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Ryanodine receptor type 1 gene mutations found in the Canadian malignant hyperthermia population

Observation de mutations génétiques des récepteurs de la ryanodine de type 1 chez les patients canadiens atteints d'hyperthermie maligne

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Abstract

Purpose *Malignant hyperthermia (MH) is an autosomal dominant pharmacogenetic disorder that is manifested on exposure of susceptible individuals to halogenated anesthetics or succinylcholine. Since MH is associated primarily with mutations in the ryanodine receptor type 1 (RYR1) gene, the purpose of this study was to determine the distribution and frequency of MH causative RyR1 mutations in the Canadian MH susceptible (MHS) population.*

Methods *In this study, we screened a representative cohort of 36 unrelated Canadian MHS individuals for RYR1 mutations by sequencing complete RYR1 transcripts and selected regions of CACNA1S transcripts. We then analyzed the correlation between caffeine-halothane contracture test (CHCT) results and RYR1 genotypes within MH families.*

Results *Eighty-six percent of patients had at least one RyR1 mutation (31 out of 36), five of which were unrelated individuals who were double-variant carriers. Fifteen of the 27 mutations identified in RYR1 were novel. Eight novel mutations, involving highly conserved amino acid residues, were predicted to be causal. Two of the mutations co-segregated with the MHS phenotype within two large independent families (a total of 79 individuals). Fourteen percent of MHS individuals (five out of 36) carried neither RYR1 nor known CACNA1S mutations.*

Conclusions *The distribution and frequency of MH causative RyR1 mutations in the Canadian MHS population are close to those of European MHS populations. Novel mutations described in this study will contribute to the worldwide pool of MH-associated mutations in the RYR1 gene, ultimately increasing the value of MH genetic diagnostic testing.*

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Résumé

Objectif *L'hyperthermie maligne (HM) est une maladie pharmacogénétique héréditaire dominante autosomique qui se manifeste lors de l'exposition des personnes susceptibles à des anesthésiques halogénés ou à la succinylcholine. Étant donné que l'HM est principalement associée aux mutations au niveau du gène des récepteurs de ryanodine de type 1 (RYR1), l'objectif de cette étude était de déterminer la distribution et la fréquence des mutations RyR1 causant une HM chez la population canadienne susceptible à l'HM (SHM).*

Méthode *Dans cette étude, nous avons examiné une cohorte représentative de 36 personnes canadiennes SHM*

sans liens familiaux pour identifier les mutations RYR1 en séquençant des transcrits complets de RYR1 et des régions choisies de transcrits de CACNA1S. Nous avons ensuite analysé la corrélation entre les résultats de l'étude de la contractilité de la cellule musculaire en présence de caféine et d'halothane (test CHCT) et les génotypes RYR1 au sein des familles d'HM.

Résultats *Quatre-vingt-six pour cent (31 sur 36) des patients ont manifesté au moins une mutation RyR1, dont cinq sans liens familiaux étaient porteurs de la double variante. Quinze des 27 mutations identifiées sur le RYR1 étaient nouvelles. Huit mutations nouvelles, y compris des acides aminés bien conservés, ont été anticipées comme étant causales. Deux des mutations se sont co-ségrégées avec le phénotype SHM dans deux vastes familles indépendantes (au total 79 personnes). Quatorze pour cent des personnes SHM (cinq sur les 36) n'étaient porteuses ni des mutations de RYR1 ni de mutations connues de CACNA1S.*

Conclusion *La distribution et la fréquence de mutations de RyR1 causatives de HM dans la population canadienne SHM sont semblables à celles de populations européennes SHM. Les nouvelles mutations décrites dans cette étude s'ajouteront au fonds mondial de mutations associées à la HM dans le gène RYR1, ce qui contribuera à augmenter la valeur du dépistage diagnostique génétique de l'HM.*

Malignant hyperthermia (MH) is an autosomal dominant pharmacogenetic disorder triggered by exposure to volatile anesthetics and/or depolarizing muscle relaxants.[1,2] MH manifests as a potentially lethal hypermetabolic crisis caused by a rapid and uncontrolled increase in myoplasmic Ca^{2+} in skeletal muscle cells.[3,4] Since MH was first recognized as an inherited condition in 1960,[5] at least six potential genetic loci for MH susceptibility have been proposed.[6] However, MH-associated mutations have been found in only two genes, the *RYR1* gene (MIM 180 901) on chromosome 19q13.1 encoding the calcium release channel of the sarcoplasmic reticulum, also known as the ryanodine receptor type 1 (RyR1), and the *CACNA1S* gene (MIM 114 208) on chromosome 1q32 encoding the α1-subunit of the voltage-gated calcium channel, also known as the dihydropyridine receptor. Both RyR1 and dihydropyridine receptors play key roles in the processes of excitation–contraction coupling and maintenance of Ca^{2+} homeostasis in skeletal muscle cells.[7] Malignant hyperthermia genetic research has shown that *RYR1* is the major causal gene for MH as well as for a congenital myopathy, central core disease (CCD).[3,6] Malignant hyperthermia-associated *RYR1* mutations have been found in 60% to 86% of MH families with diverse ethnicity,[8–11] and their distribution and frequency have been shown to be population-specific.[6] Recent improvements in molecular genetics methods have facilitated genetic analysis of the entire coding region of *RYR1*, which is ~159,000 nucleotides long and contains 106 exons that are spliced into a 15 kb *RYR1* mRNA transcript.[12] The number of MH- and CCD-associated *RYR1* mutations continues to grow and, in our current estimate, includes some 300 mutations.[13] For the second causal gene, *CACNA1S,* only three MH-associated mutations have been reported.[14-18]

Genetic testing based on advances in MH genetic research is playing an increasingly important role in MH diagnostics. It has proven to be especially useful in early diagnosis of children and patients who cannot undergo the caffeine-halothane contracture test (CHCT) for MH susceptibility because CHCT requires an invasive muscle biopsy.[19,20] Nevertheless, genetic testing for MH has low sensitivity because the European Malignant Hyperthermia Group (www.emhg.org) and the Malignant Hyperthermia Association of the United States (www.mhaus.org) currently accept only 30 well-characterized mutations as being MH causative. Development of genetic testing with high sensitivity depends on the establishment of an exhaustive collection of MH-causative mutations; hence, the continuing search for new mutations in diverse populations as well as validation of their MH causality. The objective of our study was to identify the spectrum of MH-associated mutations in the Canadian MH susceptible (MHS) population and to establish their frequency and distribution.

Methods

Patients

For this observational study, Research Ethics Board approval was obtained from each of the Universities of Toronto and Ottawa. All of the MHS patients who presented to the Malignant Hyperthermia Investigation Unit at the Department of Anesthesia, Toronto General Hospital, Toronto, Canada from 2003 to 2008 were considered to be potential subjects of the study. The following inclusion criteria were used to maximize the likelihood that each individual selected was MHS: a positive CHCT[21] in individuals with a family history of MH; abnormally high levels of serum creatine kinase and a family history of MH;[22,23] a Clinical Grading Scale Score[24] of >35 if an individual had experienced an MH episode. Of 53 individuals approached, 36 (each representing an unrelated Canadian MH family) gave written informed consent for participation in our research study on the molecular genetics of malignant hyperthermia and were included in the study. Two of the 36 individuals were referred to us from the University of Ottawa. To analyze phenotype–genotype correlations,

relatives of the chosen individuals (a total of 116 individuals) subsequently consented to enrol in the study (Table 1).

Transcript sequencing

Patient blood and/or muscle samples were collected, and nucleic acids were isolated according to published procedures.[25,26] Ribonucleic acid (RNA) isolation from blood leukocytes as well as complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) amplification of the *RYR1* transcript were performed as described previously,[27] with minor modifications. Sequencing reactions were run at the DNA Sequencing and Synthesis Facility of The Centre for Applied Genomics, Toronto, Canada.

DNA sequence analysis and genetic analysis of amino acid substitutions identified

Raw sequence data analysis (contig building and sequence comparison to the reference *RYR1* sequences GenBank accessions NM_000540.2 and NC_000019) was carried out using Sequencher 4.7 (Gene Codes, Ann Arbor, MA, USA).

Each missense DNA sequence variant identified by *RYR1* transcript sequencing was confirmed by analysis of the patient's genomic DNA. A mutation-specific genotyping assay was designed for each novel mutation, and a panel of 200-300 normal control chromosomes was screened to assess the frequency of the mutation in the general population.

Genetic characterization of the novel mutations was performed using various bioinformatics tools via HTTP interface, primarily on the Expasy Web site (www.expasy.ch, Switzerland), the site of the European Institute of Bioinformatics (www.ebi.ac.uk, UK), and the site of the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov, USA). Additional bioinformatic analysis of each novel mutation in terms of its possible effect on RyR1 protein function was carried out using three bioinformatic computer programs: PolyPhen-2[28] (http://genetics.bwh.harvard.edu/pph2/); SIFT[29] (http://sift.jcvi.org/); and PMut[30] (http://mmb2.pcb.ub.es:8080/PMut/). These programs use bioinformatic data, such as protein sequence homology, structural information, or annotations of protein functional domains, as input, and they predict the probability that an amino acid substitution will be either neutral or damaging to the protein function. The three programs we selected have been tested[31] on human disease-associated mutation datasets and on datasets of neutral amino acid substitutions and have produced predictions with false-positive error rates from 9% to 20% and false negative error rates from 21% to 31%.

Results

RyR1 mutations in the Canadian MHS population

By sequencing entire *RYR1* transcripts, we identified 28 non-synonymous nucleotide sequence changes that would result in 27 different amino acid changes (mutations) in 31 out of 36 MHS individuals. In this study of *RYR1*, our mutation detection rate was 86% (Table 1). Five unrelated individuals in this cohort were double-variant carriers.

Of the 27 RyR1 mutations identified, seven are known MH-causative mutations, five have been reported previously in association with MH, and 15 are novel. All except one of the novel mutations represent single nucleotide changes and all were heterozygous. None of the novel mutations was found in at least 200 control chromosomes.

Previously reported RyR1 mutations

Fifteen of 31 Canadian MHS individuals with identified mutations (48.4%) carried known MH causative RyR1 mutations (www.emhg.org): p.Arg163Cys (*n* = 1), p.Gly341Arg (*n* = 3), p.Arg614Cys (*n* = 4), p.Arg2163His (*n* = 2), p.Val2168Met (*n* = 1), Gly2434Arg (*n* = 4), p.R2454H (*n* = 1). One patient was a carrier of two causative mutations, p.Gly341Arg and p.Arg614Cys, that were transmitted to both of the patient's children, indicating that the mutations might be located on the same allele.

Five other unrelated patients carried recurrent mutations not yet proven to be causal of MH, p.Arg1667Cys,[32] p.Ala2200Val,[8] p.Arg2336His,[10] p.Val4849Ile,[33] p.Gly4935 Ser.[34] One individual from this group carried a novel mutation, p.Glu209Lys, in combination with a recurrent mutation, p.Arg2336His.

Novel RYR1 mutations

We identified 15 novel RyR1 mutations in 12 patients. Three patients carried two novel RyR1 mutations each: a patient from family C-16 (p.Cys64Arg/p.Ala3421Val); a patient from family C-20 (p.Arg2126Gln/p.Gly3938Asp); and a patient from family C-25 (p.Ala4185Thr/p.Val4842-Met). In addition, a patient from family C-26 carried an *RYR1* sequence variant involving two adjacent nucleotides, c.14422T>A c.14423T>A (Fig. 1). The allelic status of these changes was resolved by real-time PCR using a set of allele-specific primers (data not shown). Both mutations were located on the same allele, so that the amino acid change is predicted to be p.Phe4808Asn. It is of interest that the mutation, c.14422T>A, resulting in the amino acid change, p.Phe4808Ile, has been reported previously in association with MH.[35]

Table 1 Summary of clinical phenotype and *RYR1* transcript screening results for a cohort of patients representing Canadian MH families

Family	MH status*	CHCT results†		Exon	Nucleotide change	Amino acid change	# relatives enrolled
		Caf	Hal				
C-1	CHCT+	4.8	19.5	6	c.487C>T	p.Arg163Cys	1
C-2	CHCT+	4.8	6.2	11	c.1021G>A	p.Gly341Arg	1
C-3	CHCT+	1.3	3.4	11	c.1021G>A	p.Gly341Arg	1
C-4	CHCT+	6.5	9.2	11, 17	c.1021G>A, c.1840C>T	p.Gly341Arg, p.Arg614Cys	3
C-5	CHCT+	2.4	4	17	c.1840C>T	p.Arg614Cys	1
C-6	CGS >35	nd	nd	17	c.1840C>T	p.Arg614Cys	1
C-7	CGS >35	nd	nd	17	c.1840C>T	p.Arg614Cys	6
C-8	CHCT+	2.8	12.5	39	c.6488G>A	p.Arg2163His	1
C-9	CGS >35	nd	nd	39	c.6488G>A	p.Arg2163His	1
C-10	MH fam, CK	nd	nd	39	c.6502G>A	p.Val2168Met	1
C-11	CHCT+	1.3	5.2	45	c.7300G>A	p.Gly2434Arg	23
C-12	CHCT+	1.8	7.6	45	c.7300G>A	p.Gly2434Arg	1
C-13	CHCT+	0.1	3.3		c.7300G>A	p.Gly2434Arg	1
C-14	CHCT+	0.4	2.4	45	c.7300G>A	p.Gly2434Arg	1
C-15	CHCT+	1.3	6.2	46	c.7361G>A	p.Arg2454His	4
C-16	CHCT+	1.8	4.4	3, 68	c.190T>C,c.10262C>T	**p.Cys64Arg, p.Ala3421Val**	1
C-17	MH fam, CK	nd	nd	5	c.418G>A	**p.Ala140Thr**	3
C-18	CHCT+	2.6	6.8	7, 43	c.625G>A, c.7007G>A	**p.Glu209Lys**, p.Arg2336His	7
C-19	CHCT+	1.3	0.8	34	c.4999C>T	p.Arg1667Cys	2
C-20	CHCT+	0.2	1.7	39, 86	c.6377G>A, c.11813G>A	**p.Arg2126Gln, p.Gly3938Asp**	1
C-21	CHCT+	0.4	3.9	40	c.6599C>T	p.Ala2200Val	1
C-22	CHCT+	1.3	1	54	c.8527T>C	**p.Ser2843Pro**	2
C-23	CGS >35	nd	nd	66	c.9850T>A	**p.Trp3284Arg**	1
C-24	CGS >35	0.1	0.6	90	c.12310G>C	**p.Gly4104Arg**	1
C-25	CHCT+	0.4	1.8	90, 101	12553G>A, c.14524G>A	**p.Ala4185Thr, p.Val4842Met**	1
C-26	CGS >35	nd	nd	100	c.14422T>A, c.14423T>A	**p.Phe4808Asn**	2
C-27	CHCT+	2.1	8	101	c.14539G>C	**p.Val4847Leu**	72
C-28	CHCT+	1	6.4	101	c.14545G>A	p.Val4849Ile	1
C-29	CGS >35	nd	nd	102	c.14782A>G	**p.Ile4928Val**	2
C-30	CHCT+‡			103	c.14803G>A	p.Gly4935Ser	2
C-31	CHCT+	1.4	9.5	104	c.14968A>G	**p.Met4990Val**	1
C-32	CHCT+	1	4.8				**1**
C-33	CHCT+	0.45	2				1
C-34	CHCT+	0.35	2.2				1
C-35	CHCT+	0.1	3.1				1
C-36	CHCT+	0.05	1.3				1

*Malignant hyperthermia (MH) status of individuals selected for genetic study from corresponding families; † Results of caffeine-halothane contracture test (CHCT) according to the North American protocol[21]; Caf = contracture force in grams in the presence of 2 mM caffeine; Hal = contracture force in grams in presence of 3% halothane; ‡CHCT was done in 1974 and the results were positive

CGS >35 = MH event with MH Clinical Grading Score more than 35; CHCT+ = positive CHCT results; MH fam, CK = family history of MH and high resting creatine kinase level; nd = CHCT not done

C-1 – C-15, families with MH causative *RYR1* mutations; C-16 – C-31, families with recurrent and novel *RYR1* mutations; C-32 – C-36, families without *RYR1* mutations

Nucleotide and amino acid numbering is according to the reference sequences of GenBank accessions NM_000540.2 and NP_000531.2, respectively, with +1 corresponding to adenine of the ATG translation start codon. Novel mutations are shown in boldface

F4808N

C T A C A A C A A C T T C T T C T T T G C T

control

C T A C A A C A A C W W C T T C T T T G C T

proband
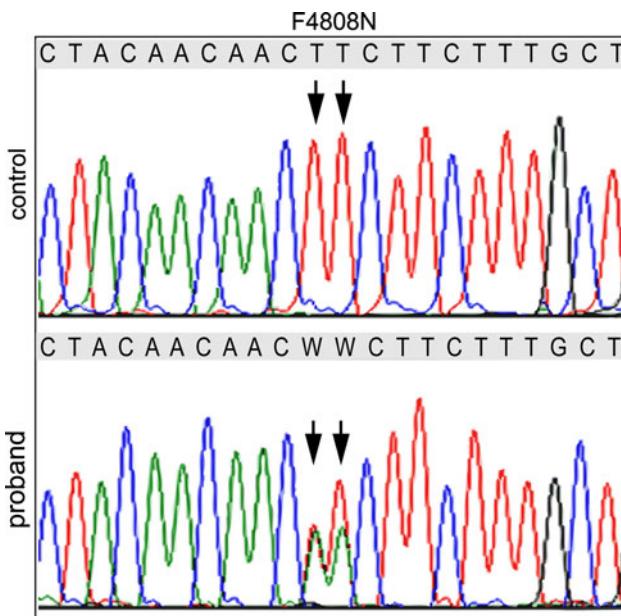
Fig. 1 DNA sequencing chromatogram illustrating the novel mutation, F4808N, resulting from the change of two consecutive nucleotides AA>TT. W denotes adenine or thymine

RYR1 polymorphisms

Three previously reported RyR1 polymorphisms, p.Pro1787Leu (rs34934920), p.Gly2060Cys (rs35364374), and p.Glu3583Gln (rs55876273) (NCBI dbSNP, www. ncbi.nlm.nih.gov/projects/SNP/), were found to be present either alone (p.Glu3583Gln, $n = 1$) or together with other mutations in four unrelated patients. In addition, four novel and 26 previously reported silent polymorphisms were detected.

Splicing variants

In line with previous reports,[6,10] RYR1 transcripts with skipped exons 70 and 83 were present in most of our samples. In addition, skipping of exons 9, 31, 66, and 94 was observed in blood RNA. In all cases, missing exons, together with their adjacent intronic regions, were resequenced directly from genomic DNA samples to confirm that their absence was not due to aberrant splicing that might have been caused by a contiguous mutation.

Patients without mutations

In five of our MHS patients from families C-32 to C-36 in Table 1, we found no RYR1 mutations. Screening of the CACNA1S gene in these patients for the presence of three known MH causative mutations, p.Arg174Trp, p.Arg1086His, and Arg1086Ser,[14–18] also gave negative results.

Protein localization and predicted effect of identified RyR1 mutations

All except one mutation involved amino acid residues that were positioned within RyR1 regions of high evolutionary conservation; furthermore, 13 mutations were mapped within known RyR1 functional domains. According to the results of bioinformatic analysis, the recurrent and novel mutations could be divided into three groups: probably causative, possibly causative, and benign (Table 2).

Phenotype–genotype analysis

Two unrelated families, C-27 and C-18, were available for segregation analysis (Fig. 2). Family C-27 is a large three-generation French–Canadian family in which three fatal MH episodes have occurred, and 38 out of 76 relatives have been diagnosed as MHS from 1993 to 2008. A novel p.Val4847Leu mutation was detected in an MHS individual from a family branch with two fatal MH episodes; the mutation segregated well with the MHS phenotype within this family branch (Fig. 2A). However, 26 MHS individuals from other branches of the family did not carry the p.Val4847Leu mutation. Of these, 21 had a positive contracture test with halothane but were negative for caffeine-induced contracture. Thus, another undetected RYR1 mutation may exist in these branches of the family. In family C-18 (Fig. 2B), the p.Arg2336His mutation was identified in combination with a novel p.Glu209Lys mutation in a proband diagnosed as MHS by CHCT. Genetic screening of the family showed that both mutations were located on the same allele and that they segregated well with the MHS phenotype. However, one discordant individual, the maternal aunt of the proband, was diagnosed as MHS but did not carry either of the two mutations.

In the remaining 29 families, the absence of CHCT data for additional family members prevented segregation analysis. Analysis of families C-19, C-22, C-26, and C-30 showed that four mutations identified in the MHS individuals (p.Arg1667Cys, p.Ser2843Pro, p.Phe4808Asn, p.Gly4935Ser, correspondingly) were all absent in relatives with negative CHCT results (MHN). In contrast, mutations p.Ala140Thr and p.Gly4104Arg, identified in probands from families C-17 and C-24, were also found in an MHN relative in each case. Moreover, the mutation p.Ile4928Val, identified in the proband from family C-29, was absent from his MHS relative.

Discussion

In this study aimed at the identification of MH-associated RYR1 mutations in the Canadian MHS population, seven

Table 2 Bioinformatic analysis of RyR1 mutations identified in Canadian malignant hyperthermia susceptible population

Amino acid change	Causal potential*			Functional Domain†	Positional conservation‡
	SIFT	Pmut	PolyPhen2		
p.Cys64Arg	Not tolerated	Pathological	Probably damaging	Yes	1
p.Arg2336His	Not tolerated	Pathological	Probably damaging	Yes	1
p.Ser2843Pro	Not tolerated	Pathological	Possibly damaging	Phosphoserine	2
p.Trp3284Arg	Not tolerated	Pathological	Probably damaging	No	1
p.Gly3938Asp	Not tolerated	Pathological	Probably damaging	Yes	1
p.Phe4808Asn	Not tolerated	Pathological	Possibly damaging	Yes	1
p.Val4847Leu	Not tolerated	Pathological	Possibly damaging	Yes	1
p.Gly4935Ser	Not tolerated	Pathological	Possibly damaging	Yes	1
p.Ala140Thr	Tolerated	Neutral	Possibly damaging	Yes	3
p.Glu209Lys	Tolerated	Pathological	Probably damaging	Yes	3
p.Arg1667Cys	Not tolerated	Pathological	Benign	No	4
p.Arg2126Gln	Not tolerated	Pathological	Benign	No	1
p.Ala2200Val	Tolerated	Pathological	Benign	Yes	2
p.Ala3421Val	Tolerated	Pathological	Benign	No	2
p.Gly4104Arg	Tolerated	Pathological	Benign	Yes	5
p.Val4842Met	Not tolerated	Neutral	Possibly damaging	Yes	1
p.Val4849Ile	Tolerated	Neutral	Possibly damaging	Yes	1
p.Ile4928Val	Not tolerated	Neutral	Possibly damaging	Yes	1
p.Met4990Val	Not tolerated	Pathological	Unknown	No	5
p.Ala4185Thr	Tolerated	Neutral	Benign	No	6

*Causal potential refers to prediction of mutation effect as made by the respective computer programs (SIFT[29], PMut[30], PolyPhen[28]): substitutions damaging to the protein function are labelled as not tolerated, pathological, or probably/possibly damaging; neutral substitutions are labelled as tolerated, neutral, or benign

†Mutation position within known RyR1 functional domains (www.ebi.ac.uk/interpro/)

‡Positional conservation of the amino acid residue affected by mutation: 1 = conserved across mammalian RyR1 and between human RyR1, RyR2, and RyR3 isoforms; 2 = conserved across mammalian RyR1 and between human RyR1 and RyR2 isoforms; 3 = conserved across mammalian RyR1 and between human RyR1 and RyR3 isoforms; 4 = conserved across only human RyR isoforms; 5 = conserved across only RyR1 in most mammals; 6 = conserved across only RyR1 in some mammals

known MH causal RyR1 mutations and 20 potentially causal mutations, including 15 novel mutations, were identified (a mutation detection rate of 86%) (Table 1). None of the MHS individuals included in the study showed any myopathic features, including individuals who carried two MH causative mutations each. This observation is consistent with a published report for clinically asymptomatic MHS patients who were homozygous or compound heterozygous for MH causative mutations[15] and with the conclusions of early exhaustive studies showing the absence of morphological abnormalities in MH muscle.[13]

The high *RYR1* mutation detection rate observed in this study can be explained by the stringent selection criteria for enrolment into the study and by the mutation screening strategy, which consisted of sequence analysis of complete *RYR1* transcripts. Comparable detection rates (70% to 86%) have been reported recently for other MHS populations[8–10] where genetic screening included the entire coding sequence of the gene. On the other hand, if only established MH causative mutations from the EMHG database (www.emhg.org) were considered, then the mutation detection rates or the diagnostic sensitivity of the genetic testing would be 42% in this study and from 28% to 33% in other studies.[8–10] This does not compare favourably with a reported sensitivity of 97% to 99% for CHCT and *in vitro* contracture test (IVCT), respectively.[36,37] Published data indicate that the frequency of MH-causative RyR1 mutations is population-specific, with only a few mutations accounting for the majority of MH cases in some populations.[6] By comparison, we found that p.Arg614Cys, p.Gly341Arg, and p.Gly2434Arg are the most frequent mutations in the Canadian MHS population, accounting for almost 35% of mutation-positive families. This outcome is not unexpected since these mutations are common in Germany, France, and the UK (Table 3), and individuals of Western European origin comprised >60% of our study group. The result also reflects the ethnic structure of the Canadian population, which consists of up to 75% of
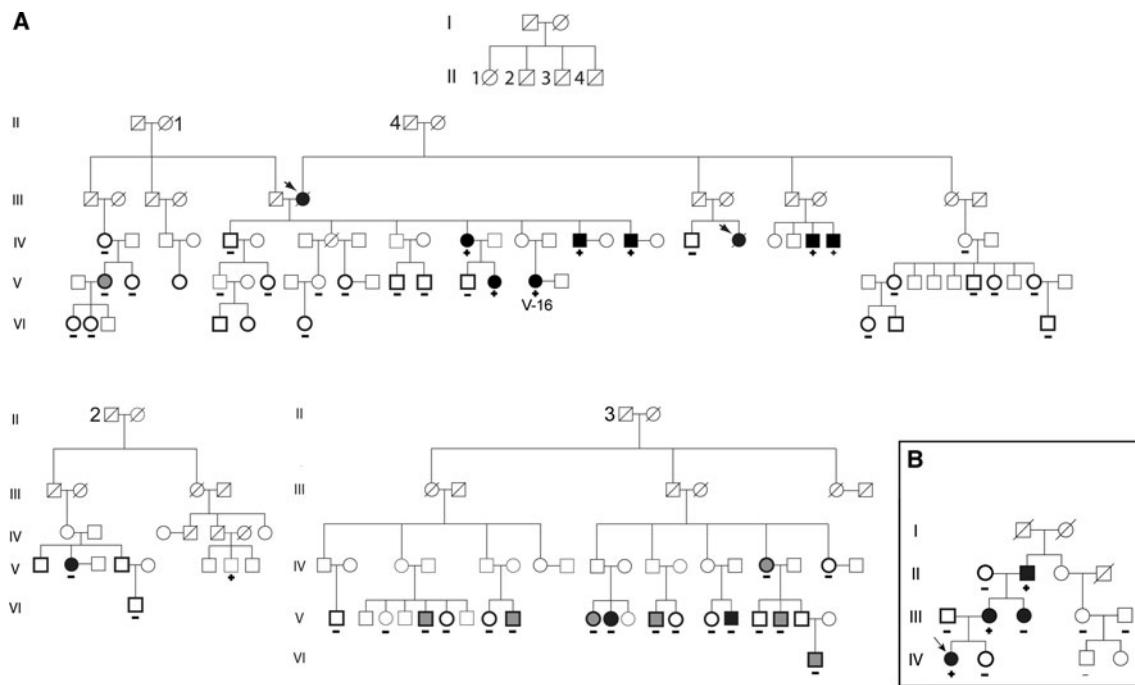
Fig. 2 *A*. Condensed pedigree of malignant hyperthermia family C-27. *B*. Pedigree of MH family C-18. Filled symbols, individuals diagnosed as MH Susceptible by caffeine-halothane contracture test (CHCT);[21] open symbols, with thick border individuals with negative CHCT; open symbols, individuals not subjected to CHCT; open symbols with diagonal line, deceased. Arrows indicate probands. Numbers from I to VI identify generations. In A, four family branches represent descendants of four siblings (generation II, individuals 1, 2,

3, 4); plus symbols indicate carriers of the p.Val4847Leu mutation; individuals with positive contracture results for halothane only are indicated by grey-filled symbols with thick border; individuals who died of malignant hyperthermia crisis are indicated by filled symbols with diagonal line. V-16 was the individual for whom screening of the complete *RYR1* transcript was done. In B, carriers of two mutations, p.Glu209Lys and p.Arg2336His, are indicated by a plus symbol; non-carriers of the mutations are indicated by a minus symbol

individuals with a genetic heritage from England, Scotland, Ireland, France, and Germany (data from the Canada Census 2006). A high proportion of mutations with unknown causal potential (55.6%) observed in this study is comparable with that found in other published studies (40% to 78%).[8–10] Although we have not yet performed functional testing of the novel RyR1 mutations, genetic analysis allowed evaluation of their potential as probably causative, possibly causative, and benign (Table 2). The group of probably causative mutations contains eight mutations: p.Cys64Arg, p.Arg2336His, p.Ser2843Pro, p.Trp3284Arg, p.Gly3938Asp, p.Phe4808Asn, p.Val4847-Leu, and p.Gly4935Ser. These mutations affected evolutionarily highly conserved amino residues within well-conserved protein regions and were predicted with high reliability to be deleterious. The MH causative nature of two mutations from this group, p.Arg2336His and p.Val4847Leu, is supported by their co-segregation with the MHS phenotype in families C-18 and C-27, respectively. In addition, p.Arg2336His found *in cis* with a novel mutation, p.Glu209Lys, in a Canadian MHS proband was recently reported to be causal for MH in several Swiss families.[10] The p.Val4847Leu that co-segregated well with an MHS phenotype within a large French–Canadian

family, C-27, is positioned within the putative transmembrane segment and maps to the HS3b "mutation hot spot" region in RyR1.[13,38] At least three other closely spaced MH/CCD mutations are located within the same transmembrane segment[33,39,40] (Fig. 3). The mutations p.Ser2843Pro and p.Gly4935Ser deserve special consideration because they affect critical amino acid residues. Ser2843, identified in an MH proband (family C-22) with positive CHCT results, has the potential of being involved in the regulation of Ca^{2+} release channel function through serine phosphorylation.[41,42] The second mutation, p.Gly4935Ser, identified in an MHS first-degree relative of the proband who had a fatal MH crisis, maps to the centre of the last transmembrane segment (amino acids 4918-4948) within the pore-forming region of RyR1[38] and a mutation hot spot, HS3b, where disease-associated mutations occur at a rate of about one in every three amino acids.[13] Furthermore, the p.Gly4935Ser was recently reported to be associated with MH in a large Brazilian pedigree.[34] Mutations involving adjacent amino acid residues, p.Ile4938Met and p.Asp4939Glu, have also been associated with MH.[43] The second group is comprised of mutations with yet unclear causal potential. Two of them, p.Ala2200Val and p.Val4842Met, each found in an MHS

Table 3 Frequencies of the most common malignant hyperthermia causative *RYR1* mutations

	p.R163C	p.G341R	p.R614C	p.T2206M	p.G2434R	p.R2454H
Canada*		9.7%	12.9%		12.9%	
UK		8%		10%	40%	
Germany			21.4%	16%	3.6%	7%
France		11%	15%		4%	
USA	9.5%				14.3%	

*Data for Canada (this paper), United Kingdom,[6] Germany,[46] France,[40] and USA[8]
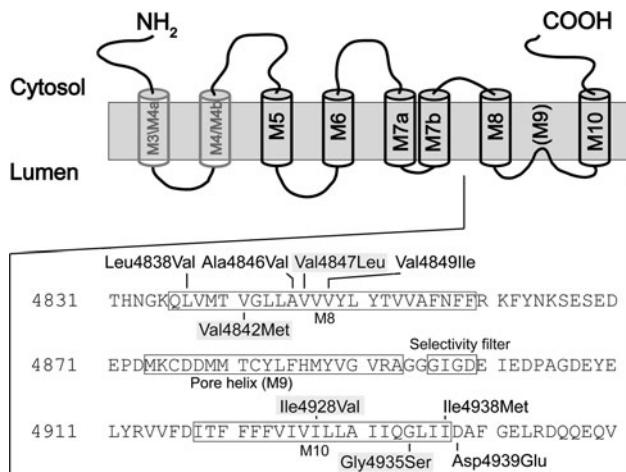
Fig. 3 Location of the malignant hyperthermia-associated mutations within the channel pore-forming region according to the proposed model of RyR1.[38] Novel mutations are indicated by grey background; M3 to M10 = transmembrane segments

first-degree relative of a proband in their families, might be considered as possibly causative. The p.Ala2200Val lies within HS2a and maps within a region in RyR1 (amino acids 1924-2446) shown to be essential for E–C coupling in skeletal muscle.[44] This mutation was linked to MH in two unrelated families from two different populations.[6] Moreover, several other MH-associated mutations are located in the same region.[45–47] The second mutation, p.Val4842Met, maps to the last transmembrane segment that forms a part of the Ca^{2+} channel pore of RyR1. It was recently found in two unrelated patients with recessive forms of CCD.[48] In contrast, another mutation from this group, p.Ala140Thr, is most likely a rare benign polymorphism similar to the only mutation from the third group, p.Ala4185Thr, which was predicted to be neutral by bioinformatic programs. This prediction is supported by the results of segregation analysis; p.Ala140Thr was identified in a second-degree relative of a proband, whereas the proband and another MHS relative did not carry this mutation. The occurrence of benign *RYR1* mutations highlights the point that knowledge of the entire spectrum of *RYR1* variants occurring in both MHS and MHN individuals would facilitate the identification of mutations that

are associated with the MHS phenotype. Unfortunately, it is not yet feasible to screen MHN individuals or the general population for *RYR1* mutations.

Despite being labelled as MHS on the basis of their CHCT results and representing families with a clear history of MH, five individuals in this study (14%) carried neither mutations in *RYR1* nor known mutations in *CACNA1S*. The absence of *RYR1* mutations in MHS individuals, together with the presence of discordant cases in families C-18 and C-27 and other MH families worldwide,[8,10] may be explained by the sensitivity and specificity of the CHCT, which identifies both false negatives and false positives at a low rate.[36,37,49] Another reason might be the possible involvement of as yet unrecognized genetic and environmental factors in the MHS phenotype. For example, mutations in calsequestrin (*CASQ1*) might be expected to cause MH, since a mouse *CASQ1* knockout line displays MHS symptoms.[50] The complex genetic nature of MH, revealed through the existence of discordant cases, is the reason why negative genetic results based on *RYR1* screening alone cannot rule out a diagnosis of MH susceptibility.

In conclusion, this study has provided insight into the spectrum of MH-associated *RYR1* mutations in the Canadian MHS population by showing that it is close to that of certain European MHS populations. Genetic analysis shows that eight novel mutations, p.Cys64Arg, p.Arg2336His, p.Ser2843Pro, p.Trp3284Arg, p.Gly3938Asp, p.Phe4808Asn, p.Val4847Leu, and p.Gly4935Ser, are good candidates for MH causative mutations. However, additional segregation data as well as functional characterization of the novel mutations are still needed to establish their role in MH. Another limitation of this study is that it included only about 20% of MH families registered in the Malignant Hyperthermia Investigation Unit database. Further genetic screening of the remaining MH families will help identify the full spectrum of MH-associated mutations in Canada. Our data corroborate some of the results reported recently in other MH populations and emphasize the fact that the rate of discovery of novel mutations remains high. This study will contribute to the worldwide pool of MH-associated mutations, ultimately increasing the value of MH genetic diagnostic testing.

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