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Scanning for Mutations of the Ryanodine Receptor (*RYR1*) Gene by Denaturing HPLC: Detection of Three Novel Malignant Hyperthermia Alleles

Angela Tammaro,[1] Adele Bracco,[1] Santolo Cozzolino,[1] Maria Esposito,[2] Antonietta Di Martino,[1] Gennaro Savoia,[1] Laura Zeuli,[1] Giulio Piluso,[3] Stefania Aurino,[2] and Vincenzo Nigro[2,3*]

Background: **Malignant hyperthermia (MH) is a fatal autosomal dominant pharmacogenetic disorder characterized by skeletal muscle hypertonicity that causes a sudden increase in body temperature after exposure to common anesthetic agents. The disease is genetically heterogeneous, with mutations in the gene encoding the skeletal muscle ryanodine receptor (*RYR1*) at 19q13.1 accounting for up to 80% of the cases. To date, at least 42 *RYR1* mutations have been described that cause MH and/or central core disease. Because the *RYR1* gene is huge, containing 106 exons, molecular tests have focused on the regions that are more frequently mutated. Thus the causative defect has been identified in only a fraction of families as linked to chromosome 19q, whereas in others it remains undetected.**

Methods: **We used denaturing HPLC (DHPLC) to analyze the *RYR1* gene. We set up conditions to scan the 27 exons to identify both known and unknown mutations in critical regions of the protein. For each exon, we analyzed members from 52 families with positive in vitro contracture test results, but without preliminary selection by linkage analysis.**

Results: **We identified seven different mutations in 11 MH families. Among them, three were novel MH alleles: Arg44Cys, Arg533Cys, and Val2117Leu.**

Conclusion: **Because of its sensitivity and speed, DHPLC could be the method of choice for the detection of unknown mutations in the *RYR1* gene.**

Malignant hyperthermia (MH;[4] OMIM 145600) is inherited as an autosomal dominant pharmacogenetic trait that predisposes individuals to a life-threatening anesthetic-drug-induced hypermetabolic syndrome *(1–3)*. Its main features include an increase in body temperature caused by generalized skeletal muscle contracture, hypercarbia, rhabdomyolysis, cardiac dysrhythmia, and renal failure. These symptoms suddenly appear on exposure to succinylcholine and volatile anesthetic agents, such as halothane. A family's disposition to MH is identified by a clinical episode of MH in the proband. In these cases, the in vitro contracture test (IVCT) of biopsied skeletal muscle represents the only generally accepted method to confirm the MH disposition *(4, 5)*. The pathobiology of MH is an abnormal increase in myoplasmic Ca^{2+}, caused by volatile anesthetics and by depolarizing muscle relaxants. Unabated, this increase in Ca^{2+} stimulates metabolic and contracture events that ultimately create acid/base and electrolyte imbalances, causing cell damage and death *(6, 7)*.

Molecular genetic studies have established the ryanodine receptor gene (*RYR1*) on chromosome 19q13.1 as the primary locus for MH susceptibility (MHS) *(8–10)* (designated the *MHS1* locus; OMIM 145600). *RYR*1 is one of the most complex genes characterized to date: it encodes a 15.3-kb mRNA and comprises 106 exons. The RyR1 protein has a subunit size of 565 kDa and forms an

[1] Malignant Hyperthermia Center, AO Cardarelli, via San Giacomo dei Capri 66, 80131 Napoli, Italy.
[2] Telethon Institute. of Genetics and Medicine (TIGEM), via Pietro Castellino 111, 80131 Napoli, Italy
[3] Dipartimento di Patologia Generale e Centro di Eccellenza sulle Malattie Cardiovascolari, Seconda Università degli Studi di Napoli, via Luigi De Crecchio 7, 80138 Napoli, Italy.
*Address correspondence to this author at: Dipartimento di Patologia Generale, Seconda Università degli Studi di Napoli, via Luigi De Crecchio 7, 80138 Napoli, Italy. Fax 39-081-5665704; e-mail vincenzo.nigro@unina2.it.
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[4] Nonstandard abbreviations: MH, malignant hyperthermia; IVCT, in vitro contracture test; RyR1, ryanodine receptor; MHS, MH susceptible; CCD, central core disease; DHPLC, denaturing HPLC; and MHE, MH equivocal.

elaborate tetrameric structure that acts as a Ca^{2+} release channel and forms a large myoplasmic "foot" structure that bridges the gap between the SR and the T-tubule in skeletal muscle *(11, 12)*. The C-terminal region of the receptor forms the transmembrane channel, which may contain 4–10 transmembrane helices *(13)*.

In addition to the *MHS1* locus, five other loci (*MHS2* to *MHS6*; OMIM 154275, 154276, 600467, 601887, and 601888, respectively) have been tentatively identified by linkage analysis *(14–17)*. A mutation in the *CACNL1A3* (*CACN1AS*) gene, which encodes the main subunit of the voltage-gated dihydropyridine receptor that interacts with the RyR1 channel, has been confirmed for the 1q locus *(18)*.

Molecular genetic methods are used with caution to diagnose MH disposition because of the genetic variability of this disease. However, sometimes genotyping can have an advantage over the standardized IVCT, which is invasive, requires patient compliance, and is labor-intensive and expensive.

To date, at least 41 missense mutations and 1 single-amino acid deletion of RyR1 have been found to segregate with the MHS trait, whereas a much smaller number of these mutations are also associated with central core disease (CCD) *(19–30)*.

Reports of discordance between mutations and IVCT phenotype have called into question the causal role of some *RYR1* mutations *(31–33)*, although the lack of specificity of the IVCT *(34–37)* or the presence of additional MH mutations *(31, 37, 38)* may account for some discrepancies.

Many methods for detecting mutations have been described, and strengths and limitations are inherent in each *(39)*. Direct DNA sequencing is considered to be the "gold standard" for the identification of mutations, albeit problems can be encountered with heterozygous alleles. We hypothesized that the diagnostic test could be made rapid and inexpensive by the use of a robust method to scan DNA samples for sequence variations/mutations before targeted sequence analysis. Denaturing HPLC (DHPLC) is such a method. DHPLC relies on the principle of heteroduplex analysis by ion-pair reversed-phase liquid chromatography under partially denaturing conditions *(40)*. Here we report three novel mutations in the N-terminal and central domains of RyR1 associated with MHS. The experimental design and our results are presented here.

Materials and Methods

PATIENTS AND DNA SPECIMENS

We recruited 52 Italian families (1–12 individuals in each family) for *RYR1* DNA analysis. Eighteen families (35%) included at least one member with episodes of hyperthermia in the course of treatment with an anesthetic. Thirty-four families (65%) were initially identified by increased serum creatine kinase concentrations from routine laboratory analysis. The IVCTs were carried out on muscle biopsies from these families, according to the criteria of the European MH group protocol. Thirty-three (63%) families had members who were susceptible to both halothane and caffeine and were designated as MHS, whereas 37% of families had members who were susceptible to either halothane or caffeine and were designated as MH equivocal (MHE).

DNA samples were collected after informed consent. Genomic DNA was extracted from blood samples either by phenol–chloroform extraction or with the Nucleon BACC1 Blood Kit (Amersham).

IVCT

The European MH diagnostic protocol *(5, 41)* was used for the IVCT. Individuals referred to our MH diagnostic center were administered MH-nontrigger anesthesia, and fascicles of the vastus medialis were taken and mounted in vitro in muscle-contracture chambers for testing. A different set of two fascicles were used for each of two tests. The first test exposed the fascicles to four increasing concentrations of halothane (0.5%, 1%, 2%, and 3%), and the second test exposed the muscle to seven increasing concentrations of caffeine, starting at a concentration of 0.5 mmol/L and ending at a concentration of 32 mmol/L. A contracture response $\geq$0.2 *g* in one or more fascicles at halothane concentrations $\leq$2% and caffeine concentrations $\leq$2 mmol/L defines a diagnosis of MHS. Patients reacting to halothane only or caffeine only are deemed equivocal and designated MHE *(42)*.

PCR PRIMER AND DNA AMPLIFICATION FOR DHPLC

Twenty-eight primer pairs were designed to amplify selected *RYR1* gene fragments (including intron/exon junctions) *(20)*. Amplified fragments were 149–428 bp in length. Primer sequences, PCR product sizes, and annealing temperatures are shown in Table 1.

PCR was performed in 25 μL containing the following: 15 pmol of each primer, 1$\times$ PCR buffer, 200 μM each deoxynucleotide triphosphate (Roche), 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 100 ng of DNA. The regions amplified included 28–68 bp of intron sequence on the 5′ side of the exon and 26–77 bp of intron sequence on the 3′ side.

PCR conditions were as follows: a denaturation step at 95 °C for 10 min; 32 cycles of 95 °C for 30 s, annealing at the primer-specific temperature for 90 s, and extension at 68 °C for 90 s, adding 3 s for each cycle; and a final extension at 68 °C for 10 min.

All PCR reactions were carried out in a PCR Express Thermalcycler (Celbio). The PCR products were stored at 4 °C before DHPLC analysis.

DHPLC ANALYSIS

DHPLC analysis was carried out with three WAVE^{TM} DNA fragment Analysis Systems (Models 3500A and 3500 HT; Transgenomic). The PCR mixture (5–8 μL) was injected in a preheated C_{18} reversed-phase column with

Table 1. PCR conditions and product sizes and DHPLC conditions for *RYR1* exons.

	PCR conditions				DHPLC conditions	
	Primers, 5′–3′					
Exon	Forward	Reverse	Product size, bp	Annealing temperature, °C	Temperature, °C	Acetonitrile gradient, % buffer B
2	CTGCAGTATTTGTGGTATCC	TGCCCCTTCAGACGCCCCTGT	230	58	67	51–58
6	AAGCCATCATCTGACAGCCACC	CAGACCTGGGAGCAGGAGGAAC	192	58	64	51–58
9	ACCCTTGACTTCACTCTCTTCTG	CCCAGCCTCTCCCTGGTCTCT	149	58	62	50–57
11	GCTCCCCTGCTAAACACACAG	CCTGGACTCAAGCGATTCTCC	279	60	66	51–58
12	CCCACTCCAGACCTCTGTCTCC	AGCACAGGAGTCACCCCAGCA	193	58	64	49–56
14	GGGTCTCCTATTGTGATGCCTC	GTTCTCAGGGAGTCAGGACTG	206	62	61	49–56
15	CAGTCCTATTGGATCTGACACCTC	TCCCACAAGCCTCTGTCCCACT	169	58	63	49–56
16	ATGGGCATGGCCGCTTCACCTC	TGGGGAGAGGCCAGCATTCAGAG	220	60	61	55–61
17	GTAGATCCTGCCCTGGTGCCTAC	TCTGGGTGTGGAGTCTCTAAGTCA	264	58	62	51–58
39	TGGTGCTCCAAGCCTTGCATTGTC	CAAAGTAAGGGGAAGGGCGGTGTC	336	58	67	52–59
40	CCTGGTGACCCCGCACACTCTG	AGCTCCCCAGCACCTGCCTGGG	180	58	64	49–56
41	GAGCTCAGGGGAGGCAGCCACAG	ACGGGGTGCCAGGAGTGGGTTC	243	63	64.5	55–61
44	GGTGGCTTCATGAGACCCCTTTCC	CAGGGAGGGCGGGTTGCCTATTG	270	60	66	55–61
45	TGCCTCCCCAACCCACCCACCTTC	TGCCTGCCCTCCCTTCCCATCCTG	183	61	64	44–60
46	TGCCCCTCTCCCTCCCTCTACTCC	AGCATCACTCCTTCGCCAAGTTCC	185	58	65	49–56
89	CAGAAGGGAGTGTTCACCGGCC	CTTGGCAACCCCACCCAAGTCC	283	60	62	57–63
90	GAGGCTCTCCAGGTCACCCCACTG	AAGCCCCAGGAGGCGAGCTTGC	428	60	64	61–67
91-1	GCTGACGGCGCCCTATCCTGTC	GCGCCGCCGCAGGCTGCGGTA	366	58	64	57–64
91-2	GCCGGGCCCTGCGAGGCCTCA	GGTGGGGTCGGGCATGCCTGCC	257	60	67	50–57
91-3	AAGAAGGTGACGGTGACCGAGCTCC	CCTCCAATCCCCCCCATCTTTCC	387	60	64	55–62
92	CCTGAGAAGCGCTTAGGGTGAGG	GGCTCCTACTCTAGGAGGGAGGCAG	191	60	63	52–58
93	GAATGAACTCATGCATTGCCTGCCC	CTCCCGGGGTGAAGAAGGTTCAGG	260	60	62	56–62
95	TGCATGTGGCAGACCCACAGATG	AGCCCCAGCCAGTGCTCCAGCAC	377	64	62.5	60–66
96	CTTCTGCTGAGACTCTGGTCCAGC	CCTGGACTGGGTGGAAACCAGG	260	60	63	56–62
98/99	CACTCCAGCTGTGTCTACACAGCC	CTGCAGAGTCCCTCCCCAGTCTG	351	60	62	59–65
100	CCCTCCAAGAGTGCTCCTCGTG	TTCACCACCCACTGCCACGCTGG	276	60	62	57–63
101	CAAGCCCAGGGCGGAGCTGACC	GCGAGAAGGAAGGGTCCCAGAG	218	64	62.5	56–62
102	TGAATGAGTGACCAGTGTGCTCC	AGCCATGGAGCTAGCCTGCCT	242	58	64	50–57

nonporous (polystyrene/divinylbenzene) particles (DNASep™ column; Transgenomic). The injected sample was then eluted by a linear acetonitrile gradient consisting of buffer A (100 mmol/L triethylammonium acetate containing 10 mL/L acetonitrile) and buffer B (100 mmol/L triethylammonium acetate containing 250 mL/L acetonitrile).

DNA was detected at 260 nm. Melting curves were predicted using the Wavemaker software (Transgenomic), but a comparison of the retention times at different temperatures was performed with control fragments. Usually, each mutation was detectable in a range of ± 1.5 °C. The typical analytical gradient time was 3.5 min with the 3500A and 2.5 min with the 3500HT, and the buffer B concentration was increased at 2% per min.

DNA SEQUENCING

The PCR products were purified from agarose with use of the Ultrafree-DA DNA extraction (Millipore) or the MinElute Gel Extraction Kit (Qiagen). Direct sequencing was performed with the Thermosequenase terminator cycle sequencing reagent set with ^{33}P-labeled dideoxynucleotide triphosphates (USB) or by automated cycle sequencing (3100 Genetic Analyzer; Applied Biosystems).

Results

Fifty-two unrelated MH families of Italian ancestry diagnosed as MHS or MHE in the IVCT test were included in this study. We carried out DNA analysis on all members (1–12 members per family). We began with the most commonly mutated exons, using a high-throughput automated method, DHPLC. This method is particularly suitable for detecting heterozygous unknown mutations with the same sensitivity as direct sequencing; in addition, it is less expensive and has high specificity. The presence of a base difference between alleles leads to the formation of heteroduplex DNA after PCR. Heteroduplex DNA is easily separated from parental homoduplex DNA in a few minutes by elution from the special reversed-phase HPLC column. In the present study, each primer pair was able to amplify an exon (or part of it, as in the case of exon 91) together with 25–50 bp of flanking intronic sequences on both sides. Table 1 shows the sequences of the primers

and the conditions of DHPLC analysis. The melting characteristics of each PCR fragment were predicted using Wavemaker software.

We designed 28 primer pairs to amplify 27 *RYR1* exons because exons 98 and 99 were coamplified, whereas exon 91 (577 bp) was split into three fragments. Amplified fragments were 149–428 bp in length. Primer sequences, PCR product sizes, and annealing temperatures are shown in Table 1. Some exons, such as exons 11 (Fig. 1) and 40, present heterogeneous melting profiles.

We identified several common and rare sequence variations in PCR products containing exons 11, 15, 39, 44, 45, and 89 and intron 91 (Table 2). This is a major drawback for the analysis because these products generate heteroduplexes that may confound the detection of true mutations. In these cases, we chose to sequence all of the heteroduplex-forming fragments to avoid missing mutations. As a result, we were able to identify a mutation in exon 15 (Arg533Cys) that generated a profile that was fully coincident with one generated by a very common polymorphism.

We found 11 mutations in the 33 MHS families (33.3%)

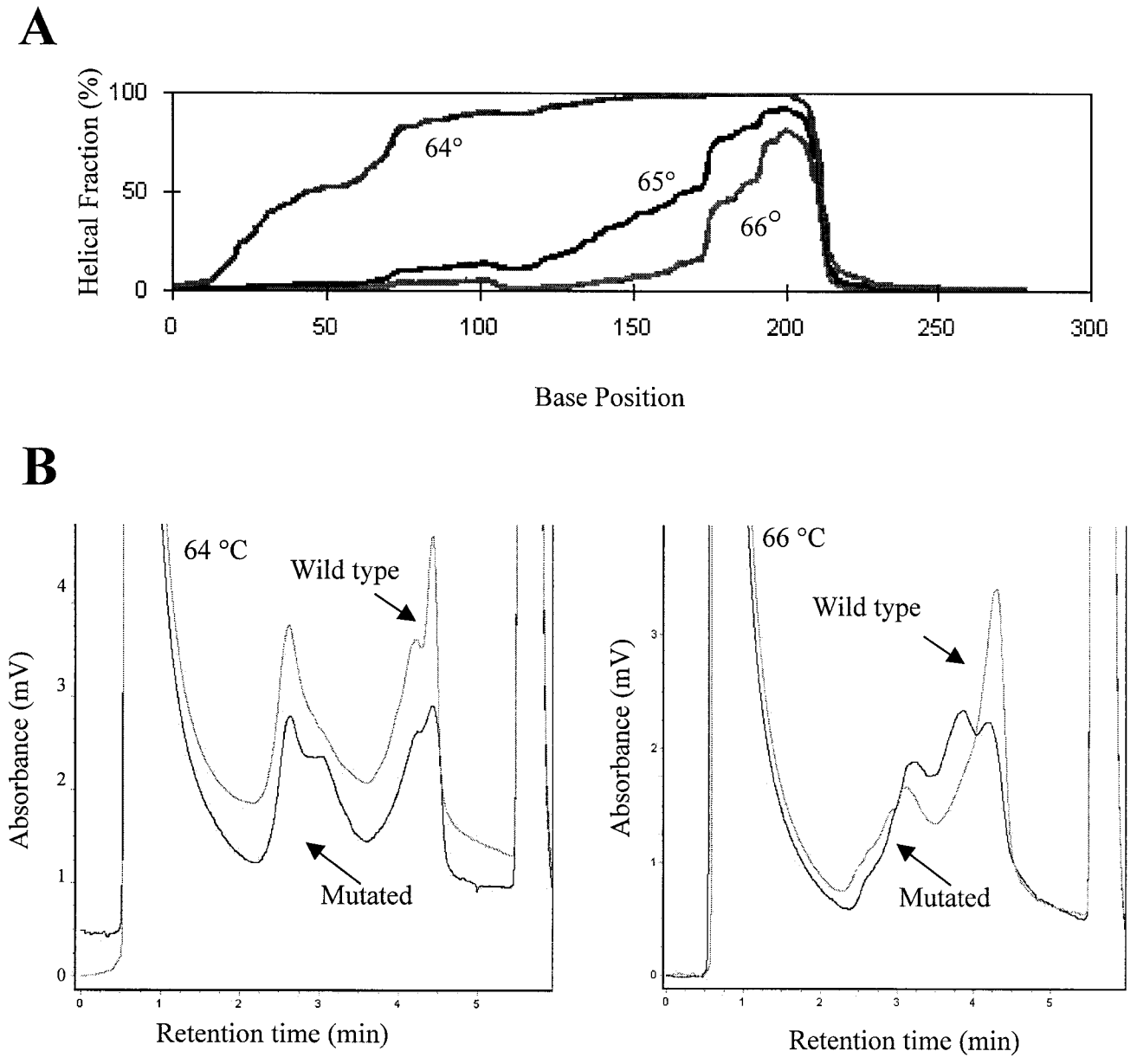


Fig. 1. Effect of column temperature on mutation detection sensitivity of DHPLC.

(*A*), melting curve profiles of exon 11, calculated at multiple temperatures by the Wavemaker software. Helical fraction (%) indicates the percentage of double-stranded DNA along the fragment. (*B*), temperature-dependent resolution of heteroduplex and homoduplex peaks in exon 11 for mutated and wild-type DNA. Elution profiles for mutated (*black line*) and wild-type DNA (*gray line*) were determined at 64 and 66 °C. At 66 °C, the differentiation improves considerably because the elution profile for mutated DNA reveals an additional peak not present in the wild-type profile.

Table 2. *RYR1* point mutations.

	Family	Exon	Nucleotide change	Amino acid change	Diagnosis
Missense mutations	147	2	130CGC→TGC	Arg44Cys	MHS
	52	15	1597CGT→TGT	Arg533Cys	MHS
	232	39	6349GTG→CTG	Val2117Leu	MHS
	115	39	3488CGC→CCC	Arg2163Pro	MHS
	90	39	6502GTG→ATG	Val2168Met	MHS
	120	39	6502GTG→ATG	Val2168Met	MHS
	62	39	6502GTG→ATG	Val2168Met	MHS
	192	45	7304CGC→CTC	Arg2435Leu	MHS
	150	45	7304CGC→CTC	Arg2435Leu	MHS
	31	45	7304CGC→CTC	Arg2435Leu	MHS
	129	46	7361CGC→CAC	Arg2454His	MHS
Polymorphisms and variants	15%	11	1077GCC→GCT	Ala359Ala	MHE-MHS
	52%	15	1668TCG→TCA	Ser556Ser	MHE-MHS
	115	39	6302ATG→AAG	Met2101Lys	MHS
	6%	44	7098CCC→CCT	Pro2366Pro	MHE
	4%	45	7260CAC→CAT	His2420His	MHS
	150	89	12120GCC→GCT	Ala4040ALa	MHS
	83	92	13438-15T→C		MHE

Table 3. IVCT results for families with novel mutations.

Individual	Contracture at 2% halothane, *g*	Halothane threshold, %	Contracture at 2 mmol/L caffeine, *g*	Caffeine threshold, mmol/L
Family 147				
I-1	1.15	0.5	1.05	1.0
II-1	0		0.0	
II-2	1.6	0.5	1.4	1.0
Family 52				
I-2	0.5	0.5	0.3	1.0
I-3	0.2	0.5	0.4	1.0
II-1	0.6	0.5	0.28	1.5
II-2	0.4	0.5	0.2	1.5
II-4	0.4	0.5	0.35	1.0
II-5	0.3	0.5	0.65	0.5
III-1	0.2	0.5	0.2	1.5
III-2	0.7	0.5	0.3	1.5
III-3	0.0		0.0	
III-4	0.4	0.5	0.2	1.5
Family 232				
II-1	1.3	0.5	1.1	1.0
II-2	0.0		0.0	
II-3	1.8	0.5	1.44	1.0

and no mutation in the 19 MHE (0%) families (Table 2). Direct sequencing of the PCR products identified three novel nucleotide substitutions, 130CGC→TGC, 1597CGT→TGT, and 6349GTG→CTG, which encode the amino acid changes Arg44Cys, Arg533Cys, and Val2117Leu, respectively. All three substitutions were fully concordant with IVCT results (Table 3) and were absent in a control population of at least 100 healthy individuals. The positions are conserved in all known vertebrate *RYR1* genes (Fig. 2). All three novel mutations were in MH/CCD region 1 or region 2 (Fig. 2).

The Arg44Cys mutation was observed in only 1 of the 52 families tested (family 147). The proband is a 27-year-old female, who at the age of 7 developed masseter spasm and increased serum creatine kinase during general anesthesia for tonsillectomy. A skeletal muscle biopsy, performed 18 years later, indicated that she was MHS. The substitution was seen in proband 234 and her father but was not observed in her brother or mother (Fig. 3A).

The Arg533Cys mutation was found in a patient who at the age of 13 years experienced a rapid increase in body temperature (41 °C) during an appendectomy under general anesthesia. The creatine kinase concentration measured in the postoperative intensive care unit was 10 710 U. Moreover, the patient had myalgic asthenia. The familial mutation completely cosegregated with the IVCT results (Fig. 3B).

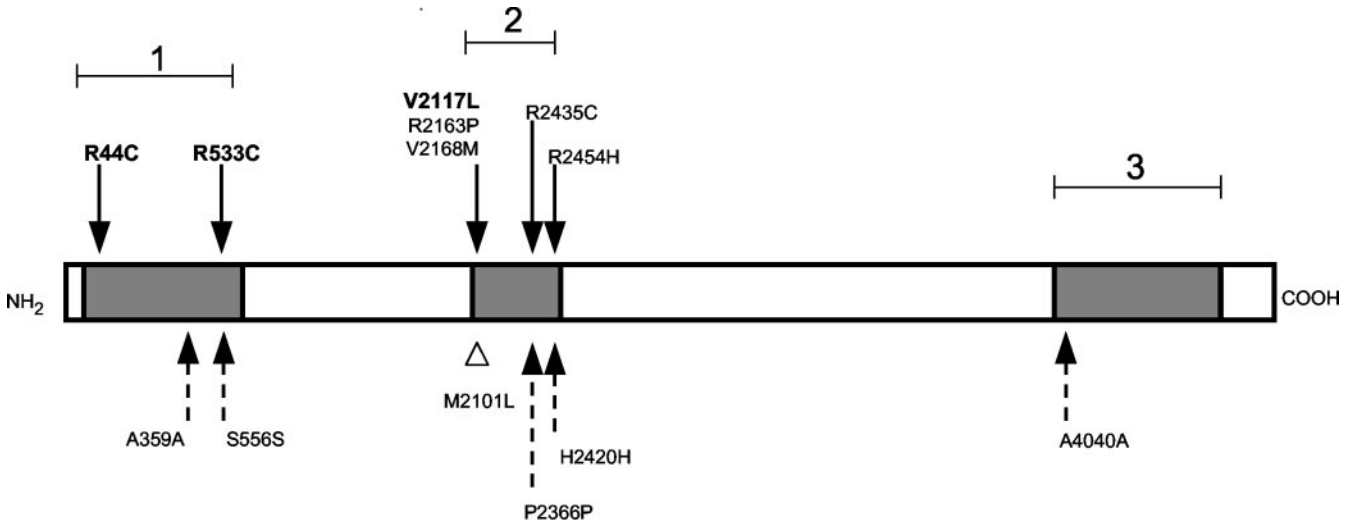
The substitution Val2117Leu was observed in family 232. The mother of the proband died from an intraoperative MH episode (Fig. 3C).

Overall, seven different *RYR1* gene mutations were detected in 11 unrelated MHS families, which accounted for ~21% of the screened population, including MHE + MHS kindreds (Table 2). The most common mutations were Val2168Met, which occurred in three families, and Arg2435Leu, which also occurred in three families, both accounting for 27%. The other five mutations were detected once in each family.

Discussion

The *RYR1* gene is unusual in size (106 exons) and thus is too complex to be fully screened for mutations. The literature reports that, to date, 42 different MH/CCD alleles have been identified *(19–30)*. Ten RyR1 mutations (24%) are between amino acids 35 and 614 (MH/CCD region 1), whereas 23 mutations (55%) are located between amino acids 2129 and 2458 (MH/CCD region 2). These are predicted to reside at the myoplasmic foot region of the protein. In addition, nine mutations (21%) have been located in a third domain (MH/CCD region 3) at the highly conserved C-terminal region, which encodes the luminal/transmembrane domain of the protein that forms the ion pore (amino acid residues 4668 and 4906). MH/CCD regions 1–3 represent hot spots for MH, although true data on the distribution of mutations can be influenced by the biased analysis of certain regions. Mutations in MH/CCD region 1 reside in exons 2, 6, 9, 11, 12, 14, 15, and 17; those in MH/CCD region 2 reside in exons 39, 40, and 44–46; and those in MH/CCD region 3 reside in exons 96 and 100–102.

We observed that direct sequencing as a first approach is unreliable for heterozygous mutations without the support of the reverse reaction; this requirement increases the costs by a factor of 2, and the high number of reactions congests the sequencing core facility. We therefore investigated the feasibility of alternative strategies for mutation scanning of MH families. Present methods of analysis involve the use of single-strand conformation polymor-



Mutation	R44C	R533C	V2117L
RYR1 human	LCLAAEGFGN**R**LCFLEPTSN	ELLASLIRGN**R**SNCALFSTN	QEDFVQSPEL**V**RAMFSLLHR
RYR1 pig	LCLAAEGFGN**R**LCFLEPTSN	EILASLIRGN**R**ANCALFSTN	QEDFVQSPEL**V**RAMFSLLHR
RYR1 rabbit	LCLAAEGFGN**R**LCFLEPTSN	ELLASLIRGN**R**ANCALFSTN	QEDYVQSPEL**V**RAMFSLLHR
RYR1 bullfrog	LCLAAEGFGN**R**LCFLEPTSN	ELLAALIRGN**R**SNCAQFSYN	QEDFVQSPEL**V**RAMFSLLHR
RYR1 fish	LCLAAEGFGN**R**LCFLEPTSN	ELLASLIRGN**R**ANCALFCDN	QESFIQNPEL**V**RMMLSLLHR
RYR2 human	LCLAAEGFGN**R**LCFLESTSN	ELLAALIRGN**R**KNCAQFSGS	QESVIEDPEL**V**RAMFVLLHR
Substitution	----------**C**---------	----------**C**---------	----------**L**---------

Fig. 2. Distribution of point mutations identified along the *RYR1* gene.

The novel MH mutations are indicated in *bold font*, whereas known mutation are indicated by *solid arrows*. The variant is represented as a *triangle*, and polymorphisms are indicated by *dashed arrows*. The three *gray areas* correspond to the hot spot regions of the *RYR1* gene. Amino acid alignments of R44, R533, and V2117 are provided for all known vertebrate *RYR1* genes and the human *RYR2*.

phisms and/or tests that identify known mutations. Compared with single-strand conformation polymorphism analysis and other methods for the discovery of unknown mutations, DHPLC presents the advantages of high sensitivity, speed, and automation *(39, 40)*. The technique detects both missense and nonsense mutations and is more sensitive to small deletions/insertions. In addition, it is well suited for the analysis of heterozygous patients because it is based on the formation of heteroduplex DNA, which is easily separated from homoduplex DNA by HPLC. This is the case for the analysis of dominant disorders in very complex genes, such as *RYR1*. The strategy of *RYR*1 testing based on DHPLC and targeted sequencing appears to be a cost-effective option. This also agrees with the cost-effectiveness study performed by Sevilla et al. *(43)* for *BRCA1* testing.

We are currently analyzing samples from many other genes, using three DHPLC instruments, and, on average, the use of a single analysis temperature is sufficient to detect any mutation in at least 80% of fragments. In the remainder of fragments, it is necessary to use a second or third analysis temperature. Under these conditions, the cost of DHPLC is at least eight times lower than that for sequencing. We identified mutations in 11 of 33 MHS families. We must take into account that 14 families (of 52) were previously tested by single-strand conformation polymorphism analysis with negative results and that one *RYR1* mutation was detected by DHPLC alone. Because in this study we included patients prescreened with negative results, the true detection rate may be underestimated. Examination of additional *RYR1* exons may increase the number of mutations detected closer to the predicted 50% of all MH families. This frequency is similar to other reports, but in Germany a higher frequency was found (69%) for the contribution of two major alleles that we did not detect in our patients *(32)*. A major general problem in the identification of novel *RYR1* mutations is the differentiation of true mutations vs private amino acid variations. When the disease is linked to the *RYR1* locus, one must take into account that all sequence variations could cosegregate with the causative mutation. Thus, in the absence of biochemical studies, each amino acid change observed only in one family should be considered as a "putative mutation". In addi-

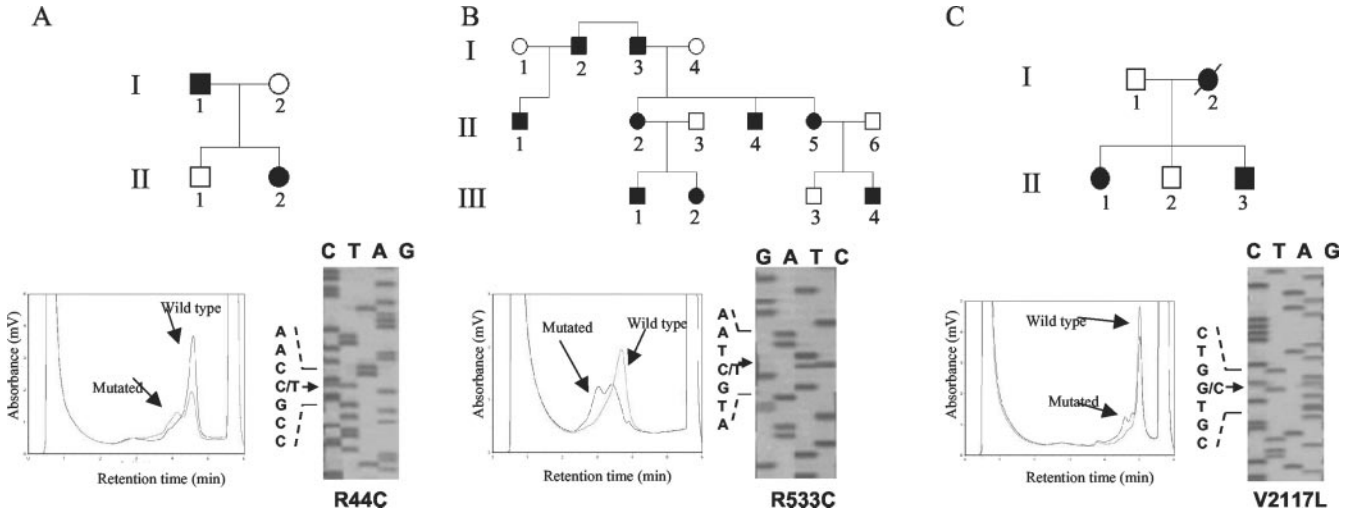


Fig. 3. Segregation, DHPLC profiles, and direct sequencing reactions for the novel MH mutations.

(*A*), kindred 147: mutation 130CGC→TGC in exon 2 (Arg44Cys). (*B*), kindred 52: mutation 1597CGT→TGT in exon 15 (Arg533Cys). (*C*), kindred 232: mutation 6349GTG→CTG in exon 39 (Val2117Leu). ● and ■ indicate individuals with a mutation; ○ and □ indicate individuals without a mutation.

tion, some mutations can give different degrees of penetrance, and this further complicates the analysis of results.

Using DHPLC, we identified polymorphisms in some PCR products. Because these generate heteroduplexes, the analysis gives rise to false positives. This problem can be eliminated by two different approaches: (*a*) sequencing all the heteroduplex-forming fragments to avoid missing mutations; and (*b*) cutting the fragment close to the mutation site. With the first approach, we were able to identify the mutation in exon 15 (Arg533Cys) despite the presence of a very common polymorphism.

Among the known mutations, 17 are missense mutations in which an arginine or a residue in MH/CCD region 1 encoded by exons 2–17 or MH/CCD region 2 encoded by exons 39–46 is mutated. Two of the three novel mutations we identified are Arg→Cys mutations that occur at a CpG dinucleotide, confirming previous observations about the nonpolymorphic nature of such changes.

In conclusion, we have shown that DHPLC is able to detect both novel and recurrent mutations in a highly complex autosomal dominant disorder, such as MH. This approach is very efficient and rapid, but requires an expensive instrument and a preliminary setup. Our approach may be best suited for the rapid processing of many samples analyzed by a centralized organization.

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