Malignant Hyperthermia in Japan

Mutation Screening of the Entire Ryanodine Receptor Type 1 Gene Coding Region by Direct Sequencing

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Background: Malignant hyperthermia (MH) is a disorder of calcium homeostasis in skeletal muscle triggered by volatile anesthetics or succinylcholine in susceptible persons. More than 100 mutations in the ryanodine receptor type 1 gene (RYR1) have been associated with MH susceptibility, central core disease, or both. RYR1 mutations may account for up to 70% of MH-susceptible cases. The authors aimed to determine the frequency and distribution of RYR1 mutations in the Japanese MH-susceptible population.

Methods: The authors selected 58 unrelated Japanese diagnosed as MH-susceptible for having an enhanced Ca2+-induced Ca2+ release rate from the sarcoplasmic reticulum on chemically skinned muscle fibers. They sequenced the entire RYR1 coding region from genomic DNA. Muscle pathology was also characterized.

Results: Seven previously reported and 26 unknown RYR1 potentially pathogenic sequence variations were identified in 33 patients (56.9%). Of these patients, 48% had cores on muscle biopsy. The mutation detection rate was higher in patients with clear enhancement of Ca2+-induced Ca2+ release rate (72.4%), whereas all patients with central core disease had RYR1 mutations. Six patients harbored potentially causative compound heterozygous sequence variations.

Conclusions: Distribution and frequency of RYR1 mutations differed markedly from those of the North American and European MH-susceptible population. Comprehensive screening of the RYR1 gene is recommended for molecular investigations in MH-susceptible individuals, because many mutations are located outside the “hot spots.” Based on the observed occurrence of compound heterozygous state, the prevalence of a possibly predisposing phenotype in the Japanese population might be as high as 1 in 2,000 people.

RYANODINE receptors (RYRs) belong to the superfamily of ion-gated channels, having three different isoforms. Functional units are homotetramers of approximately 5,000 amino acids per subunit coded by 150-Kbp-long genes. The RYR type 1 is the main isoform expressed in skeletal muscle, forming the sarcoplasmic reticulum calcium release channel. To date, more than 100 mutations in the huge gene that encodes the RYR type 1 (RYR1) have been associated with malignant hyperthermia (MH), central core disease (CCD), or both, but definitive evidence of a causative role is still lacking for most of them.

Malignant hyperthermia is a pharmacogenetic disorder in which susceptible individuals develop a hypermetabolic state with generalized muscle contracture in response to a massive calcium release from the skeletal muscle sarcoplasmic reticulum after the administration of volatile general anesthetics or the depolarizing muscle relaxant succinylcholine. The diagnosis of susceptibility to MH is achieved by the whole-muscle caffeine–halothane contracture test (IVCT) in Europe, whereas in Japan, it is determined by detecting an enhancement of the rate of calcium-induced calcium release (CICR) from the sarcoplasmic reticulum in comparison to reference values previously measured in healthy controls. The CICR test is performed on skinned fibers prepared from patients’ skeletal muscle.

On the other hand, CCD is a congenital myopathy characterized by hypotonia in infancy, different degrees of proximal dominant muscle weakness, delayed motor development, and reduced muscle bulk. CCD derives its name from the “core” structures seen in type 1 skeletal muscle fibers, which consist of amorphous central areas that lack enzyme activity on nicotinamide adenine dinucleotide-tetrazolium reductase staining. MH susceptibility and CCD are inherited mostly as autosomal dominant traits with variable penetrance. It seems clear by now that the main
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>CICR</th>
<th>Biopsy</th>
<th>Nucleotide Change</th>
<th>Exon</th>
<th>Predicted Amino Acid Change</th>
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<tr>
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<td>34</td>
<td>F</td>
<td>MHfam</td>
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<td>±</td>
<td></td>
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<td>10</td>
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<tr>
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<td>F</td>
<td>MHfam/CCD</td>
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<td>±</td>
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<td>F</td>
<td>MH</td>
<td>§</td>
<td></td>
<td>c.7522&gt;C&gt;T</td>
<td>47</td>
<td>p.R2508C</td>
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<td>37</td>
<td>F</td>
<td>CCD</td>
<td>±</td>
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<td>F</td>
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<td>±</td>
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<td>M</td>
<td>MH</td>
<td>±</td>
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<td>MH</td>
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<td>MH</td>
<td>↑ CK</td>
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<td>c.1259A_12967dup</td>
<td>91†</td>
<td>p.L4320_R4322dup</td>
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<tr>
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<td>102</td>
<td>p.A4894T</td>
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</table>

Table 1. Clinicopathologic Diagnosis and **RYR1** Phenotype of Japanese Malignant Hyperthermia–susceptible Individuals

Lines divide the table into four sections, from top to bottom: patients with one sequence variation, with two sequence variations, with nonpathogenic polymorphisms, and with no mutation. Previously reported mutations are shown in boldface, nonpathogenic polymorphisms in italics.

* Homozygous. † Nonsynonymous polymorphism in another exon. ‡ Ca²⁺−induced Ca²⁺ release test (CICR) clearly enhanced. § CICR abnormally enhanced.

**Cores.**

**CCD** = central core disease; ↑ CK = increased serum creatine kinase; LGMD2A = limb-girdle muscle dystrophy 2A; MH = malignant hyperthermia episode; MHfam = family history of MH; MmD = multimimicore disease; NA = no available biopsy; **RYR1** = ryanodine receptor type 1 gene.

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underlying pathomechanism in MH susceptibility is a dysregulation of calcium homeostasis in skeletal muscle determined by multiple interacting genetic factors, with approximately 50% of cases linked to the \textit{RYR1} and at least five other loci being apparently involved.\textsuperscript{14} In contrast, the majority—if not all—of CCD cases are due to \textit{RYR1} mutations.\textsuperscript{15}

Most of MH/CCD-related mutations have been found mainly in three regions or “hot spots”: domain 1, in the cytoplasmic aspect of the protein near the N-terminus between residues p.M1 and p.R614; domain 2, in the central region (p.R2163–p.R2458); and domain 3, in the C-terminal region (p.R4136–p.P4973) that encodes the transmembrane pore-forming segment.\textsuperscript{2} However, it is possible that the so-called hot spots might be a result of experimental bias due to insufficient investigation of other gene regions.

In an effort to develop a less invasive diagnostic test for MH susceptibility, special attention has been given to \textit{RYR1} mutation screening. In a recent study, the detection rate of potentially causative mutations in North American MH-susceptible population increased from 25% to 70% through a systematic investigation of the \textit{RYR1} entire coding region in lieu of a screening limited to the hot spots.\textsuperscript{16}

However, the vast size of the \textit{RYR1} gene and the genetic heterogeneity of the MH trait make of molecular diagnosis of MH susceptibility a painstaking task. Moreover, the incidence and distribution of mutations along the \textit{RYR1} gene in MH and in CCD patients is highly variable among populations.\textsuperscript{17} Nevertheless, molecular genetic analysis represents a useful tool in the struggle for unraveling the pathogenic background of MH and CCD. When mutation frequencies in the correspondent geographic region are determined and integrated with pedigree information,\textsuperscript{18} and the causative role of candidate mutations is functionally demonstrated \textit{in vitro},\textsuperscript{19} genetic analysis constitutes a supplement for the standard diagnostic tests in \textit{ex vivo} muscles. Considering these facts, we aimed to determine the frequency and distribution of \textit{RYR1} mutations in the Japanese MH-susceptible population.

Materials and Methods

\textbf{Patients}

This study was approved by the institutional review board of the National Institute of Neuroscience, National Institute of Neurology and Psychiatry, Tokyo, Japan. We selected 58 unrelated Japanese patients diagnosed as MH susceptible by the CICR test (i.e., enhanced CICR rate, see section titled MH Susceptibility Diagnosis: The CICR Test) from the database of our institution. The sample consisted of 33 males and 25 females ranging in age from 5 to 77 yr with a mean age of 33.5 ± 18.5 yr. Informed consent for genetic analysis was obtained from each patient or from their parents. The correspondent clinical records and biopsy specimens were reviewed—when available—and patients were classified according to the type of CICR enhancement (clear or abnormal) and according to the presence or absence of cores on muscle biopsy’s nicotinamide adenine dinucleotide-tetrazolium reductase staining.

Twenty-nine patients were recruited after experiencing an MH episode (table 1), 17 for having an MH relative, whereas the remaining 12 subjects had the CICR test under suspicion of MH susceptibility for different reasons: Three of them had been diagnosed with CCD, whereas the only remarkable finding among the others was an increase of serum creatine kinase in the absence of remarkable muscle symptoms.

\textbf{Statistical Analysis}

Association of the former categories with the presence or absence of \textit{RYR1} mutations was statistically evaluated using chi-square test. Differences between proportions were analyzed with two-tailed \(z\) test. Statistical significance was defined as \(P < 0.05\) or \(P < 0.01\), when specified.

\textbf{MH Susceptibility Diagnosis: The CICR Test}

In this test, the sarcolemma of skeletal muscle fibers is chemically destroyed, thus allowing the stimulation of calcium release from sarcoplasmic reticulum using external calcium solutions at five different concentrations. If the patient’s CICR values are 1.5 SD above the normal average values at two or more calcium concentrations in two or more skinned fibers, the CICR rate is defined as being “clearly enhanced.” Otherwise, CICR test results are reported as “abnormally enhanced” or “not enhanced” (fig. 1).

\textbf{Mutation Screening}

Genomic DNA was extracted from either muscle biopsy samples or peripheral blood lymphocytes according to previously established protocols.\textsuperscript{20} Using Web-based software,‡‡ intronic primers approximately 25 bp long were designed to amplify all 106 \textit{RYR1} exons with their flanking intron boundaries by polymerase chain reaction (PCR). The best condition for each set of primers was chosen after performing gradient temperature PCR (approximately 58°–70°C) using ExTaq polymerase with 10x ExTaq Buffer. LA Taq polymerase with 2x GC Buffer-I were used for guanine/cytosine-rich exons. The PCR amplification mix contained a total volume of 25 \(\mu\)l composed of 20–100 ng genomic DNA, 0.4 \(\mu\)M of each primer, 250 \(\mu\)M of each deoxyribonucleotide triphosphate, and 1 U enzyme diluted in an appropriate buffer volume.

\footnotesize{‡‡ Available at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Accessed February 6, 2005.}
Dimethyl sulfoxide, 0.5%, was added to the PCR mix to improve specific annealing when needed. Additional information on PCR conditions and primer sequences is available on the Anesthesiology Web site at http://www.anesthesiology.org. Enzyme/buffer kits and deoxynucleotide triphosphate for PCR reactions were purchased from Takara (Tokyo, Japan). Enzyme and deoxynucleotide triphosphate for PCR reactions were previously reported as nonpathogenic 16 was found in seven patients. In addition, another nonsynonymous polymorphism (c.5287C>T, p.P1763S) was present in two control samples but not among MH-susceptible individuals. Twenty-nine silent single nucleotide polymorphisms were in addition found (data not shown).

Of 33 potentially pathogenic sequence variations, 28 (84.8%) were private (i.e., present only in one pedigree). Two homozygous nucleotide changes were found in the same patient, the child of a consanguineous marriage. Two different heterozygous substitutions were identified in six patients as well. Interestingly, the proportion of patients with clearly enhanced CICR was significantly greater in this group than among those who carried only one plausibly pathogenic substitution (0.83 vs. 0.41, respectively; z = 2.41; P = 0.016, two tailed).

The RyR1 genotype was associated with the type of CICR enhancement and with the presence of cores on nicotinamide adenine dinucleotide-tetrazolium reductase staining in the correspondent muscle biopsies. Pathology specimens were available in 51 patients, and cores were identified in 15 of them. Patients with clearly enhanced CICR, as well as those with cores, were more likely to bear RyR1 mutations (tables 2 and 3). Besides, 13 of 27 MH-susceptible patients (48.1%) with RyR1 mutations had cores, whereas such structures were seen only in 2 patients (J-38 and J-56) among those without mutation. There was no significant difference in the incidence of mutations between patients with clinical MH events (62.1%) versus those who underwent CICR testing by virtue of familial risk only (58.8%).
Fig. 2. Distribution of sequence variations along the ryanodine receptor type 1 gene. Exons harboring sequence variations that were detected in this study are represented as solid boxes, in light or dark gray, depending on their location within or outside the hot spots, respectively. Predicted amino acid substitutions written above the exon boxes have been detected in Japan and mainland Asia (underlined if previously reported in other populations), whereas those below the boxes have been reported in Western countries. Nonsynonymous single nucleotide polymorphisms are indicated in light gray. A list of references corresponding to each sequence variation is available on the Anesthesiology Web site at http://www.anesthesiology.org. † = Detected in this study; ♦ = associated with cores. The occurrence of recurrent substitutions is written between parentheses.
Discussion

Previous genetic investigations in the distribution of the MH population either were limited to certain mutational spots or evaluated just a few pedigrees. To the best of our knowledge, this is the first report for most of the potentially pathogenic sequence variations found in our sample (26 of 33), with exception of 6 mutations formerly found in MH population of Western origin (i.e., p.R163C, p.G341R, c.1022G>A, p.R2435H, c.1598G>A, p.R533H) and 1 mutation reported in a Japanese MH-susceptible pedigree (c.14512C>T, p.R613C). In addition, the residues p.D1663 and p.R2452 were also affected as in other studies, but with different predicted amino acid substitutions. The location and distribution of mutations in our Japanese cohort differed markedly from that of MH population in Western countries. For example, in some European cohorts, p.G341R has been found in up to 10% of MH-susceptible individuals, whereas it was present only in one Japanese patient. Similarly, p.R613C is common in North America and Germany with incidences of 9% and 4%, respectively—was not found in our cohort. It is worth noting that the distribution of mutations along the RYR1 gene is not limited to the widely accepted hot spots, even though they occur more frequently in some regions of the gene. In fact, more than one third of the candidate mutations in this cohort were found outside the hot spots.

Table 2. Association between CICR Status and RYR1 Mutation Occurrence

<table>
<thead>
<tr>
<th>CICR enhancement</th>
<th>Present</th>
<th>Absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear</td>
<td>21 (72.4)</td>
<td>8 (27.6)</td>
<td>29</td>
</tr>
<tr>
<td>Abnormal</td>
<td>12 (41.4)</td>
<td>17 (58.6)</td>
<td>29</td>
</tr>
</tbody>
</table>

\[\chi^2 = 0.017; P < 0.05\]

RYR1 mutations were found more commonly in patients with clear Ca\(^{2+}\)-induced Ca\(^{2+}\) release rate (CICR enhancement) [1.5 SDs above normal range at least in two fibers at two or more [Ca\(^{2+}\)]; otherwise, it was defined as abnormal CICR enhancement]. Data are expressed as number of patients (%).

Mutations were found more commonly in patients with [1.5 SDs above normal range at least in two fibers at two or more [Ca\(^{2+}\)]; otherwise, it was defined as abnormal CICR enhancement]. Data are expressed as number of patients (%).

Table 3. Association between the Occurrence of Cores versus RYR1 Mutations

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>RYR1 Mutation</th>
<th>Present</th>
<th>Absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cores</td>
<td>13 (86.7)</td>
<td>2 (13.3)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>No cores</td>
<td>14 (38.9)</td>
<td>22 (61.1)</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

\[\chi^2 = 0.0018; P < 0.01\]

The frequency of ryanodine receptor type 1 (RYR1) mutations was higher in patients with identifiable cores on nicotinamide adenine dinucleotide-tetrazolium reductase staining of their muscle biopsies. Data are expressed as number of patients (%).

NA = no available biopsy (excluded for chi-square calculation).
very resulted from the exclusion of equivocal results on the caffeine–halothane contracture test.36

MH Susceptibility with Cores

We evaluated muscle pathology whenever available (51 of 58 cases), identifying cores—or core-like structures—in almost one third of patients. Cores were more frequently seen among individuals with RYR1 mutations in comparison to those without mutation (48.1% vs. 8.3%, respectively). From another viewpoint, RYR1 mutations were present in most of our patients with cores (86.7%), indicating that the presence of cores may be a good predictor of MH susceptibility due to RYR1 mutations. Eight patients with cores were not categorized as having CCD because their pathologic findings (e.g., just a few, rather eccentric, core-like structures with poorly defined borders) differed greatly from those seen in canonical CCD.13 In addition, they did not present any clinical signs or symptoms of myopathy.

Interestingly, we did not find any patient with the typical pathologic characteristics seen in CCD cases with mutations in domain 3 (i.e., type 2 fiber deficiency and central cores with sharp borders in most of type 1 fibers).15 Instead, we recognized just a few core-like structures in only two of the C-terminal mutation carriers (J-14 and J-51). All CCD patients included in our survey have an enhanced CICR from the sarcoplasmic reticulum. Contrastingly, CCD patients with mutations in domain 3 may exhibit normal functional results in ex vivo muscle tests.57 This suggests that the domain 3 mutations associated with MH susceptibility may be functionally different from those located in the same domain and associated with typical CCD. Furthermore, in accordance with our results, CCD-related mutations situated outside the RYR1 pore-forming region are more likely to produce hyperresponsive channels rather than excitation–contraction uncoupling mutants.3,38

MH Susceptibility without RYR1 Mutations

We did not find any RYR1 mutation in 25 patients, of whom 11 had a clinical episode of MH, 7 had a relative who experienced an MH crisis, 4 had increased creatine kinase with no other symptoms, 1 had multicore disease, and the remaining 2 were finally diagnosed with limb-girdle muscular dystrophy 2A. Despite that we may overlook other RYR1 defects by doing only direct sequencing (e.g., splicing abnormalities,39 epigenetic modifications, large deletions38 or duplications), it seems reasonable to consider the influence of another causative MH susceptibility locus in these patients.

The patients with high creatine kinase of unknown origin were originally referred by their physicians (or anesthesiologists) after preoperative routine workup to rule out the possibility of MH susceptibility. As stated previously, the CICR test is designed to detect specific abnormalities on calcium release from the sarcoplasmic reticulum, which is mainly governed by the RYR1 in skeletal muscle. Nevertheless, it may also be secondarily influenced by disturbances of calcium homeostasis in the myocyte from other sources, such as it occurs in individuals affected by neuromuscular syndromes who have positive IVCT results.40,41 This might be the case of patients J-56, J-57, and J-58, who had mild clinical manifestations of myopathy. However, it is safer to consider such patients as MH susceptible at the moment of making clinical decisions, having no additional evidence to infer their actual likelihood to experience an MH episode. Actually, we found sequence variations potentially causative of MH in two patients with high creatine kinase (J-51 and J-52), no pertinent MH history, and no remarkable signs or symptoms.

Patients with Two Candidate Mutations

Several probands whose parents both had abnormal IVCT results have been found to harbor two different causative mutations, which is defined as a compound heterozygous state.42 In previous reports, patients with compound heterozygous mutations could not be differentiated on the basis of their IVCT results.42 However, in our study, five of six patients with two candidate mutations had clear CICR enhancement. In four of them, the causative role of at least one of the mutations was demonstrated previously.28–30,52 There still remains the possibility that one of the substitutions is actually a polymorphism, as evidenced in patient J-12. Nevertheless, we could reasonably suggest that a more pronounced enhancement of CICR in these patients may be the result of having two mutations with an additive effect. In the case of patient J-51, who had cores in the muscle biopsy, we also speculate that the “core” and “MH susceptibility” traits might have resulted from one mutation each based on the location of the respective mutations within two different functional domains of the protein.15,43 Unfortunately, we were not able to confirm the state of compound heterozygosity by haplotyping or segregation analysis as reported in other investigations44,45 because no additional DNA samples from the affected families were available.

Prevalence of MH Trait in Japan

The prevalence of MH in the general population is presumed to be around 1:10,000, based on crisis reports and on its autosomal dominant pattern of inheritance.40,42 However, considering the higher-than-expected occurrence of individuals with two possibly predisposing MH alleles, we may want to question this somewhat arbitrary assumption. Having documented a series of MH susceptible patients with a compound heterozygous state, Monnier et al.42 estimated that the incidence of the MH trait in the French population might be as high as 1 in 3,000 people. Based on the size of the Japanese population (1.3 × 108) and on the probability of finding a compound heterozy-
gous MH-susceptible person according to the previously stated incidence \((10^{-4} \times 10^{-8} \times 0.25)\), we should not have identified more than one patient with compound heterozygous mutations. Taking into account only those patients with two mutations affecting well-conserved residues, we still have four cases in which a compound heterozygous state is most likely. Therefore, we estimate that the prevalence of the MH-predisposing phenotype in Japan might be closer to 1 in 2,000 people. However, this hypothesis may be confirmed only if a noninvasive test suitable for massive screening of MH susceptibility becomes available in the future.

In summary, we determined the frequency and distribution of RYR1 mutations in a large cohort, evidencing that they account for 56.9% of Japanese MH-susceptible patients. Although the causative role of many newly reported nucleotide substitutions remains to be proven, our study represents the cornerstone for establishing a protocol of molecular genetic screening as an aid for the diagnosis of MH susceptibility in Japan. The incidence of RYR1 mutations is higher in MH-susceptible patients with clear CICR enhancement and in those with identifiable cores on their muscle biopsies. The results of this work raise additional indica of the genetic heterogeneity underlying MH susceptibility. Screening the whole open reading frame of the RYR1 gene is crucial for genetic investigations in MH-susceptible individuals, because many mutations occur outside the previously known hot spots and some patients may carry two different MH-predisposing sequence variations. Finally, we think that the prevalence of MH susceptibility in the Japanese population might be significantly higher than it is believed.

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References


