Genetic Analysis with Calcium-induced Calcium Release Test in Japanese Malignant Hyperthermia Susceptible (MHS) Families

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ABSTRACT

Some genetic studies have shown a linkage between malignant hyperthermia susceptibility (MHS) and chromosome 19q or the skeletal muscle ryanodine receptor (RYR1) gene. Some types of MHS seem to be caused by an abnormality of calcium-induced calcium release (CICR). We analyzed the linkage of RYR1 gene polymorphisms in Japanese MHS families and investigated the correlation between genetic evidence of RYR1 gene mutations and an accelerated rate of CICR.

We studied 63 subjects who were referred to our institute for investigation of MHS. CICR rates were measured by the skinned fiber method in 23 subjects. DNA samples were collected from 63 individuals belonging to 22 unrelated families. Restriction fragment length polymorphism (RFLP) analyses on the RYR1 locus and hypervariable microsatellite analysis were performed.

We found one family with a linkage between acceleration of the CICR mechanism and a group of RFLPs. In CICR tests, ten of the 11 patients who had presented with fulminant MH showed accelerated rates of CICR. Analysis for the mutation C1840T, which was performed in 63 samples, did not demonstrate an alteration in any of the patients. Although we found heterozygotes in RFLP studies, we did not recognize a specific relationship between the acceleration of CICR and the RFLPs.

We suggest a linkage between the acceleration of CICR and an abnormal human RYR1 gene in MHS. These results also suggest that heterogeneity exists for MH. We conclude that genetic tests cannot replace CICR tests or caffeine-halothane contracture tests with muscle biopsy as a diagnosing test for MH in the near future.

Key words: Malignant hyperthermia, Calcium-induced calcium release, Ryanodine receptor gene

Malignant hyperthermia (MH) is an inherited pharmacogenetic disorder triggered by some inhalational anesthetics and muscle relaxants. MH susceptibility (MHS) is inherited as an autosomal dominant trait. Recent advances in molecular genetics have enabled us to study the genetic analysis of MH. McCarthy and his group reported a linkage between MH susceptible patients and chromosome 19q. MacCarthy and his group reported a linkage between MH susceptible patients and chromosome 19q. MacLenan and his group reported that the gene-encoding the skeletal muscle ryanodine receptor (RYR1) is a candidate gene for MHS. Fujii et al reported substitution of a thymine for a cytosine at nucleotide 1843 in RYR1 from MH swine. The corresponding point mutation in human RYR1 was reported as a candidate mutation in some MHS families. Thereafter, studies of the linkage between MHS and 19q chromosome or RYR1 were performed in several countries. However, most studies were of North American or European Caucasian families. Since the MH incidences which were reported previously were quite different among countries, genetic analyses of Asian people would be worth studying.

Endo et al reported the acceleration of calcium-induced calcium release (CICR) with skin fibers in MHS patients’ skeletal muscles. Ohnishi et al and Ohta reported the same phenomenon in MHS pig skeletal muscle. The CICR mechanism is one of the functions of the ryanodine receptor; MH could be caused by a functional abnormality of skeletal muscle ryanodine receptors.

Since these reports, we have used the CICR test with skin fibers of biopsied skeletal muscle specimens as a diagnostic test for MHS in Japan.
Restriction endonucleases used in this analysis.

However, the only widespread test for diagnosing MHS is the Caffeine-Halothane muscle contracture (CHC) test, with invasive muscle biopsy. If the main etiology of MH exists in an abnormality of the ryanodine receptors, the CICR test would be a better test for diagnosing MH.

Taking account of the reports of genetic studies and CICR mechanisms, we considered that linkage analysis of human RYR1 genes with the CICR test would show a relationship. In the present study, we analyzed the linkage of human RYR1 gene polymorphisms in Japanese MHS families and investigated the correlation between these results and an accelerated CICR rate, which is likely to reflect MHS.

**MATERIALS AND METHODS**

**Subjects**

We studied 63 subjects belonging to 22 unrelated families for the investigation of MHS. Twenty of the 63 subjects who were referred to our hospital, one of the test centers for diagnosing MH in Japan, had a history of some MH symptoms, and 43 were their relatives. We divided them into four groups according to their history. The first group (n=11) consisted of patients who presented the fulminant type of MH (f-MH) reaction, diagnosed as f-MH according to the criteria of Morio: the maximum body temperature (BT) ≥ 40°C, or 40°C > the maximum BT ≥ 38°C and an increased rate of BT ≥ 0.5°C/15 min with other significant clinical findings (respiratory and/or metabolic acidosis, muscle rigidity, circulatory disturbances, signs of muscle breakdown, etc.) during anesthesia. The second group (n=33) consisted of subjects who had a family history of f-MH. The third group (n=9) had episodes of MH-like reactions without severity such as f-MH. The fourth group (n=10) had a family history of MH-like reactions without severity such as f-MH. All of 63 subjects agreed to participate in the study.

DNA samples were collected from blood samples of the 63 individuals. Eleven were collected from group 1, 33 from group 2, 9 from group 3, and the remaining 10 were collected from group 4 (Table 2).

**CICR measurement protocol**

Twenty-three patients underwent skeletal muscle biopsies. A small muscle bundle about 5 mm in width and 15 mm in length was excised from each patient. We made chemically skinned fibers treated with saponin from the specimens. The rates of CICR were measured as previously reported by Kawana et al.: The general strategy of the measurements was as follows: 1) Ca²⁺ loading: the sarcoplasmic reticulum (SR) was loaded with a fixed amount of Ca²⁺ in the presence of MgATP; 2) Test: Ca²⁺ was applied to induce CICR in the absence of ATP to avoid simultaneous Ca²⁺ uptake; 3) Assay: MgATP was re-introduced and the Ca²⁺ remaining in the SR was assayed by thoroughly releasing it with a high concentration (50 mM) of caffeine. The amount of Ca²⁺ released during the test Ca²⁺ application was estimated by comparing the amount of Ca²⁺ remaining in the SR with that of a control run in which no test procedure was carried out. The rates of CICR were determined assuming single exponential decay of the Ca²⁺ content in the SR during the CICR test procedure, and were expressed by a rate constant with the dimension of min⁻¹. A CICR rate greater than the mean +2SD of that of normal subjects was judged to be “accelerated”. The others were defined as “unaccelerated”.

**Genetic analysis**

Genomic DNA was isolated from 5–10 ml of peripheral blood using proteinase K and sodium dodecyl sulfate (SDS), followed by phenol/chloroform extraction. The DNA content was quantified by agarose gel electrophoresis or spectrometry.

**Amplification of Genomic DNA**

We used four sets of oligonucleotide primers located on the RYR1 locus (Table 1). One of these sets was designed to detect a substitution of 1840 thymine for a cysteine (C1840T) in the codon for arginine 614 of the amino acid sequence, as previously reported by Otsu, and the others were designed to detect some substitutions without amino acid changes for linkage analysis, as described by Gillard. PCR was performed with

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Substitution</th>
<th>Rest.Enz.</th>
<th>PCR primers (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu 197</td>
<td>CTG→CTA</td>
<td>AlwNI</td>
<td>1Fi</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CTCTCGGACGGGGACGAT</td>
</tr>
<tr>
<td>Arg 614</td>
<td>CGC→TGC</td>
<td>RsaI</td>
<td>ATCTCTAGTGGCCCATCTTATCCGAGTGGACG*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATCTCTAGAACCTGTCAGAGATGCACTTCCATC*</td>
</tr>
<tr>
<td>Ile 1151</td>
<td>ATC→ATT</td>
<td>TaqI</td>
<td>93Fe</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATCTCTAGAGAGACCTCCAGATGAGGTTGA*</td>
</tr>
<tr>
<td>Thr 2658</td>
<td>ACG→ACA</td>
<td>BsaJI</td>
<td>3Fe</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCCCTCACCAACCACCTATGAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4Re</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TGGGCCAGAGAGTCAAATG</td>
</tr>
</tbody>
</table>

Rest.Enz.: Restriction Enzyme*:contain 9 bp XbaI linker
10–100 ng of genomic DNA in a reaction mixture of 50 µl containing 200 µM each of dNTP, 1.5 mM MgCl₂, 0.25 µM each primer, and 1 unit of Taq polymerase (Wako, Osaka, Japan) in a DNA thermal cycler (Astek, Fukuoka, Japan). The PCR reactions were carried out for 40 cycles of 1 min at 95°C, 1.5 min at 58°C, 2.5 min at 74°C, and then a 7-min extension at 74°C before returning the sample to 4°C.

**Restriction Fragment Length Polymorphism (RFLP) Analyses**

The PCR amplified product using each pair of primers was completely digested with the appropriate restriction endonuclease (Table 1) and subjected to electrophoresis on 18% polyacrylamide gels. The gels were stained with 2% ethidium bromide for 10 min and photographed under UV.

Two micrograms of DNA samples were completely digested with restriction enzyme (BanI) and subjected to electrophoresis on 0.8% agarose gels. Southern hybridization was carried out with radio-labeled HRR1 DNA probe.

**Hypervariable microsatellite analysis**

For analysis at dinucleotide repeat polymorphisms loci, D19S191 and D19S47 (Research Genetics, Huntsville, AL USA), each primer set was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD USA), and the PCR reactions were carried out with 20–100 ng of sample DNA in 12.5 µl reaction mixtures containing 200 µM each dNTP, 1.5 mM MgCl₂, 1 pmol of each primer, and 0.5 unit of Taq DNA polymerase (Wako, Osaka, Japan), for 30 cycles of 1 min at 95°C, 1 min at 52°C, and 1 min at 74°C. Three µl aliquots were subjected to electrophoresis on a 8% denaturing polyacrylamide gel and autoradiographed.

**RESULTS**

**CICR**

We performed CICR tests on 23 subjects, eleven of whom were f-MH patients, five family members of f-MH patients, and seven patients with mild MH-like reactions. We defined fifteen of them as accelerated and the remaining eight as unaccelerated. Ten of the eleven f-MH patients (90.9%) showed accelerated rates of CICR, and 3 family members and 2 unrelated subjects with mild MH-like reactions demonstrated acceleration of CICR. (Table 2)

<table>
<thead>
<tr>
<th>Category of subjects and the results of CICR test</th>
<th>number</th>
<th>CICR accelerated</th>
<th>normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>f-MH patients</td>
<td>11</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Subjects with FH of f-MH</td>
<td>33</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Patients with mild MH-like reactions</td>
<td>9</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Subjects with FH of MH-like reactions</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>63</strong></td>
<td><strong>15</strong></td>
<td><strong>8</strong></td>
</tr>
</tbody>
</table>

**Table 2.**

Genetic analysis

Analysis for a substitution of C1840T was performed in all 63 samples, but no alteration was found in any of them. In studies of the other three RFLPs, we found 14 heterozygotes in 30 subjects at the Leu 197 locus, 22 heterozygotes in 34 at Ile 1151, and 12 heterozygotes in 24 at Thr 2658. The frequencies of the heterozygote allele were almost the same as those reported by Gillard (Table 3).

We did not observe any relationship between acceleration of the CICR and C1840T point mutation. Nor did we observe a specific relationship between the acceleration of CICR and the other three silent substitutions at Leu 197, Ile 1151, and Thr 2658.

**One family represented linkage between acceleration of CICR and some RFLPs.**

We found one MHS family, which carried informative polymorphisms (Fig. 1). The proband (I-1) was a 35-year-old male. He manifested f-MH symptoms such as high body temperature (40.4°C), myoglobinuria, severe respiratory and metabolic acidosis, and ventricular arrhythmias during a neurosurgical operation under halothane anesthesia. He was treated with generalized cooling and administration of dantrolene, and recovered without any complications. First we performed a CICR test using the skinned fiber method on his daugh-

**Table 3.**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Substitution</th>
<th>AA change</th>
<th>allele frequency</th>
<th>CICR test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg 614</td>
<td>CGC→TGC</td>
<td>Arg→Cys</td>
<td>126:0</td>
<td>15</td>
</tr>
<tr>
<td>Leu 197</td>
<td>CTG→CTA</td>
<td>None</td>
<td>43:17</td>
<td>10</td>
</tr>
<tr>
<td>Ile 1151</td>
<td>ATC→ATT</td>
<td>None</td>
<td>38:30</td>
<td>11</td>
</tr>
<tr>
<td>Thr 2658</td>
<td>ACG→ACA</td>
<td>None</td>
<td>34:14</td>
<td>10</td>
</tr>
</tbody>
</table>

AA: amino acid
Fig. 1. A. A representative family pedigree showing the malignant hyperthermia status of the tested individuals, the constructed haplotypes with the tested markers. I, II represent the generation, and the number marked in the upper left of each circle or square represents the person. The proband is I-1 and he did not receive a CICR test. All of the second generation (II-1, II-2, II-3) received the CICR test. Alphabetic characters describe the haplotypes for the PCR amplified region which is listed in the left lane. Numbers describe the allele types seen in Southern blotting or microsatellite markers.

B. C. Photographs of DNA fragments produced by PCR with selected oligonucleotide primers 1Fi - 2Re (B), and 93Fe - 32Re (C), followed by digestion with A1wNI and TaqI, respectively (separation on 18% polyacrylamide gels). The tracks are aligned below the corresponding family member.

Fig. 2. Rates of Calcium release at 0, 0.3, 1.0, 3.0 and 10.0 µM of Calcium concentration solutions. Results from three members of the representative family, daughter: II-1, son: II-2, and niece: II-3, are presented with results from normal subjects (N=34), MHS patients with moderately accelerated CICR (N=23), and MHS patients with highly accelerated CICR (N=3), whom we examined previously using the same procedure. Data are presented as mean ± SD.

DISCUSSION

We describe a family which showed a relationship between RFLPs in the RYR1 region and acceleration of CICR rates in skinned fibers. In this family, we at first performed muscle biopsies on the daughter and niece of the proband for measurements of the CICR rates. We asked his son for a muscle biopsy, but he declined. Then we performed RFLP analysis on the RYR1 region and observed that the son inherited the allele from his father which was suspected to be the cause of MH. We told them the result and obtained his agreement for the muscle biopsy. Then, we showed the acceleration of CICR in his muscle. This is the first clinical evidence suggesting a linkage between the RYR1 gene and the acceleration of CICR using the skinned fiber method. In general linkage studies, the high point of the lod score (e.g. >3) suggests a linkage between a suspected genetic locus and the disease. However, because it is difficult to study many large families in Japan, we could not calculate the lod score and could not demonstrate a tight linkage between an accelerat-
ed rate of CICR and the RYR1 gene. The genetic study could not take the place of the muscle tests, both the CICR test and CHC test, as the diagnosing test for MHS. The genetic linkage analysis, however, could give us very useful information on whether or not the muscle test should be performed on a member of an MHS family, because it could be performed by only drawing a small volume of peripheral blood. We can also use genetic linkage analysis in order to persuade subjects to obtain muscle biopsy tests, as in the case described.

Although we found an informative family that suggested a correlation between the RYR1 gene and an acceleration of CICR rates, we could not find any subject who had the C1840T point mutation in the human RYR1 gene in this study. That point mutation was first reported by Fuji et al\(^{27}\) as a C1843T mutation in the porcine RYR1 gene. In studies of pigs, the alteration of C1843T in the RYR1 gene segregated with the MH phenotype\(^{34}\), and the rates of CICR in skinned fibers from MH-susceptible pig were significantly accelerated\(^{28}\). Although in human studies, there are some reports showing the C1840T mutation in human RYR1\(^{37}\), the rate at which the point mutation is segregating seems to be three to five percent of human MHS\(^{17}\). These figures were calculated using Caucasian subjects, so they might be different with Orientals. In our series, we could not find that point mutation, which would be attributed to the heterogeneity of MH.

Other alterations have been reported on RYR1 in human chromosome 19q13.1. Gillard et al reported 21 polymorphic sequence variants of the RYR1 gene, some of which we investigated in this study, and they also showed that one of the four amino acid substitutions - Arg for Gly248 - was a candidate mutation for MHS\(^{36}\). In addition to these mutations, other 15 RYR1 point mutations have been identified in MHS and central core disease pedigrees\(^{14}\), namely Cys35Arg\(^{55}\), Arg163Cys\(^{30}\), Gly341Arg\(^{30}\), Ile403Met\(^{30}\), Tyr522Ser\(^{26}\), Arg552Trp\(^{11}\), Arg614Leu\(^{34}\), Arg2162Cys\(^{36}\), Arg2162His\(^{38}\), Val2167Met\(^{10}\), Thr2205Met\(^{36}\), Gly2434Arg\(^{23}\), Arg2435His\(^{26}\), Arg2458Cys\(^{26}\), and Arg2458His\(^{26}\). Loke and MacLennan reported that these mutations accounted for the abnormality in roughly 40% of MH families\(^{14}\).

Some investigators reported the exclusion of the MHS locus from the RYR1 region and many investigators also reported linkages between other loci and MHS. Iles reported the apparent exclusion of the linkage between the MHS locus and RYR1 region of 19q13.1 using microsatellite markers\(^{8}\). Levti\(^{36}\) and Ocklers et al\(^{17}\) reported the linkage between MHS and some markers on 17q11.2-q24, and showed that the adult muscle sodium channel δ-subunit was a candidate for MHS. These reports clearly show heterogeneity in human MH.

Since Endo et al\(^{19}\), with a skinned fiber method, and Ohnishi et al\(^{20}\), with a fragmented sarcoplasmic reticulum method, reported abnormalities in the CICR in MHS, we have been carrying out the CICR test using the skinned fiber method for that purpose in Japan for about ten years\(^{20}\). Kawana et al reported that almost all of the patients with conspicuous fever (≥ 40.0°C and/or ≥ 0.5°C rise/15min) during anesthesia belong to moderately or highly accelerated rate of CICR groups\(^{20}\). The clinical criteria which they used were almost the same as the f-MH criteria used by Morico\(^{29}\). In this study, ten of eleven f-MH patients were estimated as accelerated CICR. On the other hand, only two of seven patients with MH-like reactions showed an acceleration of CICR. According to these results, most patients who suffered from clinically fulminant MH in Japan seemed to show an acceleration of CICR.

As we mentioned above, the relationship between MHS and an acceleration of CICR, and an acceleration of CICR and the C1843T point mutation of porcine RYR1 gene were clearly shown in porcine studies\(^{27,4,24}\). Recently, Otsu reported that cultured myoblastic cells transfected with mutated (C1840T) ryanodine receptor cDNA showed a higher sensitivity to caffeine for inducing Ca\(^{2+}\) release from the sarcoplasmic reticulum through the ryanodine receptor\(^{20}\). They also suggested that the modulator binding domain in the ryanodine receptor could lie between residues 2600 and 3000, and that the domain including Arg615 may play an important role in the regulation of the channel activity mediated by the ryanodine receptor. These results suggested that the C1840T mutation in the human RYR1 gene is one of the causes of the acceleration of CICR. We, however, could not find any mutation in subjects who showed an acceleration of CICR. One of the reasons is the small number of subjects that we studied. Because the channel activity is known to be regulated by many modulators, these subjects might have other kinds of abnormalities.

In conclusion, we found one Japanese MHS family with informative polymorphisms which suggested a linkage between the acceleration of CICR mechanism and the human RYR1 gene. In this family, linkage analysis of the RYR1 gene was very useful for further examination, including muscle biopsy. However, acceleration of CICR caused by cytosine 1840 to thymine point mutation was not demonstrated in 63 Japanese subjects who were suspected to be MHS. These results suggest that one of causative alterations of CICR acceleration may occur in or near the RYR1 gene. We could not establish a tight linkage between polymorphisms in the human RYR1 gene locus and MHS diagnosed by the acceleration of CICR. Although at present the genetic test cannot replace the CICR tests or CHC tests with muscle biopsy as a diagnostic test for MH, molecular diag-
nosis of MHS will be developed in the future.

ACKNOWLEDGEMENTS

The authors thank Professor David H. MacLennan of the University of Toronto for his helpful comments on the manuscript and for his kind donation of several DNA probes and PCR primers to us. We would also like to thank Ms. Ikuko Fukuba of the laboratory of 1st Department of Surgery for her excellent technical assistance. This work was partly supported by grants-in-aid from the Ministry of Education, Science and Culture of Japan (No.08407052: Osafumi Yuge, No.09771157: Yasuhiro Maehara) and by a research grant from the Labor Welfare Corporation of Japan.

(Received September 16, 1998)
(Accepted January 6, 1999)

REFERENCES


