

North American Malignant Hyperthermia Population

Screening of the Ryanodine Receptor Gene and Identification of Novel Mutations

Nyamkhishig Sambuughin, Ph.D.,* Yoshitatsu Sei, M.D., Ph.D.,† Kathleen L. Gallagher, M.S.,‡ Hadley W. Wyre,§ Daniel Madsen, M.D.,|| Thomas E. Nelson, Ph.D.,# Jeffrey E. Fletcher, Ph.D.,** Henry Rosenberg, M.D.,†† Sheila M. Muldoon, M.D.‡‡

Background: Malignant hyperthermia (MH) is a disorder of skeletal muscle manifested as a life-threatening hypermetabolic crisis in susceptible individuals after exposure to inhalational anesthetics and depolarizing muscle relaxants. Mutations in the gene encoding the skeletal muscle ryanodine receptor (*RYR1*) are considered a common cause of the disorder, and, to date, more than 20 *RYR1* mutations have been reported in European and Canadian families. Some studies suggest that differences may exist in the frequencies and distribution of mutations in the *RYR1* gene between European and North American MH families the frequency and distribution of mutations in the *RYR1* gene.

Methods: Skeletal muscle samples from 73 unrelated individuals diagnosed as MH susceptible according to the North American MH caffeine-halothane contracture test were studied. Genomic DNA of MH-susceptible patients was investigated by polymerase chain reaction-based restriction fragment length polymorphism, single-strand conformation polymorphism, and sequencing analysis. The majority of known *RYR1* mutations were analyzed using the restriction fragment length polymorphism method, whereas new mutations were searched by single-strand conformation polymorphism in exons 12, 15, 39, 40, 44, 45, and 46 of the gene.

Results: Seven known *RYR1* mutations (Arg163Cys, Gly248Arg, Arg614Cys, Val2168Met, Thr2206Met, Gly2434Arg, and Arg2454His) were detected at frequencies of 2.7, 1.4, 1.4, 1.4, 1.4, 5.5, and 4.1%, respectively. In addition, three novel amino acid substitutions (Val2214Ile, Ala2367Thr, and Asp2431Asn) were detected at frequency of 1.4% each. These 10 mutations account for 21.9% of the North American MH-susceptible population.

Conclusion: Three novel candidate mutations in the *RYR1* gene were identified in these MH patients. The frequency and distribution of *RYR1* mutations observed in this North American MH population was markedly different from that previ-

ously identified in Europe. Larger-scale studies are necessary to clarify the type and frequency of mutations in *RYR1* associated with MH in North American families.

MALIGNANT hyperthermia (MH) is a disorder of skeletal muscle manifested as a life-threatening hypermetabolic crisis in susceptible individuals after exposure to inhalational anesthetics and the depolarizing muscle relaxant succinylcholine. One or more signs, including tachycardia, arrhythmia, acidosis, hyperthermia, generalized skeletal muscle contracture, and rhabdomyolysis, characterize an MH episode. From retrospective studies it is known that the incidence is higher in children than in adults: 1 in 15,000 anesthetic administrations in children versus 1 in 50,000 in adults.¹

Malignant hyperthermia is inherited as an autosomal dominant trait with reduced penetrance. With the exception of patients with central core disease (CCD), the typical MH patient appears clinically normal until exposed to triggering anesthetic agents, which renders presurgical diagnosis difficult. For the diagnosis of MH, the *in vitro* contracture test and the caffeine-halothane contracture test (CHCT) have been developed and standardized by European² and North American³ Malignant Hyperthermia Groups, respectively. Patients who test positive for these tests are considered to be MH susceptible (MHS).

The evidence to date clearly indicates that abnormal calcium regulation in skeletal muscle is the underlying pathophysiologic defect in MH and CCD. The skeletal muscle calcium release channel, *i.e.*, the ryanodine receptor (RYR) plays a pivotal role in myoplasmic calcium control, allowing the release of calcium stored in the sarcoplasmic reticulum to be available for electromechanical coupling and thus contraction. RYR is a 560-kd protein of 5,000 amino acids that assemble into a homotetramer and thereby forms a functioning calcium release channel. It is well recognized that mutations in the amino acid sequence of this channel protein can lead to dysfunction.

There are three isoforms of the RYR (RYR1, RYR2, and RYR3) encoded by separate genes on chromosomes 19, 1, and 15, respectively. The isoform in skeletal muscle is RYR1, which forms a functional unit with the voltage-dependent calcium channel of the cell membrane and is a key component in excitation contraction in skeletal muscle. Although MH appears to be a genetically heter-

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* Associate Staff Scientist, Barrow Neurological Institute, Phoenix, Arizona.
† Assistant Professor, ‡ Research Biologist, § Summer Student, || Medical Student, ¶¶ Professor, Department of Anesthesiology, Uniformed Services University of the Health Sciences. # Professor, Wake Forest University School of Medicine, Winston-Salem, North Carolina. ** Professor, Trinity Communications, Inc., Conshohocken, Pennsylvania. †† Professor, Thomas Jefferson University-Jefferson Medical College, Philadelphia, Pennsylvania.

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Address reprint requests to Dr. Muldoon: Department of Anesthesiology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799. Address electronic mail to: smuldoon@usuhs.mil. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

ogenous disorder, molecular genetic studies in humans have identified *RYR1* on chromosome 19 as the primary locus for MH in approximately 50% of MH patients.⁴ However, the human *RYR1* gene is one of the largest most complex genes consisting of 106 exons and remains incompletely understood.⁵

To date, more than 20 *RYR1* mutations have been reported in MH families, and some of them are also expressed as CCD, which has characteristic clinical and histopathologic features.⁶⁻⁸ The majority of *RYR1* mutations causing MH and CCD are clustered in two regions of the *RYR1* gene, the N-terminal region and the central region. Both of these regions are highly conserved across species.^{6,8} Thus, the N-terminal and the central regions of the *RYR1* gene are the main targets in mutation screening of MH populations.

Alternative candidate genes linked to MH have been located on chromosomes 1q31, 3q13, and 7q11-21.⁹⁻¹¹ The MH locus on chromosome 1q31 contains the *CACNLIA3* gene coding for the α_1 subunit of the dihydropyridine receptor, which acts as a voltage sensor for the RYR. Two MH causative mutations in this gene have been identified.^{12,13} No other gene associated with MH has been identified so far.

Screening for mutations in the *RYR1* gene among North American MH families has been performed previously,¹⁴⁻¹⁶ but these studies were limited in number of MH families and individuals and in the number of *RYR1* gene mutations tested. Here we report a more systematic screening of the *RYR1* gene among a North American MH population.

Materials and Methods

Skeletal Muscle Samples from Malignant Hypertbermia-Susceptible Persons

Skeletal muscle samples from 73 unrelated individuals were collected over a period of 3 yr and stored at -70°C . The samples were obtained from patients who had been diagnosed as MHS by the CHCT test at three North American MH diagnostic centers (Uniformed Services University of the Health Sciences, Bethesda, MD; Wake Forest University School of Medicine, Winston-Salem, NC; and Thomas Jefferson University-Jefferson Medical College, Philadelphia, PA). Patients had been referred for CHCT because of either a positive family history of MH or development of signs of MH during anesthesia. Diagnosis of MH was performed according to the North American CHCT³ protocol. Individuals were diagnosed as MHS if any one of three muscle strips exposed to halothane or caffeine exceeded diagnostic threshold. The threshold values for a positive test are greater than or equal to 0.7 g contracture response to 3% halothane or greater than or equal to 0.3 g contracture to 2 mM caffeine as recommended by the North American MH Group.¹⁷ Because this was a retrospective study on

stored muscle samples, not all patients could be contacted to give consent for genetic analysis. Therefore, the institutional review boards at the three participating institutions (Uniformed Services University of the Health Sciences, Bethesda, MD; Wake Forest University School of Medicine, Winston-Salem, NC; and Thomas Jefferson University-Jefferson Medical College, Philadelphia, PA) determined that clinical and demographic data were permitted only in terms of reasons for referral, MH diagnostic status, and sample identification for patients who could not be contacted.

RYR1 Mutational Analysis

Genomic DNA was extracted from stored muscle tissue or peripheral blood taken from MHS patients using the QIAamp DNA mini kit (Qiagen, Valencia, CA) or the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Amplification was performed during standard polymerase chain reaction (PCR) conditions: denaturation at 95°C for 3 min followed by 28-35 cycles of 94°C for 30 s, annealing at primer specific temperatures for 30 s, extension at 72°C for 30 s, with a final 10-min extension at 72°C . The PCR mix contained 100 ng of genomic DNA, 1.5-3.0 mM MgCl_2 , buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl; Perkin Elmer, Foster City, CA), 20 pmol of each primer, 250 mM of each dinucleotide triphosphate (dNTP), and 0.3 units of AmpliTaq (Perkin Elmer) in a final volume of 20-30 μl . For PCR we also used Master Amp PCR Pre-Mix D (Epicentre Technologies, Madison, WI) or Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN). The primer sets were either developed in the laboratory using Oligo (Molecular Biology Insights Inc., Plymouth, MN) software or chosen from the literature and covered the N-terminal and the central domains of the *RYR1* gene.^{18,19}

Screening for mutations was performed either by restriction fragment length polymorphism or single-strand conformation polymorphism analysis. PCR fragments were digested with 3-4 units of restriction enzyme (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. The digested products were resolved in a 3% NuSieve GTG[®] low melting agarose gel (FMC BioProducts, Rockland, ME) or 20% polyacrylamide TBE gel (Novex, San Diego, CA) depending on their sizes. For single-strand conformation polymorphism analysis, PCR products were mixed with formamide in a ratio of 1:3 and, after denaturing for 5 min, were loaded onto 20% polyacrylamide gel. The samples were then run overnight at 45 V in a cold room, and the denatured single-stranded DNA bands were visualized by staining with 1 $\mu\text{g}/\text{ml}$ ethidium bromide. Direct sequencing of PCR product was performed when changes in restriction site or single-strand DNA bands were detected. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and directly

Table 1. Mutations in the N Terminal and Central Regions of the *RYR1* Gene among North American MHS Individuals

| <i>RYR1</i> Gene Exon | Mutation | Effect on Protein | Number of Unrelated Patients with Mutation | Frequency of Mutation (%) |
|-----------------------|----------|-------------------|--|---------------------------|
| N-terminal region | | | | |
| 6 | C487T | Arg163Cys | 2 | 2.7 |
| 9 | G742A | Gly248Arg | 1 | 1.4 |
| 17 | C1840T | Arg614Cys | 1 | 1.4 |
| Central region | | | | |
| 39 | G6502A | Val2168Met | 1 | 1.4 |
| 40 | C6617T | Thr2206Met | 1 | 1.4 |
| 40 | G6640A* | Val2214Ile* | 1 | 1.4 |
| 44 | G7099A* | Ala2367Thr* | 1 | 1.4 |
| 45 | G7291A* | Asp2431Asn* | 1 | 1.4 |
| 45 | G7303A | Gly2434Arg | 4 | 5.5 |
| 46 | G7361A | Arg2454His | 3 | 4.1 |

* Novel mutations identified in this study.

MHS = malignant hyperthermia susceptible.

sequenced using the Big Dye Terminator Cycle Sequencing kit on ABI 310 Genetic Analyzer (Perkin Elmer) as described by the manufacturer.

Results

Screening of Known *RYR1* Mutations

We initially screened 20 of 22 known *RYR1* mutations using restriction fragment length polymorphism, single-strand conformation polymorphism, or both methods.^{6,18,19} The Tyr522Ser and Ile4898Thr mutations reported within the C-terminal region of the *RYR1* gene were not analyzed in this study as they are associated with CCD and appear to be very rare.^{19,20} Of the 20

previously reported mutations in the *RYR1* gene, seven were detected in North American MH population: Arg163Cys, Gly248Arg, Arg614Cys, Val2168Met, Thr2206Met, Gly2434Arg, and Arg2454His (table 1).

Novel Mutations in Central Domain of the *RYR1* Gene

The central domain of the *RYR1* gene, particularly exons 39, 40, and 44–46, are the most studied mutational sites within the *RYR1* gene, and more than 50% of all known *RYR1* mutations have been detected in these exons.^{8,13} We analyzed exons 12, 15, 39, 40, and 44–46 by single-strand conformation polymorphism and detected unique single-strand DNA patterns in three MH

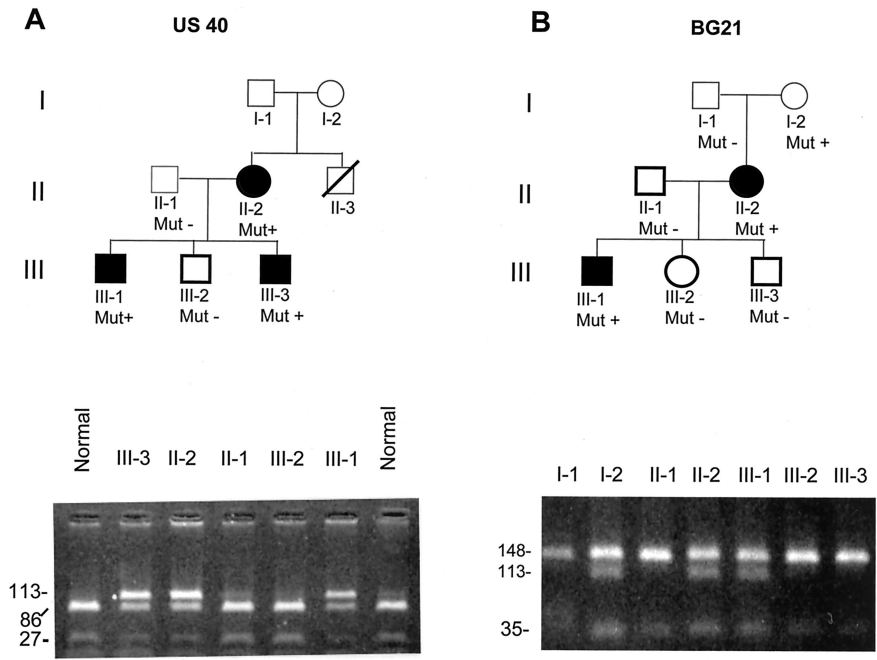
Table 2. Phenotype and Genotype Data of North American MHS Individuals

| Patient ID* | <i>RYR1</i> Mutation | CHCT Data† | | Reasons for Referral of Patient | |
|-------------|----------------------|-----------------------|-------------------------|----------------------------------|----------------|
| | | 2 mM Caffeine Max (g) | 3% Halothane/MH Max (g) | Episode (Suspected or Confirmed) | Family History |
| US19 | Arg163Cys | 3.0 | 8.4 | Yes | No |
| TJ55 | Arg163Cys | 1.1 | 3.6 | No | Yes |
| TJ34 | Gly248Arg | 0.3 | 3.15 | No | Yes |
| TJ65 | Arg614Cys | 0.3 | 2.3 | No | Yes |
| US40-1 | Val2168Met | 3.1 | 8.6 | No | Yes |
| US40-2‡ | Val2168Met | 1.6 | 8.7 | No | Yes |
| TJ48 | Thr2206Met | 1.7 | 3.5 | Yes | Yes |
| TJ27 | Val2214Ile§ | 0.2 | 1.6 | Yes | Yes |
| TJ1 | Ala2367Thr§ | 0.5 | 1.1 | Yes | No |
| US41 | Asp2431Asn§ | 0.7 | 6.0 | No | Yes |
| US47-1 | Gly2434Arg | 2.2 | 7.4 | No | Yes |
| US47-2‡ | Gly2434Arg | 0.0 | 3.4 | No | Yes |
| US57 | Gly2434Arg | 2.2 | 6.3 | Yes | No |
| TJ40 | Gly2434Arg | 0.04 | 6.0 | Yes | Yes |
| BG21-1 | Gly2434Arg | 4.67 | 4.55 | Yes | Yes |
| BG21-2‡ | Gly2434Arg | 0.34 | 5.68 | Yes | Yes |
| TJ61 | Arg2454His | 0.4 | 3.0 | Yes | No |
| TJ62 | Arg2454His | 0.1 | 0.9 | Yes | No |
| TJ63 | Arg2454His | 1.1 | 2.3 | No | Yes |

* Samples coded according to the participating biopsy centers: US (Uniformed Services University of the Health Sciences), TJ (Thomas Jefferson University), and BG (Wake Forest University). † The values given for each subject are the maximum of the caffeine–halothane contracture test (CHCT) responses (see Methods). ‡ Family members are indented under probands. § Novel mutations identified in this study.

MHS = malignant hyperthermia susceptible.

Fig. 1. (A) Segregation of Val2168Met (G6502A) mutation in the US40 pedigree. The referred patient, US40 (III-1), and his mother (II-2) and brother (III-3) had positive caffeine-halothane contracture test (CHCT) results. His mother (II-2) and maternal uncle (II-3) had clinical episodes of malignant hyperthermia (MH). BsgI digestion of the 113-base pair (bp) polymerase chain reaction product yielded fragments of 86 and 27 bp for normal DNA and fragments of 86 and 27 bp plus a single undigested fragment of 113 bp for DNA containing the heterozygous mutation. BsgI restriction endonuclease analysis revealed that all three CHCT-positive individuals (II-2, III-1, and III-3) had Val2168Met (G6502A), whereas II-1 and III-2 (CHCT-negative) had no mutation. (B) Segregation of Gly2434Arg (G7303A) mutation in the BG21 pedigree. Proband BG21 (III-1) had a clinical episode of MH. Proband (III-1) and his mother (II-2) showed positive CHCT results, whereas his father (II-1) and two brothers (III-2 and III-3) had negative CHCT results. DdeI digestion of the 183-bp polymerase chain reaction fragment yielded fragments of 148 and 35 bp for normal DNA and fragments of 148 and 35 bp plus a fragment of 113 bp for DNA containing the heterozygous mutation. DdeI restriction endonuclease analysis indicated that CHCT-positive II-2 and III-1 had Gly2434Arg (G7303A), whereas CHCT-negative II-1, III-2, and III-3 had no mutation. Symbols: bold lines = individuals who underwent CHCT; filled = CHCT-positive; open = CHCT-negative.



patients (table 1). Direct sequencing of the PCR products identified nucleotide substitutions at G6640A, G7099A, and G7291A, which results in amino acid changes Val2214Ile, Ala2367Thr, and Asp2431Asn. All of these alterations were sequenced in reverse directions, and nucleotide substitutions were confirmed. Segregation analyses of novel substitutions were not provided within the families as family members were not available for the study. All of the new substitutions could be detected using restriction enzymes Mae II for Val2214Ile, FauI for Ala2367Thr, and Taq I for Asp2431Asn. Analysis of these sites with restriction fragment length polymorphism was extended to 134–158 normal chromosomes, and none displayed the presence of novel substitutions.

Distribution of RYR1 Mutations in North American Malignant Hyperthermia Population

Overall, 10 different RYR1 gene mutations were detected in 16 unrelated MHS individuals, which accounts for approximately 21.9% of the screened population (table 1). The most common mutation was Gly2434Arg, occurring in four patients and accounting for 5.5% of the population, followed by Arg2454His in three patients (4.1%) and Arg163Cys in two patients (2.7%). The other seven mutations were detected in single patients.

Phenotype and Genotype Correlation

Caffeine-halothane contracture test data for MHS patients with RYR1 mutations are shown in table 2. Each subject bearing an RYR1 mutation showed strong posi-

tive CHCT results, particularly for 3% halothane. Four patients with RYR1 mutations were negative for the 2-mm caffeine test according to North American CHCT standards.¹⁷ Wide variations in contracture phenotype were observed within genotypes. For example, with the Gly2434Arg mutation, the halothane phenotype is similar among MHS subjects (3.4–7.4 g), whereas the caffeine sensitivity varies from 0.0 to 4.7 g (table 2).

Family members tested by CHCT were available for three MH patients with an RYR1 mutation, and complete phenotype and genotype correlation was seen in these families (table 2). In the family US40, Val2168Met (G6502A) mutation was segregated with CHCT results (fig. 1). The referred patient, US40 (III-1), a 30-yr-old man, underwent CHCT and was found to be positive for MHS. The mother (II-2) and brother (III-3) also had positive CHCT results in 1981 and 1988, respectively. There is a report of an intraoperative MH episode (death) in his maternal uncle (II-3). All three CHCT-positive individuals (II-2, III-1, and III-3) had Val2168Met (G6502A), whereas II-1 and III-2 (CHCT-negative) had no mutation (fig. 1). Gly2434Arg (G7303A) mutation was also cosegregated with MH phenotype in the family of BG21 (fig. 1). In the family BG21, both proband (III-1) and his mother (II-2) were CHCT-positive and showed the Gly2434Arg mutation. Proband's father (II-1) and two brothers (III-2 and III-3) were negative for both CHCT and Gly2434Arg mutation. The same Gly2434Arg mutation also cosegregated with positive CHCT phenotype in two members of the family US47 (table 2, data not presented in fig. 1).

Discussion

To assess the frequency and distribution of *RYR1* gene mutations in a North American MHS population, we screened muscle samples from 73 unrelated individuals diagnosed as MHS according to the North American MH guidelines. We found 10 different *RYR1* gene mutations in 16 unrelated MHS individuals; seven of the mutations had been previously described, but three are novel mutations identified for the first time in this study. Overall, these mutations account for 21.9% of the North American screened population, which is similar to results obtained in recent studies of 105 German and 57 Italian MHS individuals.^{18,19}

All patients with an *RYR1* mutation were highly positive for halothane in the CHCT, and most were positive to caffeine. However, variations in contracture phenotype, even within a genotype (e.g., Gly2434Arg), were observed across the screened patients. Possible reasons for variability of CHCT data include diagnostic center-to-center test results, and other genetic or environmental factors. Statistical correlation between CHCT phenotypes and RyR1 genotype was not studied because of the small number of subjects carrying the same mutation or lack of family members for the study. Therefore, larger-scale studies are needed to assess genotype-phenotype relations for the North American MHS population.

We found three novel *RYR1* missense mutations, G6640A (Val2214Ile), G7099A (Ala2367Thr), and G7291A (Asp2431Asn), in the *RYR1*. Although linkage and gene expression studies are needed to define causality of mutations for hereditary diseases, we consider these novel amino acid substitutions as strong candidate mutations for MH susceptibility. The novel substitutions met two criteria that suggest they are not likely to be polymorphisms, namely, none were found among 134–158 normal control chromosomes, and each substitution occurred in conserved amino acid residues.⁸

Regional differences in frequencies for common MHS mutations are observed across Western Europe.^{6,13} Frequencies of *RYR1* gene mutations detected in this North American MHS population were markedly different from those found in western Europe. Thus, the Arg614Cys and Val2168Met mutations, which are common in Germany (9%) and Switzerland (6%), respectively, were rare in our MHS population (only 1.4% each). Moreover, the Gly314Arg mutation, which is common in Ireland, England, and France (6%), was not detected in our population. In contrast, 4.1% of our MHS population had Arg2454His, which has been seen in only 1% of MHS families in Europe. The mutation common for both continents was Gly2434Arg, occurring in 4–7% of European and 5.5% of North American MHS families. It is likely that marked variability in frequency of *RYR1* gene mutations between North American and European families is a result of regional and population differences. However,

the North American MHS population was more heterogeneous in the types of observed *RYR1* gene mutations (allelic heterogeneity), which may be explained by heterogeneity of North American population in general.

Although genetic linkage analysis shows that 50–80% of European MHS families are linked to the *RYR1* gene, mutations are reported in only 25–40% of MHS families so far.^{6,18,19} The majority of *RYR1* gene mutations are clustered in the N-terminal region with amino acid residues from 35 to 614 and in the central region from 2163 to 2458. Most genetic screening studies target these two regions, which account for only approximately one fourth of the entire coding region of the *RYR1* gene. Thus, the absence of *RYR1* mutations in the rest of the screened population might be explained either by a mutation located outside the two regions analyzed in the current study or by the involvement of another gene. Recent genetic studies reveal a new region of the *RYR1* gene where MHS-CCD mutations may cluster.^{20–23} With regard to other MH candidate genes, only one other gene, that for the α_1 -subunit of the dihydropyridine receptor, has mutations linked to MHS.¹² Recent data obtained by Stewart *et al.*²⁴ suggest that the dihydropyridine receptor gene Arg1086His mutation is present in only 1% of a large North American MHS population. The two mutations, Arg1086His and Arg1086Gly, reported in the dihydropyridine receptor gene^{12,13} were not detected in our 73 MHS individuals (data not shown). Thus, dihydropyridine receptor mutations associated with MH appear to be very rare.

Mutational screening analysis of MH candidate genes is an important step for the future development of simplified genetic testing and for understanding phenotype-genotype relation in MH. Continuous and collaborative efforts involving sharing of tissue samples, clinical data, and CHCT results are needed to achieve progress in MH genetics.

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