

Screening of the ryanodine receptor gene in 105 malignant hyperthermia families: novel mutations and concordance with the *in vitro* contracture test

Annebill Brandt¹, Lothar Schleithoff¹, Karin Jurkat-Rott¹, Werner Klingler¹, Christoph Baur^{1,2} and Frank Lehmann-Horn^{1,+}

¹Department of Applied Physiology and ²Department of Anesthesiology, University of Ulm, Albert-Einstein-Allee 11, 89069 Ulm, Germany

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Malignant hyperthermia (MH) in man is an autosomal dominant disorder of skeletal muscle Ca²⁺-regulation. During anesthesia in predisposed individuals, it is triggered by volatile anesthetics and depolarizing muscle relaxants. In >50% of the families, MH susceptibility is linked to the gene encoding the skeletal muscle ryanodine receptor (RYR1), the calcium release channel of the sarcoplasmic reticulum, on chromosome 19q12–13.2. To date, 21 RYR1 mutations have been identified in a number of pedigrees. Four of them are also associated with central core disease (CCD), a congenital myopathy. Screening for these 21 mutations in 105 MH families including 10 CCD families phenotyped by the *in vitro* contracture test (IVCT) according to the European protocol revealed the following approximate distribution: 9% Arg-614-Cys, 1% Arg-614-Leu, 1% Arg-2163-Cys, 1% Val-2168-Met, 3% Thr-2206-Met and 7% Gly-2434-Arg. In one CCD family, the disease was caused by a recently reported MH mutation, Arg-2454-His. Two novel mutations, Thr-2206-Arg and Arg-2454-Cys were detected, each in a single pedigree. In the 109 individuals of the 25 families with RYR1 mutations cosegregation between genetic result and IVCT was almost perfect, only three genotypes were discordant with the IVCT phenotypes, suggesting a true sensitivity of 98.5% and a specificity of minimally 81.8% for this test. Screening of the transmembraneous region of RYR1 did not yield a new mutation confirming the cytosolic portion of the protein to be of main functional importance for disease pathogenesis.

INTRODUCTION

Malignant hyperthermia (MH) is an autosomal dominant, potentially lethal pharmacogenetic predisposition which is considered to be one of the main causes of death during anesthesia. Triggering by volatile anesthetics and depolarizing muscle relaxants in susceptible patients leads to an abnormally

high release of intracellular Ca²⁺ in skeletal muscle (1). Clinically, accelerated muscle metabolism, muscle contractures ranging from masseter spasms to generalized rigidity, tachycardia, metabolic acidosis and hyperthermia are then observed (2,3). Classification of the severity of the symptoms is performed according to Larach *et al.* (4), whereby a score of at least 50 is considered to be a fulminant crisis.

As susceptible individuals are inconspicuous in the absence of triggering agents, a provocative diagnostic test on excised muscle bundles, the *in vitro* contracture test (IVCT), was introduced by the European Malignant Hyperpyrexia Group (EMHG) (5). This test differentiates between clearly MH susceptible (MHS) and MH normal (MHN) individuals and defines a third MH equivocal (MHE) group of unclear status. In a population of 105 patients who had experienced an anesthetic crisis with a Larach score of 50 and above, the test showed a sensitivity of 99% and a specificity of 93.6% based on 202 controls (6). Methodologically, MH risk is classified either as MHS, if muscle specimens exhibit a contracture of ≥ 2 mN after separate application of both ≤ 2.0 mM caffeine and ≤ 0.44 mM (2 vol %) halothane, as MHN for smaller contractures, and as MHE, if a muscle specimen reacts pathologically to one substance only.

Genetically, in >50% of the affected families, MH is caused by mutations in the ryanodine receptor of skeletal muscle (RYR1) encoded by a gene on chromosome 19q12–13.2 (7,8). Functionally, RYR1 controls intracellular calcium release initiating contraction upon interaction with the voltage-dependent dihydropyridine receptor. It is one of the largest known proteins with 2200 kDa corresponding to 5000 amino acids encoded by 106 exons making genetic screening very difficult (9). Until now, 21 RYR1 mutations causing MH have been described accounting for only ~20% of the families. Four further loci have been identified (10), but only for one of these has a second causative gene, the $\alpha 1$ subunit of the dihydropyridine receptor, been identified and only in a single pedigree (11). Therefore, for practical purposes, the RYR1 gene remains a main target for genetic analysis.

This is also true for an allelic congenital myopathy, central core disease (CCD), histologically characterized by degenerative central cores which consist of damaged myofibrils along the full length of muscle fibers. CCD patients are usually MH susceptible (12), but unlike other MH patients they present with muscle

⁺To whom correspondence should be addressed. Tel: +49 731 50 23250; Fax: +49 731 50 23260; Email: frank.lehmann-horn@medizin.uni-ulm.de

hypotonia and weakness, contractures and bone abnormalities. Of the 21 known mutations in RYR1, four are associated with CCD.

Until now, genetic testing for MH susceptibility has not been able to replace IVCT phenotyping. One reason for this is lack of large epidemiological studies correlating test results with the presence of RYR1 mutations. This study describes mutation frequency in a large population of 95 German MH and 10 CCD families along with concordance with EMHG IVCT and, by these data, determines sensitivity and specificity of the *in vitro* test on a genetic basis for the first time. Novel mutations are described in addition to the screening of an RYR1 region of special functional interest: the transmembraneous segments located in the sarco-plasmic reticulum membrane.

RESULTS

Screening for known mutations

Screening of 105 unrelated MHS individuals for all known RYR1 mutations (Table 1) revealed base exchanges in 23 MH families resulting in the following amino acid substitutions: Arg-614-Cys in nine patients, Arg-614-Leu in one, Arg-2163-Cys in one, Val-2168-Met in one, Thr-2206-Met in three, Gly-2434-Arg in seven and Arg-2454-His in one. In the corresponding pedigrees, genetic data were concordant with phenotyping by IVCT, except in three individuals (Table 2). The remaining 14 known mutations, i.e. Cys-35-Arg, Arg-163-Cys, Gly-248-Arg, Gly-341-Arg, Ile-403-Met, Tyr-522-Ser, Arg-533-His, Arg-552-Trp, Arg-2163-His, Arg-2163-Pro, Arg-2435-His, Arg-2435-Leu, Arg-2458-Cys and Arg-2458-His were screened for using 12 different positive controls, but could not be found in this patient group.

Novel MH mutation at nucleotide position 6617

An abnormal electrophoresis mobility of single-stranded DNA of exon 40 was detected in a patient of MH33. This aberrant SSCA pattern was different from that of Thr-2206-Met. Direct sequencing of the PCR product yielded a novel C→G transition at nucleotide position 6617 predicting a Thr-2206-Arg substitution (Fig. 1A). A forward primer with the G at the 5' end was established. Genomic DNA of the affected family members was amplified with the specific primer while no PCR product was generated for the unaffected family members and 80 controls. The large pedigree of MH 33 shows complete segregation of the IVCT phenotype with the mutation (Fig. 2) generating a two point lod score of 3.5 at a recombination frequency of $\Theta = 0.0$. Of interest is that the strabism occurring in seven family members did not cosegregate.

Novel MH mutation at nucleotide position 7360

A further novel mutation could be detected by *Bsh1236I* restriction analysis of exon 46. Loss of the cleavage site in a patient of MH 5 was due to a C→T transition at nucleotide 7360 predicting an Arg-2454-Cys substitution (Fig. 1B). Again, the mutation shows complete segregation with the IVCT phenotype and is not found in 80 controls.

MH mutation at nucleotide position 7361 causing CCD

We detected another mutation in a CCD family (MH89) by restriction analysis, Arg-2454-His, described recently (13) to

cause MH. Direct PCR sequencing showed the underlying G→A transition at nucleotide position 7361.

Mutation screening in the transmembraneous region of RYR1

Fifteen additional CCD families (total 120 families) were included in the SSCA screening for mutations in the transmembraneous putative pore region of the RYR1 in the exons listed at the bottom of Table 1. Several aberrant bands were detected, but sequencing of the PCR products did not reveal any base exchanges leading to amino acid substitutions.

IVCT concordance

Of the 109 patients in the families in which we identified mutations, the genetic status of 99 showed perfect concordance with IVCT result, while discordances were found for only three individuals (Table 2). Of the remaining seven individuals tested as MHE, one MHEh individual was a carrier of Arg-614-Cys, and all other MHEh individuals and one MHEc individual did not have a mutation. Two individuals, with neither mutation nor disease haplotype in a family carrying the mutation Gly-2434-Arg, revealed positive IVCT results. In contrast, one individual who tested negative in the IVCT carried both the Arg-614-Cys mutation and the disease haplotype. Of the 65 mutation carriers, 64 had MHS or MHE test results corresponding to a sensitivity of 98.5% (Table 2). On the other hand, of the 44 genetically defined unaffected persons, 36 had MHN test results corresponding to a specificity of minimally 81.8%, should neither of the two putative false positive MHS test results be associated with an additional, unknown mutation.

Linkage to RYR1

Linkage data were only performed in families with a detected RYR1 mutation and were completely concordant with mutational screening results (Fig. 2).

DISCUSSION

Epidemiology

In the following, we consider the two novel amino acid changes Thr-2206-Arg and Arg-2454-Cys as mutations because they cosegregate with the MHS status in the two affected families, are not found in 80 unrelated controls (160 different chromosomes) and concern residues for which MH-causing mutations are already known—especially since they may be considered to cause the more severe chemical changes in the sense of gain or loss of charge. Under this reasonable assumption, our results suggest that there may be regional differences in the occurrence of RYR1 mutations. In the mainly German population studied here, we observed ~9% Arg-614-Cys, 7% Gly-2434-Arg, 3% Thr-2206-Met, and 1% each of Arg-614-Leu, Arg-2163-Cys, Val-2168-Met and Arg-2454-His and the two novel mutations Thr-2206-Arg and Arg-2454-Cys. While most mutations are obviously rare, the frequency of Arg-614-Cys is noticeably higher than described in the literature [here 9 versus 4% (14)]. This is also true for Gly-2434-Arg [here 7 versus 4% (14)]. On the other hand, it is noteworthy that Gly-341-Arg did not appear in our MH patient group at all whereas it is described in 6% of Irish MH families

Table 1. Polymerase chain reaction primer and restriction enzymes detecting the RYR1 mutations causing CCD and/or MH [residue numbering according to Manning *et al.* (14) and J. Loke and D. MacLennan, Toronto, personal communication]

Exon	5'→3' primer	Size (bp)	T_a (°C)	Nucleotide	Amino acids	Disease state	Methods	Reference
1	F: tgg tcc tgc agt gca gcg R: ccg aag ccc tcg gcg acc agg ^a	70	65	T103C	Cys-35-Arg	MH	<i>AccI</i> gain	(22)
6	F: tcc aag cag agg tct gaa gga gaa R: agc gct cgg agg aga cac tga caa	76	54	C487G	Arg-163-Cys	MH;CCD	<i>Bsh1236I</i> loss	(23)
9	F: tgc tga cag tga tga cca gcg cag R: ctg att ctc agt ggc tcc agc ctc	237	60	G742A	Gly-248-Arg	MH	SSCA	(24)
11	F: gcc ccc ctg aga tca agt cc ^a R: gtc tgg agc ggc ata ggt gag	86	63	G1021A	Gly-341-Arg	MH	<i>MspI</i> loss, SSCA	(25,26)
12	F: cat gga cga cgc act gtc g R: tgg ttg tat agg cca ttg gtg ct	90	57	C1209G	Ile-403-Met	CCD	<i>DpmII</i> loss	(23)
14	F: tac acc act gct gcc cac ttt R: gac ccc tac cta gga gct c ^a	100	60	A1565C	Tyr-522-Ser	MH;CCD	<i>DdeI</i> loss	(27)
15	F: ctc agc ttc tct aat ccg tg R: tac agg acc tcc agg atg c	196	58	G1598A	Arg-533-His	MH	SSCA	T. McCarthy, personal communication
15	F: teg tag caa ctg tgc cct c R: tac agg acc tcc agg atg c	182	60	C1654T	Arg-552-Trp	MH	<i>AlwI</i> loss	(28)
17	F: gag ctc tag gtc ctg gac R: aga gcc aga cct ggt gac	149	57	C1840T	Arg-614-Cys	MH	<i>RsaI</i> loss	(29)
17	F: gag ctc tag gtc ctg gac R: aga gcc aga cct ggt gac	149	57	G1841T	Arg-614-Leu	MH	SSCA	(30)
39	F: tcc tcc gtg gaa gac acc R: cga tgc tct gga tca tga g	109	56	C6487T	Arg-2163-Cys	MH	<i>BsrBI</i> loss	(14)
39	F: tcc tcc gtg gaa gac acc R: cga tgc tct gga tca tga g	109	56	G6488A	Arg-2163-His	MH;CCD	<i>BsrBI</i> loss	(14)
39	F: tcc tcc gtg gaa gac acc R: cga tgc tct gga tca tga g	109	56	G6488C	Arg-2163-Pro	MH	<i>BsrBI</i> loss	T. McCarthy, personal communication
39	F: tcc tcc gtg gaa gac acc R: cga tgc tct gga tca tga g	109	56	G6502A	Val-2168-Met	MH	<i>NlaIII</i> gain	(14)
40	F: cgt gca cag gaa cat cat g R: ctc acc ttg gac tgc ccg	129	59	C6617T	Thr-2206-Met	MH	SSCA	(14)
40	F: cgt gca cag gaa cat cat g R: ctc acc ttg gac tgc ccg	129	59	C6617G	Thr-2206-Arg	MH	SSCA	This study
45	F: ttc cct gca gct ttg gtg R: ggg tct cac atg cat ctc	130	57	G7303A	Gly-2434-Arg	MH	<i>AlwNI</i> gain	(31)
45	F: ttc cct gca gct ttg gtg R: ggg tct cac atg cat ctc	130	59	G7307A	Arg-2435-His	MH;CCD	<i>HgaI</i> loss	(32)
45	F: ttc cct gca gct ttg gtg R: ggg tct cac atg cat ctc	130	59	G7307T	Arg-2435-Leu	MH	<i>HgaI</i> loss	(13)
46	F: cta atc caa gcc ggc aag g R: ccc tet gca cct ttg ccc	131	61	G7361A	Arg-2454-His	MH	<i>Bsh1236I</i> loss	(13)
46	F: cta atc caa gcc ggc aag g R: ccc tet gca cct ttg ccc	131	61	C7360T	Arg-2454-Cys	MH	<i>Bsh1236I</i> loss	(31)
46	F: cta atc caa gcc ggc aag g R: ccc tet gca cct ttg ccc	131	61	C7372T	Arg-2458-Cys	MH	<i>BsrBI</i> loss	(33)
46	F: cta atc caa gcc ggc aag g R: ccc tet gca cct ttg ccc	131	61	G7373A	Arg-2458-His	MH	<i>BsrBI</i> loss	(33)
87	F: cgc atc gtg atc cct gat c R: ctc ggg tga cca act gac c	267	60				SSCA	
88	F: gct ggg tag gtg agg agg R: gtt gca gat ggg gtt gag g	287	60				SSCA	
91	F: gag ttt ctg cga gga cac c R: gag cag cgc cgc cac tg	307	60				SSCA	
94	F: ctg tct gtg gcg ett tct c R: ctt cag tgg agg aac cct g	223	60				SSCA	
95	F: gtg gca gac cca cag atg R: cag aac cat gcc atg ggc	312	58				SSCA	
100	F: gta ccc tcc aag agt gct c R: gcc ctt atc cct tea cca c	290	60				SSCA	
101	F: ggc cat cct gcc cca gc R: gcc tcc cga cgc act gg	261	56				SSCA	
102	F: gtc ttc agc cct gcc tat c R: gcc atg gag cta gcc tgc	244	60				SSCA	

Additionally, information about the eight exons encoding the transmembraneous segments of RYR1 in the sarcoplasmic reticulum is shown.

^aPCR created restriction site.

Table 2. Concordance of the genetic results with the IVCT

Mutation	No. of families	Mutation				No mutation			
		MHS	MHE _h	MHE _c	MHN	MHN	MHE _h	MHE _c	MHS
Arg-614-Cys	9	18	1		1	11		1	
Arg-614-Leu	1	1							
Arg-2163-Cys	1	3				1			
Val-2168-Met	1	4				3			
Thr-2206-Met	3	4				1	1		
Thr-2206-Arg	1	6				12	2		
Gly-2434-Arg	7	23				5	1		2
Arg-2454-His	1	1				1			
Arg-2454-Cys	1	3				2	1		
Total	25	63	1		1	36	5	1	2

(14). Data of an EMHG meeting protocol (1995) showed this mutation in 5% of the British MH families, but it is reported only in one of 48 screened Danish immigrant MH families and in none of 44 Swedish MH families (15).

Phenotype and mutation severity

The Arg-2454-His mutation was first detected in an MH family (13). In one CCD family presenting with foot deformations, scoliosis and weakness of trunk muscles, we found the same amino acid change. This could imply greater severity of functional defect than produced by most of the other MH-causing mutations due to presence of clinical symptoms even in absence of triggering agents.

Similar correlations between clinical severity and very low trigger threshold values (0.5 mM for caffeine and 0.5% for halothane; Table 3) were found for the novel MH-causing mutation, Thr-2206-Arg, in which affected individuals showed clinical signs of myopathy but histologically no central cores (family MH 33).

In contrast, as suggested by the MHE_h and MHN test results of mutation carriers, Arg-614-Cys had higher thresholds and smaller contractures when compared with the data of all other mutations. Altogether, however, mutation severity is not the only decisive factor for clinical symptoms, but the mechanism of malfunction may be equally important as suggested by the very high halothane and caffeine contractures and quite low trigger threshold values found for the other novel mutation, Arg-2454-Cys, associated with MH without myopathy (MH 5).

Functional significance

Effects of MH-causing mutations on RYR1 function include both disturbances of excitation–contraction coupling (16,17) and/or intracellular calcium homeostasis (18,19). A putative hot spot for mutations could be the highly conserved transmembraneous segments presumably associated to the pore region because a leaky pore and subsequent intracellular calcium elevation would be an ideal possible pathogenetic mechanism both for MH crises as well as CCD myopathy. Surprisingly, screening for mutations in this region of the RYR1 did not reveal a single amino acid change. While low sensitivity of the screening method applied (SSCA) is likely a reason, another possible explanation might be the association with a different perhaps myopathic phenotype. In

any case, the occurrence of MH-causing mutations exclusively within the cytosolic region indicates a disturbance of RYR1 regulation regardless of whether by ligands or by direct interaction with the dihydropyridine receptor to be the mechanism underlying MH.

IVCT versus genetic data

In the 109 individuals of the 25 families with identified mutations studied here, there was almost perfect cosegregation of linkage/mutation analysis and IVCT results with only three exceptions: two non-carriers of the mutation in a Gly-2434-Arg MH family revealed borderline positive IVCT results, while an Arg-614-Cys carrier was tested as MHN. Remarkably, calculation of test parameters based on these genetic data yielded similar sensitivity (64 concordancies among 65 mutation carriers = 98.5%) but lower specificity (36 concordancies among 44 non-carriers = 81.8%) than values based on the Larach score [99.0 and 93.6% respectively (6)]. This is obviously associated with the apparent low specificity of halothane in the IVCT in our study in which MHE_h was only associated to a mutation in one of six cases. When contemplating only the caffeine contracture, sensitivity was equal 96.9% and the specificity rose to 93.2% corresponding to literature values. This is surprising, as our data is based mainly on the two most frequently occurring mutations Arg-614-Cys and Gly-2434-Arg. Therefore, these two may be considered to be representative for the severity of MH in the general population, namely the very population on which the IVCT quality parameters were originally calculated by Ordning *et al.* (6).

CONCLUSIONS

Genetic studies are a valuable supplement to MH diagnostic testing and can replace IVCT for typing individuals of families with known mutations, i.e. children. Interpretation of these results must be performed with care, though, because lack of the particular mutation segregating in the family does not exclude absence of further independent unknown mutations. Additionally, genetic screening is not yet suitable for routine diagnostics due to the low incidence of each mutation and the vastness of the gene. IVCT according to the EMHG is a reliable test even when comparing it with the MH-underlying genotype; it shows both high sensitivity and specificity, despite variable clinical expressivity during anesthesia. Next to resolving further genetic

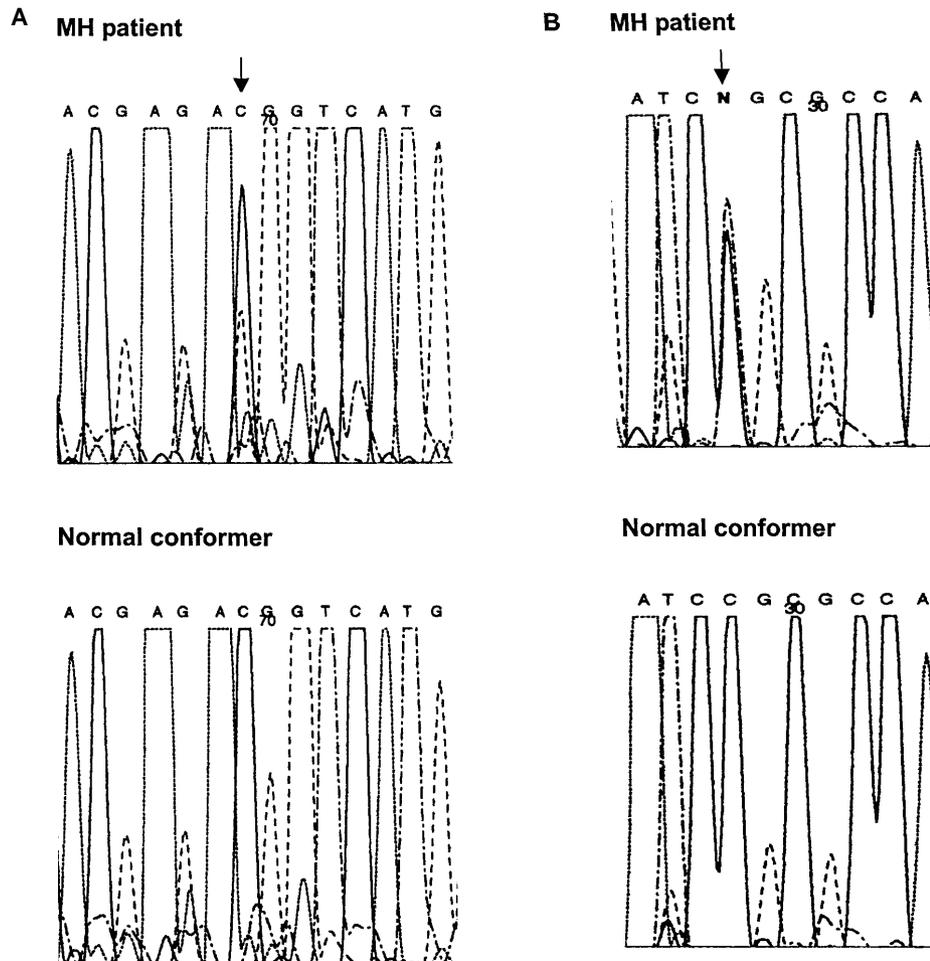


Figure 1. (A) Comparison of the RYR1 sequences of an MHS and an MHN family member of MH 33. Note a C→G base exchange predicting Thr-2206-Arg. (B) Comparison of the RYR1 sequences of an MHS and an MHN family member of MH 5. Note the C→T transition predicting Arg-2454-Cys.

causes underlying MH susceptibility, less invasive provocative tests should be developed.

MATERIALS AND METHODS

Pedigrees and IVCT phenotyping

In this study, nine CCD and 95 MH pedigrees of Western European nationality (96 German, four Austrian, two Swiss, one French and one Italian family) were collected in each of which at least one individual had experienced an anesthetic crisis. At least one further family member was tested to be MHS by IVCT according to the EMGH standardized protocol (5). Additionally, one CCD family with positive IVCT and positive histology but no known crisis was included so that our screening involved a total of 105 families.

MH crises and clinical symptoms

The first two of the following case reports are related to MH families in which we detected novel mutations; the third relates to a CCD family with a mutation described previously for MH.

MH33. Patient 309 had been operated on under general anesthesia using ether and succinylcholine three times without complication before he suffered from a fulminant MH crisis in 1985 at the age of 33 years. In the latter anesthesia during a meniscus operation, thiopental, halothane and succinylcholine were employed. Thirty minutes after initiation, hyperthermia (42.6°C), tachycardia (120 beats/min) and cyanosis were observed. Also, the patient had generalized muscle spasms and arrhythmia, while blood pressure remained normal. Dantrolene (1 mg/kg) was administered i.v. and external cooling of the body initiated. At a ventilation rate of 15 l/min, the blood pH was 7.59, the pCO₂ 23 mmHg and serum K⁺ 5.7 mM. Cardiac arrest occurred 1.5 h after intubation. The patient was successfully reanimated but died from kidney insufficiency 4 days later.

The patient's nephew suffered from a congenital myopathy with muscle hypotonia (floppy infant syndrome), clump-foot, finger contracture, high arch, inguinal hernia and hepatomegaly. The inguinal hernia was corrected under general anesthesia without complications. In 1983 at the age of 8 months, he was hospitalized because of pneumonia. One evening apnea and cardiac arrest occurred without warning. His temperature was elevated to 41.4°C, which was not treatable with antipyretics;

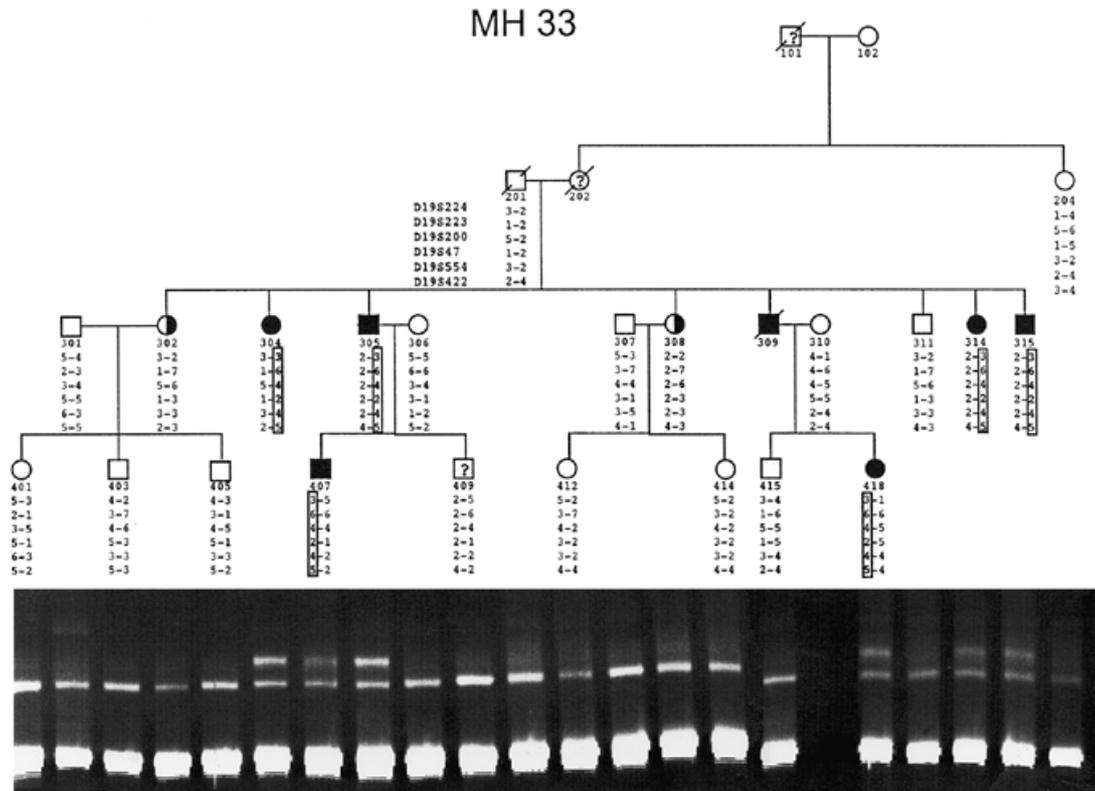


Figure 2. Pedigree of MH 33 heterozygous for the novel RYR1 mutation Thr-2206-Arg with haplotypes and corresponding 6% polyacrylamide/2% agarose gel. (A) The SSCA pattern shows the aberrant band pattern of the affected family members. (B) Forward primer with the base exchange C/G as the last base was used. PCR products were obtained only in MHS individuals. The IVCT results are indicated by filled (MHS), open (MHN) and half-filled symbols (MHE_h).

Table 3. Comparison of average caffeine and halothane thresholds and contractures for the different mutations in order to allow approximate assumption of mutation severity

Mutation	Caffeine		Halothane	
	Threshold (mM)	Contracture (mN) at 2 mM	Threshold (%)	Contracture (mN) at 2%
Arg-614-Cys	1.84 ± 0.18 (n = 25)	6.17 ± 1.34 (n = 25)	1.17 ± 0.12 (n = 27)	10.09 ± 1.51 (n = 24)
Arg-614-Leu	0.75 ± 0.18 (n = 2)	8.00 ± 1.41 (n = 2)	0.50 ± 0.00 (n = 2)	11.00 ± 2.12 (n = 2)
Arg-2163-Cys	1.50 ± 0.35 (n = 2)	6.25 ± 1.24 (n = 2)	1.50 ± 0.35 (n = 2)	11.55 ± 1.80 (n = 2)
Val-2168-Met	2.13 ± 0.19 (n = 8)	–	0.63 ± 0.08 (n = 8)	–
Thr-2206-Met	1.25 ± 0.18 (n = 2)	7.80 ± 3.81 (n = 2)	0.75 ± 0.18 (n = 2)	7.00 ± 1.27 (n = 2)
Thr-2206-Arg	0.50 ± 0.00 (n = 2)	–	0.50 ± 0.00 (n = 1)	–
Gly-2434-Arg	1.98 ± 0.18 (n = 22)	3.81 ± 0.74 (n = 21)	1.48 ± 0.13 (n = 22)	6.77 ± 1.23 (n = 22)
Arg-2454-Cys	1.00 ± 0.00 (n = 2)	6.00 ± 0.00 (n = 2)	0.50 ± 0.00 (n = 2)	12.00 ± 0.00 (n = 2)

n, number of tested muscle bundles.
Numbers indicate the mean ± SEM.

tachycardia (250 beats/min) and metabolic acidosis occurred. The patient died. Histological investigations of a muscle biopsy did not show any cores or other abnormalities.

MH5. A family member underwent an eye operation at the age of 7 years. During intubation, the masseter did not relax leading to two applications of 150 mg succinylcholine. Generalized muscle spasms occurred so that anesthesia was interrupted. The patient was suspected of having MH. During the first 2 h after terminating

anesthesia, a maximal tachycardia of 160 beats/min was observed. Maximal creatine kinase (CK) elevation was 5800 U/l and myoglobinuria was detected. Hours later, the patient presented muscle pain and weakness and general pain. Histological investigations showed no central cores.

MH89/CCD. The investigated patient had a progressive contracture of the Achilles tendon, muscle weakness and progressive lordosis. Muscle biopsies performed at the age of

10, 13, 19 and 22 years exhibited typical central cores. Myosonography revealed an increased echo intensity. Serum CK level was elevated to 644 U/l. A sister of the patient suffered from several foot deformations like pes equinovarus and a shortened Achilles tendon. She had a mild scoliosis and weakness of trunk muscles. Within the scope of a fourth foot operation under general anesthesia with pentothal penthrane 1966 at the age of 20, an epileptic seizure characterized by clonic contractions occurred. After subsequent cardiac arrest, the patient died. Further data about temperature, CK, etc. were not available.

Polymerase chain reaction (PCR)

Genomic DNA of each index patient was extracted from EDTA whole blood using the Cruachem ISOLATE 2 DNA extraction kit. PCR amplification was performed under standard PCR conditions [denaturation at 96°C for 1 min and 94°C for 4 min followed by 35 cycles of 94°C for 30 s, annealing at primer-specific annealing temperatures (Table 1) for 45 s, extension at 72°C for 30 s and a final extension of 72°C for 2 min]. The reaction mix contained 50 ng DNA, 50 pmol of each PCR primer (Table 1), 600 µmol dNTPs, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ and 1 U *Taq* polymerase (Amersham Pharmacia Biotech, Freiburg, Germany) in a final volume of 50 µl.

Mutation screening

Screening for mutations listed in Table 1 was performed either by restriction analysis or SSCA. For the former, PCR products were precipitated with ethanol and redissolved in 20 µl sterile H₂O. A reaction mix containing 10 µl PCR fragment, 3 U restriction enzyme (New England Biolabs, Schwalbach, Germany) (Table 1), 2 µl reaction buffer (10×; New England Biolabs) and aqua bidest filled up to a final volume of 20 µl was incubated at 37°C for 2–3 h. The digestion products were separated by agarose or polyacrylamide gel electrophoresis depending on their sizes and finally visualized by staining with 1 µg/ml ethidium bromide.

For the latter SSCA, PCR products were precipitated with ethanol and redissolved in 10 µl sterile H₂O. Precipitated PCR products (10 µl) were diluted with 2 µl of gel loading dye and denatured at 95°C for 8 min. The denatured single-stranded DNA was stored on ice water, loaded onto a 6% polyacrylamide gel and run at 300 V at 4/12°C for 4 h in buffer containing 89 mM Tris, 89 mM boric acid and 2 mM EDTA (pH 8.3). SSCA bands were visualized by staining with 1 µg/ml ethidium bromide.

Sequencing

Direct PCR sequencing was performed when an aberrant SSCA pattern or a change in digestion site was detected. PCR products containing the mutation were precipitated with ethanol, redissolved in 20 µl sterile H₂O and purified on a 6% polyacrylamide gel. Single bands were cut out and eluted in 50 µl sterile H₂O at 80°C for 30 min. Sequencing was performed with an Amersham Pharmacia Biotech cycle sequencing kit as described by the manufacturer (20) on a 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Linkage analysis

The microsatellite repeat markers (Cy-5 labeled) for the RYR1 locus (D19S191, D19S220, D19S224, D19S223, an intragenic marker RYR1, D19S190, D19S200, D19S47, D19S554,

D19S421, D19S422) were amplified by PCR and separated on denaturing polyacrylamide gels on an ALF Express (Amersham Pharmacia Biotech). LOD scores were calculated with the M-LINK computer program (21).

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