, 57°C 90s, 28X	143-189	15	Weber *et al* (1993)

Table 3.3 Primers for amplification of microsatellite markers on chromosome 19q used in linkage analysis. Initial denaturation for all primers was 94°C for 4 min and final extension (72°C) was 10 minutes.

* For this marker the situation is different, because radioisotope has not been used and allele separation was carried out using low melting agarose, 2% Metaphor agarose or 2.5 % Nusieve in 1XTBE. Initial denaturation using this primer was 94°C for 3 min.

Fig 3.14 Autoradiograph of allele segregation using the chromosome 19q *RYR1* microsatellite marker. Two alleles, 1 and 2, are observed. These alleles are shown in lanes 1 to 8. Lanes 1, 3, 7 and 8 show allele 2, lanes 2, 4 and 5 show two alleles and lane 6 is a control and shows no DNA.

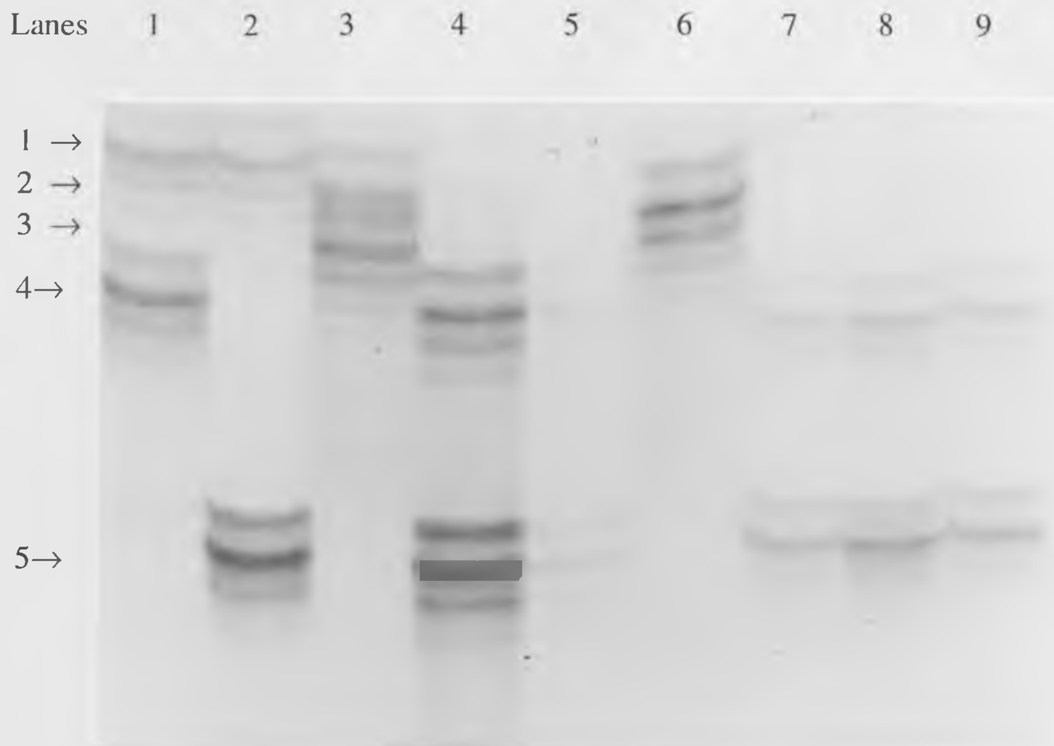


Fig 3.15 Autoradiograph of allele segregation of the chromosome 19q marker, *D19S178*. Five alleles of this marker are observed in lanes 1 to 9. Lane 1 contains two bands, 1 and 4, lane 2 two bands, 1 and 5, lane 3 two bands, 2 and 3, lanes 4 and 5 contain bands 4 and 5, lane 6 shows only band 3 and lanes 7, 8 and 9 contain the same bands, 4 and 5.

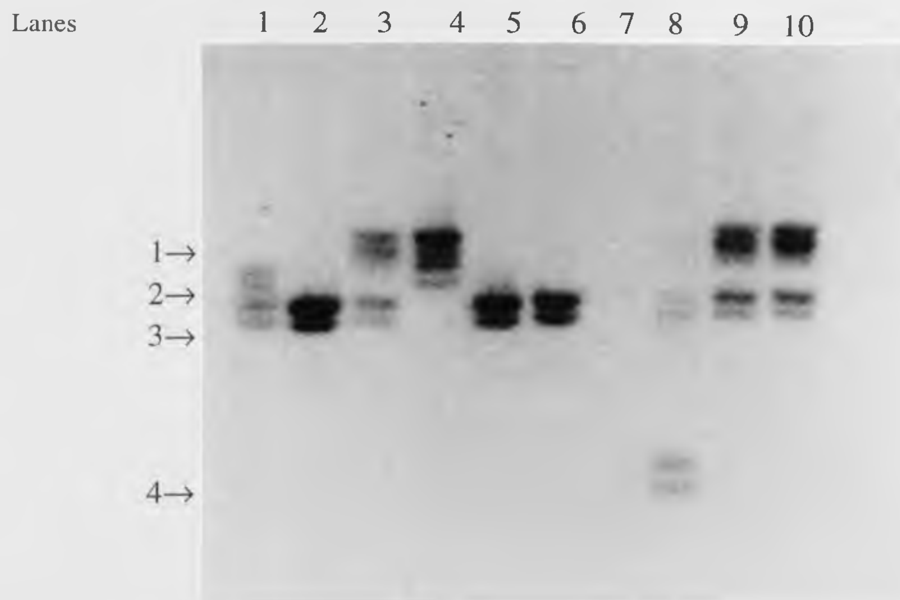


Fig 3.16 Autoradiograph of chromosome 19q marker, *WUT1.9*.

Four alleles of this marker are observed in lanes 1 to 10. Lanes 2, 5 and 6 are homozygote for allele 2 and lanes 3, 9 and 10 show two alleles, 1 and 3. Lane 1 shows alleles 2 and 3 but lane 4 shows alleles 1 and 2. Lane 7 shows no DNA.

3.4.1.2 Primers for chromosomes 7q, 3q, 2p and 1q

These primers with details and PCR conditions are described in tables 3.4 (for chromosome 7q), 3.5 (for chromosome 3q), 3.6 (for chromosome 1q) and 3.7 for chromosome 2p respectively. In addition these loci are shown on fig 3.17 for chromosome 7q (Matise *et al* 1994; Iles *et al* 1994; Gyapay *et al* 1994; Dib *et al* 1996). One sample of allele segregation for chromosome 7q markers, *D7S849* is shown in Figure 3.18.

Markers for chromosome 3q are illustrated in fig 3.19 (Matise *et al* 1994; O'Connell *et al* 1994; Gyapay *et al* 1994; Sudbrak *et al* 1995; Dib *et al* 1996) and one sample of allele segregation for *D3S1302* marker is represented in Figure 3.20.

Chromosome 1q markers (Gyapay *et al* 1994; Dib *et al* 1996) and one sample of allele segregation for *D1S252* marker are shown in figs 3.21 and 3.22 respectively.

Primers and PCR conditions for chromosome 2p and also one sample of allele segregation for *D2S139* marker are represented in table 3.7 and fig 3.23 respectively.

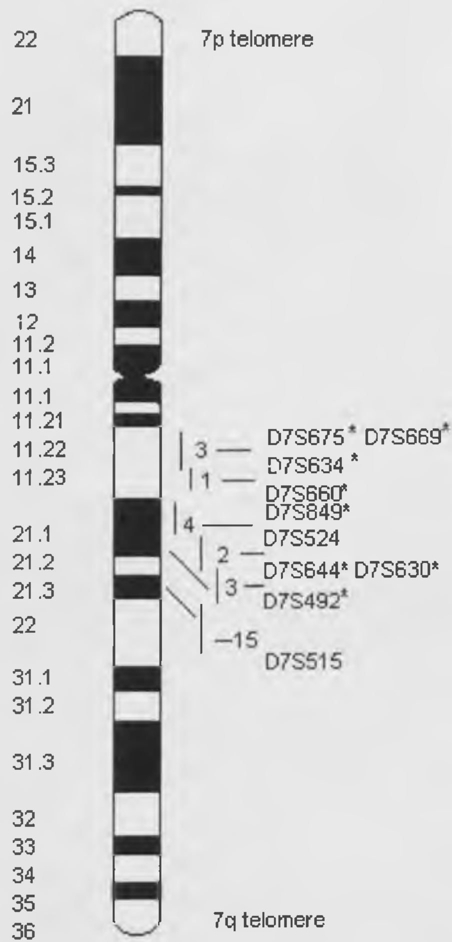


Fig 3.17 Chromosome 7q markers which were used in haplotype segregation and linkage analysis.

* : Indicates the markers used by Iles *et al* (1994).

Locus	Location	Primer sequences	PCR conditions	Product size (bp)	Number of alleles	Ref.
D7S675	7q11.22	For GGAATNAAAGTATTACCGTATAGGA Rev TTGCTGTCCAGGTGCC	94°C 40s, 55°C 30s, 72°C 1min	201-299	5	Gyapay *et al* (1994) Dib *et al* (1996)
D7S634	7q11.22	For AATCCTCCAAATGAAACAGA Rev CTCAGTACACGTTGCTGGTA	94°C 40s, 55°C 30s, 72°C 1min	136-148	7	Gyapay *et al* (1994) Dib *et al* (1996)
D7S660	7q11.23-21.1	For TAGGCCAACACTGGGG Rev AGCTTGATAGTGGGAATCATTT	94°C 40s, 55°C 30s, 72°C 1min	189-197	4	Gyapay *et al* (1994) Dib *et al* (1996)
D7S849	7q11.23-q21.1	For AAGGCCTGTTAAAAATCACC Rev GACCCTGGGCAAGTCATTA	94°C 40s, 55°C 30s, 72°C 1min	138-166	8	Iles *et al* (1994)
D7S524	7q21.1	For AAGTAATGCAAAACAGCCTTGA For ACCCACTGAAAAGATTTGTGTC	94°C 40s, 55°C 30s, 72°C 1min	234-246	7	Gyapay *et al* (1994) Dib *et al* (1996)
D7S515	7q21.3	For GGGAGTTACTACCCTCACTTAATG Rev GGACTGGGCAGCAAAG	94°C 40s, 55°C 30s, 72°C 1min.	128-190	10	Gyapay *et al* (1994) Dib *et al* (1996)

Table 3.4 Primers for amplification of microsatellite markers on chromosome 7q used in linkage analysis. The initial denaturation for all primers was 94°C and final extension at 72°C was 10 mintues.

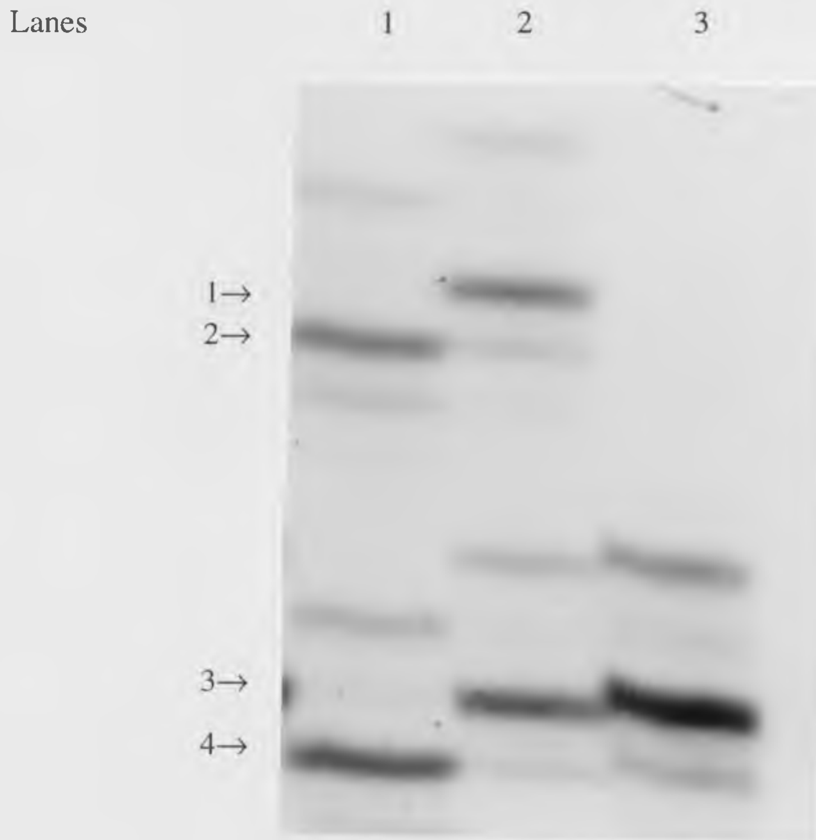


Fig 3.18 Autoradiograph of allele segregation of chromosome 7q marker, *D7S849*.

Four alleles for this marker are observed which are shown in lanes 1 to 3. Lane 1 shows bands 2 and 4 and lane 2 shows bands 1 and 3. Lane 3 is homozygous for band 3.

Fig 3.19 Chromosome 3q markers which were used in linkage analysis.

* : Indicates markers used by Sudbrak *et al* (1995) from the region 3q13 in mapping a further malignant hyperthermia susceptibility locus.

Locus	Locatio n	Primer sequences	PCR conditions	Product size (bp)	Number of alleles	References
D3S1271	3q11.1	For TGATTGGAGGTGGTAGAGGT Rev AGCTATCATGTAGAAAAGCAGCA	94°C 40s, 55°C 30s, 72C 1min	146-158	5	Gyapay *et al* (1994) Dib *et al* (1996)
D3S1302	3q11.2	For CTGAGTTTCAGTTTCCTTATCT Rev ACCTACTAAGTCCCCAGC	94°C 40s, 55°C 30s, 72°C 1 min	123-143	6	Gyapay *et al* (1994) Dib *et al* (1996)
D3S1278	3q11.2	For GGACACATGCTCCTGGAA Rev TGCACTACAGGGCAGTTG	94°C 40s, 55°C 30s, 72°C 1 min	203-231	13	Gyapay *et al* (1994) Dib *et al* (1996)
D3S3654	3q13.1	For CAGGCTACTTGTCTTATCTTTTG Rev GATCTTGTAGCTATTCCAGTTTG	94°C 40s, 55°C 30s, 72°C 1 min	157-167	10	Gyapay *et al* (1994) Dib *et al* (1996)
D3S1303	3q13.1	For CAGACAATGGCTTCCAAAAGTA Rev CAAACTTAGGGTTGTTTCCTCAC	94°C 40s, 55°C 30s, 72°C 1 min	169-220	10	Gyapay *et al* (1994) Dib *et al* (1996)
D3S1309	3q21	For CTTTGGGGAATCATTAGTCTGT Rev ATGAGAATTGTCATGGTGC	94°C 40s, 55°C 30s, 72°C 1 min	132-150	8	Gyapay *et al* (1994) Dib *et al* (1996)
D3S1279	3q23	For CACCATCTGTGTGGTATTGG Rev GACCTATTTTGGTTAACAATTTAGA	94°C 40s, 55°C 30s, 72°C 1 min	264-282	10	Gyapay *et al* (1994) Dib *et al* (1996)
D3S3638	3q13.1	For TCCACATTGGACAACAAA Rev ACCGAATAGTCTTGCACTG	94°C 40s, 55°C 30s, 72°C 1 min	149-163	5	Gyapay *et al* (1994) Dib *et al* (1996)

Table 3.5 Primers for amplification of microsatellite markers on chromosome 3q used in linkage analysis. The initial temperature was 94°C for all markers and final extension at 72°C was 10 minutes.

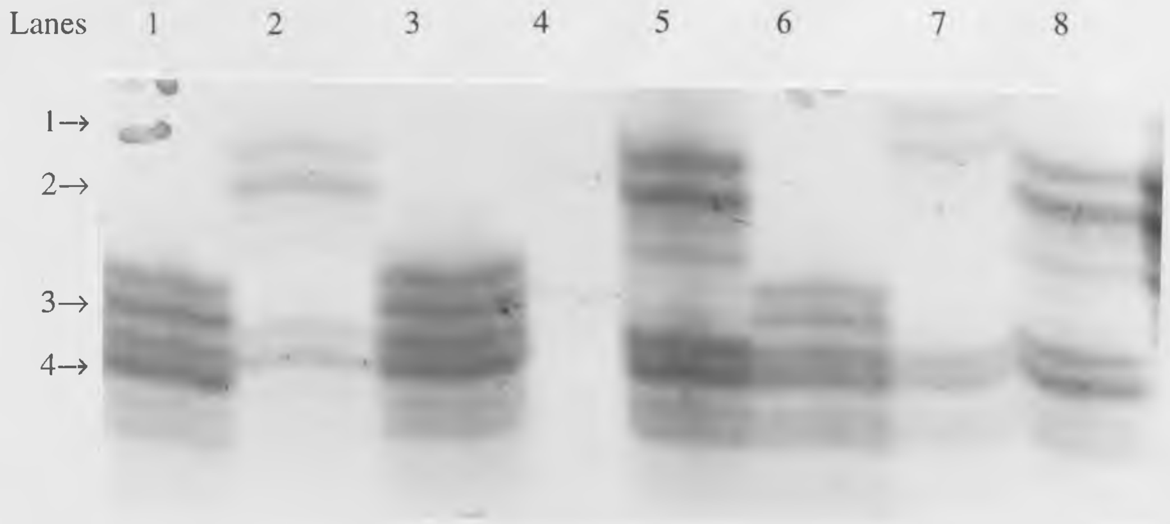


Fig 3.20 Autoradiograph of chromosome 3q marker, *D3S1302*.

Four alleles are shown in lanes 1 to 8. Lanes 1, 3 and 6 show bands 3 and 4 but bands 2 and 4 are observed in lanes 2, 5 and 8. Lane 7 contains two bands, 1 and 4.

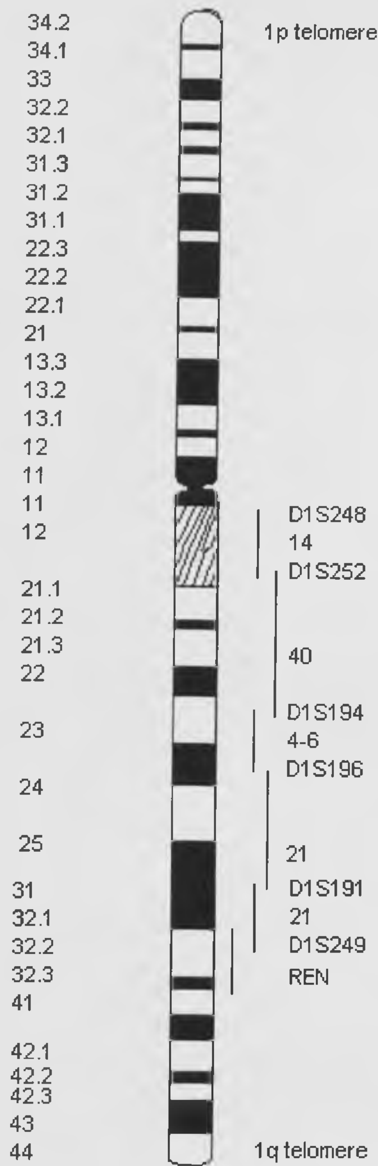


Fig 3.21 Chromosome 1q markers used in haplotype segregation and linkage analysis.

The *REN* gene encodes the α_1-subunit of the calcium channel dihydropyridine receptor. It is proposed as a candidate gene in malignant hyperthermia susceptibility. The *D1S249* marker is close to region 1q31 where the *REN* gene is located.

Locus	Location	Primer sequences	PCR conditions	Products size (bp)	Number of alleles	References
D1S248	1q11	For GATCTGATTAGTATTGTCTGCTTGA Rev GCTTTATAGGAGGTATCTTTNTGTG	94°C 40s, 55°C 30s, 72°C 1min	191-211	11	Gyapay *et al* (1994) Dib *et al* (1996)
D1S252	1q12	For AGCTTTTTACTCTTAACCTATTCAT Rev GCAGAGAACATGTGTATTAATGA	94°C 40s, 55°C 30s, 72°C 1min	99-119	8	Gyapay *et al* (1994) Dib *et al* (1996)
D1S194	1q23	For AGCTAGGCTGTAAGTTTTCTGCTC Rev GTCTCTTGCTGGACTGGGA	94°C 40s, 55°C 30s, 72°C 1min.	233-239	4	Gyapay *et al* (1994) Dib *et al* (1996)
D1S196	1q23-q24	For GGCTGTGGGTGTTTCTCCTA Rev AGCTCTCATGNCTTTACATTCT	94°C 40s, 55°C 30s, 72°C 1min.	267-279	5	Gyapay *et al* (1994) Dib *et al* (1996)
D1S191	1q31	For GGCTGTGGGTGTTTCTCCTA Rev AGCTCTCATGNCTTTACATTCT	94°C 40s, 55°C 30s, 72°C 1min.	153-169	8	Gyapay *et al* (1994) Dib *et al* (1996)
D1S249	1q31-q32.1	For TGGCATGTCTTTGAAGGAAT Rev TGGTTGTAGATGAGACTGGC	94°C 40s, 55°C 30s, 72°C	155-185	15	Gyapay *et al* (1994) Dib *et al* (1996)

Table 3.6 Primers for amplification of microsatellite markers on chromosome 1q used in linkage analysis. The initial denaturation temperature was 94°C for four minuets for all markers and final extension was 10 minutes at 72C°.

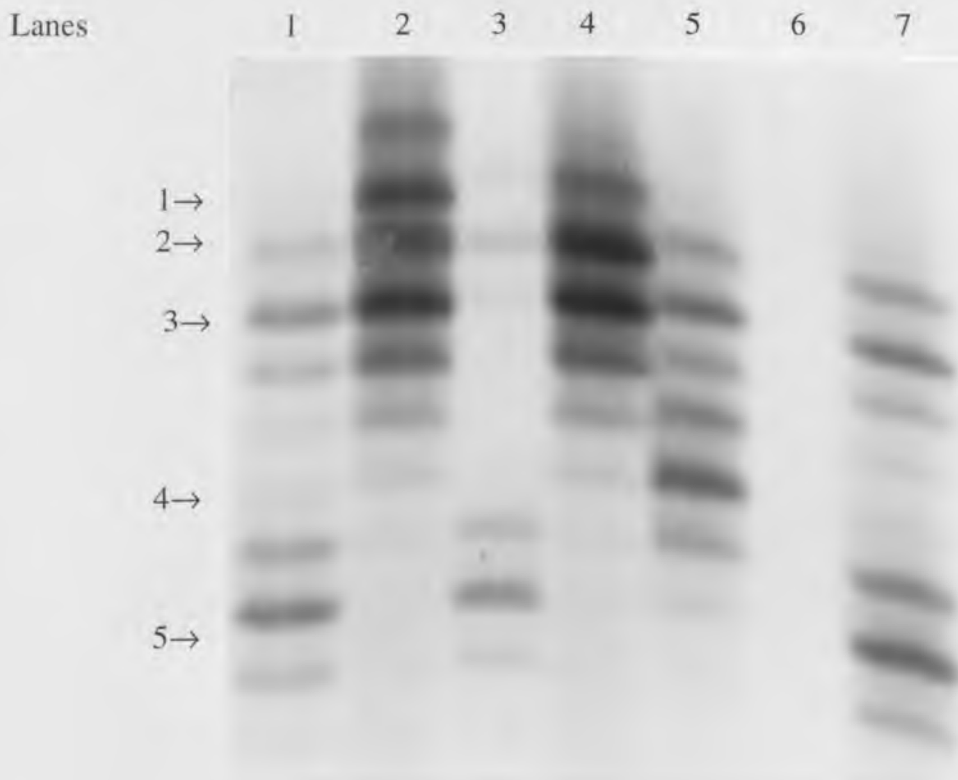


Fig 3.22 Autoradiograph of allele segregation of the chromosome 1q marker, D1S252.

Five alleles of this marker are shown in lanes 1 to 7. Lane 6 is control with no DNA. The alleles are numbered at the left hand side of the photograph.

locus	Location	Sequences	PCR condition	Product size (bp)	No. of allele s	Ref.
D2S139	2p11.1	For AGCTCAAAGCAAATGCATGC Rev AAATTGCGAAACTGTGGCTT	94C 40s, 58C 30s, 28X	175-197	9	Gyapay *et al* 1994 Matise et al 1994 Dib *et al* 1996
D2S398	2p22	For TTGCAGCGAGACAAGATCACACTA Rev TGATGCCCTCATAACAAAGAGTTC	94C 40s, 58C 30s, 28X	113-139	10	Gyapay *et al* 1994 Dib *et al* 1996

Table 3.7 Primers for amplification of microsatellite markers on chromosome 2p used in linkage analysis. The initial denaturation was carried out at 94°C for 5 min and final extension was 10 minutes at 72°C.

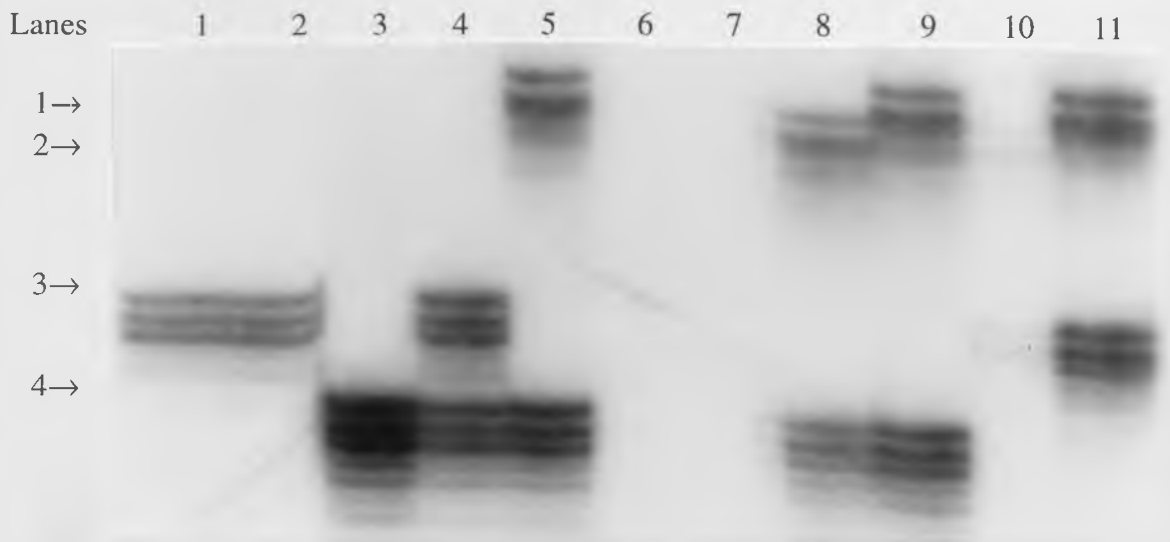


Fig 3.23 Autoradiograph of chromosome 2p allele segregation.
The *D2S139* marker shows four alleles which are observed in different lanes. Lanes 6 and 7 have no DNA but different alleles are observed in lanes 1 to 10.

3.5 Mutation screening in *RYR1* gene

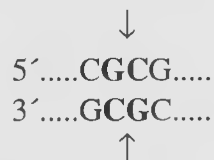
Three mutations, C487T, G1021A and C1840T were investigated using genomic DNA samples from MHS, MHE and MHN individuals. Screening was carried out using PCR amplification and restriction enzyme digestion. The conditions and primers used are described in table 3.8.

3.5.1 Primers for mutation screening

Details of primers used for amplifying genomic DNA in the region of three *RYR1* gene mutations investigated in this study are shown in table 3.8.

3.5.2. T substitution at C487

This survey was carried out by amplification of the 76 base pair fragment which spans the region of the C487T mutation (table 3.8) (Quane *et al* 1993). By restriction analysis due to base substitution (mutant sequence) the specific site for the *Bst*UI restriction enzyme (Bio-lab) is absent. The normal specific site for enzyme action is :



The C487 to T mutation (CGCG→CGTG) deletes a *Bst*UI restriction site and so in mutant samples there is no cleavage with this enzyme. Normal individuals yield two bands at 44 and 32bp. Mutation carriers yield three bands, two at 44 and 32bp from their normal *RYR1* allele and a 76bp fragment

RYR1 mutations	Sequence of primers 5′→3′	PCR condition	Location	Length of product
C487T Forward Reverse	TCCAAGCAGAGGTCTGAAGGAGAA AGCGCTCGGAGGAGACACTGACAA	94°C 1min, 58°C 1min, 72°C 1min, 30 cycles	forward at 457TC... and reverse at 532AG… on cDNA	76bp
G1021A Forward Reverse	GCC CCC CTG AGA TCA AGT CC CAG TCC TGA GGC CAC ATG CTG	94°C 1min, 58°C 1min, 72°C 1min 30 cycles	forward at 1001GC... and reverse at 1061GT on cDNA	62bp
C1840T Forward Reverse	GTTCCCTGTGTGTGTGTGCAATGGTG AGCCAGGGAGCAAGTTCTCAGTAAT	94°C 45s 58°C 90s 30 cycles	forward at 1808GT...and reverse at 1881AG…on cDNA	74bp

Table 3.8 The sequences, PCR conditions, and locations of primers used in *RYR1* gene mutation screening.

corresponding to the uncleaved PCR product. 15µl of the 76bp PCR product was digested using 2 units *Bst*UI (New England, Bio-Labs) in a total volume of 20µl using the 1X manufacture's buffer (New England, Bio-Labs). Digests were performed at 60°C for 2 hours. 10-15µl samples of digestion products were mixed with loading buffer (0.5mM xylene cyanol, 1mM bromophenol blue, 20% Ficoll 400) and resolved on 4% agarose gels (3% low melting high resolution agarose and 1% normal agarose) at 70 volts for 50-60 minutes. DNA was stained with ethidium bromide and viewed with ultraviolet light and photographed. An example of a gel for typing the C487T mutation is illustrated in fig 3.24.

3.5.3 G1021 to A

At nucleotide 1021 the normal **G** is converted to an **A** in mutated samples. This mutation does not change any known restriction sites Then the diagnosis of heterozygous individuals was possible using single-stranded conformation polymorphism (SSCP) (Quane *et al* 1994). Therefore a primer (Adeokun *et al* submitted 1997) was designed to create a restriction site. This forward primer includes a base substitution C for A at position 1019. This change creates a specific site (CCGG) for *Msp* I (restriction enzyme) digestion in the normal PCR products (1021G) which is absent in products generated from the mutant 1021A (CCAG) DNA.

1001 GCCCCCCTGAGATCAAGTACGG \ CAGCATGTGGCCTCAGGACTG 1061

5′ GCCCCCCTGAGATCAAGTCC (engineered primer) GTCGTACACCGGAGTCCTGAC

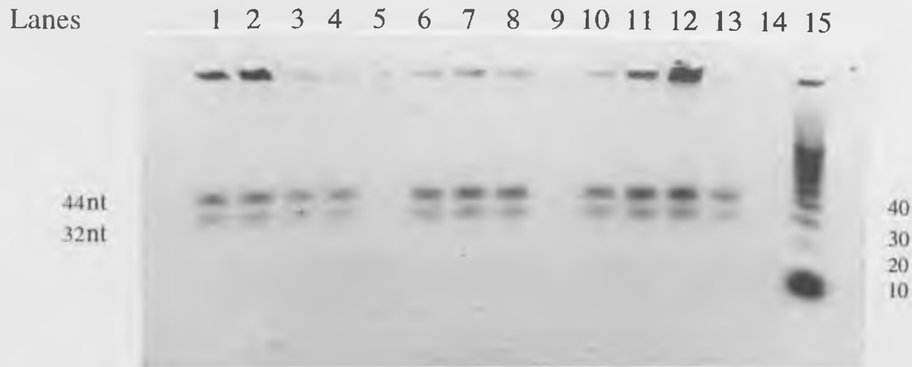


Fig 3.24 C487T mutation screening.
The 76 nucleotide PCR product yields a 44 and a 33 nucleotide fragments from C487 (normal) chromosomes after *Bst* UI enzyme digestion; the 76 nucleotide fragment is not digested from 487T chromosomes. This mutation was not observed in UK MH families. Lanes 5 and 9 ave no DNA and lane 14 shows a 10bp ladder.

At position 1019 in engineered primer **A** at normal sequence is changed to **C** and this substitution creates CCGG sequence which is specific site for *Msp* I restriction enzyme.

PCR was performed as table 3.8. PCR products were digested by using *Msp* I restriction endonuclease enzyme(Bio-Lab). 2-3 units of enzyme in 1X buffer 2 (50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT and pH 7.9)(New England, Bio-Labs) was added to 15μl PCR product and incubated at 37°C for 2 hours. The 62bp products were cleaved in 21bp and 41bp fragments in normal G1021 alleles but three bands, 21bp, 41bp and 62bp in mutation carriers of 1021A alleles. 10-15μl of digested products were analysed on 4% agarose gel (3% Nusieve and 1% normal agarose) at 70 volts for 50-60 min. DNA was stained with the ethidium bromide and viewed with ultraviolet light and photographed. An example of a gel for typing the G1021A mutation is illustrated in fig 3.25.

3.5.4 Pig mutation (substitution of C1840 to T)

Genomic DNA was amplified using primers (Genosys) which hybridise at nucleotides 1808 and 1881 (table 3.8) and generate a fragment of 74bp. The C1840T mutation deletes a *Rsa*1 restriction site (GTAC→GTAT) and so in mutant samples there is no cleavage with this enzyme. The specific restriction site for *Rsa*1 is :

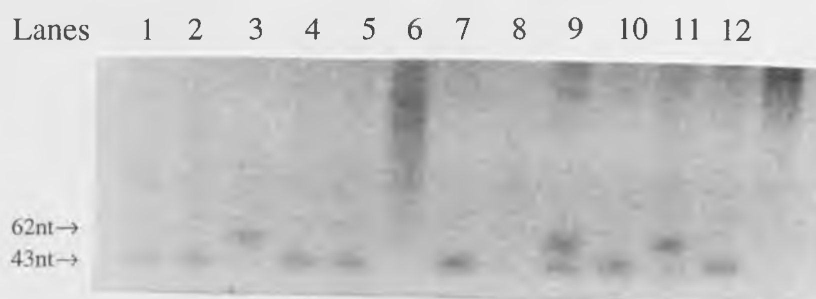
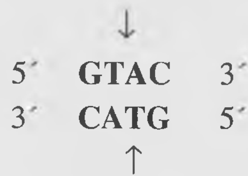


Fig 3.25 G1021A mutation screening.
The 62 nucleotide PCR product yields a 43 nucleotide fragment from G1021 chromosomes after *Msp*I digestion; the 62 nucleotide fragment is not digested from 1021A chromosomes. Lanes 3, 9 and 11 are heterozygous for 1021 mutation (G base substituted by A), Lane 6 has no DNA but the others lanes show normal sequence for the 1021 mutation



Due to the lack of a restriction site in mutant chromosomes, MHS samples generate three bands, 74, 41 and 33bp but two bands, 41 and 33 from normal DNA. 10µl of the 74bp PCR product was digested using 1µl of *Rsa*I (Promega) 1X buffer. Digests were performed at 37°C for two hours. The 10-15µl of digested products were mixed with loading buffer (Xylene cyanol; bromophenol blue, Ficoll 400) and run on 4% agarose, a mix of 3% Nusieve (Flowgen) and 1% normal agarose (Boehringer Mannheim) at 70 volts for 50-60 minutes. DNA bands were stained with ethidium bromide and viewed with ultraviolet light and photographed. An example of a gel for typing the C1840T mutation is illustrated in fig 3.26.

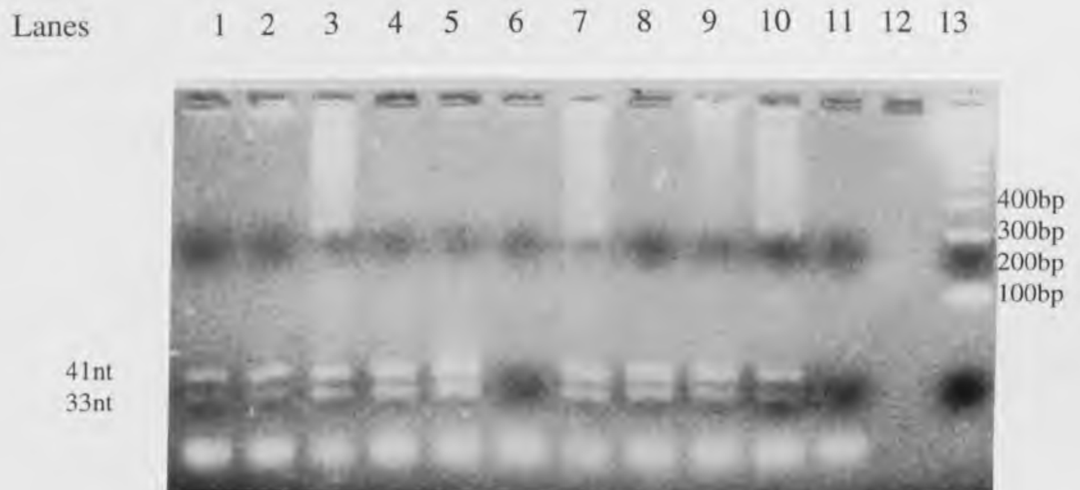


Fig 3.26 C1840T mutation screening.

The 74 nucleotide PCR product yields 41 and 33 nucleotide fragments from C1840 chromosomes with *Rsa*I digestion. The 74 nucleotide fragment is not digested from 1840T chromosomes. This mutation was not observed in screened samples from UK MH families. Lanes 6, 11 and 12 show no DNA. The marker used is 100bp ladder.

Chapter four

Results and Discussion of Linkage Analysis

Because of heterogeneity individual families are considered independently as chromosome 19q linked and non 19q linked.

The probable MHS gene candidate *RYR1* and two distal flanking markers (*D19S47* and *D19S178*) and four proximal flanking markers (*D19S49*, *D19S191*, *D19S220* and *WUT1.9*) were typed in four families.

For chromosome 19q six families, LMH07, LMH12, LMH15, LMH16, LMH17 and LMH18, which had been investigated by IVCT were studied. Details of typing these seven microsatellites (for families LMH07 and LMH12 the microsatellites were different as the major part of marker typing for these two families had been previously carried out by Dr J. Curran) and their haplotypes for chromosome 19q13.1 are summarised for each of these pedigrees in figures 4.1 to 4.6. However, families used in the project will be discussed in the following results. The investigated families are classified as results achieved using markers from chromosomes 19q, 7q, 3q, 1q and 2p.

4.1 Linkage analysis of MHS with markers from chromosome 19q

Family **LMH07** composed three MHS, two MHE, two MHN and one person who is not tested using IVCT. Eight individuals have been typed for six chromosome 19q linkage markers (*D19S179, D19S191, WUT1.9*, *RYR1, D19S190*

and *D19S47*) in family LMH07. Results of marker segregation are shown in fig 4.1 The high risk haplotype 2-2-3-2-2 is shared between MHS and MHE individuals and one MHN, III.1. This individual, excluding the most proximal marker *D19S179,* has inherited the high risk haplotype despite that she showed no contracture responses using 3% halothane and 2mM caffeine. In addition the proband III.2 is a recombinant for *D19S47* the most distal marker used. Despite the similarity in haplotypes in MHS and MHE individuals the lod scores for this family mostly are low for 19q region markers except for *D19S179* which is +0.6 at $\theta = 0.00$. Lod score results for this pedigree are listed in table 4 1.

Family LMH07 was first surveyed for chromosome 19q markers (some parts of this practical work for chromosome 19q markers for this family was carried out by Dr J. Curran) and excluded as a chromosome 19-linked family.

Family **LMH 12** has been investigated for linkage between MH susceptibility and markers (*D19S191, WUT1.9, RYR1* and *D19S47*) from chromosome 19q13.1. The pedigree of family LMH12 showing segregation of these markers is illustrated in fig 4.2. The proband, III.3, had positive contracture to halothane but he is negative for caffeine contracture in IVCT and so is classified as $MHE_{(h)}$. In addition IVCT indicated $MHE_{(h)}$ status for his sister, III.1 and his uncle, II.3. His father II.1 and another uncle II.5 are MHS. The high risk haplotype, 1-3-2-6 is observed in two MHS and one MHN, III.2 individuals but it is not shared with $MHE_{(h)}$ individuals and the reminding MHN individuals.

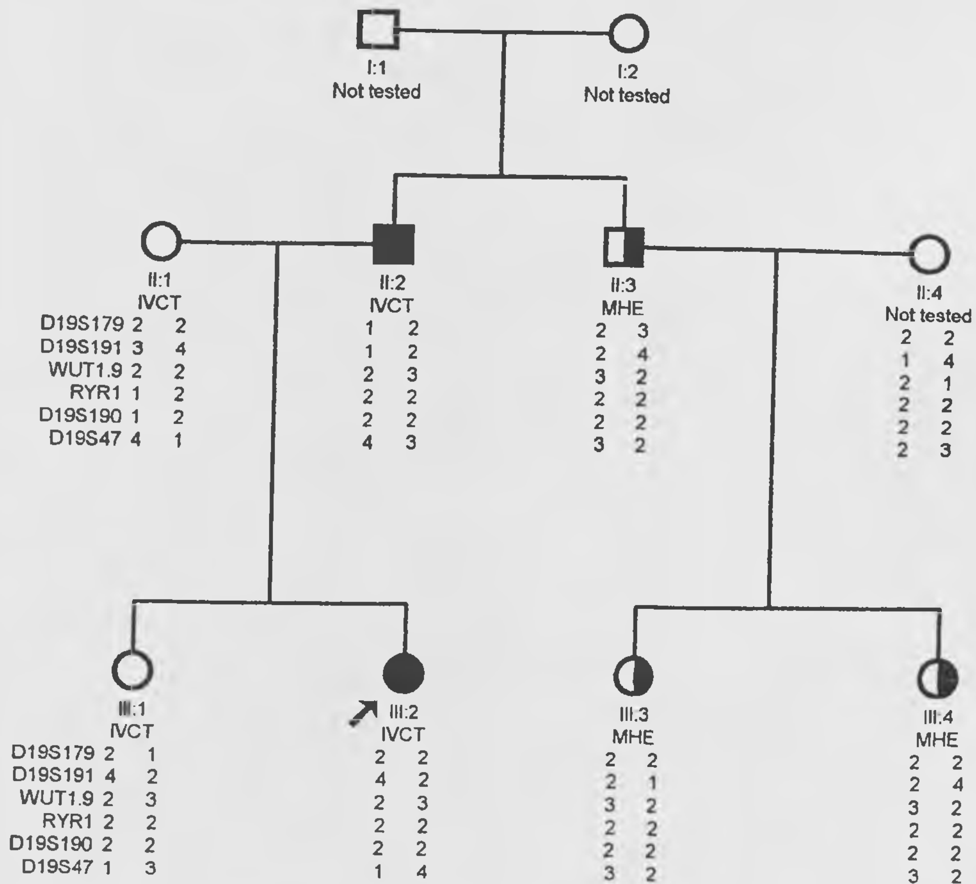


Fig 4.1 Marker segregation of chromosome 19q in family LMH07.

The markers used are *D19S179, D19S191, WUT1.9, RYR1, D19S190* and *D19S47* respectively. Although the III.1 is MHN, she received high risk haplotype 2-2-3-2-2-3 except with recombination for *D19S179*. The high risk haplotype is shared between MHS and MHE individuals. The individuals III.3 and III.4 although are MHE and MHS respectively but they inherited the same haplotype from their father. Maybe for these persons it can be concluded that the maternal genotype has additive effects on susceptibility.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.1	0.15	0.2	0.25	0.3	0.5
D19179	0.60	0.50	0.42	0.33	0.25	0.17	0.11	0.00
D19S191	-0.35	-0.23	-0.16	-0.11	-0.08	-0.06	0.04	0.00
WUT1.9	-0.33	-0.20	-0.12	-0.07	-0.04	-0.02	-0.01	0.00
RYR1	0.10	0.07	0.05	0.30	0.01	0.00	0.00	0.00
D19S190	0.10	0.09	0.07	0.05	0.04	0.03	0.02	0.00
D19S47	-0.32	-0.18	-0.09	-0.04	-0.01	-0.00	0.00	0.00

Table 4.1 The two point lod scores results of linkage analysis between chromosome 19q markers and malignant hyperthermia susceptibility in family **LMH07**.

III.2 is a definite MHN with no positive response to halothane and caffeine using high concentrations. In LMH12 the lod score for all markers are -0.00 at θ = 0.00. These results are shown in table 4.2. Based on this results and lod score calculation the suggestion for lack of linkage to chromosome 19q13.1 and exclusion of it is not impossible.

In family **LMH 15** twelve persons were tested for marker segregation analysis. The proband of this family IV.2 is $MHE_{(h)}$ but his haplotype is similar to the other MHS individuals III.2, III.3 and IV.1 (fig 4.3). Where data exist the high risk haplotype (4-5-2-3-2-3-5) is common between all four MHS individuals and it is not transmitted to the three MHN offspring of III.3 and the MHN daughter of III.2. The two point lod score results for this family are mostly positive at θ = 0.00 except for *D19S178* although they are not very significant. These results are shown in table 4.3. Apparently, despite the incomplete results the co-segregation of chromosome 19q haplotypes associated with the MH susceptibility looks almost significant and acceptable.

In family LMH15 as shown in fig 4.3 the marker haplotype, 4-5-2-3-2-3-3 shared between MHS and MHE individuals shows no recombination. These linkage data also suggest that the individual II.3 who is not tested by IVCT may be MHS or perhaps MHE because she carries the high risk haplotype which appears to co-segregate with MH susceptibility. Therefore it may be suggested that the high risk haplotype is transferred from II.3 to subsequent generations.

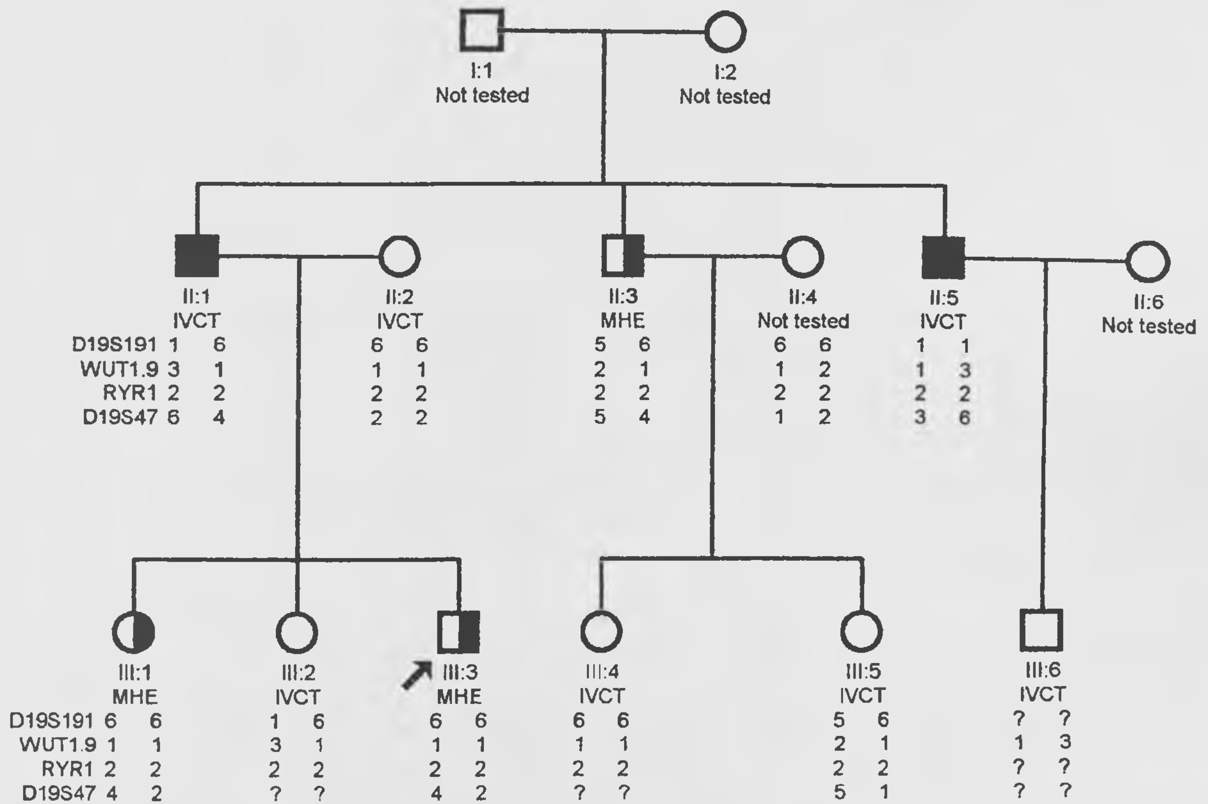


Fig 4.2 Marker segregation of chromosome 19q in family LMH12.

The used markers are *D19S191, WUT1.9, RYR1*, and *D19S47* respectively. The proband of this family, III.3, is MHE and he presented weak responses to halothane (at 2%). Apparently the haplotype 1-3-2-6 is shared between MHS individuals and MHN III.2 but is not observed in MHE persons. Perhaps it is possible to conclude that the MH gene in this family is not linked to chromosome 19q markers.

LOD SCORES								
Recombination fraction→ Markers↓	0.0	0.05	0.10	0.15	0.20	0.25	0.30	0.5
D19S191	-0.01	-0.01	-0.00	-0.00	-0.00	-0.00	0.00	0.00
WUT1.9	-0.00	-0.00	0.00	-0.00	0.00	0.00	0.00	0.00
RYR1	-0.00	-0.00	-0.01	-0.00	-0.00	-0.00	-0.00	0.00
D19S47	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 4.2 The lod scores of family **LMH12** resulted from linkage analysis of chromosome 19q markers and malignant hyperthermia susceptibility.

Based on the lack of high risk haplotype in MHN individuals it may be concluded that in this family the MH gene is linked to chromosome 19q despite the low two point lod scores which are presented in table 4.3. The low lod scores result in part from the MHE status of individual IV.2, who despite the good contracture on expose to halothane (1% halothane for static and dynamic tests, fig 3.5) and inheritance of high risk haplotype is entered in analysis as unknown status (50% chance for being susceptible compared to 98% for MHS individuals). But if we assume the IV.2 individual is MHS the lod scores will significantly increase as shown in table 4.3a. In addition to the incomplete haplotypes and the homozygosity of some markers, *D19S191* and *D19S220* also affect informativeness and reduce the lod scores.

The **LMH 16** family (fig 4.4) as well as family LMH15 has a shared haplotype and a clear co-segregation of the 19q3.1 haplotype with MHS susceptibility, 1-6-5-1-2-4-4. This high risk haplotype is observed in MHE, (II.4 and II.6) and MHS individuals. Despite the good and common high risk haplotype shared in MHS and $MHE_{(h)}$ individuals one recombination is observed in III.2, who has no positive contracture at 2% halothane and 4mM caffeine concentrations. This high risk haplotype with one recombination for *D19S178* marker is shown in III.2. She has two $MHE_{(h)}$ sisters and her mother is affected (MHS). It seems unlikely that this is due to an error in IVCT typing because III.2 showed no positive contracture at more than 2% halothane and 4mM caffeine whereas her MHS mother II.2 has good contracture in 1% static halothane and 0.5% dynamic halothane as well as in 2mM caffeine.

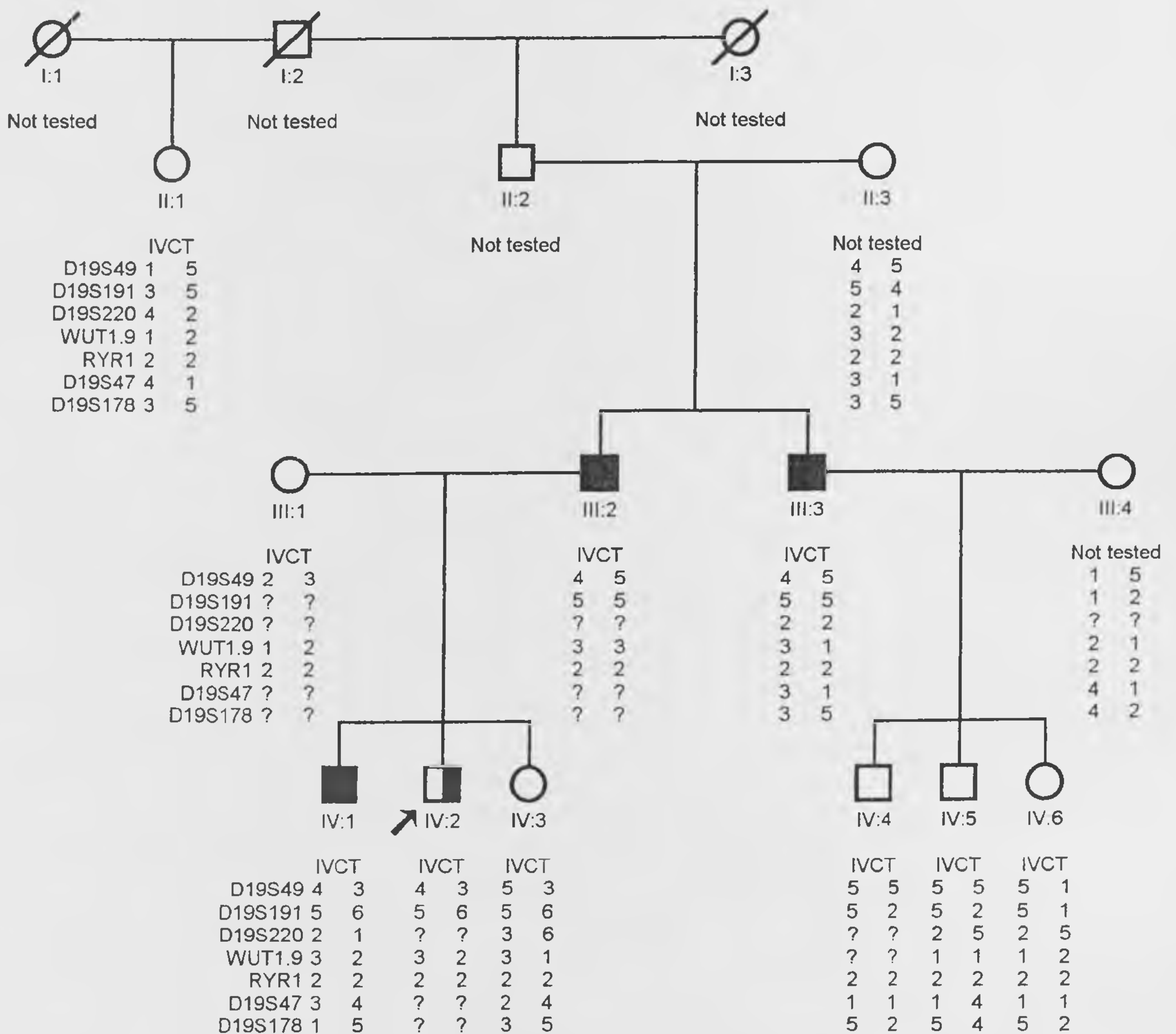


Fig 4.3 Marker segregation of chromosome 19q in family LMH15.

The markers used are *D19S49, D19S191, D19S220, WUT1.9, RYR1, D19S47* and *D19S178* respectively. Apparently the high risk haplotype for this family is 4-5-2-3-2-3-3 which is common (although not completely) between MHS and MHE individuals. This haplotype is not observed in MHN individuals. Individual II.3 who is not tested by IVCT possibly is not MHN as she inherited the haplotype which co-segregates with susceptibility to malignant hyperthermia.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.2	0.25	0.3	0.5
D19S49	0.87	0.74	0.60	0.47	0.36	0.25	0.16	0.00
D19S191	0.05	0.04	0.03	0.02	0.01	0.01	0.00	0.00
D19S220	0.12	0.10	0.09	0.07	0.05	0.04	0.03	0.00
WUT1.9	0.26	0.22	0.17	0.13	0.09	0.06	0.04	0.00
RYR1	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D19S47	0.73	0.61	0.49	0.38	0.28	0.20	0.12	0.00
D19178	-0.11	-0.03	0.00	0.02	0.03	0.03	0.02	0.00

Table 4.3 The results of two point linkage analysis between MH and markers on chromosome 19q in family **LMH15**. As shown in table despite the segregation of high risk haplotype only two markers *D19S49* and *D19S47* have high lod scores. Although the lod scores are not very significant there is no reason for excluding of this family from chromosome 19q.

LOD SCORES				
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.5
D19S49	1.62	1.44	1.26	0.00
D19S191	0.14	0.11	0.09	0.00
D19S220	0.31	0.27	0.23	0.00
WUT1.9	0.56	0.48	0.40	0.00
RYR1	-0.02	-0.01	-0.01	0.00
D19S47	1.17	1.02	0.88	0.00
D19178	-0.52	-0.12	0.01	0.00

Table 4.3a The results of two point linkage analysis between MH susceptibility and chromosome 19q markers in family **LMH15**. As shown in table if assume the IV.3 is MHS the lod scores will increase significantly.

Furthermore, in this family the $MHE_{(h)}$ individuals, II.4 and II.6, have MHS progeny III-4, III-5 (proband) who show good contracture to halothane and caffeine. Linkage analysis for LMH 16 show low lod scores (two point) for chromosome 19q markers and are mostly negative except for *D19S178* for which the lod score is +0.92 at $\theta = 0.00$ (two point lod scores at table 4.4).

The *D19S178* marker is reported to be close to the *LIPE* gene which is a candidate in malignant hyperthermia susceptibility (Levitt *et al* 1995). Support for a close relation between MH locus and *D19S178* is observed in LMH 16. For the MHN individual III.2 carrying the high risk haplotypes a few alternative explanations will be considered. The lack of penetrance due to modifying genes, misclassification and/or mislabelling are the simplest suggestions for this apparent recombinant. *RYR1* gene mutation analysis can be useful for this individual since if these mutations are observed in LMH16 family. The low lod scores, for two point analysis for this family can be due to allele homozygosity and lack of fully informative meioses and $MHE_{(h)}$ individuals otherwise when the MHE individual supposed to be MHS the lod scores are increased (table 4.4a).

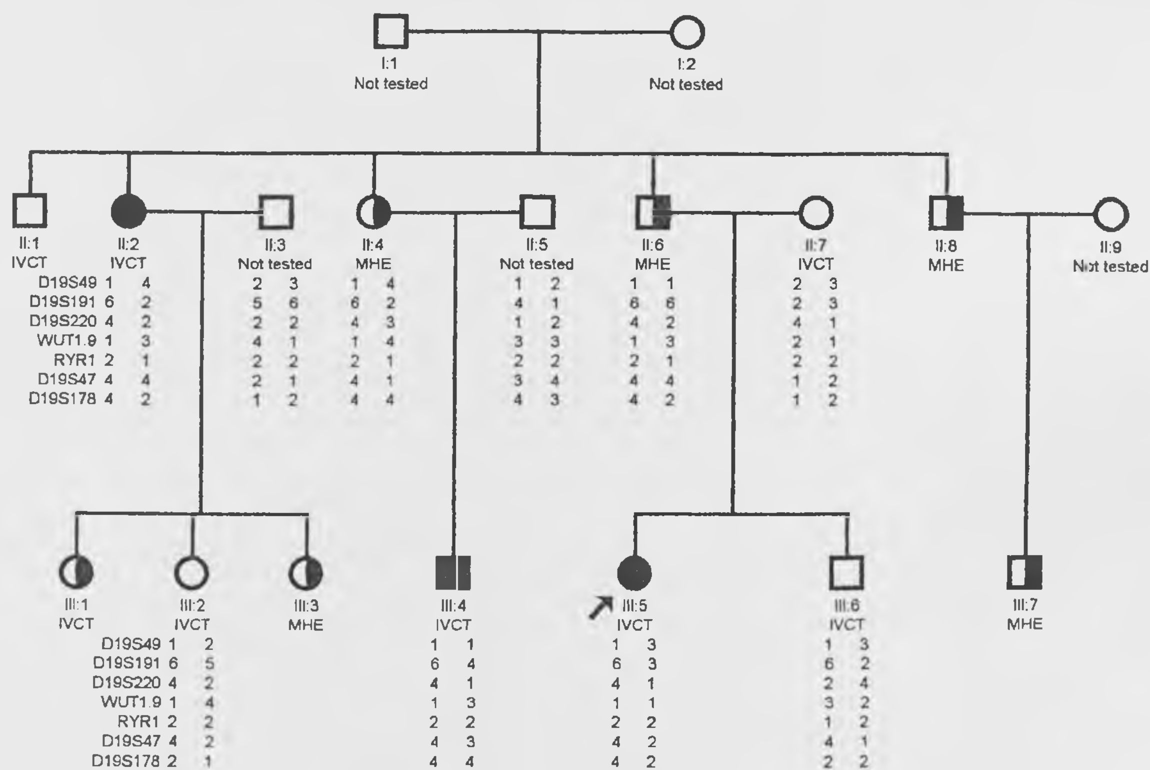


Fig 4.4 Marker segregation of chromosome 19q in family LMH 16.

The markers used are *D19S49, D19S191, D19S220, WUT1.9, RYR1, D19S47* and *D19S178* respectively. The high risk haplotype 1-6-5-1-2-4-4 is shared between MHS and MHE individuals with one recombination for individual III.2 who is MHN. This person has not shown any contracture using high amounts of halothane and caffeine.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.20	0.25	0.3	0.5
D19S49	-0.64	-0.37	-0.23	-0.15	-0.10	-0.06	-0.03	0.00
D19S191	-0.63	-0.36	-0.22	-0.14	-0.09	-0.05	-0.03	0.00
D19S220	-0.38	-0.08	0.01	0.04	0.04	0.03	0.02	0.00
WUT1.9	-0.41	-0.11	-0.01	0.02	0.02	0.02	0.01	0.00
RYR1	-0.43	-0.12	-0.11	0.03	0.04	0.04	0.03	0.00
D19S47	0.40	0.33	0.26	0.19	0.14	0.09	0.05	0.00
D19178	0.92	0.80	0.67	0.55	0.42	0.31	0.20	0.00

Table 4.4 The results of pairwise linkage analysis between MH susceptibility and chromosome 19 markers using family **LMH 16**. The lod scores are given in different recombination fraction. As it is shown despite the clear high risk haplotype in MHS individuals and lack of in MHN persons there is not a significant lod score.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.20	0.25	0.3	0.5
D19S49	0.62	0.53	0.43	0.34	0.25	0.17	0.10	0.00
D19S191	0.66	0.56	0.46	0.36	0.27	0.18	0.11	0.00
D19S220	1.21	1.07	0.93	0.78	0.63	0.48	0.33	0.00
WUT1.9	1.16	1.03	0.89	0.74	0.60	0.45	0.32	0.00
RYR1	1.13	1.00	0.86	0.73	0.60	0.45	0.32	0.00
D19S47	0.47	0.40	0.32	0.24	0.17	0.11	0.07	0.00
D19178	-0.65	-0.19	-0.05	-0.00	0.01	0.01	0.00	0.00

Table 4.4a The results of pairwise linkage analysis between MH susceptibility and chromosome 19 markers using family **LMH 16** when assume the MHE and III.2 individuals are MHS. The lod scores are given in different recombination fraction. With this situation the lod scores for main markers (*D19S220*, *WUT1.9* and *RYR1* significantly is increased. As it is shown the high risk haplotype in MHS individuals is more acceptable.

Family **LMH 17** is composed of two parts with two different probands and two different haplotypes which segregate with MH susceptibility. In the right part or large section except for one marker, *D19S49* in individual III.7 the same haplotype co-segregates with the MHS phenotype. So except for *D19S49* individual III.7, there are no recombinants out of six opportunities for recombination. Recombination between *D19S49* and *RYR1* is not unexpected because *D19S49* is the most proximal marker of this linkage group and because of the distance, approximately 14 cM between *D19S49*, and the main linkage group of markers, *D19S191*, to *RYR1* (fig 3.13). Thus simple inspection suggests that in this part of pedigree MHS is linked to *RYR1*.

In the left hand part of the pedigree the IV.1 is the MHS proband with contracture responses at low concentration of halothane and caffeine. But she has inherited susceptibility to MH from her susceptible father who is not related to the right hand side of family as shown in fig 4.5 and appears probably affected by different haplotype which is not common in MHS individuals of this family. She probably has inherited the high risk haplotype, 2-1-1-2-1-4-5 which may be is high risk haplotype in the relatives of III.2.

The other proband of this family, IV.4 although not tested by IVCT, has inherited the similar high risk haplotype, 4-4-4-2-2-3-5 which is common to all the MHS individuals in the right hand side of the pedigree. His two brothers, IV.2 and IV.3 show strong contracture responses to halothane (0.5%) and caffeine (fig 3.7). In linkage analysis apparently the lod scores for three markers *D19S191, D19S220* and *D19S47* are around 1 although the lod score for the other

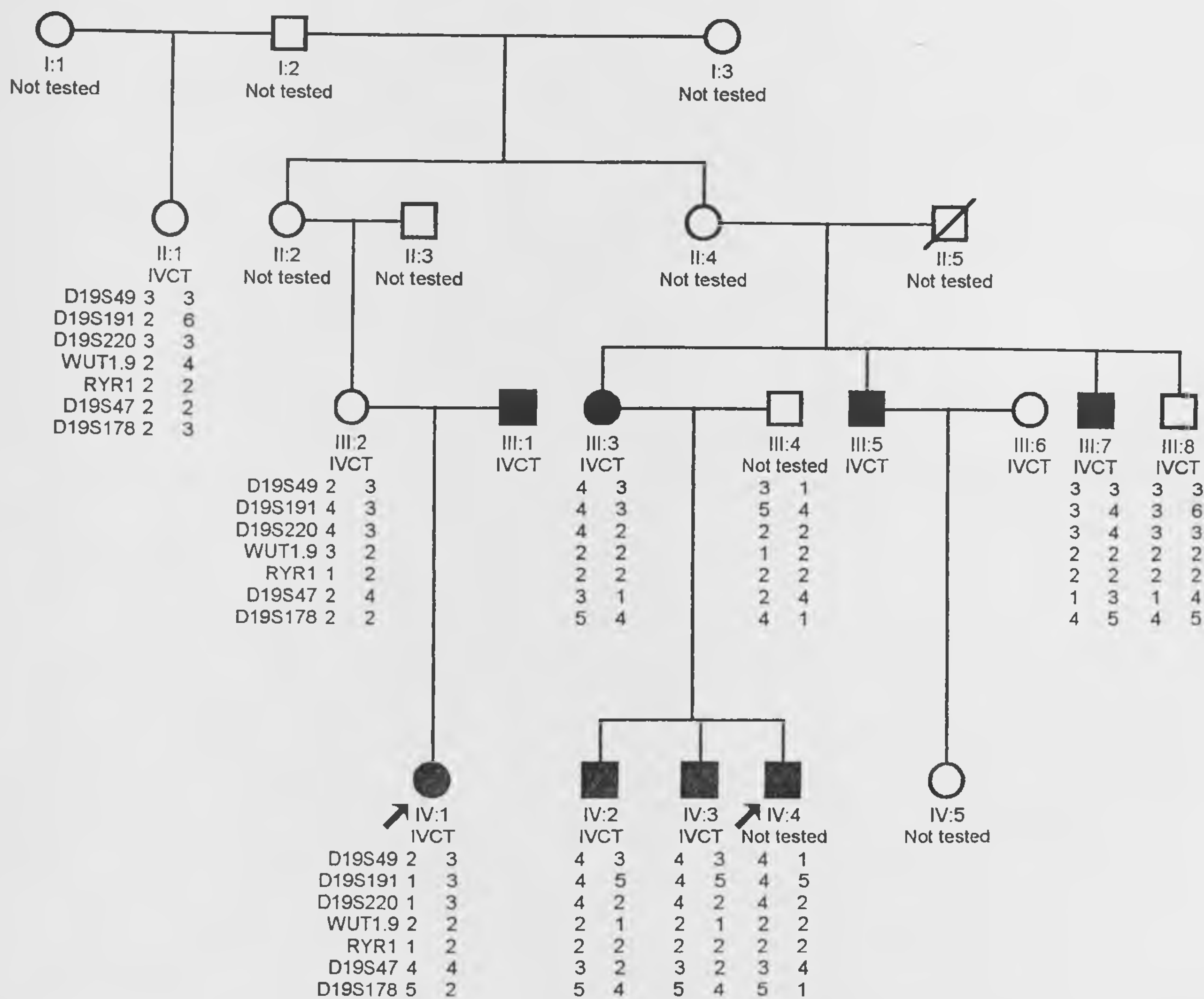


Fig 4.5 Marker segregation of chromosome 19q in family LMH17.

The markers used are *D19S49, D19S191, D19S220, WUT1.9, RYR1, D19S47* and *D19S178* respectively. This family shows co-segregation of the high risk haplotype with malignant hyperthermia susceptibility. The shared haplotype with one recombination for D19S49 at individual III.7, is 4-4-4-2-2-3-5.

four markers *D19S49, WUT 1.9, RYR1* and *D19S178* at $\theta = 0.00$ are negative. The lod score results are presented in table 4.5. Based on the results and condition of IV.1, the suggestion of another locus is not impossible although other reasons such as a different haplotype of chromosome 19q like independent pedigree, cannot be rejected. However, in an explanation of this family based on the two genes model in MH susceptibility the lod score calculation without individual IV-1 will be increased as presented in table 4.5a.

In conclusion family LMH17 like family LMH15 may be supposed to be a 19q-linked family and the low and minus lod scores using two point method referred to un-informativeness of some primers (tables 4.5 and 4.5a).

In family **LMH 18** haplotype analysis has been carried out for 9 individuals, one MHN, four MHS, two MHE and two individuals who have not been tested by IVCT. Markers *D19S49, D19S191, D19S220, WUT1.9, RYR1, D19S47* and *D19S178* co-segregate with the MHS phenotype through the entire pedigree, and support linkage between the MHS locus in MHS and MHE individuals and the 19q13.1 region. In LMH 18 the high risk haplotype, 1-1-4-1-2-3-2 is shared between MHS individuals and this looks to be a 19q linked family based on the results of a two point lod scores analysis presented in table 4.6.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.20	0.25	0.3	0.5
D19S49	-0.73	-0.27	-0.11	-0.04	-0.00	0.01	0.02	0.00
D19S191	0.75	0.67	0.59	0.49	0.39	0.29	0.19	0.00
D19S220	0.83	0.74	0.64	0.53	0.42	0.31	0.21	0.00
WUT1.9	-0.39	-0.29	-0.21	-0.16	-0.11	-0.07	-0.04	0.00
RYR1	-0.16	-0.13	-0.10	-0.07	-0.05	-0.04	-0.02	0.00
D19S47	0.95	0.82	0.70	0.57	0.44	0.19	0.20	0.00
D19S178	-0.18	-0.06	-0.01	0.01	0.02	0.02	0.02	0.00

Table 4.5 The results of two point linkage analysis between MH and markers on chromosome 19q13.2 in family **LMH 17**. As shown in the table the two markers *D19S191* and *D19S220* closer to the *RYR1* gene have higher lod scores with MH locus as well as the distal locus *D19S47*.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.1	0.15	0.2	0.25	0.3	0.5
D19S49	-0.48	0.07	0.03	0.07	0.07	0.04	0.01	0.00
D19S191	1.00	0.95	0.87	0.77	0.65	0.52	0.40	0.00
D19S220	1.22	1.13	1.09	0.89	0.75	0.61	0.46	0.00
WUT1.9	-0.33	-0.26	-0.20	-0.15	-0.11	-0.07	0.04	0.00
RYR1	-0.17	-0.14	-0.11	-0.08	-0.06	-0.04	-0.03	0.00
D19S47	1.32	1.20	1.03	0.87	0.69	0.52	0.36	0.00
D19S178	0.06	0.20	0.24	0.24	0.21	0.17	0.13	0.00

Table 4.5a The results of two point linkage analysis between MH locus and markers on chromosome 19q in family **LMH 17** without individual IV.1. As supposed the lod scores is increased when the individual IV.1 is not entered in calculation.

The high risk haplotype order shows (fig 4.6) no recombination with MH susceptibility in MHS patients and one $MHE_{(h)}$ individual (II.4). Individual III.4 who is $MHE_{(h)}$ has inherited a different haplotype from her mother $MHE_{(h)}$ compared with her MHS sister, III.5. This haplotype is not observed in MHS or MHN individuals (except for *D19S49* and *D19S191*). The proband's mother (II.4), is $MHE_{(h)}$ and she has the same haplotype as MHS individuals. This situation may arise for different reasons. For example despite the proband's mother (II.4) being $MHE_{(h)}$ she shows strong positive contracture responses to halothane at 0.5% as well as her daughter, III.5 but the situation for III.4 using IVCT is different. He shows contracture at 2% halothane which is close to the border between MHN and MHE and he does not show any positive response at concentrations of caffeine under 8mM which is a high concentration. These concentrations of halothane and caffeine suggest doubt about the assignment of the status for III.4 and the condition for making a strong decision in classifying III.4 as MHN or $MHE_{(h)}$. An alternative explanation may be described by the same gene with different high risk haplotypes if we suppose that the individual II.3 is MHS. Another possible explanation involves two causative genes but the probability of this is lower compared to above explanations because of lack of enough data for this family and also low frequency of MH individuals, although finding of two genes in one family is not impossible. The lod scores at $\theta = 0.00$ for three markers, *D19S220*, *D19S47* and *D19S178* are positive, +1.05, +1.4 and +1.17 respectively as presented in table 4.6 . Apparently the low lod scores are related to markers which are not informative (*RYR1*).

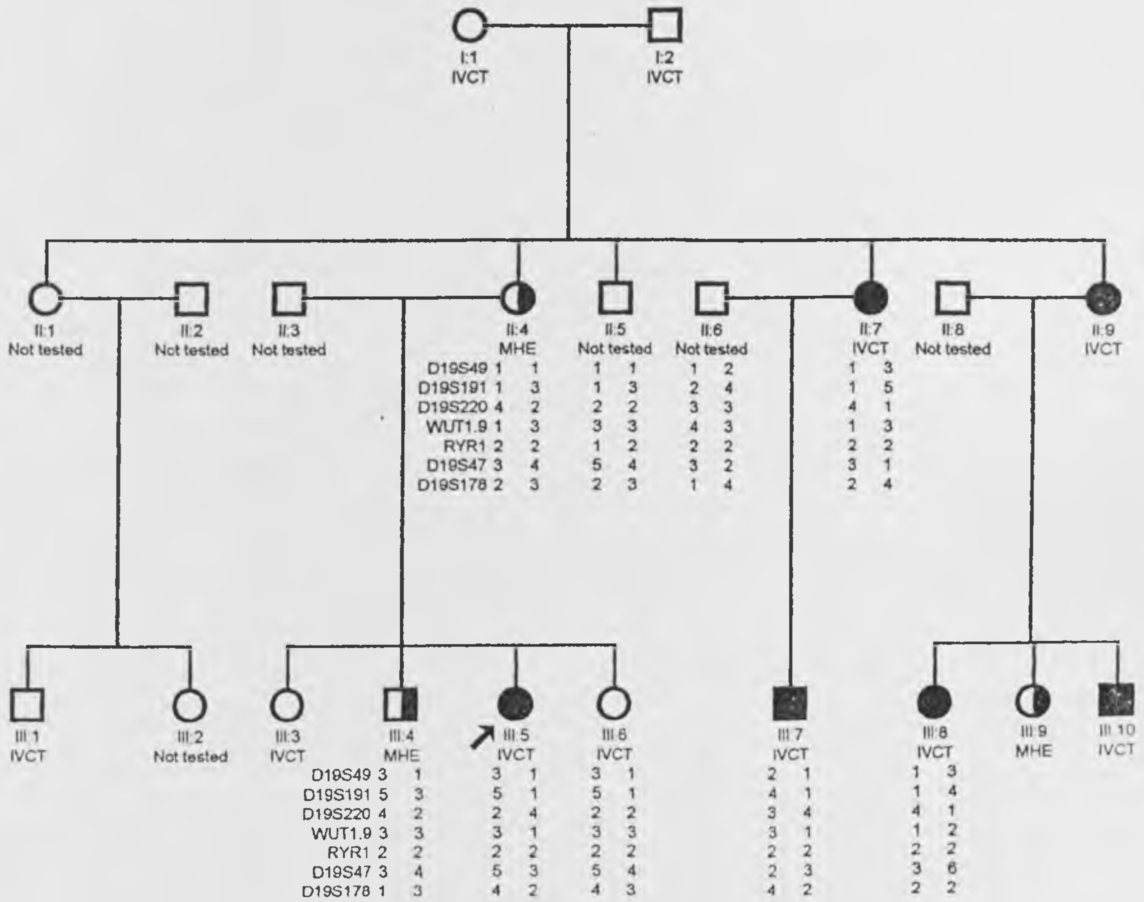


Fig 4.6 Marker segregation of chromosome 19q in LMH18 family.

The markers used are *D19S49, D19S191, D19S220, WUT1.9, RYR1, D19S47* and *D19S178* respectively. Except for individual III.4 (MHE), the high risk haplotype, 1-1-4-1-2-3-2 is shared between MHS and MHE individuals. This individual presented weak responses to halothane and caffeine at 2% and 8mM respectively (fig 3.8) and is probably not MH susceptible.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.20	0.025	0.30	0.5
D19S49	0.17	0.12	0.08	0.05	0.03	0.01	0.00	0.00
D19S191	-0.37	-0.09	-0.00	0.03	0.04	0.03	0.02	0.00
D19S220	1.05	0.91	0.77	0.63	0.50	0.35	0.23	0.00
WUT1.9	-0.09	0.30	0.36	0.35	0.030	0.24	0.16	0.00
RYR1	0.34	0.30	0.23	0.19	0.14	0.10	0.06	0.00
D19S47	1.40	1.25	1.09	0.92	0.75	0.57	0.40	0.00
D19S178	1.17	1.03	0.89	0.74	0.60	0.44	0.30	0.00

Table 4.6 Lod score table of two point linkage analysis between MH locus and chromosome 19 markers of family **LMH 18**. The two markers, *D19S220* and *D19S47* which are rather close to *RYR1* marker have relatively high lod scores compared with other markers and then may close linkage between the MH locus and these two loci.

In conclusion it may be supposed that family LMH18 is 19-linked and probably that individual III.4 is not MH susceptible.

4.2 Conclusion of Chromosome 19q Results

In conclusion it is probable that MH susceptibility is linked to *RYR1* in families LMH15, LMH16, LMH17 and LMH18. There is not strong evidence for exclusion of these families from the 19q region using two point and multipoint linkage analysis.

Based on these results a few points should be considered. In general, although the marker segregation looks to show a correlation with MH locus on 19q in LMH15, LMH16, LMH17, LMH18 the high number of $MHE_{(h)}$ individuals with haplotypes similar to MHS individuals, but entered in the calculations as "unknown status", result in a reduction of lod score information. In addition homozygosity for some markers especially for *RYR1* and *WUT1.9* also results in lod score reduction. The decision in classification of sample as MHS, MHN or $MHE_{(h)}$ is another factor, and misclassification in IVCT or molecular genetic analysis or mislabelling of samples (maybe in LMH16 and LMH18) can be involved in linkage analysis.

The other point which cannot be ignored is the size of families, and the number of generations available for analysis. Perhaps spreading of the search to more members of the pedigrees and completing of haplotypes especially for new IVCT typed members of pedigrees could give rise to increased lod scores and make interesting haplotypes.

In the other part of the linkage analysis, for non 19 linked families the LMH07 and LMH12 families at a brief glance appear not to be linked to the chromosome 19q markers. The linkage analysis results provide less support for linkage of MH susceptibility to chromosome 19q in family LMH07. This family shows one MHN recombination using chromosome 19q markers. Altogether the results of this investigation for linkage to chromosome 19q are inconclusive. With concern to discussion for the mentioned pedigrees (LMH15, 16, 17 and LMH18) this suggestion is not impossible (of course the situation for LMH12 is different). In family LMH12 there is clearer evidence that MH susceptibility is not linked to *RYR1* gene. However acceptance of linkage of LMH12 to 19q gives rise to two cases which do not follow this conclusion. In LMH12 the individual II.5 who is MHS with a different haplotype from the high risk haplotype and individual III.4 who is MHN with the high risk haplotype, 6-1-2-4 are two cases with a low probability against this explanation. If we arrange the haplotype 1-3-2-6 for MHS individuals then there will not be any difficulty in conclusion and excluding it from chromosome 19 markers and the description of the marker distribution.

In family LMH07 despite the common haplotype between MHS and MHE individuals based on the marker segregation and lod scores, linkage to chromosome 19q needs more evidence to be more clear. In LMH07 the appearance for linkage to 19q are more acceptable than the LMH12. The high risk haplotype is shared between susceptible individuals. But the high level of homozygosity for *D19S179*, *D19S190* and *RYR1* and the recombination in III.1 may exclude this family from chromosome 19q although for the reason described

for the III-2 in LMH 16 this is not impossible. In summary the exact conclusion for LMH12 for linkage to chromosome 19q needs more attempts and more evidence but supposing linkage of LMH07 to chromosome 19q may be possible with reasonable features and clear situation.

4.3 Linkage data with other chromosomal regions, 7q, 3q, 1q and 2p

Four families LMH03, LMH07, LMH12 and AN2 have been identified as non 19-linked families as a result of these investigations and previous studies (families LMH07 and LMH12, data are shown in figures 4.1 and tables 4.2; family LMH03 by Dr. J. Curran and AN2 by EMHG). The families LMH03 and AN2 have been investigated previously for marker segregation using markers from chromosomes 19q, 17q, 7q and 3q and excluded from these candidate regions. In this study family LMH07 is investigated for chromosome 3q and 7q and LMH12 was studied using markers from chromosomes 7q, 3q and 1q. The two other families LMH03 and AN2 were investigated using markers from chromosome 2p as a part of a genomic search.

4.3.1 Linkage analysis with markers from chromosome 7q

In families LMH07 and LMH12 marker segregation for six markers (*D7S675, D7S634, D7S660, D7S849, D7S524, D7S515*) from chromosome 7q11.23-q21.3 close to the *CACNLA2* locus are illustrated in figs 4.7 and 4.8 for LMH07 and LMH12 respectively. In family **LMH07** the haplotypes are varied to different alleles and there is not a shared high risk haplotype between MHS or even MHE individuals.

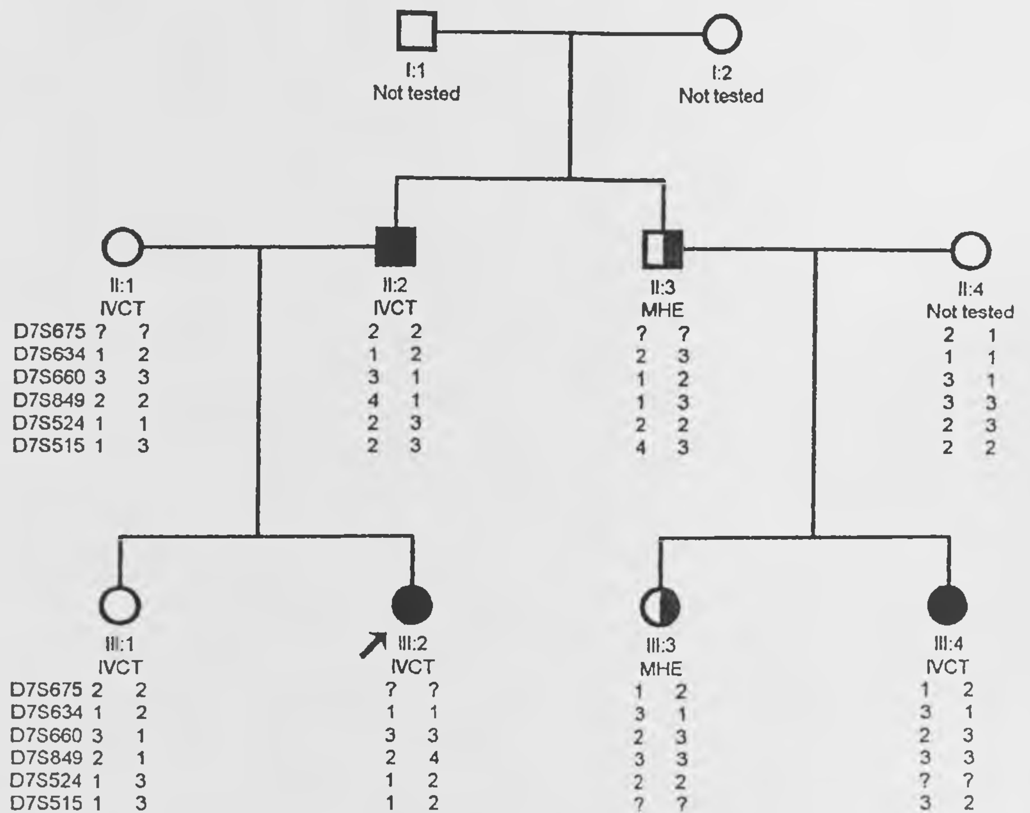


Fig 4.7 Marker segregation of chromosome 7q in family LMH07.

The markers used are *D7S675, D7S634, D7S660, D7S849 D7S524* and *D7S515* respectively. There is no high risk haplotype that co-segregates with malignant hyperthermia susceptibility and using two point linkage analysis shows no linkage to chromosome 7q (table 4.7).

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.1	0.15	0.2	0.25	0.3	0.5
D7S675	0.05	0.04	0.03	0.02	0.01	0.01	0.00	0.00
D7S634	-0.36	-0.21	-0.13	-0.07	-0.04	-0.01	-0.00	0.00
D7S660	-0.32	-0.12	-0.02	0.01	0.03	0.04	0.03	0.00
D7S849	-0.32	-0.10	-0.00	0.03	0.05	0.05	0.04	0.00
D7S524	0.36	0.30	0.23	0.18	0.13	0.08	0.05	0.00
D7S515	-0.32	-0.26	-0.20	-0.14	-0.10	-0.06	-0.03	0.00

Table **4.7** The two point linkage analysis results of chromosome 7q markers and MH susceptibility in family **LMH07**. Except for the locus *D7S524* most lod scores are negative or close to zero.

In conclusion as results are shown (fig 4.7 and table 4.7) there is no evidence of linkage between MH susceptibility and markers from chromosome 7q.

In family **LMH 12** (fig 4.8) the MHS individual II.1 has three children, two III.1 and III.3 (proband) are $MHE_{(h)}$ and one is MHN (III.2). They have all inherited the same haplotype (3-3-3-1-3-3) from their father. In addition the daughters of II-3 (MHE) who are both MHN have received different haplotypes from their father. This means that there is no common 7q marker haplotype which co-segregates with MH susceptibility in this family. Simple inspection of the pedigrees reveals that there is no common haplotype in MHS individuals in either pedigree. The results of chromosome 7q marker linkage analysis in the family LMH12 using two point lod score calculation, are summarised in tables 4.8.

Then for these two families there is no evidence to support linkage between MHS and markers from chromosome 7q. Although for excluding the MH susceptibility locus from chromosome 7q the lod score of -2 is required. These results are consistent with 27 other non 19-linked families data from European MH centres which are reported in EMHG (Genetics Section) meeting in 1995. But they are contrary to the observation of Iles *et al* (1994) who found good evidence for linkage to this region of chromosome 7q in a single three-generation MHS family from central Europe. The candidate region on chromosome 7q (Iles *et al* 1994) was mapped between q11.23 to q21.1 using the *D7S849* and *D7S524, D7S492, D7S634, D7S660, D7S669, D7S675, D7S644* and *D7S630* markers (fig 3.13) with a maximum lod score of +2.91. The co-segregation of marker *D7S849* which is

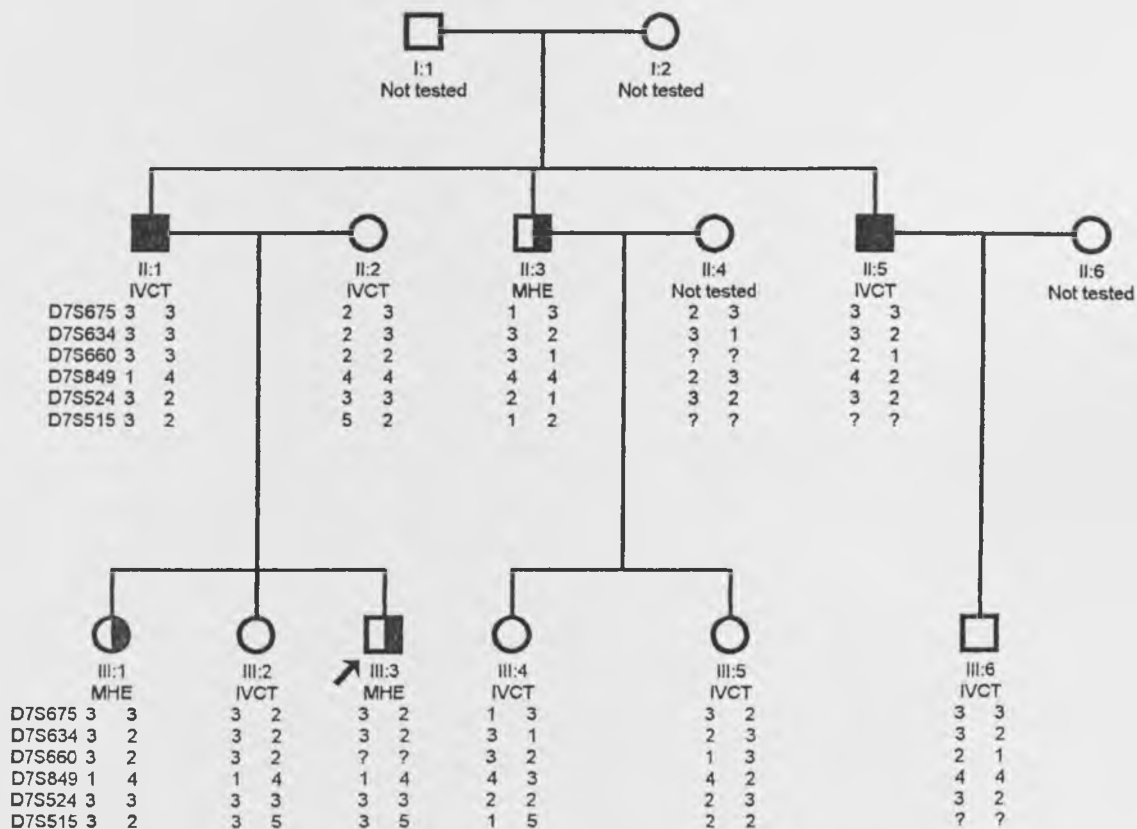


Fig 4.8. Marker segregation of chromosome 7q in LMH12 family.

The markers used are *D7S675, D7S634, D7S660, D7S849 D7S524* and *D7S515* respectively. Allele segregation using these markers does not show co-segregation with susceptibility to malignant hyperthermia.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.50
D7S675	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
D7S634	-0.01	-0.01	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D7S660	-0.01	-0.01	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D7S849	-0.01	-0.01	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D7S524	-0.00	-0.00	-0.00	0.00	0.00	0.00	0.00	0.00
D7S515	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	0.00

Table 4.8 The two point lod scores of linkage analysis between chromosome 7q markers and MH susceptibility in family **LMH12**. As with family LMH07 most of the lod scores are low and there is no significant suggestion of linkage between MH susceptibility and chromosome 7q markers in this family.

close to a gene encoding the α_2/δ subunit of DHPR (dihydropyridine receptor) and MH susceptibility resulted in a claim for another MHS locus in this region.

4.3.2 *Linkage analysis with markers from chromosome 3q*

Allele segregation for chromosome 3q markers, *D3S1271, D3S1302, D3S1278, D3S3654, D3S1309, D3S1303, D3S1279* and *D3S3638* in families LMH07 and LMH 12 were investigated for linkage analysis between these markers and the MH susceptibility locus (*D3S1279* was not typed in family LMH07).

Haplotype analysis for eight markers of chromosome 3q which span the region 3q11-1 to q23 (fig 3.19) shows no common high risk haplotype and no association with the MH locus in either family (figs 4.9 and 4.10). Marker segregation for chromosome 3q in comparison with the results of chromosome 19q markers have not a specific haplotype in family **LMH 07** as well as markers of chromosome 7q. In family LMH07 both III.1 and III.2 have inherited the same haplotype from their father. The proband III.2 has two recombination (for *D3S1302* and *D3S1303* markers) and III.1 shows one crossing over for *D3S1309*. The daughters of $MHE_{(h)}$ individual, II.3, have inherited the same the haplotype (1-1-2-2-6-1-3) from their father although one of them is MHE (III.3) and the other one is MHS (III.4). Based on these observations it is unlikely that the MH locus in LMH07 is linked to chromosome 3q. Pairwise lod score data between MHS and these markers supports this conclusion (table 4.9).

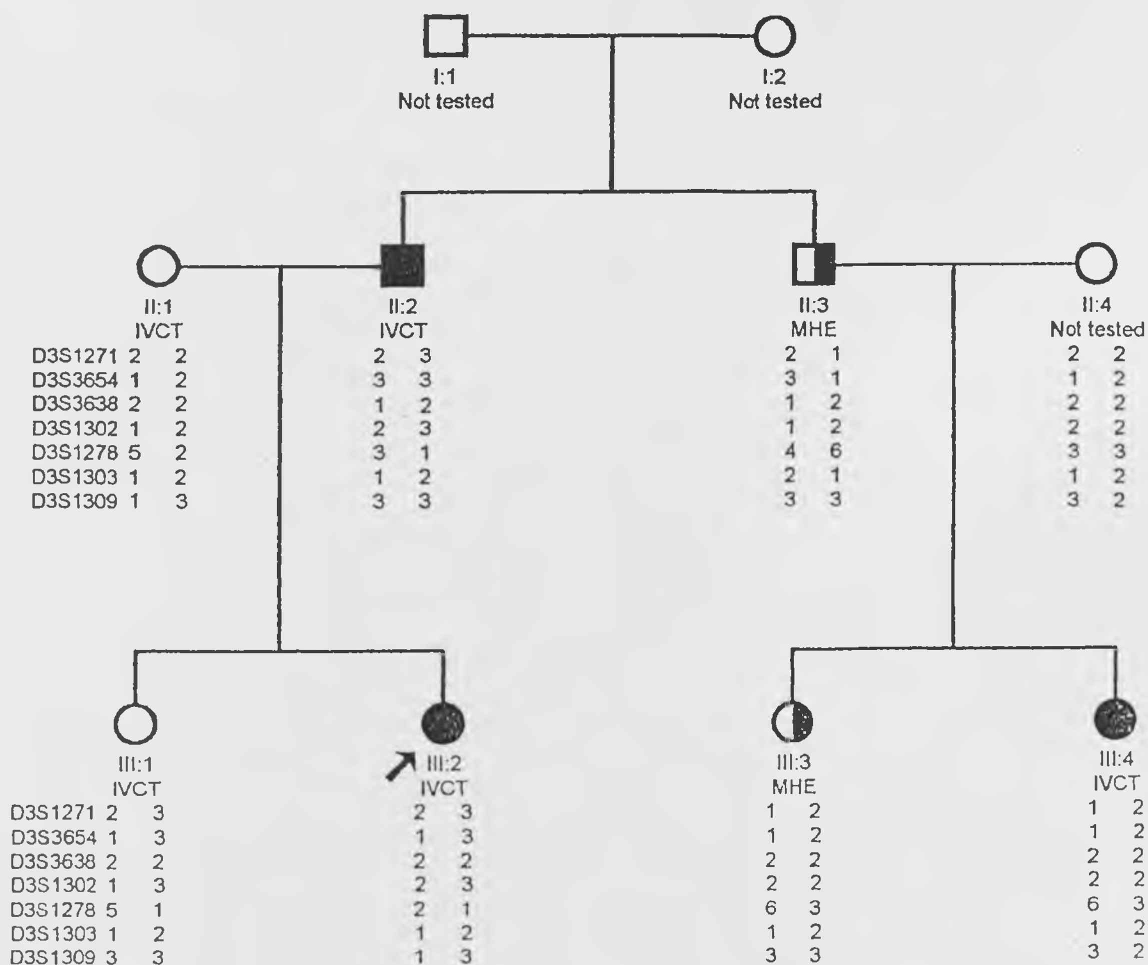


Fig 4.9 Marker segregation of chromosome 3q in family LMH07.

The markers used are *D3S1271, D3S3654, D3S3638, D3S1302, D3S1278, D3S1303* and *D3S1309* respectively. This family does not show any correlation between MH susceptibility and the segregation of these markers.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.1	0.15	0.2	0.25	0.3	0.5
D3S1271	-0.49	-0.36	-0.30	-0.22	-0.15	-0.10	-0.06	0.00
D3S3654	0.16	0.14	0.11	0.09	0.06	0.04	0.03	0.00
D3S3638	-0.34	-0.21	-0.13	-0.08	-0.05	-0.03	-0.01	0.00
D3S1302	-0.34	-0.23	-0.16	-0.12	-0.08	-0.06	-0.04	0.00
D3S1278	-0.41	-0.37	-0.31	-0.24	-0.18	-0.12	-0.08	0.00
D3S1303	-0.32	-0.18	-0.11	-0.07	-0.05	-0.03	-0.02	0.00
D3S1309	0.16	0.13	0.11	0.08	0.06	0.04	0.03	0.00

Table 4.9 The two point linkage analysis results between MH susceptibility and chromosome 3q markers in family **LMH07**. As shown in the table the lod scores are low and do not provide any evidence for linkage of MHS to chromosome 3q.

In family **LMH12** eight chromosome 3q markers (*D3S1271, D3S1302, D3S1278, D3S3654, D3S1309, D3S1303, D3S1279*, and *D3S3638*) have been investigated. The results are presented in fig 4.10. As with the last pedigree (LMH07, fig 4.9) there is not a common haplotype in the MHS individuals. Individuals III.1 (MHEh), III.2 (MHN) and III.3 (MHEh) have inherited the same haplotype form their father II.1 who is MHS. Furthermore the daughters of II.3 have received different haplotypes from their $MHE_{(h)}$ father although both are MHN. This simple inspection indicates that MHS is not linked to 3q13.3 in this family. Lod score analysis support this conclusion for different markers. At θ = 0.00 the most lod scores are near to zero and as listed in table 4.10 there is no significant result for linkage to chromosome 3q.

The chromosome 3q13.1 region in a collaborative genome search by the EMHG (Genetic Section) using hypervariable microsatellites was reported to be linked to MH susceptibility in one German family which had previously been excluded from 19q linkage and linkage putative MHS loci on 17q and 7q (Sudbrak *et al* 1995). In this present project using these eight chromosome 3q markers there was no correlation between 3q markers and MHS locus. These results agree with other results for 18 non 19-linked families reported in EMHG (Genetics Section) meeting from European centres in 1995. Of course this piece of work, although it covers the candidate region, cannot exclude the supposed MHS locus from this region because of the high distance between markers and since only eight marker were used for long arm of chromosome 3q.

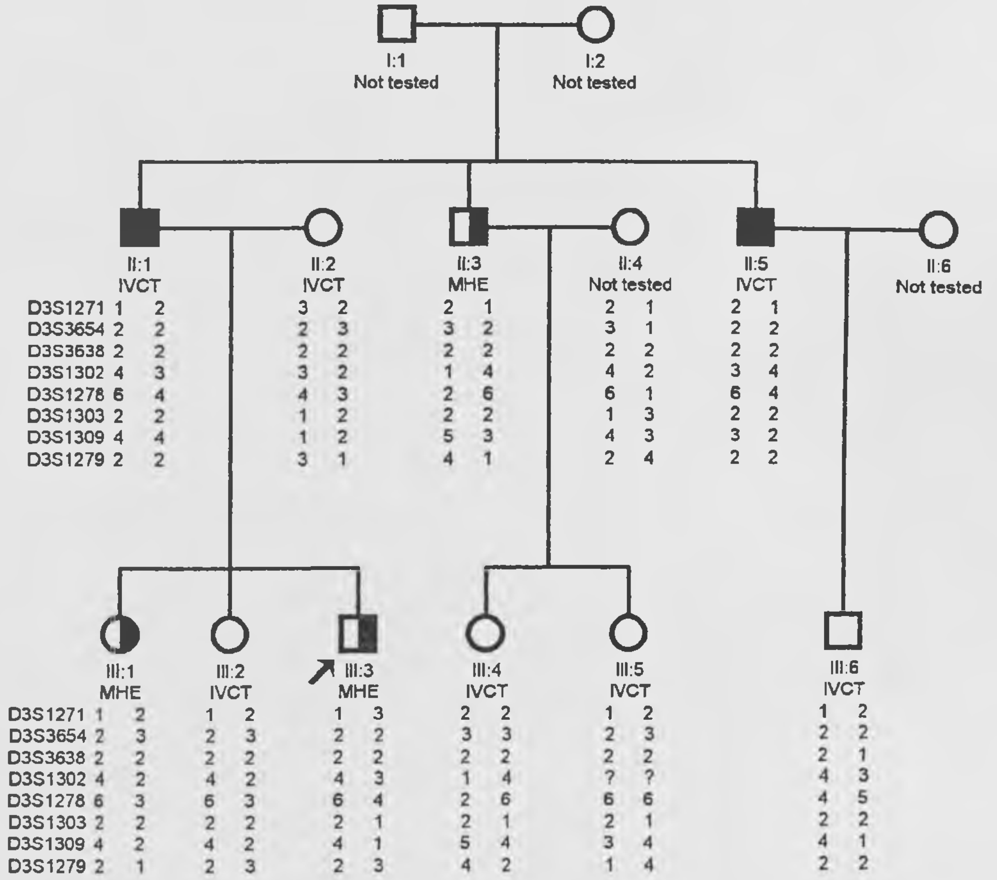


Fig 4.10 Marker segregation of chromosome 3q in LMH12 family.

The markers used are *D3S1271, D3S3654, D3S3638, D3S1302, D3S1278, D3S1303, D3S1309* and *D3S1279* respectively. Allele segregation using these eight markers does not show co-segregation of a high risk haplotype with malignant hyperthermia susceptibility.

LOD SCORES								
Recombination fraction → Markers↓	0.00	0.05	0.10	0.15	0.20	0.25	0.3	0.5
D3S1271	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D3S3654	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D3S3638	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00
D3S1302	0.03	0.02	0.02	-0.00	0.00	0.00	0.00	0.00
D3S1278	0.01	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D3S1303	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D3S1309	-0.01	-0.01	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D3S1279	0.02	0.02	0.01	0.01	0.00	0.00	0.00	0.03

Table 4.10 Two point lod scores for linkage analysis between chromosome 3q markers and MH susceptibility using eight markers in family **LMH12**. These results do not provide evidence for linkage of MHS to chromosome 3q markers in this family.

4.3.3 *Linkage analysis with markers from chromosome 1q*

In family LMH12 six chromosome 1q markers (*D1S248, D1S252, D1S194, D1S196, D1S191* and *D1S249*) have been typed which span the region from 1q12 to 1q32.1. The segregation of these markers is shown in fig 4.11. No significant haplotype is associated with MH susceptibility locus. Haplotype analysis in the progeny of II.1 shows a similar order for III.1 and III.3 who are $MHE_{(h)}$ and MHS respectively. The great distance between the marker loci (fig 3.21) is likely to result in a high rate of crossing over between them as observed in pedigree LMH12 and shown in fig 4.11.

The gene encoding the α_1 subunit of DHPR (a candidate protein in MH susceptibility) is located at 1q31-q32 very close to the *D1S249* locus used in this analysis. The lod scores for pairwise linkage analysis between MH susceptibility and these markers on chromosome 1q are presented in table 4.11 and all around zero at $\theta = 0.00$ and therefore indicate that there is no linkage between MHS locus and these 1q marker loci. These results are consistent with other results for four non 19-linked families reported in Genetics Section of EMHG meeting in 1995.

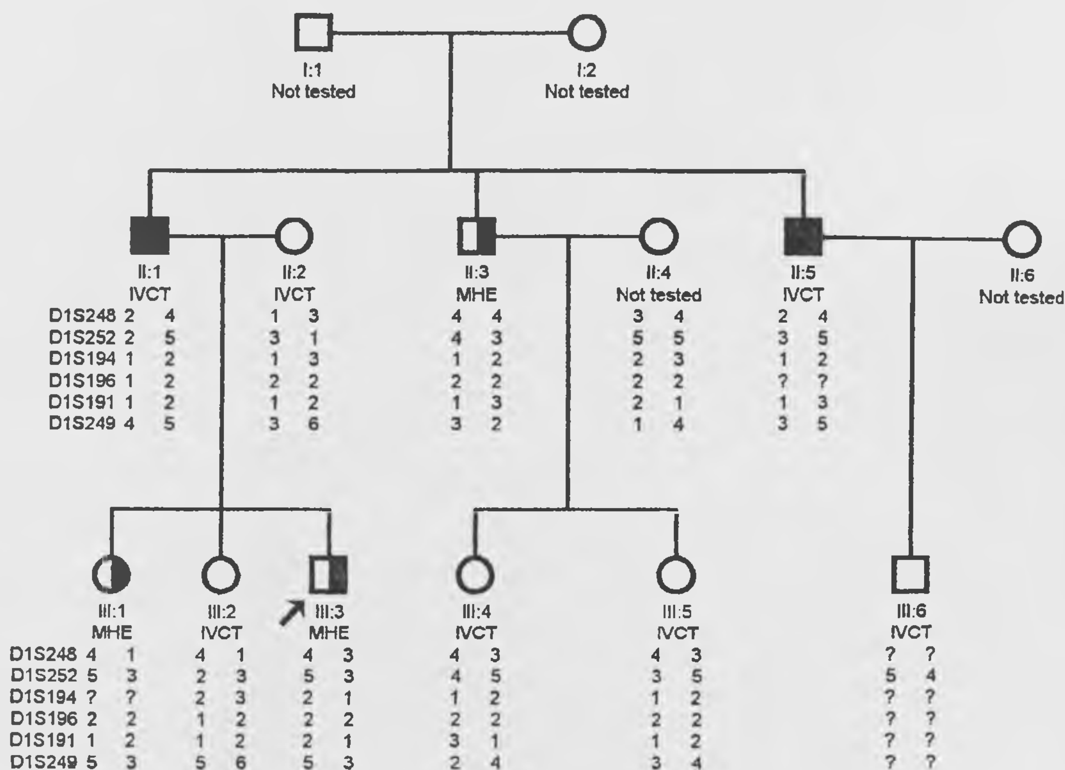


Fig 4.11 Marker segregation of chromosome 1q in family LMH12.

The used markers are *D1S248, D1S252, D1S194, D1S196, D1S191,* and *D1S249* respectively. As observed for chromosomes 7q and 3q there is no co-segregation between susceptibility to MH and the candidate region on chromosome 1q.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.5
D1S248	0.02	0.02	0.01	0.01	0.00	0.00	0.00	0.00
D1S252	-0.01	-0.01	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D1S194	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D1S196	-0.00	-0.00	-0.00	-0.00	-0.00	0.00	0.00	0.00
D1S191	-0.01	-0.01	-0.00	-0.00	-0.00	-0.00	-0.00	0.00
D1S249	-0.01	-0.01	-0.00	-0.00	-0.00	-0.00	-0.00	0.00

Table 4.11 The two point lod scores of linkage analysis between chromosome 1q markers and MH susceptibility in family **LMH12**. The lod scores are very low and can not be used in proving or excluding the MH locus from this family.

4.3.4 linkage analysis with markers from chromosome 2p

Preliminary results from the genomic search project (collaborative project of the European Malignant Hyperthermia Group, Genetic Section) suggested that there may be an MH susceptibility locus on chromosome 2p. Genotyping of two candidate markers, *D2S398* and *D2S139* was carried out for two families, AN2 and LMH03 which had been excluded from linkage to chromosomes 19q, 17q and 7q indicated by the genomic search.

For these two markers there is no common haplotype in MHS individuals; however, a shared haplotype is not expected since the distance between these markers, approximately 80cM is very great. The *D2S398* and *D2S139* markers are not linked to MH susceptibility locus in family **LMH03**. The LMH03 yields negative lod scores for both markers, -3.23 and -5.18 *D2S139* and *D2S398* respectively at zero recombination fraction and low positive lod scores at $\theta = 0.15$ and more (table 4.12).

In family **AN2** one allele of the *D2S139* marker (allele 2) is co-inherited with MH susceptibility except with one recombination in individual II.12 who is MHN (fig 4.13). The lod scores for linkage between MHS and this marker in family AN2 are presented in table 4.13 and the high lod score is approximately 1 at $\theta = 0.00$ although it is -2.20 for *D2S398* and then this excludes close linkage to MH susceptibility locus. Based on the results in family AN2 typing of more

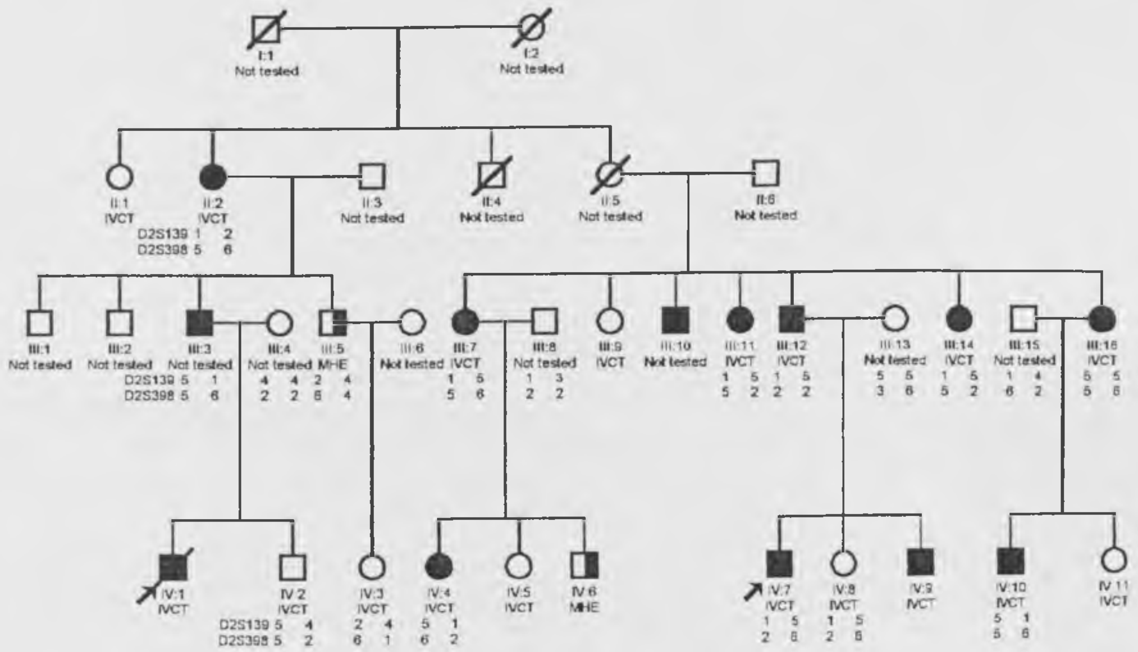


Fig 4.12. Segregation of chromosome 2 markers in LMH03 family.

The markers used are *D2S139* and *D2S398*. Allele segregation using two markers shows no shared haplotype between MH susceptible individuals.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.5
D2S139	-3.23	-0.54	-0.13	0.05	0.13	0.016	0.15	0.00
D2S398	-5.18	-1.45	-0.84	-0.52	-0.33	0.21	-0.13	0.00

Table 4.12 Lod scores of two point linkage analysis between MH susceptibility locus and chromosome 2 markers *D2S139* and *D2S398* in family **LMH 03**.

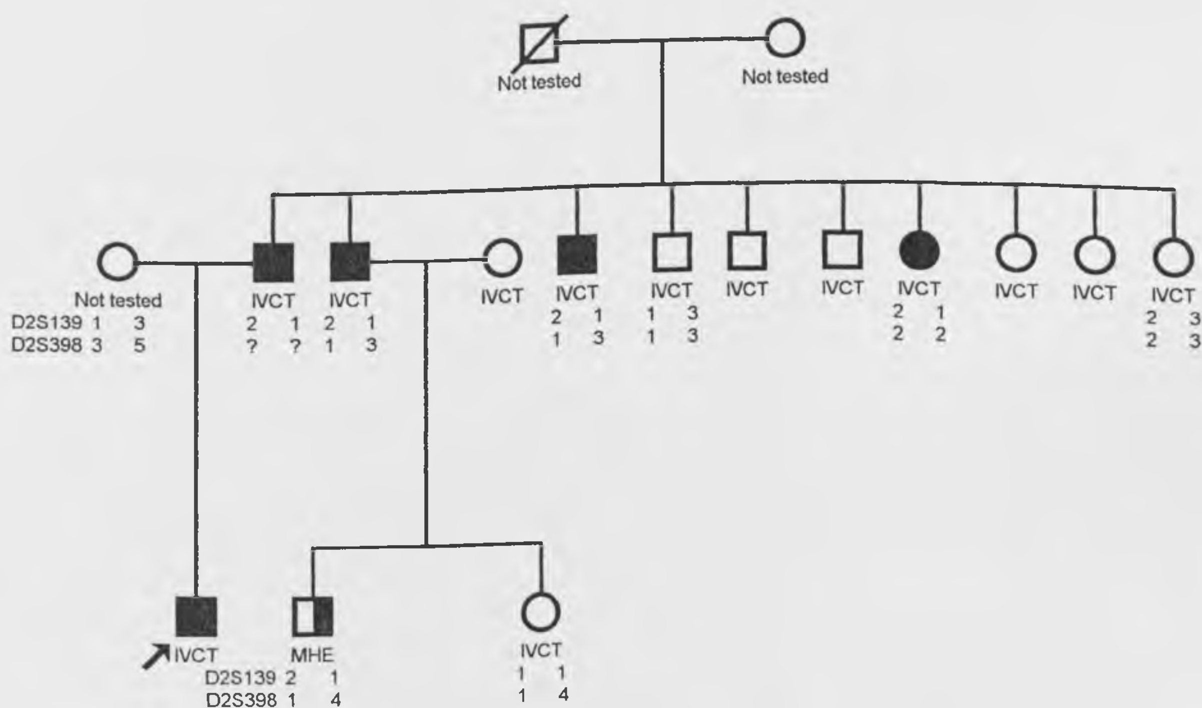


Fig 4.13 Segregation of chromosome 2p markers in family AN2.

The markers used are *D2S139* and *D2S398*. Except for allele 2 *D2S139* (with one recombination at individual II.12) there was no correlation of markers used with MH susceptibility.

LOD SCORES								
Recombination fraction→ Markers↓	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.5
D2S139	0.99	0.90	0.80	0.70	0.59	0.47	0.35	0.00
D2S398	-2.20	-1.18	-0.76	-0.51	-0.34	-0.22	-0.13	0.00

Table 4.13 Lod score results of linkage analysis between MHS and two markers from chromosome 2p, *D2S139* and *D2S398* in family **AN2**.

markers close to *D2S139* seems to be necessary to demonstrate or exclude linkage of MHS to chromosome 2p in this family.

4.4 Conclusion of linkage data with other chromosomal regions

In conclusion regarding the linkage data of this project for chromosomes 7q, 3q and 1q and chromosome 2, despite the reports by Iles *et al* 1994 (for chromosome 7q with lod score less than 3) and Sudbrak *et al* 1995 (for chromosome 3q with lod score more than 3) there was no evidence to support their finding in UK families. Although except for chromosome 2p (for LMH03 and AN2 families) there is not strong evidence (lod score -2) to exclude these families from chromosomes 7q ,3q and 1q. In addition linkage analysis in two families LMH07 and LMH12 using chromosome 7q markers two more UK MH families have been investigated and no linkage was found to chromosome 7q (EMHGGS 1995).

Furthermore investigation for linkage between MH susceptibility and chromosome 7q markers using 27 MH European families have shown no linkage for this candidate region. In addition, further studies in MH units (European countries) using chromosome 3q markers for 18 MH families gave the same results as UK families, namely lack of any linkage to the proposed candidate region. This project's result for chromosome 1q agree with an investigation of four more European MH families which reported no linkage to the 1q candidate region (EMHG Genetics Section, 1995). There is no considerable marker segregation or lod score for chromosome 2p except for one marker (AN2 family, fig 4.13).

Finally although it is not possible to exclude the families LMH07 and LMH12 from candidate regions on chromosomes 7q, 3q, 1q and LMH03 and AN2 from chromosome 2 as well as there in is not possible evidence to suppose any linkage to these candidate regions.

Chapter five

Results and Discussion of Mutation Detection in *RYR1* gene

5.1 Results of Mutation detection

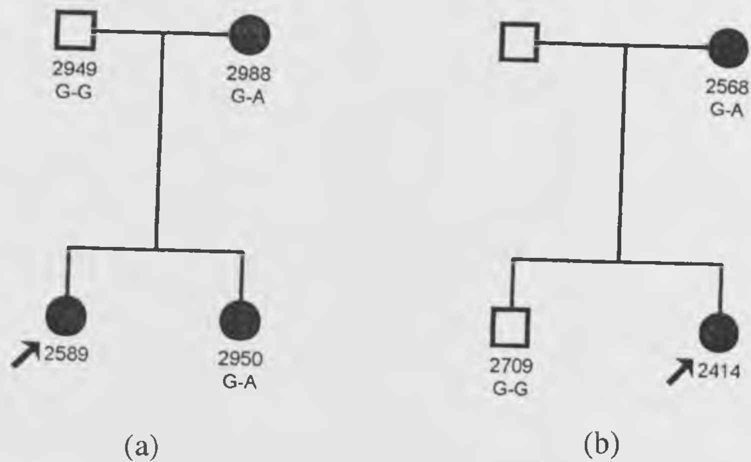
5.1.1 C487 substitution to T

31 unrelated MHN, 102 unrelated MHS and 2 unrelated MHE individuals were tested for this mutation. These data are shown in table 5.1. There is no evidence for the presence of the C487 to T mutation in UK MH population.

Furthermore this survey was carried out in 9 MHS, 2 MHN and 2 MHE individuals diagnosed as having central core disease (CCD). This mutation was not detected in CCD individuals. These results are presented in table 5.2.

5.1.2 G1021 to A mutation

100 unrelated MHS individuals, 130 unrelated MHN individuals and also four unrelated MHE individuals from UK MHS families were investigated for the presence of the G1021A mutation. The results of this investigation are shown in table 5.3. This mutation is recognised in 10 patients from six different MHS families who were heterozygous for the G1021 site. In addition 11 MHS / CCD, 6 MHN / CCD and 3 MHE / CCD samples were tested for this mutation. There was no mutant individual in the CCD families. These results are summarised in table 5.4. Three of seven families who are heterozygote for G1021A mutation are shown in figs 5.1 and 5.2.



(a) (b)

Families : Qua-Man Kel-Por

Fig 5.1 Two pedigrees which are heterozygous for G1021A mutation.

G-G and G-A genotypes respectively show normal and mutant sequences for the G1021A mutation. This base substitution was observed in individuals who are susceptible to malignant hyperthermia. The individuals with number have been tested by IVCT and unnumbered individuals have not been IVCT tested

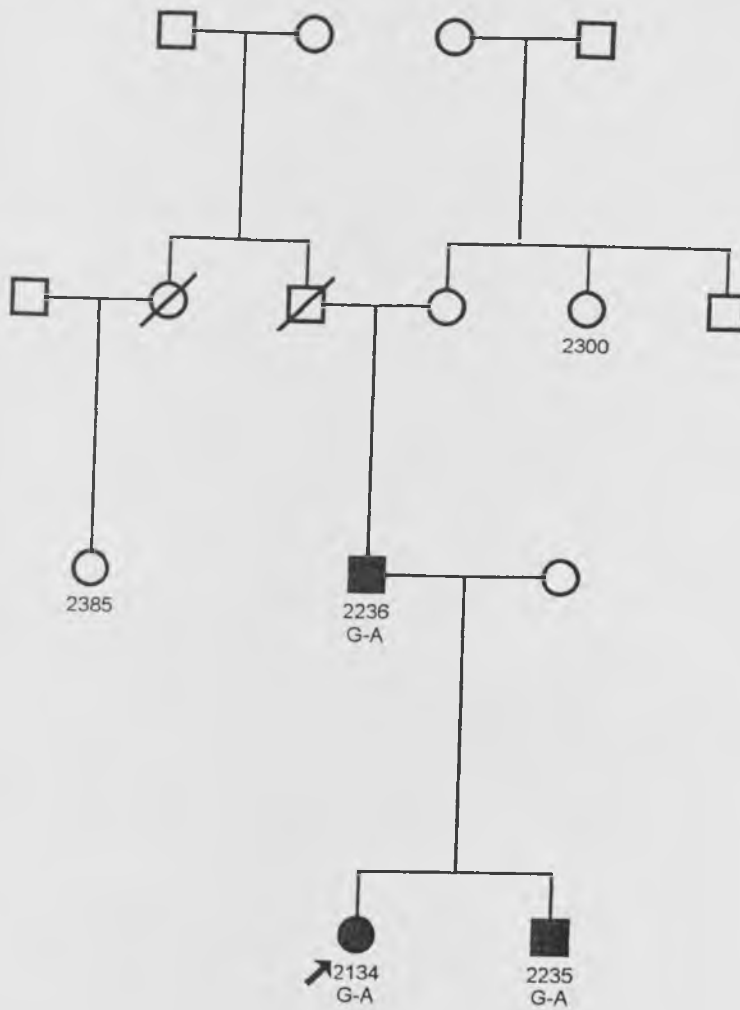


Fig 5.2 Pedigree of family Lea-Sus which segregates for malignant hyperthermia susceptibility and the G1021A mutation.

The individuals with number have been tested using IVCT and vice versa.

Table 5.1 Results of C487T mutation detection in UK MHS families.

This mutation is not observed in 102 MHS, 31 MHN and 2 MHE individuals.

MH status	C487T heterozygotes	C487 homozygotes	Total
MHS	0	102	102
MHN	0	31	31
$MHE_{(h)}$	0	2	2
Total	0	135	135

Table 5.2 Results of C487T mutation detection in UK MH/CCD families.

MH status	C487T heterozygotes	C487 homozygotes	Total
MHS	0	9	9
MHN	0	2	2
$MHE_{(h)}$	0	1	1
Total	0	0	12

Table 5.3 Results of G1021A mutation screening in British MH families in the *RYR1* gene.

MH status	G1021A heterozygotes	G1021 homozygotes	Total
MHS	10	134	144
MHN	0	157	157
$MHE_{(h)}$	0	10	10
Total	10	301	311

Table 5.4 Results of G1021A mutation screening in British MH/CCD families in the *RYR1* gene.

MH status	G1021A heterozygotes	G1021 homozygotes	Total
MHS	0	10	10
MHN	0	6	6
$MHE_{(h)}$	0	1	1
Total	0	17	17

5.1.3 C1840 to T results

38 samples from MH families (20 MHS, 15 MHN and 3 MHE and unknown status) were tested for the C1840T mutation. This mutation was not detected in any of the individuals tested. The results are summarised in table 5.5.

5.2 Mutation analysis study

The mutation detection for three known mutations of the *RYR1* gene (C487T, G1021A and C1840T) from the eight potentially causative mutations reported in the *RYR1* gene (in MHS and CCD individuals, table 1.1) was carried out using DNA samples from different MHS families and also from CCD patients with or without MHS. Some of these families (MH families) had been shown to have an MH locus linked to 19q.

5.2.1 C487T mutation

The search for the C487T mutation was followed using 135 samples from MHS, $MHE_{(h)}$ and MHN individuals. This mutation may be causative in CCD induction and MH susceptibility (Quane *et al* 1993). This mutation is reported (Quane *et al* 1993) in one MHS family and one CCD family. The results of this work in UK samples tested for this mutation revealed no mutant sample in MHS, MHN, MHE and CCD samples (table 5.1). This mutation in a survey of 48 Danish MHS families is reported in only one family (Fagerlund *et al* 1994). The frequency of this mutation in MH families is low, and supposed to be around 3% in the European population (West 1996) or lower, 1% in the North American population (Fletcher 1995).

Table 5.5 The results of a mutation search (the C1840 to T mutation) in different samples from UK MH families.

MH status	C1840T heterozygotes	C1840 homozygotes	Total
MHS	0	20	20
MHN	0	15	15
$MHE_{(h)}$ and unknown status	0	3	3
Total	0	38	38

5.2.2 C1840T mutation

The search for the C1840T mutation was carried out in some samples from MHS and MHN individuals from UK families. The results show no mutant samples on enzyme digestion (fig 3.26) consistent with Curran *et al* (1993) who reported no mutant in one hundred British unrelated MHS individuals. There is no overlap in these data. Of course the frequency of this mutation is not high and supposed to be approximately 5% in European families (West 1996).

Finally it is important to beware of association of this mutation with susceptibility to MH as no unity was reported between IVCT results, C1840T mutation and susceptibility to MH by Fletcher *et al* 1995; Deufel *et al* 1995 and Serfas *et al* 1996. These studies describe the correlation between MH susceptibility and the *RYR1* C1840T mutation with different features. Fletcher *et al* (1995b) reported 18 uneventful anaesthetics in one subject with the *RYR1* C1840T mutation. They supposed that either additional modulating factors must contribute to the syndrome or the presence of the C1840T mutation is not a causative factor in MH. However, in another study in pigs it is supposed that the presence of the C1840T mutation alone might not be sufficient to consistently cause MH in pigs (Fletcher *et al* 1993). In another family which is reported by Deufel *et al* (1995) the *RYR1* C1840T mutation is present in one branch of the family but not in other. In fact there are two independent C1840T transitions in the one branch as judged by the flanking 19q haplotypes and homozygosity of one individual. In addition there is the further complication that the C1840T mutation is also present in an individual who clearly types as MHN. Serfas *et al* (1996)

observed a Canadian family in which there was disconcordance between the *RYR1* C1840T mutation and CHCT (caffeine halothane contracture test) assignment of MH susceptibility. They attributed this observation to two errors in the biopsy testing.

In summary based on the different reports from different centres it is supposed this mutation indicates a geographical distribution (West 1996).

5.2.3 G1021A mutation

The survey for detection of the G1021A mutation was carried out using 234 samples (one hundred samples from unrelated MHS individuals, one hundred thirty unrelated MHN samples and four MHEh samples). These results indicate that 10 individuals from seven unrelated MHS families are heterozygous for this mutation and carry a base substitution of A instead G (table 5.3). The pedigrees of these six families are small, three of these families are shown in figs 5.1 and 5.2. In addition to these MH individuals 17 CCD individuals, ten MHS, 6 MHN and one $MHE_{(h)}$, were screened for this mutation. The results from these CCD samples show no positive individual for this mutation (table 5.4).

The G1021A mutation (Gly341 to Arg amino acid substitution) mutation perhaps is the most common mutation so far reported in the *RYR1* gene in MHS individuals. However this mutation is observed in one non 19q linked family LMH03 (Adeokun *et al* 1997). In LMH03 the mutation is inherited within the pedigree and is only present in MHS individuals but did not show complete co-segregation with MH susceptibility. This mutation was not observed in five

individuals of this family who are MHS. Three different explanations for correlation between MH susceptibility and *RYR1* G1021A mutation for this family are proposed by the authors. The frequency of this base substitution has been estimated to be 10% of all MHS families (Quane *et al* 1994a) and in comparison with the C1840T mutation it has not been observed in MHN samples. The association of this mutation with MHS individuals (here in 140 samples) and lack of it in MHN individuals (150 samples) as well as similar results in another centre in the UK (Adeokun *et al* 1997) in addition to the reports of Quane *et al* (1994a) strongly indicates a causative role for the G1021A as a causative mutation in MH susceptibility. The interesting point in Adeokun *et al* (1997) report in LMH03 family was the co-segregation of this mutation in individuals who show chromosome 19q-linked haplotypes and lack of this mutation in other part of this family which does not show linkage to chromosome 19q.

In conclusion in UK families the two *RYR1* mutations, C487T and C1840T are believed to be at very low frequency, less than 3%. The results of investigation in European MH families for these two mutations show 3 heterozygotes in 246 MHS individuals and 22 in 463 MHS individuals for C487T and C1840T mutations respectively (European Malignant Hyperthermia Group Genetics Section, Dec. 1995). But the *RYR1* G1021A mutation is found at greater frequency, around 7% in MHS individuals in the UK population (20 mutants in 400 MH individuals) and probably is a causative mutation in MH susceptibility. In CCD patients although the C487T mutation is reported in a few families (Quane *et al* 1994) we could not find any evidence in our samples. These results agree with West's suggestion (1996) on the geographical distribution of *RYR1* mutations and

it may be supposed that approximately between 15 to 20% of MH families (19q linked families) carry one of the eight mentioned *RYR1* mutations (table 1.1). The possibility of finding more *RYR1* mutations which could account for more MHS individuals is considerable and necessary.

Chapter SIX

Conclusion and future work

6.1 Critical points and future work

Two approaches to the elucidation of the molecular defects underlying MHS were taken in this project. At first this project started on a logical path which is stated from main source or malignant hyperthermia susceptible individuals. Using DNA samples of MH families, screening for causative genes or candidate loci was continued by linkage analysis. Finding linkage to the *RYR1* gene and following this path using mutation screening was the next step in the molecular analysis of malignant hyperthermia. Although this project finished at this step it could have been continued by sequencing of individuals who are susceptible and are heterozygous for *RYR1* mutations proving the relation between MH susceptibility and causative mutations.

Ensuring the absence of these mutations in normal people and providing the transgenic animals are essential in finding more about the pathogenic role of these mutations in malignant hyperthermia susceptibility especially finding more about defects in *RYR1* gene and malignant hyperthermia susceptibility. Up to now the exact mechanism of excitation-contraction coupling and the role of volatile anaesthetics and also muscle relaxants in Ca^{2+} release is not known. Then as an interdisciplinary project it needs to be associated with electrophysiology and studies on calcium currents in muscle fibres. Although it is reported that some

factors such as the calcium pump are not involved (chapter one) in MH susceptibility, as Morgan and Sedensky (1995) reported in *C. elegans*, mutations which are not involved in *RYR1* may give rise to contractile responses. However, following genomic search for MH causative loci, using the new achievements from ion channels (especially calcium currents) is necessary. The new survey on triggering agent may be another project which can be involved in MH susceptibility.

In the second part of this project few points could effect on the results. Firstly it may be supposed that the project did not follow a specific path. Following the chromosome 19q project for the LMH15, 16, 17 and LMH18 families, the choice of families LMH07 and LMH12 for screening the other candidate loci was made after excluding 19q for these families. The future work for these families may be supposed to be the collection of more data using chromosome 19q markers in new members. Marker segregation and linkage analysis for these two families, especially for family LMH07, and using markers from chromosomes 7q, 3q and 1q were ambiguous, more data needs to be entered in this project for localisation of the MH locus to candidate regions or exclusion of it. The other point which should be considered in future work is more attention to the markers for a candidate region which should be based on more data. The study using two distantly spaced markers for chromosome 2p was a " shot in the dark ". The two markers with high distance apart on a big chromosome appears not to give rise to reasonable results. Using more markers spread over the candidate region for chromosome 19q especially for *LIPE* gene could be an interesting study for the

future in addition to a review of results and completion of these results. The more important point in this part is focusing on the IVCT results and making a strong decision on classification of MH groups and if it is possible, of course it should be possible to draw pedigrees focusing on the IVCT results with less doubts and more accurate data.

6.2 General conclusion

A brief review and conclusion of practical work and analysis of results make it possible to classify the achievements in two sections, linkage analysis results and mutation search. Results of linkage analysis based on the investigated pedigrees are classified in two main groups, linkage data with markers on chromosome 19q and linkage data with other chromosomal regions. In the first group the families LMH15 and LMH17 despite the low lod scores show co-segregation of high risk haplotypes in MHS and $MHE_{(h)}$ individuals and show unambiguous linkage between MHS and markers from chromosome 19q. The other three families, LMH07, LMH16 and LMH18 show linkage between MHS and markers from chromosome 19q in most members with one MHN inconsistency in the two first pedigrees and one MHE recombination in LMH18. The LMH12 is a family for which conclusion on linkage between MHS locus and markers from chromosome 19q cannot be drawn and this locus is proposed to be unlinked to chromosome 19q. Despite more candidate regions from reports on other chromosomes, 7q, 3q, 1q and 2p (chapter one), these linkage analysis results do not confirm these candidate loci and do not consistently support any linkage to these regions. Families LMH07 and LMH12 do not show significant lod scores or

shared high risk haplotypes between MHS and MHE individuals to prove linkage to candidate regions on chromosomes 7q, 3q and 1q (only family LMH12) although there is no strong evidence for exclusion of these families from these candidate regions. For these two families it is not reasonable to develop more linkage analysis using the reported candidate regions. It seems acceptable to extend the pedigrees (LMH15, LMH16, LMH17, LMH18 and LMH07) for further linkage analysis. The satisfactory classification of MH individuals using IVCT and selection of appropriate markers for linkage analysis are important for the future of this study.

Mutation testing was carried out for three mutations, C487T, G1021A C1840T. The C487T was not found in any samples in MHS, MHE and MHN individuals in UK MH and CCD families and it might be supposed that the frequency of this mutation in UK MH families is less than 3%. The results of the C1840T mutation screening were similar to C487T and are consistent with another report (Curran *et al* 1993) from UK families where this mutation was not detected in any of the individuals tested. The only *RYR1* mutation detected in UK MH families is the G1021A mutation. The frequency of this mutation is deduced to be around 6% in UK MH families. These results are consistent with West's suggestion (1996) that the MH mutations are geographically distributed.

Finally despite the reports on genetical diagnosis of malignant hyperthermia susceptibility status (Healy *et al* 1991; 1996; Wallace *et al* 1996), the time for using these techniques is too early and using these methods in diagnosis is at high risk of misleading diagnoses. This is specially true where the linkage analysis is

based on IVCT results which are not reproducible and may give false results (Hopkins *et al* 1994; Serfas *et al* 1996). On the other hand the DNA-based test for chromosome 19-linked families in humans for MH diagnosis as reported by Fujii *et al* (1991) and Otsu *et al* (1991) in pigs is not useable and confident for human. In addition lack of co-segregation of *RYR1* gene mutations with linkage analysis results and chromosome 19q haplotypes in some pedigrees are not unusual. The report of Adeokun *et al* (1997) on co-segregation of G1021A mutation in some MHS individuals and lack of co-segregation in others in one pedigree is a good example which confirm that DNA tests are not reliable based on present MH knowledge (Fletcher *et al* 1995; Deufel *et al* 1995). Finally the results presented here indicate that more understanding is needed before DNA tests (linkage analysis and mutation typing) for MH status diagnosis could be considered reliable. Then in conclusion the in vitro contracture testing, IVCT remains the gold standard technique in diagnostic methods for MH susceptibility (Ball *et al* 1991; Larach 1993).

At the end with confidence based on the biochemical, physiological and genetical achievements it is logical to state that the *RYR1* gene is the main key to solve the mystery of malignant hyperthermia susceptibility. This study shows five out of six families in addition to the large branch of the LMH03 family (Ball *et al* 1993) and the recent report (Curran *et al* in press) show that more than 60% UK families are linked to chromosome 19q and this gives rise to suppose that a defect in the *RYR1* gene is the main reason to causing their malignant hyperthermia susceptibility. In rare cases which do not show linkage to chromosome 19q possibly the genetic background and environmental factors can affect the

phenotype as modifiers, enhancers or inhibitors. Then the main axis for future work will be based on finding out more about the *RYR1* gene and its protein product. For example more mutation screening and introducing the mutant gene into a line of normal animals to observe whether the transgenic animal develops disease. Eventually it will be necessary to find out more about the relationship between the calcium release channel and other factors mentioned in chapter one.

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