Malignant Hyperthermia in North America

Genetic Screening of the Three Hot Spots in the Type I Ryanodine Receptor Gene

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Background: Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle, manifested as a life-threatening hypermetabolic crisis after exposure to anesthetics. Type 1 ryanodine receptor 1 is the primary gene responsible for susceptibility to MH as well as central core disease, a congenital myopathy that predisposes susceptibility to MH. More than 40 mutations in the RyR1 gene cluster in three coding regions: the N-terminus, central, and C-terminus regions. However, the frequency of mutations in each region has not been studied in the North American MH-susceptible population.

Methods: The authors tested 124 unrelated patients with MH susceptibility for the presence of mutations in the N-terminus (exons 2, 6, 9, 11, 12, and 17), central (exons 39, 40, 44, 45, and 46), and C-terminus (exons 95, 100, 101, and 102) regions.

Results: Fourteen mutations have been identified in 29 of 124 MH-susceptible patients (23%). Approximately 70% of the mutations, which include a novel mutation, Ala 2437val, were in the central region. In 8 patients (28%), mutations were identified in the N-terminus region. Screening the C-terminus region yielded a novel mutation, Leu4824Pro, in a single patient with a diagnosis of central core disease.

Conclusions: The detection rate for mutations is only 23% by screening mutations (or exons) listed in the 2002 North American consensus panel. The implications from this study suggest that testing the central region first is currently the most effective screening strategy for the North American population. Screening more exons in the three hot spots may be needed to find an accurate frequency of mutations in the RyR1 gene.

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MALIGNANT hyperthermia (MH) is a life-threatening pharmacogenetic disorder triggered by administration of commonly used volatile anesthetics (halothane, enflurane, isoflurane, desflurane, and sevoflurane), the depolarizing muscle relaxant succinylcholine, or both.1 To diagnose an individual’s risk of susceptibility to MH (MHS), the in vitro contracture test and the caffeine-halothane contracture test (CHCT) have been developed and standardized by European2 and North American MH study groups,3 respectively. However, because the tests require an invasive surgical biopsy of the leg muscle and travel to specialized centers, development of an alternative noninvasive test is a priority. In the past decade, significant progress in MH genetic research has been made, and genetic test for MHS has become a focus of interest among MH researchers, patients, and their families.

Malignant hyperthermia is caused by a fundamental defect in the ability of muscle to adequately regulate its myoplasmic Ca2+ concentration.4 Consistent with this observation, genetic studies have shown the ryanodine receptor 1 (RyR1, Ca2+ channel of skeletal muscle) on chromosome 19q13.1 (MHS1 locus; OMIM 145600) is the primary locus for susceptibility to MH.5,6 Genetic linkage studies estimate that more than 50% of cases are associated with the RyR1 gene.7 If the specificity of phenotyping is taken into account, the RyR1 gene may be involved in an even higher percentage of MHS families.8 Therefore, despite a high level of genetic heterogeneity, the RyR1 gene is the focus for development of genetic testing.8,9 Accordingly, with the caution that this testing is not currently feasible to diagnose MH risk, the European MH group has recently established guidelines for MH genetic testing using the RyR1 gene.10 Using the European guidelines, the MH genetics group in North America has also recently developed a consensus panel of mutations for genetic screening.11

To date, more than 40 RyR1 mutations have been reported in MHS families, and some of them are also associated with central core disease (CCD; OMIM 117000), a rare congenital myopathy that may predispose individuals to MH.12 The majority of RyR1 mutations causing MH and CCD are clustered in three regions of the RyR1 gene8, a region close to the N-terminus (MH/CCD1; amino acid 35–614), a central region (MH/CCD2; amino acid 2163–2458), and the C-terminus of the gene (MH/CCD3; transmembrane and pore region.
of the receptor). Since a mutation, Ile4898Thr, was found in the MH/CCD3 region, more than 12 mutations have been identified in families with CCD in the MH3 region. However, the frequency of these mutations in MHS families and in each region is currently unknown.

The objective of this interinstitutional collaborative study was to study the frequency of the mutations in the three “hot spot” regions of the RyR1 gene among North American MHS patients. We have previously reported frequency of mutations in MH/CCD1 and MH/CCD2 regions. Here, we increased the number of MHS patients and for the first time conducted the screening of exons 95, 100, 101, and 102 to determine the frequency of mutations in the C-terminus MH/CCD3 region using a denaturing high-performance liquid chromatography (DHPLC) method. We also performed a functional study to measure Ca$^{2+}$ responses to RyR1 agonists for a novel MH/CCD3 mutation using B lymphocytes. Based on results from this screening, we discuss frequency of mutations in the three hot spot regions and the strategy for the mutation screening in the RyR1 gene for North American MHS families.

Materials and Methods

Patients

We studied 124 unrelated patients with a diagnosis of MHS. Diagnosis of MHS was provided according to North American MH CHCT. Studies were approved by the Institutional Review Board of participating biopsy centers (Uniformed Services University of the Health Sciences, Bethesda, Maryland; University of California Davis, Davis, California; Thomas Jefferson University-Jefferson Medical College, Philadelphia, Pennsylvania; Wake Forest University, Winston Salem, North Carolina), and informed consent was obtained from each patient.

RyR1 Mutation Analysis

Genomic DNA was extracted from muscle tissue, peripheral blood, or buccal cells using a DNA purification kit (Qiagen, Valencia, CA) or the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Amplification was performed under standard polymerase chain reaction (PCR) conditions: denaturation at 95°C for 3 min followed by 45 cycles of 94°C for 30 s, annealing at primer specific temperatures (table 1) for 30 s, and 72°C for 30 s, with a final 7-min extension at 72°C. The PCR mixture contained 100 ng genomic DNA, 1.5–3.0 mM MgCl$_2$, Perkin Elmer PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 20 pmol of each primer, 250 μM of each deoxyribonucleoside triphosphate, and 0.3 U AmpliTaq (Perkin Elmer, Foster City, CA) in a final volume of 20–30 μl. For PCR, we also used Master Amp PCR Pre-Mix D (Epicenter Technologies, Madison, WI) and Expand long PCR System (Boehringer Mannheim, Indianapolis, IN). Primers used in the study have been published or designed and synthesized in our laboratory (table 1).

Screening for mutations was performed by restriction fragment length polymorphism, single-strand conformation polymorphism (SSCP), or DHPLC analysis to test for the presence of known mutations in MH/CCD1 (exons 2, 6, 9, 11, 12, and 17), MH/CCD2 (exons 39, 40, 44, 45, and 46), and MH/CCD3 (exons 95, 100, 101, and 102) regions. PCR fragments were digested with 3–4 U restriction enzyme (New England Biolabs, Beverly, MA) according to manufacturer instructions. The digested products were resolved in a 3% NuSieve GTG™ low-melting agarose gel (FMC BioProducts, Rockland, ME) or 20% polyacrylamide Novex TBE gel (Invitrogen, Carlsbad, CA) depending on their sizes. For SSCP analysis, PCR products were mixed with formamide in a ratio of 1:3 and, after denaturing for 5 min, were loaded onto a 20% polyacrylamide gel. The samples were to run overnight at 45 V and at 4°C. Denatured single-stranded DNA bands were visualized by staining with 1 μg/ml ethidium bromide. DHPLC analysis was performed using the Transgenicome WAVE DNA fragment analysis system (Transgenicome, Omaha, NE). Direct sequencing of PCR product was performed when changes in restriction site, single-strand DNA bands, or heteroduplex were detected. After purification using a QIAquick PCR Purification Kit (Qiagen), the PCR products were directly sequenced using the Big DyeTerminator Cycle Sequencing Kit and analyzed on an automated fluorescence sequence apparatus (ABI 310 Genetic Analyzer; Perkin Elmer).

Calcium Response in B Cells

Relative changes in [Ca$^{2+}$]i in B cells were derived from changes in the fluorescence intensity of fluo-3 in peripheral mononuclear cells stained with phycoerythrin-conjugated anti-CD19 mAB. The measurements were performed using FACScan (Becton-Dickinson, San Jose, CA) as previously described. The percentage of fluo-3+ cells relative to unstimulated baseline was calculated and analyzed using the Consort 30 program (Becton-Dickinson).

Results

Screening MH/CCD1 and MH/CCD2 Regions for RyR1 Mutations

One hundred twenty-four unrelated MHS patients were screened for the presence of mutations in MH/CCD1 (exons 2, 6, 9, 11, 12, and 17) and MH/CCD2 (exons 39, 40, 44, 45, and 46) regions using restriction fragment length polymorphism, SSCP, or DHPLC methods. A total of 13 RyR1 mutations (12 known and 1 novel) were detected in
28 patients in these two regions (table 1). The identified known mutations were Arg163Cys, Gly248Arg, Arg614Cys, Arg2163Cys, Val2168Met, Thr2206Met, Ala2350Thr, Ala2367Thr, Asp2431Asn, Gly2434Arg, and Arg2454His (table 2). The most common mutation was Gly2434Arg, occurring among 4.7% of the studied patients, followed by Arg2454His (3.1%) and Arg614Cys (3.1%). One patient was found to have a novel nucleotide substitution, C7301T, in exon 45 (table 2). To determine whether this change was a normal polymorphism, we examined 108 chromosomes from healthy individuals by DHPLC and found that the mutation was absent. Direct sequencing of the patient sample in both 5'-to-3' and 3'-to-5' directions confirmed nucleotide substitution C7310T, which resulted in Ala2437Val amino acid change (fig. 1). This Ala at 2437 is evolutionally conserved, as shown in Drosophila melanog and Caenorhabditis elegans (fig. 1C). The Ala2437Val mutation was found in a patient who, in his fifties, experienced a reaction suggestive of MH during surgery on his wrist during general anesthesia. The anesthetic agent was isoflurane following use of succinylcholine for tracheal intubation. After 1 h of anesthesia, there was an unexplained hypercapnia. The creatine kinase concentration was 40,000 U after surgery. CHCT conducted 10 yr after the episode recorded a contracture of more than 3.0 g in response to 3% halothane. The muscle histology test did not reveal any specific abnormalities.

**Screening MH/CCD3 Region for RYR1 Mutations**

We used a DHPLC method to test 124 MHS samples for the presence of mutations in exons 95, 100, 101, and 102. The DHPLC screening and direct DNA sequencing confirmed three different nucleotide substitutions: C-to-A substitution within intron 99 (~47 from the first nucleotide of exon 100); C-to-T substitution at nucleotide 14589, which preserves Phe at amino acid 4863; and T-to-C transition at nucleotide 14471, which causes a Leu4824Pro substitution (fig. 2). Absence of Leu4824Pro was confirmed in 108 normal chromosomes by screen-
ing exon 100 using DHPLC (fig. 2A) followed by DNA sequencing (fig. 2B). The substituted residue is also evolutionally conserved (fig. 2C). The patient, who carried Leu4824Pro, was a muscular 34-yr-old white man who had a maternal male first cousin who died of MH. The first cousin’s brother also had a positive diagnosis by CHCT. The CHCT results in the patient showed contraction tensions of 11.8, 12.2, and 8.9 g in three separate muscle strips after 3% halothane and 4.1, 4.6, and 5.2 g.

Table 2. Distribution of the Identified Mutations in North American Malignant Hyperthermia–susceptible Population

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutations Found in This Study</th>
<th>Number of Patients</th>
<th>Number of Patients in Each MH/CCD Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Arg163Cys</td>
<td>3</td>
<td>Total 8 patients in MH/CCD1 region</td>
</tr>
<tr>
<td>9</td>
<td>Gly248Arg</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Arg614Cys</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>Arg2163Cys</td>
<td>2</td>
<td>Total 20 patients in MH/CCD2 region</td>
</tr>
<tr>
<td>39</td>
<td>Val2168Met</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Thr2206Met</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>Ala2350Thr</td>
<td>1</td>
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<td>44</td>
<td>Ala2367Thr</td>
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<td>45</td>
<td>Asp2431Asn</td>
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<tr>
<td>45</td>
<td>Gly2434Arg</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Ala2437Val</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Arg2454His</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Leu4824Pro*</td>
<td>1</td>
<td>Total 1 patient in MH/CCD 3 region</td>
</tr>
</tbody>
</table>

Total 29 patients in 124 MHS patients screened

* Novel mutations reported in this study.

MH/CCD1 = the N-terminus of the RyR1 gene (amino acid 35–614); MH/CCD2 = a central region of the RyR1 gene (amino acid 2163–2458); MH/CCD3 = the C-terminus of the RyR1 gene (transmembrane and pore region of the receptor); MHS = malignant hyperthermia susceptible.

Fig. 1. (A) A heteroduplex pattern of C7310T mutant elucidated by the denaturing high-performance liquid chromatography method (Transgenomics Wave, Omaha, NE). A superimposed image shows four denaturing high-performance liquid chromatography patterns from three wild types and from the patient with the mutant. (B) Identification of the novel mutation by sequencing analysis. Nucleotide sequence indicates C-to-T nucleotide exchange at 7310. (C) Aligned amino acid sequences of ryanodine receptor (RYR) genes from different species. Sequences are derived from their GeneBank entries (human RyR1, NM 000540; human RyR2, NM 001035; human RyR3, AJ001515; frog RyR1, D21070; fish RyR1, U97329; Drosophila melanogaster RyR homolog, AE003835; Caenorhabditis elegans RyR, D45899). The position of the mutation Ala2437Val is indicated as an arrow, and its numbering corresponds to the human RyR1 sequence. A horizontal dash means identical to a corresponding amino acid of human RyR1. Amino acid sequences were aligned using the MegAlign software (DNASTAR, Inc., Madison, WI).

Fig. 2. (A) A heteroduplex pattern of T14471C acquired by the denaturing high-performance liquid chromatography method (Transgenomics Wave, Omaha, NE). A superimposed image shows four denaturing high-performance liquid chromatography patterns from three wild types and the patient with the mutant. (B) Identification of the novel mutation by sequencing analysis. Nucleotide sequence indicates T-to-C nucleotide exchange at 14471. (C) Aligned amino acid sequences of ryanodine receptor (RYR) genes from different species. Sequences are derived from their GeneBank entries (access numbers for the displayed genes are listed in fig. 1 legend). The position of the mutation Leu4824Pro is indicated as an arrow, and its numbering corresponds to the human RyR1 sequence. A horizontal dash means identical to a corresponding amino acid of human RyR1. Amino acid sequences were aligned using the MegAlign software (DNASTAR, Inc., Madison, WI).
after 2 ms caffeine. Histopathologic examination from the muscle biopsy indicated central core myopathy. Because the B lymphocytes have been shown to express the functional RyR1 channel, we examined Ca\(^{2+}\) response using B lymphocytes from this patient (US53). Measurement of Ca\(^{2+}\) responses to the RyR1 agonists caffeine and 4-chloro-m-cresol (4-CmC) indicated that the Ca\(^{2+}\) release was significantly greater as compared with B lymphocytes from control subjects (fig. 3A).

**Overall Detection Rate from Each MH/CCD Region**

Using restriction fragment length polymorphism, SSCP, or DHPLC methods, we screened three selected MH/CCD regions for the RYR1 mutations, which included 15 exons and at least 25 known mutations. A total of 14 RYR1 mutations (12 known and 2 novel) were detected in 29 patients, estimating an incidence of 23% (table 2). Overall, 20 of the 29 mutations (69%), including a novel mutation, Ala 2437Val, originated from the MH/CCD2 region. In 8 patients (28%), mutations were identified in MH/CCD1 region. Screening the MH/CCD3 region yielded a novel mutation, Leu4824Pro, in a single patient with a diagnosis of CCD.

**Discussion**

Numerous genetic studies have identified more than 40 missense mutations and several deletions linked to MH susceptibility. The mutations are clustered in three regions: the N-terminal MH/CCD1, the central MH/CCD2, and the C-terminus MH/CCD3. In all three MH regions, mutations are also associated with CCD, a congenital myopathy that usually predisposes MHS. Until recently, seven mutations have been identified in the N-terminal and the central region of the gene as being causative of CCD. However, the C-terminal MH/CCD3 region has now been found as the major hot spot for CCD-causing mutations; to date, 15 mutations have been identified in this MH3 region. As a result, the number of mutations in the MH/CCD3 region is approximately one third of all the mutations identified in the RYR1. We studied the MH/CCD3 region because frequency of mutations in the C-terminus region has not been extensively studied for the MHS population. Among 124 unrelated MHS patients, we have found only one MH/CCD3 mutation in a single MHS patient. This Leu4824Pro mutation in exon 100 is in close proximity to Arg4825Cys that is reported to be associated with CCD. Leu4824Pro is in close proximity to Arg4825Cys that is reported to be associated with CCD.

There was evidence to suggest functional abnormalities in the RyR1 of patient US53, who carried the Leu4824Pro. As we previously reported, the functional RyR1 channels are expressed in B lymphocytes and have been used to study phenotypic function as well as genotype of the RYR1 for MH and CCD. We observed that the intracellular concentrations of Ca\(^{2+}\) in the B cells of patient US53 were significantly higher than those in B cells from controls in response to caffeine and 4-CmC (fig. 3A). This augmented Ca\(^{2+}\) response was similar to those reported for MH mutations in the MH/CCD1 and 2 regions, such as Arg614Cys and Val2168Met, respectively, but in contrast to the Ca\(^{2+}\) response found in some CCD mutations in the MH/CCD3 region. Four MH/CCD3 mutations, Arg4861His, Arg4893Trp, Ile4898Thr, and Gly4899Arg, have been all shown to cause spontaneous leakage from the RyR1 Ca\(^{2+}\) pool and reduced Ca\(^{2+}\) release by 4-CmC in Epstein-
Barr Virus–transformed B-cell lines.\textsuperscript{14} It has also been recently shown that the two latter mutations cause excitation–contraction uncoupling.\textsuperscript{20} Because contracile force in normal excitation–contraction coupling is proportional to myoplasmic Ca\textsuperscript{2+} concentrations, reduced RyR1-mediated Ca\textsuperscript{2+} release due to leaky Ca\textsuperscript{2+} stores, excitation–contraction uncoupling, or both may explain muscle weakness in patients with CCD. Unlike with these four mutations, we found no spontaneous leakage of Ca\textsuperscript{2+} but enhanced response to 4CmC in B cells from patient US53. Consistent with this finding, patient US53 exhibited no muscle weakness and had large contractile responses to the RyR1 agonists. Therefore, there may be different functional abnormalities in the \textit{RYRI} mutants that are associated with different subsets of CCD pathologies. Interestingly, the mutations, which have been found to cause leaky Ca\textsuperscript{2+}, are localized in the sarcoplasmic luminal domain between M8 and M10, according to recent studies that the two latter mutations cause mutations in the MH/CCD3 region have been associated with CCD. This is consistent with the fact that most of the North American MHS population, using the current screening protocol, only 1 in 124 patients was found to have Arg2454His, which has been identified in only a single family in Europe.\textsuperscript{9} As a result, frequencies for mutations in the MH/CCD1 region were relatively higher in Europe than in North America. The status of mutations in the MH/CCD3 region in Europe has not been well studied. In the North American MHS population, using the current screening protocol, only 1 in 124 patients was found to have a mutation in MH/CCD3, and this was associated with CCD. This is consistent with the fact that most of the mutations in the MH/CCD3 region have been associated with CCD.\textsuperscript{12} However, it should be noted that inclusion of more exons for screening may lead to identification of novel mutations, as shown in a recent report by Galli \textit{et al.}\textsuperscript{29} Classifying new mutations in the screening panel is a constant challenge for developing genetic testing for MH. Although discovery of new mutations may change our statistics, using our current protocol, mutations in the MH/CCD2 region, within exons 39, 40, 44, 45, and 46, accounts for 72% of the MHS patients with positive mutations. Therefore, results from the current study suggest that initial screening for mutations in the MH/CCD2 region may be the most time- and cost-effective strategy for the North American MHS population at this time. We found the DHPLC method highly effective for screening mutations in the \textit{RYRI} gene, especially for the six exons in the MH/CCD2 regions. Further, the DHPLC method produced more accurate results and was more time efficient to perform than SSCP in screening the MH/CCD2 region. However, more investigations are needed to discuss sensitivity and specificity of DHPLC.

The MH genetics working group in North America encourages use of genetic information from the \textit{RYRI} gene to develop genetic testing for MH and CCD.\textsuperscript{11} Considering the complex etiology of MH, use of genetic information for MH cannot replace CHCT as the standard diagnostic test for MH in the near future. However, in those families in which a familial causative mutation is identified, genetic screening of other members of the family can be beneficial. For this reason, the North American MH genetics working group recommends continuous and collaborative research efforts to advance genetic screening and to make appropriate revisions/recommendations to the mutation panel for molecular genetic studies of MH as well as CCD.

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