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Report

Single-Amino-Acid Deletion in the *RYR1* Gene, Associated with Malignant Hyperthermia Susceptibility and Unusual Contraction Phenotype

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Malignant hyperthermia (MH) is an anesthetic-drug–induced, life-threatening hypermetabolic syndrome caused by abnormal calcium regulation in skeletal muscle. Often inherited as an autosomal dominant trait, MH has linkage to 30 different mutations in the *RYR1* gene, which encodes a calcium-release–channel protein found in the sarcoplasmic reticulum membrane in skeletal muscle. All published *RYR1* mutations exclusively represent single-nucleotide changes. The present report documents, in exon 44 of *RYR1* in two unrelated, MH-susceptible families, a 3-bp deletion that results in deletion of a conserved glutamic acid at position 2347. This is the first deletion, in *RYR1,* found to be associated with MH susceptibility. MH susceptibility was confirmed among some family members by in vitro diagnostic pharmacological contracture testing of biopsied skeletal muscle. Although a single-amino-acid deletion appears to be a subtle change in the protein, the deletion of Glu2347 from *RYR1* produces an unusually large electrically evoked contraction tension in MH-positive individuals, suggesting that this deletion produces an alteration in skeletal-muscle calcium regulation, even in the absence of pharmacological agents.

Malignant hyperthermia (MH [MIM 145600]) is often inherited as an autosomal dominant pharmacogenetic trait that predisposes individuals to a life-threatening anesthetic-drug–induced hypermetabolic syndrome (Nelson and Flewellen 1983). A family's predisposition to MH is identified by a clinical episode of MH in the proband, and other family members can be screened by in vitro pharmacological contracture testing of biopsied skeletal muscle (Larach 1989). The pathobiology of MH is an abnormal increase in myoplasmic [Ca^{++}], caused by volatile anesthetics and by depolarizing muscle relaxants. Unabated, this rise in [Ca^{++}] stimulates metabolic and contracture events that ultimately create acid/base and electrolyte disturbances, causing cell destruction and death. The occurrence of MH in species other than humans has provided investigational animal models that have advanced our knowledge about this disease. Missense mutations in the gene encoding the skeletal-muscle ryanodine receptor, RYR1 (MIM 180901), have been linked to MH in the animal models for the pig (Gly614Arg [Fujii et al. 1991]) and for the dog (Val547Ala [Roberts et al., in press]) (also see GenBank). The search for similar mutations in MH-susceptible human families has presently led to the discovery of 30 *RYR1* missense mutations associated with MH in ~50% of affected families (Brown et al. 2000; Chamley et al. 2000; Jurkat-Rott et al. 2000; McCarty et al. 2000; Monnier et al. 2000; Sambuughin et al., in press-*a*, in press-*b*). All *RYR1* mutations associated with MH are missense mutations, and we now report the first deletion of a single amino acid, in *RYR1,* cosegregating with MH, as well as its expression in two unrelated MH-affected families.

The North American MH diagnostic protocol (Larach 1989; Allen et al. 1998) was utilized for the in vitro skeletal-muscle–contracture tests. Subjects referred to our MH diagnostic center are administered MH-nontrigger anesthesia, and fascicles of the vastus lateralis are taken and mounted in vitro in muscle-contracture chambers for testing. A different set of three fascicles are used for each of two tests. The first test exposes the fascicles to 3% halothane, and the second test exposes the muscle to increasing concentrations of caffeine, starting at a concentration of 0.5 mM and ending at a concentration

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Table 1

Skeletal-Muscle Phenotypes among Subjects with Deletion in *RYR1*

Subject	Halothane, 3% Contracture (g)	Caffeine, 2-mM Contracture (g)	Electrically Evoked Twitch (g)
Family 1:			
Individual 3	.7	.07	12.8
Individual 4	5.8	1.69	24.6
Family 2:			
Individual 3	2.5	.52	29.1
Individual 4	3.9	2.2	19.2

NOTE.—Tension value represents the maximum response among three fascicles tested, for each variable.

of 32 mM. A contracture response of ⩾0.7 g to 3% halothane or ⩾0.3 g at caffeine concentrations of ⩽2 mM, in one or more fascicles, is sufficient for an MH-positive diagnosis.

Family 1 is represented by a proband who developed life-threatening signs of MH when, at 9 years of age, she was anesthetized for a tonsillectomy. Anesthesia was induced with thiopental sodium and was maintained with halothane. On injection of succinylcholine (20 mg iv) to facilitate intubation of the trachea, the patient developed global skeletal-muscle rigidity, and the mouth could not be opened. The body temperature rose from 37°C to 40.3°C over a period of 20 min. No data regarding blood gases, end-tidal carbon dioxide, or other metabolic indicators were available. The proband was administered fluids, recovered without sequelae, and received a clinical diagnosis of MH positive. In 1980, the proband underwent a muscle biopsy to test for calcium uptake and for actomyosin ATPase activity in thin section (Allen et al. 1986). These tests for MH were subsequently discontinued because of reliability problems, but in this proband the results were reported as "unquestionably positive." Two of the proband's three brothers underwent muscle biopsies to test for MH-diagnostic contracture, and both had unequivocally MH-positive contracture responses to caffeine and to halothane (table 1). Part of the in vitro MH muscle-contracture protocol involves optimizing the length of muscle fascicles to obtain maximum electrically evoked contraction tension. We observed unusually large contraction tensions in the muscle fascicles of these two individuals (table 1).

Family 2 is represented by a proband who developed signs of MH when, at 15 years of age, she was anesthetized for maxillary-mandibular advancement–augmentation genioplasty. Anesthesia was induced with propofol (140 mg) and fentanyl (3.5 μg), and nasotracheal intubation was accomplished after topical application of lidocaine; anesthesia was maintained with isoflurane. During the first 60 min after induction of anesthesia, no signs of MH were evident. For the period of 60–150 min after induction of anesthesia, elevations in end-tidal carbon dioxide (70 mmHg maximum) and in body temperature (38.9°C maximum) were associated with an arterial-blood acidosis (pH 7.22, pCO_2 53, and pO_2 414) and base deficit (−5.4). A clinical diagnosis of MH was made, and an MH treatment protocol, including dantrolene administration, was instituted. The signs of MH reversed during the next 40 min, and the proband survived without sequelae. The proband and her sister subsequently underwent muscle biopsies, and each had unequivocally MH-positive in vitro contracture responses to caffeine and to halothane (table 1). Abnormally large electrically evoked in vitro contraction tensions were also observed in muscle of the proband and of her sister (table 1).

Genetic screening was performed on exons 39–46 of *RYR1,* in both probands, since >50% of MH-associated *RYR1* mutations occur within this region. When the SSCP screening method was used, a unique but similar pattern of single-strand DNA was detected in the probands, and this occurred within a PCR product from exon 44. PCR was performed with primers RYR44F (5′-

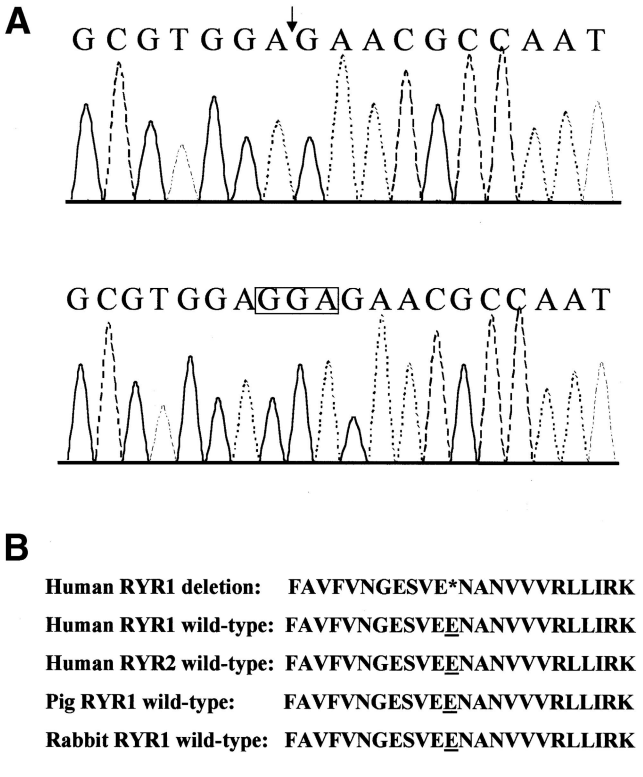

Figure 1 *A,* Sequence analysis of cloned PCR product, revealing a 3-bp deletion (GGA). The upper chromatogram shows a sequence of DNA, from the proband in family 1, with the deletion. The arrow indicates the site of the deletion. The lower chromatogram shows a sequence of DNA, from individual 1 in family 2, without the deletion. *B,* Amino acid comparison of *RYR1* region flanking the deleted residue. The asterisk (*) indicates glutamic acid (E) deleted at 2347.

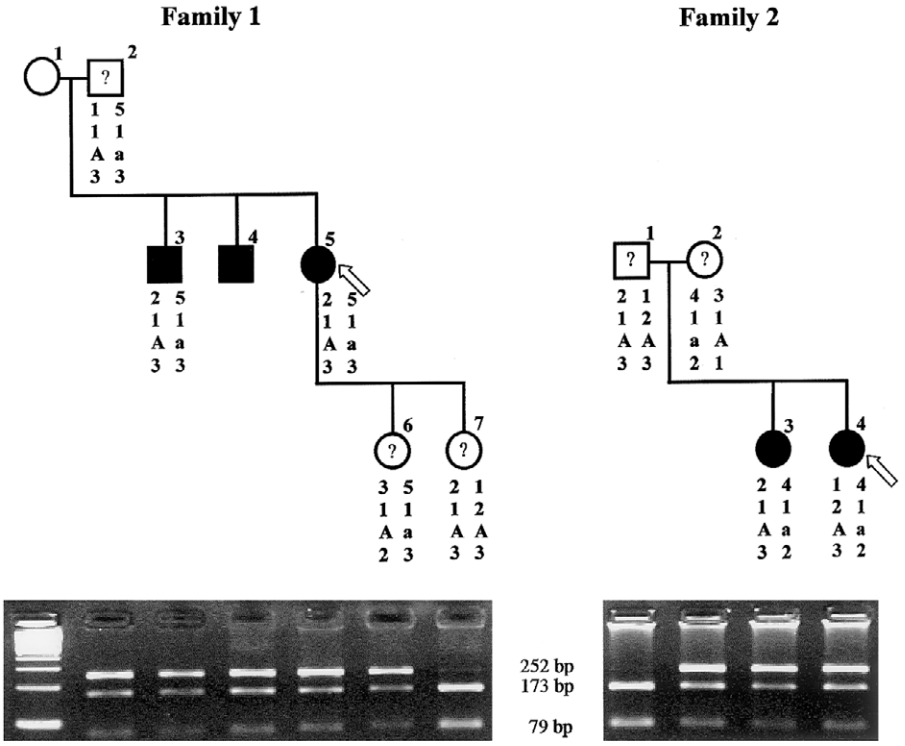

Figure 2 Pedigrees of two families with MH-positive-plus phenotype. Blackened circles and squares denote patients who had either a clinical episode of MH or positive muscle-biopsy contracture-test results; the unblackened empty circle denotes a family member who was not studied; circles and square with a question mark denote family members studied genetically but in whom contracture testing was not performed; arrows indicate the probands. Haplotypes were constructed on the basis of the markers, with the following order: *D20S220* (*top*), an intragenic *Taq*I polymorphism in *RYR1* exon 20, presence (a) or absence (A) of the deletion, and *D20S47*. Segregation of the deletion was analyzed by *Bse*RI digestion of a 252-bp PCR product of exon 44, as shown on the agarose gel below the pedigrees. The deletion was detected by loss of a *Bse*RI site, resulting in the generation of 252-bp, 173-bp, and 79-bp DNA fragments. The first lane on the left represents a 100-bp marker ladder.

gggaggtctctgatggtg-3′) and RYR44R (5′-cgggagactcactg-ctcg-3′), by use of MasterAmp 2X PCR Premix D (Epicentre Technologies), for 1 cycle of 94°C for 3 min and 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s (for *RYR1* exon 44 sequence, see GenBank). Direct sequencing of PCR product from the probands was performed by use of a QIAquick PCR purification kit (Qiagen) and a BigDye Terminator Cycle Sequencing Kit (ABI). The sequence alterations in exon 44 were detected, in both probands, as overlapping nucleotides, which indicate either a deletion or an insertion mutation type. To ensure the nature and the location of the sequence alteration, we cloned PCR product, using a TOPO TA cloning kit (Invitrogen), and sequenced 10 clones for each proband. As shown in figure 1*A,* sequencing of cloned PCR product revealed a deletion of GGA nucleotides in exon 44. Surprisingly, the type and the location of the deletion were the same in all MH-positive members of both families (fig. 1*A*). This deletion leads to an in-frame loss, at position 2347, of glutamic acid, an amino acid that is conserved in *RYR1* across different species (fig. 1*B*). The deletion eliminates a restriction site for the enzyme *Bse*RI, which allows for detection by restriction-enzyme analysis. DNA obtained from the members of both families was screened for the deletion, and figure 2 shows the results for the two pedigrees. All four members of the two families who were diagnosed as MH-positive by in vitro contracture test each had a deletion (fig. 2).

Since a deletion has never been reported in *RYR1* and since both probands were referred to the same MH diagnostic center, we investigated the possibility that these two families are related, and we genotyped some members of these two families by using additional genetic markers—*Taq*I polymorphism in exon 20 of *RYR1,* *D20S220,* and *D20S47* (fig. 2). Relative order and genetic distance (in cM) between markers within *RYR1* is *D20S220*-1-*RYR1*-0-*D20S47* (Center for Medical Genetics, Marshfield Medical Research Foundation). Although an intragenic marker in *RYR1* was not informative, two highly polymorphic microsatellite markers tightly linked to *RYR1* show cosegregation of different alleles with GGA/*RYR1* deletion, in each family. Thus, each family has a unique haplotype associated with an

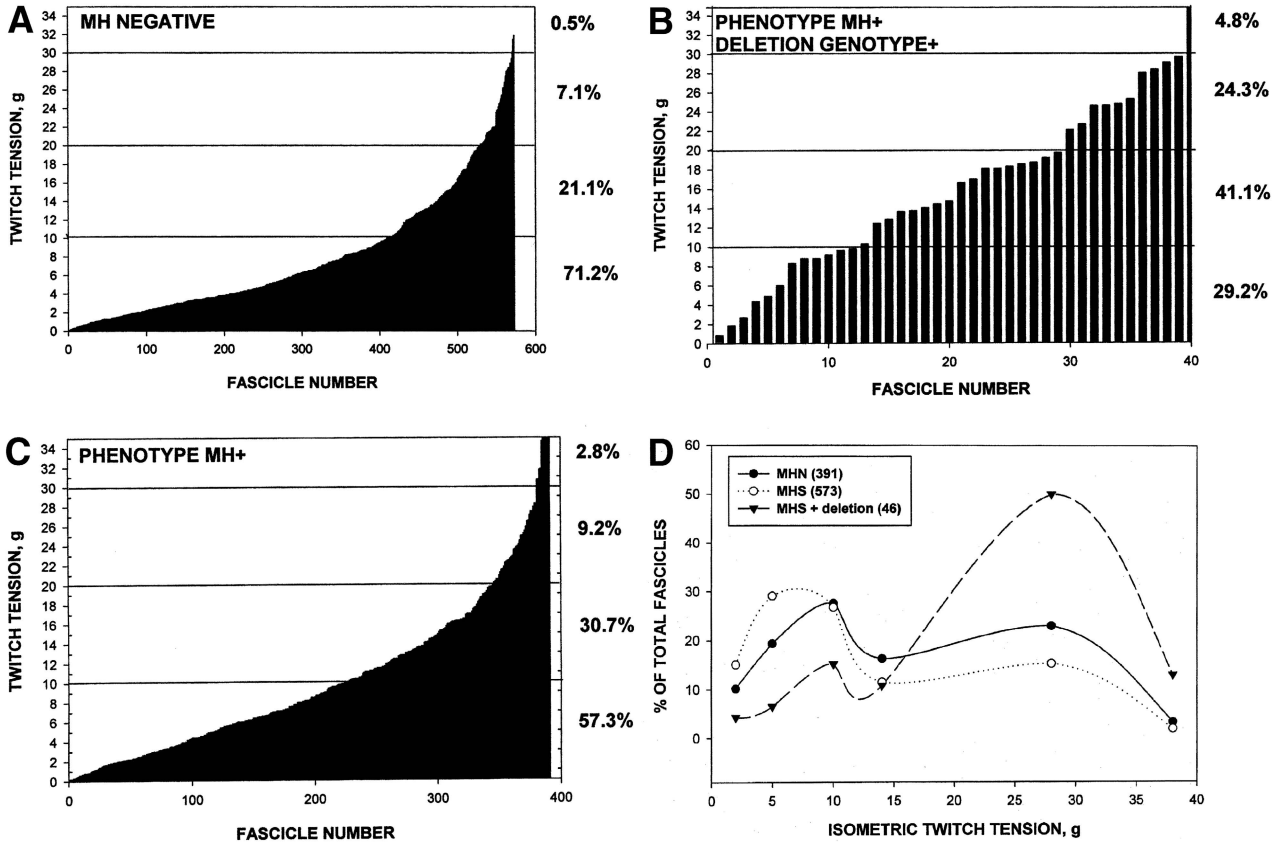

Figure 3 *A–C,* Distribution of in vitro electrically evoked contractions in skeletal muscle from groups that are MH negative (*A*), MH positive plus (*B*), and MH positive with and/or without deletion (*C*). Individual fascicle responses were graphed in order of increasing contraction tension (in g force). Not all individuals in MH-positive groups have been screened for deletion, which, therefore, may or may not be present. To the right of the graphs, the percentages of total fascicles in given ranges of tension level are denoted. *D,* Distribution of isometric-twitch force in fascicles 391, 573, and 46, forming, respectively, MH-negative (*solid curve/filled circles*), MH-positive (*dotted curve/open circles*), and MH-positive-plus (*dashed curve/triangles*) phenotypes in *RYR1*. Force ranges are 0–2 g, 2–5 g, 5–10 g, 10–14 g, 14–28 g, and 28–38 g. Each group has a bimodal distribution of twitch forces, with most MH-negative and MH-positive twitch forces occurring in the range of 2–14 g and with the MH-positive-plus twitch forces showing a higher percentage of forces in the range of 14–28 g.

MH-positive phenotype and a GGA/*RYR1* deletion, which shows that the two families are unrelated. Among 76 contracture-phenotyped unrelated MH-susceptible individuals, 4 members of two families were found to have the 2347 deletion, accounting for a 2.6% incidence. The presence of the 2347 deletion in two unrelated MH-affected families increases the likelihood that this deletion is a significant cause of MH. In addition, this deletion was not found among 148 normal chromosomes.

The phenotypic hallmark of all MH-positive members of both families who have the 2347 deletion was unusually large contraction tensions in the muscle fascicles. We compared twitch-tension–value data between three groups of individuals from our diagnostic center: MH negative, MH positive with and/or without deletion, and MH positive with deletion (MH positive plus). Not all individuals in the group with the MH-positive phenotype have undergone complete genetic screening and, therefore, may or may not have the deletion. Analyses of the data show that contraction tension differs ($P < .001$) among groups, with means ± SD of 8.0 ± 6.7 g ($n = 573$), 10.3 ± 7.9 g ($n = 391$), and 16.5 ± 9.0 g ($n = 41$) among fascicles from subjects with an MH-negative, MH-positive, and MH-positive-plus phenotype, respectively. The distributions of electrically evoked muscle-contraction tensions among these groups illustrate these differences (fig. 3).

The RYR1 calcium-release channel is the main pathway by which Ca^{++} is released from the sarcoplasmic reticulum (SR) membrane to initiate contraction in skeletal muscle. The reverse of this is muscle relaxation, via both inactivation (closure) of the channel and ATP-dependent pumping of calcium back into the SR. *RYR1* mutations with linkage to MH are thought to cause an abnormal opening of the calcium-release channel when it is exposed to certain anesthetic drugs (Tong et al. 1997). With the exception of patients with *RYR1* mutations associated with central core disease (MIM

117000), the majority of patients with MH show no clinical manifestations of the mutation until the muscle is exposed to anesthetic drugs. Studies on the contraction kinetics of isolated human-MH skeletal muscle have not revealed any abnormalities, in the absence of drugs (Etchrivi 1998). It may be that the MH phenotype is expressed only in the presence of drugs. The glutamic acid deletion at position 2347 in *RYR1* may have produced an *RYR1* defect that is detected in the absence of drugs. All four individuals with the 2347 deletion had in vitro electrically evoked muscle contractions that were unusually large and that were significantly different from contraction tensions in muscle from MH-susceptible and MH-insusceptible individuals. This finding may be a hallmark of an MH muscle response, in the absence of drug effects, that distinguishes the individual's susceptibility to MH.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Center for Medical Genetics, Marshfield Medical Research Foundation, http://research.marshfieldclinic.org/genetics/ (for chromosome 19 markers)

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for human skeletal-muscle *RYR1* exon 44 sequence [accession number U48477], RYR1 sequence [accession number J05200], cardiac-muscle RYR2 sequence [accession number X98330], dog skeletal-muscle RYR1 sequence [accession numbers AF302182 and AF302129], and pig skeletal-muscle RYR1 sequence [accession number M91452])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for MH [MIM 145600], human RYR1 [MIM 180901], and central core disease [MIM 117000])

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