

Results of Contracture Tests with Halothane, Caffeine, and Ryanodine Depend on Different Malignant Hyperthermia-associated Ryanodine Receptor Gene Mutations

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Background: More than 20 mutations in the gene encoding for the ryanodine receptor (RYR1), a Ca^{2+} release channel of the skeletal muscle sarcoplasmic reticulum, have been found to be associated with malignant hyperthermia (MH). This study was designed to investigate the effects of different mutations in the RYR1 gene on contracture development in *in vitro* contracture tests (IVCT) with halothane, caffeine, and ryanodine.

Methods: Ninety-three MH-susceptible (MHS) patients, diagnosed by the standard IVCT with halothane and caffeine, were included in this prospective study. Surplus muscle specimens were used for an IVCT with 1 μ M ryanodine. The contracture course during the ryanodine IVCT was described by the attainment of different time points: onset time of contracture and times when contracture reached 2 mN or 10 mN. In addition, all patients were screened for mutations of the RYR1 gene.

Results: In 36 patients, four different mutations of the RYR1 gene (C487-T, G1021-A, C1840-T, G7300-A) were found. The IVCT threshold concentrations of halothane and caffeine were lower in patients with the C487-T mutation compared with patients without a detected mutation in the RYR1 gene. In the IVCT with ryanodine, contracture levels of 2 mN and 10 mN were reached earlier in muscle specimens from patients with C487-T, C1840-T, and G7300-A mutations compared with specimens from patients with the G1021-A mutation and patients without detected mutation in the RYR1 gene.

Conclusions: The differences between the groups in the halothane and caffeine IVCT threshold concentrations and in the time course of contracture development in the ryanodine IVCT underline the hypothesis that certain mutations in the RYR1 gene could make the ryanodine receptor more sensitive to specific ligands. This may be an explanation for varying clinical symptoms of MH crisis in humans.

MALIGNANT hyperthermia (MH) is an autosomal inherited myopathy that is usually triggered by volatile anesthetic agents and depolarizing muscle relaxants.¹ It is widely accepted that susceptibility to MH is caused by an abnormal calcium metabolism within the skeletal muscle fiber. Calcium release from intracellular stores during anesthetic-induced MH crisis results in high myoplasmic calcium concentrations in skeletal muscle cells and subsequent muscle contractures and hypermetabolism.²

The site of the defect in MH is supposed to be the skeletal muscle ryanodine receptor, which is the calcium

release channel of skeletal muscle sarcoplasmic reticulum. To date, more than 20 point mutations in the gene encoding the human skeletal muscle ryanodine receptor (RYR1) have been identified to be tightly linked with MH susceptibility in humans.³ Linkage to the RYR1 gene has been detected in more than 50% of European MH families, but the genetic defect in MH-susceptible (MHS) patients from other families is unclear yet. Further, it is still a matter of debate whether different mutations in RYR1 have influence on presentation of MH during *in vivo* and *in vitro* conditions.⁴

Susceptibility to MH is diagnosed by *in vitro* contracture tests (IVCT), according to standardized test protocols of the European and the North American MH Groups.^{5,6} The standard IVCT are performed with halothane and caffeine. Differences in results of these IVCT among several families with MH indicated genetic influences on the variability of this test procedure.⁴ Therefore, it could be speculated that different mutations have an important impact on *in vitro* presentation of MH. However, the effect of certain mutations in the RYR1 gene on IVCT results was not investigated until now. Ryanodine, a specific and potent ligand of the ryanodine receptor, induces contracture development in skeletal muscles, and an IVCT with ryanodine for diagnosis of MH was developed in the past decade.^{7,8} The current study was designed to test the hypothesis that different mutations of the RYR1 gene modulate the effects of halothane, caffeine, and ryanodine in the IVCT.

Materials and Methods

After approval by the local ethics committee, written informed consent for the performed investigations was obtained from the patients or their parents, as appropriate. Three hundred twenty-two patients, aged 4-71 yr, with clinical suspicion of MH were examined in this prospective study. Before the following investigations, each patient was scored according to the Clinical Grading Scale (CGS).⁹

Adult muscle bundles were excised during regional anesthesia (3-in-1 nerve block) with 40 ml of prilocaine 1%, supplemented by sedation with midazolam, if required. Biopsies in children were obtained during trigger-free general anesthesia using propofol, alfentanil, and nitrous oxide in oxygen. Muscle bundles were excised carefully from the vastus lateralis and immediately

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placed in Krebs-Ringer's solution (constituents [mM]: NaCl, 118.1; KCl, 3.4; CaCl₂, 2.5; MgSO₄, 0.8; KH₂PO₄, 1.2; NaHCO₃, 25.0; glucose, 11.1) equilibrated with carbogen (95% oxygen, 5% carbon dioxide).

All *in vitro* investigations were performed within 5 h maximum after muscle biopsy. Muscle bundles for IVCT were split into 6–10 specimens (length, 12–25 mm; width, 2–3 mm; weight, 85–210 mg). Only viable muscle specimens with a twitch response of 10 mN or greater to supramaximal stimulation were used for IVCT. Each muscle sample was secured with silk sutures to a fixed point and connected with a force displacement transducer (Lectromed, Welwyn Garden City, UK). The specimens were suspended in a 20-ml tissue bath perfused with Krebs-Ringer's solution bubbled with carbogen continuously. Temperature was constant at 37°C, and pH was 7.4. The muscles were stimulated electrically with square waves to achieve a supramaximal response by an HSE Stimulator type 215/I (Hugo Sachs Elektronik, March, Germany) with a duration of 1 ms and a frequency of 0.2 Hz. Contracture curves were displayed on a Linseis L2200 II (Selb, Germany) and recorded with a computer-based data evaluation program (MusCo, RS BioMedTech, Sinzing, Germany). The resting length of the specimens was measured before testing, and the initial baseline tension before testing was achieved by stretching the samples slowly (4 mm/s) to $150 \pm 10\%$ of the resting length.

The patients were first classified by the standard IVCT according to the procedure of the European MH Group (EMHG).⁵ Halothane was added to the carbogen with a Draeger vaporizer (Luebeck, Germany), and the concentration of halothane was measured with an anesthetic gas monitor (Normac®, Datex, Helsinki, Finland) and confirmed by gas chromatography periodically. Caffeine (Sigma, Deisenhofen, Germany) was dissolved fresh before each investigation in carboxygenated Krebs-Ringer's solution and administered directly to the tissue bath. In each patient, a minimum of two muscle specimens were tested with halothane and caffeine, respectively. The MH status was defined by the halothane and caffeine thresholds as follows: MHS = muscle contractures 2 mN or greater at a halothane concentration of 0.44 mM or less *and* a caffeine concentration of 2 mM or less; MH normal (MHN) = muscle contractures less 2 mN at a halothane concentration of 0.44 mM or less *and* a caffeine concentration of 2 mM or less; MH equivocal (MHE) = muscle contractures of 2 mN or greater at a halothane concentration of 0.44 mM or less *or* a caffeine concentration of 2 mM or less.

Patients diagnosed as MHN and MHE were excluded from this investigation. If available, viable muscle specimens from MHS patients surplus to diagnostic requirements were used for the ryanodine contracture test. The conditions for this IVCT were in compliance with the procedures of the EMHG.⁵ After achieving at least 10

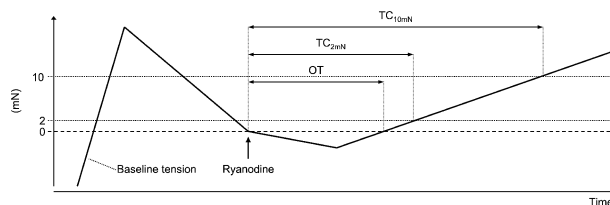


Fig. 1. Diagram of the record from a ryanodine contracture test to show determination of the three end-points evaluated in the present study. OT (min): time when contracture reached predrug level; TC_{2mN} (min): time when contracture reached 2 mN above predrug level; TC_{10mN} (min): time when contracture reached 10 mN above predrug level (Adapted from Hopkins *et al.*⁸).

min of stable baseline tension, ryanodine (high purity ryanodine, Calbiochem, La Jolla, CA) was added as a bolus directly to the tissue bath to obtain a concentration of 1 μM .^{10,11} The contracture course after ryanodine administration was defined as described previously and is presented in figure 1¹⁰: (1) onset time of contracture (OT; min); (2) time when contracture reached 2 mN above predrug level (TC_{2mN}; min); and (3) time when contracture reached 10 mN above predrug level (TC_{10mN}; min).

All patients were screened for mutations in two hot spot regions of the RYR1 gene. Constitutional DNA was obtained from leukocytes extracted from 10 ml of blood and subjected to polymerase chain reaction (PCR) as described previously,¹² with the aid of primers specific for exons 2, 6, 9, 11, 14, 17, 39, 45, and 46 of the RYR1 gene. Two methods were applied to screen for known and new mutations in these RYR1 gene exons, where mutations have been reported before, *i.e.*, single-strand conformation polymorphism and direct sequencing of several amplified products spanning the selected coding regions of the gene. The single-strand conformation polymorphism was performed in a part of this sample according to the method by Singh *et al.*¹³, except that silver staining was conducted on polyacrylamide gels to monitor the single strands instead of the incorporation of P³² and autoradiography. PCR products corresponding to aberrant bands and all of these PCR products were sequenced using an automated fluorescence system (ABI, Prism 310, Perkin-Elmer, Applied Biosystems Inc., Fremont, CA) to confirm the wild type and the sequences of the mutation sites. In a few cases, restriction digestion of the PCR products with specific enzymes was used to confirm results.

Statistical evaluation was performed using a computer-based program (StatView 4.57, Abacus Concepts, Berkeley, CA). All data are presented as median and ranges. Statistical analysis of demographic and contracture data were calculated using Kruskal-Wallis analysis of variance (ANOVA) and *post hoc* Mann-Whitney U test. Results were considered significant if *P* values were less than 0.05.

Table 1. Demographic and Clinical Characteristics of 93 MH-susceptible (MHS) Patients

Mutation	n	Number of Families	Male/Female	Age (yr)	Height (cm)	Weight (kg)	CK (U/l)	CGS
None	57	47	39/18	24 (5–59)	173 (102–191)	70 (18–106)	61 (18–2295)	15 (0–43)
C487-T	15	5	7/8	27 (6–52)	166 (117–180)	60 (23–89)	69 (23–495)	15 (5–43)
G1021-A	8	4	5/3	28 (21–57)	179 (169–185)	71 (58–91)	72 (24–406)	15 (0–15)
C1840-T	7	4	6/1	27 (7–48)	175 (114–180)	65 (20–89)	102 (25–295)	15 (15–65)
G7300-A	6	2	5/1	34 (9–47)	172 (145–188)	77 (32–85)	39 (30–175)	15 (0–18)

Values are median and ranges.
CGS = Clinical Grading Scale.⁹

Results

Three hundred twenty-two patients with clinical suspicion of MH were checked on MH susceptibility by IVCT with halothane and caffeine. Ninety-three patients were diagnosed as MHS, 44 patients as MHE, and 185 patients as MHN according to the EMHG criteria. The MHE and MHN patients were excluded from further examinations. Viable muscle specimens (twitch ≥ 10 mN) surplus to diagnostic requirements were available to perform the IVCT with ryanodine in 64 of 93 MHS patients.

Malignant hyperthermia-associated RYR1 mutations were detected in 36 of 93 MHS patients *via* RYR1 gene mutation screening. In 57 patients, none of the known mutations in two hot spot regions of the RYR1 gene were detected. These patients belonged to 47 families, and a maximum of two patients came from the same family. The C487-T mutation was found in 15 patients (five families; four - four - three - three - one patient[s] per family), G1021-A mutation in 8 patients (four families, two - two - two - two patients per family), C1840-T mutation in 7 patients (four families, two - two - two - one patient[s] per family), and G7300-A mutation in 6 patients (two families, four - two patients per family). In addition, screening for neuromuscular diseases led to the discovery of histologic signs for central core disease (CCD) in three patients with C487-T mutation. The demographic and clinical characteristics of the 93 MHS patients divided to genetic status are summarized in table 1.

Muscle specimens of all patients developed contractures to administration of halothane in concentrations of

0.44 mM or less and to caffeine in concentrations of 2 mM or less, according to the inclusion criteria. The threshold data for the IVCT with halothane and caffeine are summarized in table 2. The halothane and caffeine threshold concentrations were comparable in muscle specimens of patients with G-1021-A, C1840-T, or G7300-A mutations or without a detected mutation. In patients with C487-T mutation, the halothane threshold of 0.11 mM (0.11–0.44 mM) was significantly lower compared to patients without detected mutation with a threshold concentration of 0.44 mM (0.11–0.44 mM). The caffeine threshold concentration of 1.0 mM (0.5–2.0 mM) was lower in patients with C487-T mutation compared with thresholds of 2.0 mM (1.5–2.0 mM) or 1.5 mM (0.5–2.0 mM) in patients with G1021-A mutation or without a detected mutation.

All muscle specimens developed marked contractures after bolus administration of 1 μM ryanodine. The data describing the contracture course in the skeletal muscle specimens during the IVCT with ryanodine are summarized in table 3. The OT was comparable in all groups. A contracture level of 2 mN (TC_{2mN}) above predrug baseline tension was reached in patients with the C487-T mutation in 8.0 min (1.4–13.0 min), with the C1840-T mutation in 8.0 min (3.5–9.1 min), and with G7300-A mutation in 6.8 min (4.2–8.4 min). This was faster compared to patients with the G1021-A mutation or without detected mutation, who reached TC_{2mN} after 11.8 min (7.3–23.0 min) or 11.3 min (2.0–18.5 min), respectively. A contracture level of 10 mN (TC_{10mN}) above predrug baseline tension was also reached significantly faster in

Table 2. Thresholds of Halothane and Caffeine in Skeletal Muscle Specimens from 93 MH-susceptible (MHS) Patients

Mutation	n	Halothane [mM]	Caffeine [mM]
None	57	0.44 (0.11–0.44)	1.5 (0.5–2.0)
C487-T	15	0.11 (0.11–0.44)*	1.0 (0.5–2.0)*†
G1021-A	8	0.33 (0.11–0.44)	2.0 (1.5–2.0)
C1840-T	7	0.22 (0.11–0.44)	1.5 (0.5–2.0)
G7300-A	6	0.22 (0.22–0.44)	1.5 (1.0–2.0)

Values are median and ranges.

* P < 0.05 versus none mutation; † P < 0.05 versus G1021A mutation; ‡ to reach a contracture ≥ 2 mN.

Table 3. Contracture Course after Bolus Administration of 1 μM Ryanodine in Skeletal Muscle Specimens from 64 MH Susceptible (MHS) Patients

Mutation	n	OT (min)	TC _{2mN} (min)	TC _{10mN} (min)
None	34	5.8 (1.0–9.1)	11.3 (2.0–18.5)	16.8 (3.0–28.3)
C487-T	10	5.0 (0.6–8.6)	8.0 (1.4–13.0)*†	11.0 (3.0–20.4)*†
G1021-A	8	8.9 (2.9–20.5)	11.8 (7.3–23.0)	26.8 (18.2–38.2)
C1840-T	7	4.5 (2.1–6.7)	8.0 (3.5–9.1)*†	14.0 (5.2–17.0)*†
G7300-A	5	4.1 (2.3–6.2)	6.8 (4.2–8.4)*†	11.2 (8.6–14.5)*†

Values are median and ranges.

OT = onset time of contracture development; TC_{2mN} = time to reach a contracture level of 2 mN; TC_{10mN} = time to reach a contracture level of 10 mN; * P < 0.05 versus none mutation; † P < 0.05 versus G1021A mutation.

patients with C487-T mutation, C1840-T mutation, or G7300-A mutation compared with patients with G1021-A mutation or without detected mutation. TC_{10mN} was reached after 11.0 min (3.0–20.4 min) in patients with C487-T mutation, after 14.0 min (5.2–17.0 min) with C1840-T mutation, and after 11.2 min (8.6–14.5 min) with G7300-A mutation. The TC_{10mN} was reached after 26.8 min (18.2–38.2 min) in patients with G1021-A mutation and after 16.8 min (3.0–28.3 min) in patients without detected MH-associated mutation.

Discussion

It is widely accepted that susceptibility to MH is caused by abnormal calcium metabolism within the skeletal muscle fiber.¹ A rapid and sustained calcium release of intracellular stores results in high myoplasmic calcium concentrations during an anesthetic-induced MH crisis in skeletal muscle cells. This effect is accountable for muscle contractures and for hypermetabolism. The alterations in MHS muscle fiber Ca^{2+} homeostasis could result from a defect in any of the mechanisms regulating myoplasmic Ca^{2+} concentration and excitation-contraction coupling.²

In more than 50% of the MH families, linkage of the autosomal-dominant MHS trait to the gene encoding the human skeletal muscle RYR1 can be found. To date, more than 20 point mutations in RYR1 associated with MH susceptibility have been identified in humans.^{3,14} Four of these mutations are also associated with CCD.¹⁴ However, it is still a matter of debate whether and how these mutations in the RYR1 affect the underlying steps that trigger a fulminant MH episode and the severity of the clinical symptoms. Further, the influence of different RYR1 gene mutations on the effects of certain substances in skeletal muscles during *in vitro* conditions is unknown.

Susceptibility to MH is diagnosed by IVCT with halothane and caffeine, according to standardized test protocols of the European and the North American MH Groups.^{5,6} Urwyler *et al.*⁴ were the first to describe genetic influences on the variability of halothane and caffeine IVCT results in 1994 by comparison of different families. Persons from four MHS families were tested by halothane and caffeine IVCT, and the test results of overall 36 MHS family members were compared. Significant differences in contracture development at the halothane or the caffeine IVCT and change of twitch force during the IVCT were found between the four families. The authors concluded that genetic differences between the families could be responsible for the observed differences in the IVCT. However, the accordance of genetic markers within a family was only suggested but not proved by mutation screening in this study. Another study from Islander *et al.*¹⁵ investigated monozygote

twins with suspected MH susceptibility. A satisfactory reproducibility of IVCT results between the twins was found in this investigation. Therefore, it could be suggested that genetic factors have a distinct effect on halothane and caffeine IVCT results.

To date, only a few studies have been performed to investigate modulations of intracellular functions by MH-associated RYR1 gene mutations. For instance, the C1840-T and G7300-A mutations provide the channel increased sensitivity to calcium, increased ryanodine binding activity, and less inhibitory effect of calmodulin, rendering the calcium release channel overactive.¹⁶ In cell culture, 17 mutations corresponding to human MH mutations were made in rabbit RYR1 cDNA and transfected into HEK-293 cells.^{17,18} Ca^{2+} release in these cells was more sensitive to low concentrations of caffeine and halothane compared with wild type cells. Further, a strong correlation for caffeine and a weaker correlation for halothane were found between induction of Ca^{2+} release by these substances and halothane and caffeine IVCT results. CCD is usually closely associated with MH. After engineering different CCD mutations into rabbit RYR1 cDNA and expression in myotubes from RYR1-knockout mice, the functional effects of the CCD mutations in the cytoplasmic region of RYR1 were investigated.¹⁹ The CCD mutations enhanced RYR1 sensitivity to activation by voltage, leading to increased SR Ca^{2+} leak, SR store depletion, and a reduction in voltage-gated Ca^{2+} release. Recently, cultured myotubes from four MH patients with G7300-A mutation and three control patients were investigated to prove whether cultivated muscle cells from MHS patients can be distinguished pharmacologically from those from control subjects.²⁰ Resting intracellular calcium concentration of MH myotubes was similar to those of control subjects. However, ryanodine-induced calcium release was faster and more distinct in MHS cells compared with those in control subjects.

Ryanodine, a plant alkaloid from *Ryania speciosa*, is a specific ligand at the calcium release channel of the sarcoplasmic reticulum. Several studies have shown that an IVCT with ryanodine enables a specific evaluation of MH susceptibility,^{7,10,21–24} and in 1993 the EMHG decided to include the ryanodine IVCT into the standard MH test protocol. Results of an European multicenter evaluation of this protocol showed that the ryanodine IVCT was highly sensitive and specific compared with the halothane and caffeine IVCT and enabled the reduction of the rate of MHE results within an individual test center.⁸ However, the results between several test centers differed, although a determination of common cut-off levels for the IVCT with ryanodine for differentiation between the diagnostic groups was not possible. These observations may, on the one hand, be the result of differences in laboratory performance between the centers. On the other hand it could also be speculated that the genetic composition within the study population varied between

the test centers, leading to different results in the ryanodine IVCT.

The aim of our study was to test the hypothesis that the results of halothane, caffeine, and ryanodine IVCT depend on mutations in the RYR1 gene. By comparison of IVCT results of patients with different MH-associated mutations, a relationship between genotype and functional impairment in skeletal muscle contracture responses of MHS patients could be studied. Therefore, MHS patients with four different MH-associated mutations and patients without any detected mutation were compared by their IVCT results. Skeletal muscle specimens from patients with C487-T mutation compared with patients without detected mutation required lower concentration of halothane and caffeine to initiate contracture development. Patients with a C487-T, C1840-T, or G7300-A mutation developed faster defined contracture levels in the ryanodine IVCT compared with patients with a G1021-A mutation or without a detected mutation. IVCT results could depend on different factors like patients age, the muscle sample size, or the muscle fiber type composition, and false-positive IVCT results are likely. Further, other genetic factors than the four detected mutations may have an influence on IVCT and may disturb the results of this study. However, in this study the differences between the groups were more distinct in the IVCT with ryanodine compared with halothane and caffeine. This may be an effect of different end-points of the tests (time *vs.* threshold concentration), or it may be that the ryanodine test was found to be more specific than IVCT with halothane or caffeine.¹¹

The frequencies of the various MH-associated mutations apparently vary across Europe. The G1021-A mutation is present in approximately 6% of Irish, English, and French families²⁵ but is rare in Northern Europe.²⁶ The C1840-T mutation is with 9% more common in German families,²⁶ whereas the G6502-A mutation is common in Swiss families but is relatively rare elsewhere.^{25,26} Regional variability of MH-associated mutations and mutation-dependency of ryanodine IVCT results may explain several discrepancies in MH, *e.g.*, contracture test results, rate of MHE diagnoses, and so on.

The clinical syndrome of MH is extremely variable.¹ Only a few patients develop a fulminant form of MH with an acute and rapid, life-threatening course of symptoms. The majority of patients with a disposition to MH only develop mild or abortive forms. The reasons for the different courses are still not fully understood. Connections with the patients' age,²⁷ specific anesthetic agents (*e.g.*, different volatile anesthetics or succinylcholine),²⁸ and promoting factors such as increased sympathetic tonus were described or suspected.²⁹ However, a genetic influence on clinical course of MH has been postulated. The current study demonstrated a genotype-related *in vitro* effect, which may be another hint for an analogous *in vivo* phenomenon.

Even during *in vitro* conditions various discrepancies in MH require clarification. Center-to-center differences in halothane and caffeine contracture test results were found in nearly every multicenter study of recent years.^{5,8,30} One of the markers for these differences is the rate of MHE results within one laboratory, which varies between 5% and 20% of all tested patients. This variety may be a problem of deficient standardization of certain variables of the IVCT, *e.g.*, type of muscle, tissue bath temperature, viability criterion, and technical equipment.^{8,31} Further, one of the results of the European multicenter evaluation of the IVCT with ryanodine was a good intracenter but an insufficient intercenter comparability of ryanodine IVCT results.⁸ Regional variability of MH-associated mutations and mutation dependence of halothane, caffeine, and ryanodine IVCT results may be an explanation for this observation as well.

The results of the present study provide evidence that different MH-associated mutations in the RYR1 gene had a distinct effect on ryanodine-induced calcium release on a cellular level. Therefore, development of tests for MH susceptibility based on cell culture should be confirmed with different MH-associated mutations.^{20,32} This study was carried out in one center with a few investigated patients. Confirmation of these data in a multicenter study and with more patients will harden the results. Thus, a correlation between clinical presentation of fulminant MH crisis and different RYR1 gene mutations may also be possible.

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