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Identification of Heterozygous and Homozygous Individuals with the Novel RYR1 Mutation Cys35Arg in a Large Kindred

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Background: Malignant hyperthermia (MH) is a potentially fatal, often autosomal dominant, disorder of skeletal muscle and is triggered in susceptible people by all commonly used inhalational anesthetics. In this article, the authors describe a malignant hyperthermia susceptible (MHS) kindred in which both parents of the proband are MHS and are first-degree cousins. Haplotype analysis in this kindred with chromosome 19 linked markers revealed that the proband and another sibling were homozygous for the affected RYR1 allele.

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Methods: Eighteen members of this large pedigree were investigated, with a clinical examination for signs of a myopathy, a caffeine halothane contracture test, a histo-enzymologic study on the muscle biopsies, and linkage analysis on genomic DNA isolated from family blood samples. RYR1 cDNA was amplified by polymerase chain reaction and was cloned and sequenced, facilitating mutation detection.

Results: Linkage analysis demonstrated linkage between RYR1-linked markers and MH susceptibility in this family. DNA sequencing identified a T to C transition at nucleotide position 103, resulting in the substitution of an arginine for cysteine 35, representing the most N-terminal mutation reported to date in the RYR1 gene. This mutation segregates fully with the MHS trait, generating a lod score of 4.65 in favor of linkage to MHS at a recombination frequency of 0.0.

Conclusions: The proband in this kindred is the first reported homozygote to have presented with an MH episode. The homozygotes in this pedigree do not have an overt myopathy. The sensitivity of muscle samples to caffeine clearly distinguished the two homozygotes from other heterozygous-susceptible individuals. No clear differentiation was observed with the halothane contracture results. (Key words: Genetic factors: mutations. Hyperthermia: malignant. Genotype. Homozygotes.)

MALIGNANT hyperthermia (MH) is a potentially fatal disorder triggered in susceptible patients by the administration of halogenated inhalational anesthetics and depolarizing muscle relaxants during anesthesia.¹ Signs include skeletal muscle rigidity, metabolic acidosis, hyperkalemia, cardiac dysrhythmia, and hyperthermia.¹ The frequency of MH has been reported, in adults, to be approximately 1 per 50,000-100,000 anesthetics, and, in children, to be 1 per 15,000 anesthetics.² Malignant hyperthermia susceptibility (MHS) is determined by the caffeine-halothane contracture test (CHCT) on strips of skeletal muscle biopsies. Contractures from MHS individuals display an increased sensitivity to halothane and caffeine when compared with healthy muscle.

From the study of the porcine equivalent of MH, porcine stress syndrome, the underlying biochemical defect appears to be the unregulated release of calcium ions from the sarcoplasmic reticulum into the myoplasm of skeletal muscle cells.³ Initial genetic studies mapped a locus for MHS to chromosome 19q12-13.2

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in human families.⁴ The gene for the skeletal muscle sarcoplasmic reticulum calcium release channel, RYR1 (ryanodine receptor), was mapped to this region and was proposed as a candidate gene.⁵ The RYR1 mutation Arg614Cys has previously been found to be causative in all cases of porcine MH, and has been detected in 5–10% of all human MHS families.^{6,7} Screening other MH families led to the identification of seven other RYR1 mutations that cosegregate with MH susceptibility: Arg163Cys, Gly248Arg, Gly341Arg, Ile403Met, Tyr522Ser, Gly2433Arg, and Arg2434His.^{8–14}

Here, we describe a novel mutation Cys35Arg in the RYR1 gene in a consanguineous MHS kindred in which two of the MHS individuals are homozygotes.

Materials and Methods

MHS Diagnosis

The proband, individual IV:6, who is of Sicilian origin, suffered a suspected MH episode in 1979 when he was 5 yr old while undergoing a general anesthetic for a diagnostic bronchoscopy. After induction of general anesthesia with halothane, succinylcholine was administered for muscle relaxation. The patient immediately experienced severe masseter spasm, rendering tracheal intubation impossible. Tachycardia (150 beats/min) rapidly developed, along with tachypnea and generalized rigidity. The volatile anesthetic was discontinued, and the lungs were hyperventilated with 100% oxygen. Although dantrolene was not readily available at that time, he survived the episode and made an uneventful recovery. Postoperative creatine kinase (CK) was increased (3,253 IU/L). At the age of 16 yr old, he was referred to the Lille MH diagnostic center to evaluate MH susceptibility by CHCT. No indication of muscle disease was found on clinical examination, and his resting CK level was normal. There was no history of muscular disease or unexpected anesthetic reactions in the family. No patient complained of muscle faintness or muscle cramps. Eighteen patients from this pedigree were investigated by physical examination, CHCT (the diagnosis of MH susceptibility was based on skeletal muscle contractures by the CHCT performed according to the European malignant hyperpyrexia group protocol),¹⁵ histo-enzymologic study, and DNA analysis.

Linkage Analysis Studies

Genomic DNA was isolated from blood preserved in edetic acid with informed consent from patients. Link-

age analysis was performed using the Linkage 5.2 program.¹⁶ Members of the pedigree were genotyped using chromosome 19 microsatellite markers flanking the RYR1 locus (D19S191, D19S220, D19S190, and D19S223).

Examination for DNA Polymorphisms Using Single Stranded Conformational Polymorphism and Direct DNA Sequencing of Polymerase Chain Reaction Products and Conditions for the Polymerase Chain Reaction

Following RNA extraction and cDNA synthesis from a muscle sample obtained from the proband, the entire RYR1 cDNA was amplified by polymerase chain reaction in short overlapping segments for single stranded conformational polymorphism analysis,¹⁷ but we were unsuccessful in identifying a RYR1 mutation in the proband.

We then investigated the proband's RYR1 cDNA, sequencing cloned RYR1 polymerase chain reaction fragments. Initially, we chose to clone and sequence the first 2,000 base pairs (bp) of the RYR1 cDNA, because the majority of previously described mutations are located in this region.¹² Total RNA was extracted from a 100-mg muscle biopsy sample obtained from individual IV:6 by guanidinium thiocyanate extraction.¹⁸ First-strand cDNA synthesis was carried out using 200 ng random hexamers, 1 µg total RNA, and 15 units of avian myeloblastosis virus reverse transcriptase in a final volume of 20 µl. Polymerase chain reaction primers were designed using the published human RYR1 cDNA sequence.¹⁹ Overlapping 500 bp products were amplified by polymerase chain reaction using the first strand cDNA as a template. Ligation into the TA cloning vector pCRII (In Vitrogen Corporations, San Diego, CA) was carried out according to the manufacturer's instructions.²⁰ Recombinant plasmids were sequenced using the T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden).

To amplify the region bearing the mutation from genomic DNA, the cDNA sequence of the first 500 bp was determined by standard dideoxy sequencing methods and revealed the presence of a substitution of C for T at nucleotide 103 in 4 independent clones (fig. 1). This transition mutation results in the replacement of a cysteine with an arginine at codon position 35. We amplified the smallest region possible to avoid inclusion of unknown intronic DNA. Polymerase chain reaction primers 78F (5'-CGTGCTCAAGGAGCAGCTCAAGCT-3') and 105R (5'-CCGAAGCCCTCGGCGACCAGG-3')

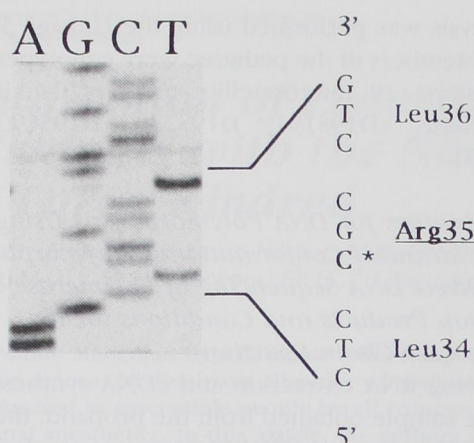


Fig. 1. Nucleotide and deduced amino acid sequence of the RYR1 cDNA from individual IV:6. The T103C mutation (indicated by the asterisk) changes the cysteine 35 codon TGC to an arginine codon CGC.

were designed to amplify the mutation site and a flanking base on either side. Polymerase chain reaction amplification of genomic DNA and cDNA yielded the expected 48 bp product, demonstrating that no intron existed in the region amplified using the primers selected.

Amplification of the region flanking the T103C mutation was achieved using primers 78F and 105R and resulted in a 48 bp amplicon. To abolish a common Acil site, eight bases 3' to the mutation Acil site created by the T103C exchange, a guanine at position 16 of the 105R primer was replaced with an adenosine (underlined in primer sequence). Amplification was carried out using a hot start of 95°C for 5 min, followed by 32 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. Cycling was followed by a final extension of 72°C for 10 min.

Mutation Detection

The presence of the T103C transition creates a new Acil site in the amplified product, which results after digestion, in two fragments, of 25 bp and 23 bp. Amplified DNA products in which the mutation is absent are not cleaved by Acil. Products amplified from patients heterozygous for the mutation should yield fragments of 48 bp, 25 bp and 23 bp, whereas products from patients homozygous for the mutation should yield fragments of 25 bp and 23 bp (fig. 2). Digestion products were electrophoresed through 18% polyacrylamide gels, stained with ethidium bromide, and visualized by ultraviolet irradiation. This restriction enzyme assay was

used to test for segregation of the mutation in the MHS family, and to test for the presence of the mutation in unrelated affected and healthy individuals.

Results

The CHCT and serum CK values of the family members are shown in table 1. The MHS pedigree is shown in figure 3 and is unusual in that the parents, III:7 and III:8, both identified as MHS, are first-degree cousins. No specific myopathic signs were found on histo-enzymologic examination. The CHCT values are presented under four headings; the tension generated in each muscle strip at 2% halothane and 2 mM caffeine, respectively, and the concentration of halothane and caffeine required to generate a threshold tension of 0.2 g. The biopsies from the two homozygote members were the most sensitive of all samples to caffeine. Both had the lowest threshold concentration of caffeine (0.5 mM) and had the strongest contractures at a 2 mM concentration. The two homozygotes and three heterozygotes reached the threshold tension at the lowest amount of

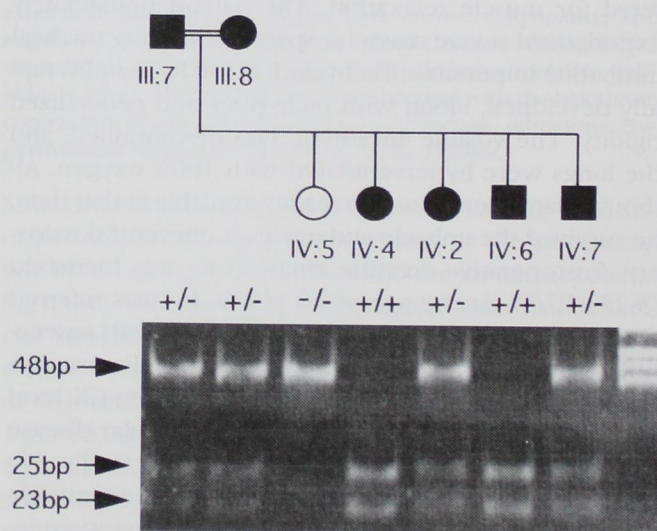


Fig. 2. Detection of the T103C mutation by Acil restriction endonuclease digestion. The 48 base pair (bp) polymerase chain reaction fragment amplified by primer pair 78F and 105R is cleaved into two fragments of 25 bp and 23 bp if the mutation is present. Fragments amplified from samples homozygous for the mutation are completely cleaved by Acil digestion. Heterozygous samples yield the two digestion products as well as the 48 bp polymerase chain reaction product. Amplification products from normal samples are not cleaved by Acil and appear as a single band of 48 bp. Squares = males; circles = females; ■ = MHS; □ = MHN; +/+ = homozygous for mutation; +/- = heterozygous for mutation; -/- = normal.

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Table 1. Results of *In Vitro* Caffeine-Halothane Contracture Test

Individual*	Age (yr)	Serum CK (IU/L) (n < 120)	Threshold Concentration†		Maximal Contracture‡		Status§
			Halothane (vol %)	Caffeine (mM)	2% Halothane	2 mM Caffeine	
III7	43	51	2.0	2.0	1.05	0.3	MHS
III8	45	121	1.0	1.5	1.1	0.45	MHS
III9	39	51	>3.0	4.0	0.0	0.0	MHN
III10	46	95	0.5	2.0	1.6	0.25	MHS
III12	40	75	2.0	>3.0	0.2	0.0	MHEh
IV2	25	53	2.0	2.0	0.85	0.6	MHS
IV4¶	24	55	0.5	0.5	4.5	3.0	MHS
IV5	22	44	>3.0	3.0	0.05	0.0	MHN
IV6¶**	16	54	0.5	0.5	1.6	1.2	MHS
IV7	14	109	2.0	2.0	0.35	0.2	MHS
IV10	25	50	0.5	2.0	1.1	0.45	MHS
IV11	22	47	0.5	1.5	2.3	0.65	MHS
IV12	20	43	2.0	2.0	0.65	0.2	MHS
IV13	14	32	1.0	1.0	0.2	0.2	MHS
IV14	10	53	>2.0	4.0	0.1	0.0	MHN
IV15	17	44	>3.0	4.0	0.1	0.0	MHN
IV16	16	78	>3.0	8.0	0.01	0.0	MHN
IV19	44	85	2.0	2.0	0.4	0.2	MHS

MHN = halothane threshold > 2.0% and caffeine concentration > 2.0 mM; MHEh = only halothane threshold lowered; MHS = malignant hyperthermia susceptible.

* Proband and members of the family at risk of MH were tested according to the European Malignant Hyperthermia Group protocol.

† At which a contracture > 0.2 g was obtained.

‡ Maximal force g contracture at 2.0% halothane or 2.0 mM caffeine.

§ MHS halothane threshold ≤ 2.0 vol% and caffeine threshold ≤ 2.0 mM.

¶ Homozygote.

** Proband.

halothane used (0.5% v/v). Homozygote IV:4 achieved the highest muscle tension at 2% halothane volume. However the next highest tension of 2.3 g was achieved by the heterozygote IV:11. Homozygote IV:6 had the third highest contracture at 2% halothane. Therefore, the two homozygotes could only be differentiated from other susceptible heterozygous family members by caffeine sensitivity.

Linkage analysis generated a maximal pairwise lod score of 3.7 and 3.26 in favor of linkage to MHS for the D19S220 and D19S223 markers, respectively, at a recombination fraction of 0.00. All affected individuals share a common haplotype; 13-8-2-9 for the D19S191, D19S220, D19S190, and D19S223 markers, respectively (fig. 3). This result indicates that the RYR1 gene is linked to MH in the MHS individuals in this pedigree. Haplotype analysis indicates that the proband, IV:6, and his sibling, IV:4, were homozygous for the mutant allele, having inherited the affected paternal and maternal haplotypes (fig. 3).

A search for the eight known MH mutations was negative. DNA sequencing identified a T to C transition at nucleotide position 103, resulting in the substitution of an arginine for cysteine 35, representing the most N-terminal MHS mutation reported to date in the RYR1 gene (fig. 1).

Mutation analysis in the pedigree shows that the mutant allele is present in the 13-8-2-9 haplotype and segregates fully with the MHS phenotype, generating a lod score of 4.65 in favor of linkage. The amplified products are completely cleaved to 25 bp and 23 bp fragments in the proband IV:6 and his sibling IV:4, confirming that these two individuals are homozygous for the Cys35Arg mutation. The consanguineous parents III:7 and III:8 are heterozygous for the mutation (fig. 2). The mutation was not detected in 200 normal chromosomes, or in 65 unrelated MHS samples.

Discussion

Linkage analysis has identified two members of a MHS pedigree who have inherited identical affected RYR1

Family name : MARINO

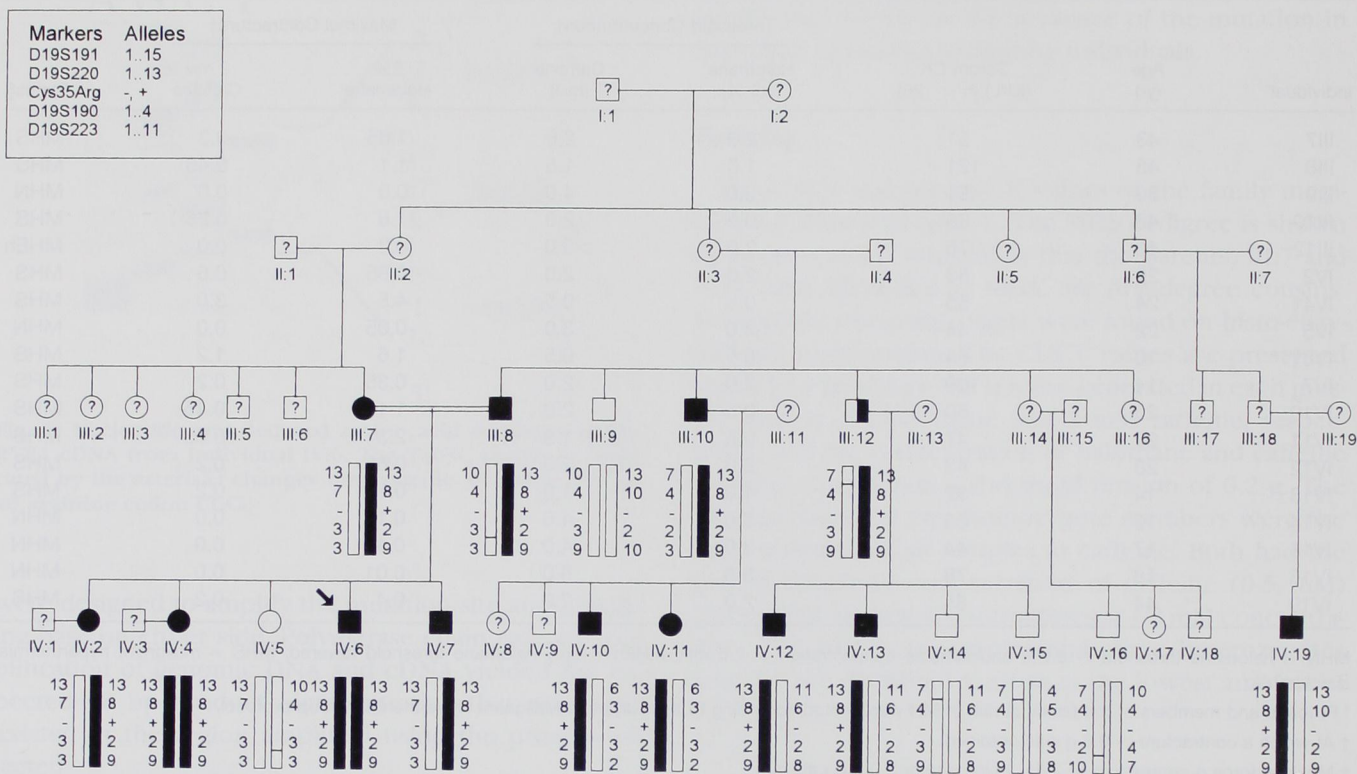


Fig. 3. Family pedigree showing the malignant hyperthermia status of the tested individuals, the constructed haplotypes with the tested markers, and the presence of the Cys35Arg mutation. Arrow denotes the proband; circles = females; squares = males; ■ = CHCT positive (MHS); □ = CHCT negative (MHN); ft = CHCT equivocal (MHEh); ? = not tested with CHCT. The results of typing with the four markers for the loci D19S191, D19S220, D19S190, D19S223, and segregation of MH status with the Cys35Arg mutation are shown.

haplotypes, indicating that these individuals are homozygous for a RYR1 mutation. RYR1 cDNA of a known homozygote was cloned and partially sequenced, resulting in the detection of a novel T103C transition, resulting in the substitution of cysteine 35 for an arginine. This is the most N terminal mutation detected, to date. The mutation was not present in 200 normal chromosomes, indicating that the change is not a polymorphism. The exchange was not detected in 65 MH-susceptible individuals and appears to be unique to this particular family. The cysteine 35 residue is otherwise conserved across 8 RYR receptors so far sequenced from 5 species, including human and *Drosophila* (fig. 4).

This is the first report of a genotyped homozygous proband arising from consanguineous parents. An MHS patient homozygous for a known mutation (Arg614Cys) was reported recently.⁷ However, in this pedigree, there are many discrepancies between the CHCT results

and haplotypes of markers for the MHS1/RYR1 region, including the C1840T transition: (1) all MHS patients do not have the mutation, casting doubt on the causality of this mutation in this family; (2) the haplotype of the MHS patient reported to be homozygous for the mutation is not compatible with the inheritance of an

		Cys35
		*
Human	Ryrl	TVLKEQLKL C LAAEGFGNRLCFLE
Pig	Ryrl	TVLKEQLKL C LAAEGFGNRLCFLE
Rabbit	Ryrl	TVLKEQLKL C LAAEGFGNRLCFLE
Rabbit	Ryr2	TIHKEQKQL C LAAEGFGNRLCFLE
Rabbit	Ryr3	TVHKEQRKF C LAAEGLGNRLCFLE
Bullfrog	Ryr α	TIRKENLKM C MGVEGFGNRLCYLE
Bullfrog	Ryr β	TIHKEQRKF C LAAEGLGNRLCFLE
D. melanogaster	Ryr	TG----ERV C LAAEGFGNRHCFLE

Fig. 4. Amino acid alignment of the human RYR1 protein and related isoforms flanking the Cys35Arg mutation site. The substituted amino acid residue (*) is conserved in all eight proteins.

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identical set of alleles from her parents; (3) one false-negative CHCT result must have occurred in this pedigree; (4) the haplotype of the father of the homozygote is reconstructed because the father is deceased. In our pedigree, the haplotypes of the two potential MHS homozygotes are compatible with such an inheritance, especially because their parents are directly related. There are no discrepancies between the CHCT results and the haplotypes of markers for the MH locus. A lod score $\geq +3$ has been found in this pedigree, strongly suggesting linkage to the RYR1 gene.

The presence of the two MHS RYR1 alleles in the homozygotes clearly influenced the caffeine CHCT results, because it was possible to distinguish heterozygotes from homozygotes by comparing the level of contracture achieved at any caffeine concentration (table 1). However, no clear differentiation could be achieved using the halothane CHCT results. This outcome may be explained by the fact that halothane and caffeine are known to have different modes of action on calcium release in skeletal muscle cells. Whereas the caffeine results differentiated the homozygotes from the rest of the family, this test alone was not sufficient to detect susceptibility in all heterozygous family members, because one MHE(h) individual was recorded.

Depending on the amino acid substitution and location within the protein primary structure, a mutation in the RYR1 gene can give rise to a range of phenotypes. Some human mutations appear to have no effect on phenotype in the absence of triggering agents. In other cases, the mutation is manifested even in the absence of triggering agents. Certain susceptible patients complain of persistent muscle cramps, with associated elevated blood CK values indicating excessive muscle contractures and damage.^{21,22} The presence of the Arg615-Cys mutation in swine is associated with muscle hypertrophy.⁶ In this case, the mutated calcium channel appears to open for longer than normal periods of time, releasing extra amounts of calcium during the contracture process, which could lead to hypertrophy.^{6,23} A more deleterious change to the channel structure might allow the release of even greater amounts of calcium into the myoplasm, which could give rise to many of the characteristic features observed in central core disease cases: loss of mitochondria, Z disk streaming, and muscle atrophy.¹¹ It has been suggested that the inheritance of homozygous dominant alleles is associated with a more severe phenotype.²⁴ One MHS patient with a chronic myopathy was reported in a pedigree in which both parents were typed MHS by the CHCT,

suggesting that the patient was either a homozygote or a compound heterozygote.²⁵ Subsequently, homozygosity in this patient was ruled out, and it is likely she has inherited two independent, and not necessarily identical, MHS mutations. The current mutation does not appear to have any effect on phenotype in the absence of halothane or caffeine, because both homozygous patients had no signs of muscle abnormality, and had normal serum CK values. This indicates that the Cys35Arg substitution alters myoplasmic calcium regulation in a distinct manner from other mutations that may be detected in the absence of triggering agents. Further biochemical and electrophysiologic characterization will be necessary to elucidate the mechanistic nature of these mutations on RYR1 channel function and correlation with the observed phenotype in affected MHS individuals.

Individual IV-19 was tested according to the European malignant hyperpyrexia group protocol by Dr. L. Heytens (Antwerp MH diagnostic unit, Belgium), and individual III-12 was tested by Dr. Cozzolino (Naples MH diagnostic unit, Italy.) We are grateful to both of them for supplying the CHCT results.

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