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# *Comparison of the Segregation of the* RYR*1 C1840T Mutation with Segregation of the Caffeine/Halothane Contracture Test Results for Malignant Hyperthermia Susceptibility in a Large Manitoba Mennonite Family*

*Kimberly D. Serfas, B.Sc.,* Deepak Bose, M.D. Ph.D.,† Leena Patel, M.D.,‡ Klaus Wrogemann, M.D., Ph.D.,§ Michael S. Phillips, B.Sc.,‖ David H. MacLennan, Ph.D.,# Cheryl R. Greenberg, M.D., C.M.***

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

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

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

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

This article is accompanied by a Highlight. Please see this issue of ANESTHESIOLOGY, page 29A.

* Graduate Student, Department of Human Genetics, University of Manitoba.

† Professor, Departments of Pharmacology and Therapeutics, Anesthesiology, and Internal Medicine, University of Manitoba.

‡ Associate Professor, Department of Anesthesiology, University of Manitoba.

§ Professor, Departments of Biochemistry and Molecular Biology and Human Genetics, University of Manitoba.

‖ Graduate Student, Banting and Best Department of Medical Research, University of Toronto.

# University Professor, Banting and Best Department of Medical Research, University of Toronto.

** Professor, Departments of Pediatrics and Child Health and Human Genetics, University of Manitoba.

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Address reprints requests to Dr. Greenberg: Department of Human Genetics, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada, R3E 0W3. Address electronic mail to: greenbec@bldghsc.lan1.UManitoba.ca.





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**segregating in the family, or that there are errors in the CHCT. Because there is strong evidence supporting the causal nature of the Arg614Cys mutation, the discordant persons are not closely related within the pedigree as they would be if a second MH mutation were segregating, and the CHCT is not 100% accurate, we propose that the observed discordance between DNA test results and CHCT assignment in this kindred results from two false-positive diagnoses by the CHCT. (Key words: Anesthetics, volatile: halothane. Caffeine/halothane contracture test. Calcium release channel (ryanodine receptor). Malignant hyperthermia susceptibility testing. Mutation analysis. Neuromuscular blocking agent: succinylcholine. *RYR*1 C1840T mutation testing.)**

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

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

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

A primary MH defect has been proposed to involve abnormal gating of the calcium release channel (ryanodine receptor) of human and porcine skeletal muscle sarcoplasmic reticulum.[10–16] Genetic studies also support *RYR*1, the gene encoding the skeletal muscle isoform of the ryanodine receptor, as a causal gene for MH in humans[17–26] and porcine stress syndrome in pigs.[27–28] In the MHS pig, the substitution of T for C at position 1843 in *RYR*1, resulting in the substitution of cysteine for arginine 615 in the ryanodine receptor, was the only amino acid difference detected in a comparison with a normal animal.[27] This mutation cosegregated with MHS in more than 450 animals from 6 breeds of selectively inbred pigs with a lod score of 101.75 at a recombination fraction $\theta = 0.00$.[28] This strongly implicated it as the causal mutation for porcine MH. The corresponding human C1840T mutation (Arg614Cys) has been linked to MH in unrelated families.[19,20] The mutation, located in exon 17 of *RYR*1, eliminates a *Rsa*I restriction endonuclease site, providing the basis for diagnosis of at-risk individuals.[19]

Linkage of MH to *RYR*1 has been possible in only 30–50% of all cases studied[29] and, in one case, lack of linkage of the Arg614Cys mutation to MH was reported in a complex MH family.[30] There are at least three possible reasons why the Arg614Cys mutation or other *RYR*1 mutations may not segregate with MH in all cases. First, there may be no linkage. Second, more than one MH allele may be segregating in the family. Third, there may be linkage, but inaccurate phenotypic assessment may prevent the demonstration of linkage.

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

## Methods

### *Patients and Caffeine/Halothane Contracture Testing*

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

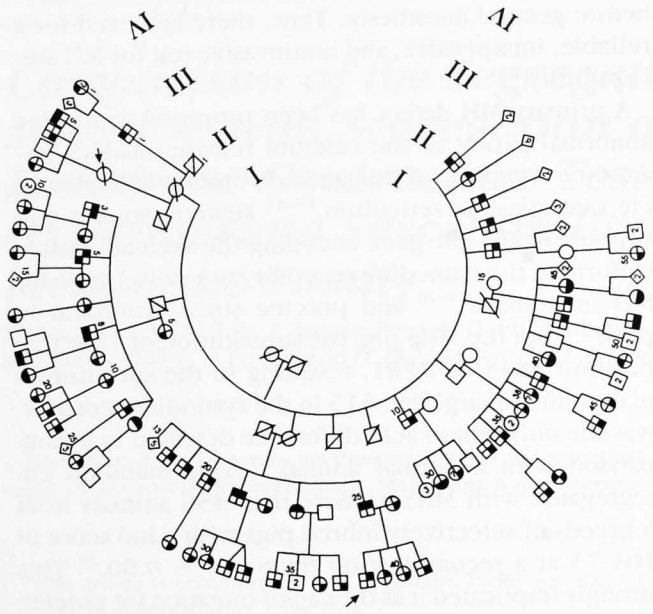

**Fig. 1. Partial pedigree of large Manitoba kindred with 2 persons (↗) with documented malignant hyperthermia crisis. (◐) = malignant hyperthermia normal by CHCT; (◔) = malignant hyperthermia susceptible by CHCT; (◍) = malignant hyperthermia status by CHCT unknown; (◓) = C1840T mutation present; (◒)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

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

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

### *Mutation Analysis*

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

## Results

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

**Table 1. Positive Caffeine/Halothane Contracture Testing Criteria**

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





Table 2. Malignant Hyperthe

| ID No. | Date of Biopsy |
|---|---|
| III-3 | 22/5/87 |
| III-5 | 9/12/91 |
| III-8 | 12/5/89 |
| III-12 | 4/6/92 |
| III-21 | 24/8/93 |
| III-23 | 20/2/90 |
| III-25 | 7/12/90 |
| III-30 | 21/1/86 |
| III-31 | 14/12/92 |
| III-38 | 12/5/92 |
| III-40 | 5/5/83 |
| III-44 | 28/8/92 |
| IV-1 | 11/5/93 |
| IV-5† | 4/6/86 |
| IV-18 | 8/12/87 |
| IV-19 | 21/3/90 |
| IV-38 | 6/12/89 |
| IV-43 | No biopsy |
| IV-51 | 7/4/89 |
| IV-52 | 26/8/86 |
| | 13/5/86 |

CSC = concentration of caffeine t
of poor muscle quality; + = C184
* Abnormal test result.
† Done in Calgary.

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of III-31, III-38, and IV
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table 2.

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

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

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

* Abnormal test result.

† Done in Calgary.

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

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

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

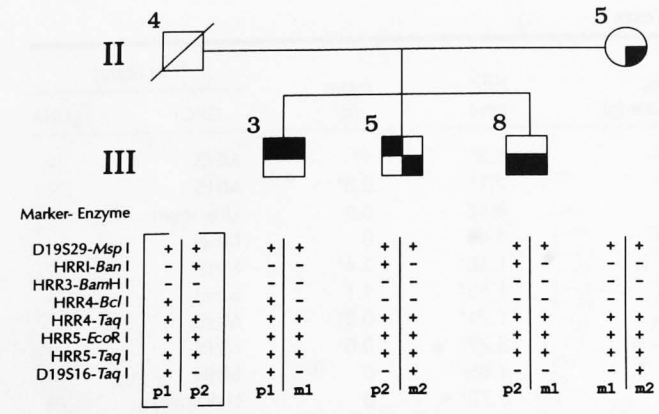


**Fig. 2. Haplotypes of a partial nuclear family showing inheritance of the same low risk paternal haplotype p2 by subjects III-5 and III-8, and inheritance of the high risk paternal haplotype P1 by their MHS sib III-3. Alleles are as described[16] and are presented as haplotypes. "−" indicates absence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

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

## Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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