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Detection of a Novel Ryanodine Receptor Subtype 1 Mutation (R328W) in a Malignant Hyperthermia Family by Sequencing of a Leukocyte Transcript

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Background: **To determine whether malignant hyperthermia (MH) susceptibility in a Canadian pedigree is associated with a mutation in the ryanodine receptor subtype 1 (*RYR1*) gene, the complete RYR1 transcript obtained from the leukocytes of one MH-susceptible family member was sequenced, using a newly developed protocol.**

Methods: **RNA was extracted from leukocytes and converted into complementary DNA. Overlapping fragments of *RYR1* complementary DNA were amplified by the polymerase chain reaction and used for double-strand sequencing to find a single mutation likely to be causal of MH susceptibility. Inheritance of the mutation in the family was studied by restriction endonuclease analysis and/or sequencing of genomic DNA and compared to available caffeine halothane contracture test data. The mutation was introduced into rabbit *RYR1* complementary DNA, the complementary DNA was expressed in human embryonic kidney line 293 cells, and Ca^{2+} release by the mutant Ca^{2+} release channel was measured following the addition of caffeine and halothane.**

Results: **A novel arginine 328 to tryptophan mutation in *RYR1* was detected by direct sequencing of the *RYR1* transcript from leukocytes of one MH-susceptible individual. A causal role for this mutation in MH is indicated by cosegregation of the mutation with the MH-susceptible phenotype within the family and by the demonstration that the mutant channel has increased sensitivity to both caffeine and halothane.**

Conclusions: **The feasibility of using complete *RYR1* transcripts from leukocytes for sequence analysis offers an efficient and noninvasive method for scanning *RYR1* for novel mutations.**

MOLECULAR genetic and linkage studies have shown unambiguously that the skeletal muscle ryanodine receptor (RyR1) plays a crucial role in the pathogenesis of malignant hyperthermia (MH) as well as of the related myopathy, central core disease (CCD). Though defects in another gene could influence predisposition to MH/CCD, linkage has been demonstrated only to the genes encoding the α subunit of the sodium channel, *SCN4A*, and the α-1 subunit of the L-type calcium channel, *CACNA1S*, in a few families.[1–4] By contrast, linkage to *RYR1* has been established for many MH/CCD families from various populations.[5,6] Reevaluation of a recent analysis of 20 large MH-susceptible (MHS) families,[7] based on the assumption that a single recombinant in a family could be explained by the less than 100% accuracy of the Caffeine–Halothane Contracture Test (CHCT), showed that more than 85% of the families investigated could be linked to *RYR1*.[8] More stringent selection of MHS patients increased the level of mutation identification in *RYR1* to 70% of MHS index cases, even if only a part of the *RYR1* gene was screened.[9]

The total number of *RYR1* mutations associated with MHS or CCD now exceeds 50.[9–12] The mutation frequencies vary, depending on the MH population investigated,[6] and many mutations have been found in only a single MH family. Neomutations in the *RYR1* gene have been revealed in approximately 10% of CCD families investigated.[13] In light of the high level of allelic heterogeneity in *RYR1*, it is clear that molecular genetic testing cannot yet be reduced to the automated detection of a few frequent mutations, but, rather, must involve a complete *RYR1* sequence scan.

The search for novel mutations in the *RYR1* gene is hampered by the large size of the gene, approximately 160 kb, and its complex structure: the 15 kb *RYR1* transcript is built up through the splicing of 106 exons with an average size of 144 base pairs (bp).[14] The majority of known MH and CCD mutations, however, occur within three regions of the *RYR1* gene: MH/CCD region 1 covers exons 2–17, region 2 covers exons 39–46, and region 3 covers exons 100–104. Consequently, mutation screening is usually limited to these regions of the gene, which comprise less than 30% of the RYR1 transcript.[8,9,13,15]

In the accompanying article,[16] we reported a method for the amplification of the complete *RYR1* transcript from muscle samples as eight overlapping polymerase chain reaction (PCR) fragments. Direct sequencing of these fragments allowed the efficient identification of all polymorphisms present in the transcript. Taking advantage of the fact that B lymphocytes also express *RYR1*,[17] we demonstrated that the method could be used with

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Readers interested in this topic should also see the article presented on page 289 of this issue (Kraev N, Loke JCP, Kraev A, MacLennan DH: Protocol for the sequence analysis of ryanodine receptor subtype 1 gene transcripts from human leukocytes. ANESTHESIOLOGY 2003; 99:289–96).

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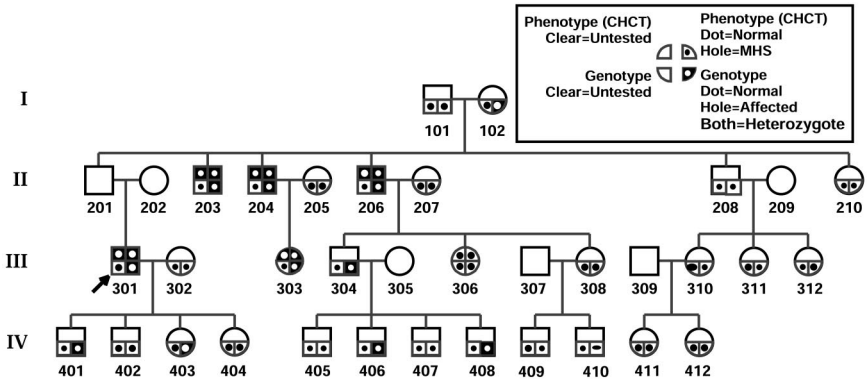

Fig. 1. The pedigree of a Canadian malignant hyperthermia family and correlation of the presence of the R328W mutation with caffeine–halothane contracture test (CHCT) results. MHS = malignant hyperthermia susceptible.

some modifications for the amplification and analysis of complete *RYR1* transcripts from leukocytes isolated from fresh clinical blood samples.

Here, we report the detailed characterization of a novel Arg328Trp mutation in *RYR1* that was detected by the direct sequencing of the *RYR1* transcript from leukocytes of one MHS individual. The causative role for this mutation in MH was confirmed by cosegregation of the mutation with the positive MH phenotype within the family and by functional analysis.

Materials and Methods

Following Institutional Review Board (University of Toronto, Toronto, Ontario, Canada) approval and written and informed consent, 30 members, representing four generations, of one Canadian family were available for this study (fig. 1). The proband of the family (301) had a perioperative reaction in 1978, diagnosed clinically as an MH crisis. Although the original medical records were destroyed, remaining records indicate that he experienced generalized body rigidity, an elevated temperature, and an elevated creatine phosphokinase concentration. Five of his relatives (203, 204, 206, 303, 306) underwent CHCT in accordance with the standards of the North American Malignant Hyperthermia Group protocol.[18] The CHCT results are listed in table 1.

Because muscle samples were no longer available from any of the family members tested, this family was chosen for analysis by *RYR1* sequencing from leukocyte messenger RNA.

Blood collection from individual 303, RNA extraction from leukocytes, and subsequent *RYR1* transcript sequencing were performed exactly as described in the accompanying article.[16] Genomic DNA was also extracted from blood using QIAamp DNA Blood Midi Kit (QIAGEN Inc., Mississauga, Ontario, Canada).

To screen for the presence of the C982T mutation in genomic DNA, a 395-bp fragment of the *RYR1* gene spanning the part of exon 11 in which C982 is located and part of the adjacent intron 11 was PCR amplified from genomic DNA samples using forward (5'-GGAGTGGGAGCCACCTGCGCT-3') and reverse (5'-CCACAGTCCTGAGGCCACATGCTGC-3') primers. PCR was performed in 50 μl of 1X PCR Gold Buffer (Applied Biosystems, Foster City, CA) with 150 ng genomic DNA, 5% dimethyl sulfoxide, 0.3 μM of each primer, 0.2 mM deoxynucleotide triphosphates, 2.5 mM $MgCl_2$, and 1.5 units of AmpliTaq Gold polymerase (Applied Biosystems). Cycling parameters were as follows: denaturation at 95°C for 10 min, five cycles of touchdown from 70°C to 60°C with denaturation at 95°C for 15 s, and annealing–extension at 70°C to 60°C for 30 s followed by 25 cycles of 95°C for 15 s, annealing at 72°C for 30 s, and a final extension step at 70°C for 10 min, held at 4°C.

Polymerase chain reaction products were purified using QIAquick PCR purification kit (QIAGEN Inc.) and screened for the presence of the C982T mutation, either by direct sequencing or by restriction fragment length

Table 1. Malignant Hyperthermia Status of Family Members Included in the Study

Patient	Diagnosis	Contracture (g) at 2 mM Caffeine	Contracture(s) at Halothane		CSC (mM Caffeine)	CSC-H (mM Caffiene)
			3%	2%		
203	MHS	4.2	7.4			
204	MHS	2.15	2.6			
206	MHS	1.2	8.4			
303	MHS	2.2	6.4			
306	MHN			0	5.35	3.82

CSC = caffeine-specific contracture; CSC-H = caffeine-specific contracture in halothane; MHN = malignant hyperthermia–negative; MHS = malignant hyperthermia–susceptible.

polymorphism pattern analysis after digestion with the restriction endonuclease *Aci*I (New England BioLabs, Beverly, MA). The restriction fragments were resolved in 10% polyacrylamide gel and stained with SYBR Green 1 Nucleic Acid Gel Stain (Roche Diagnostics GmbH, Basel, Switzerland).

The functional effect of the C982T mutation was studied by comparing the effect of caffeine and halothane on Ca^{2+} release of wild-type and mutant RyR1 proteins expressed in human embryonic kidney line 293 cells essentially as described.[19,20] Briefly, caffeine and halothane sensitivity was measured by intracellular calcium fluorescence photometry at 340/380 nm using Fura-2-AM indicator; dose–response curves were constructed by measuring peak amplitudes of the fluorescence ratio (340/380 nm), which, after background subtraction, were normalized against a maximal response produced by 10 mM caffeine. Data normalization was necessary to compare individual samples to the response of halothane due to different samples expressing ryanodine receptor proteins at different levels. For each drug concentration, data from six independent experiments were averaged and expressed as mean ± SD. Linear regression analysis was performed using Origin software (Microcal Software Ltd., Northampton, MA). Data were compared using the unpaired Student *t* test, with statistical significance accepted at $P < 0.05$.[20]

Genetic linkage analysis was performed using LINKAGE (Jurg Ott, Rockefeller University, New York, NY) version 5.1 and FASTLINK (Alejandro Schäffer, National Institutes for Health Research, Bethesda, MD) packages.[21,22] The genetic model that was used assumed autosomal dominant inheritance, an affected frequency of 1:10,000, near-complete penetrance of 0.98, a low phenocopy rate of 0.02, and a recombination fraction of 0. Statistical significance favoring linkage was accepted for logarithm of odds scores of 3.0 or greater.

Results

We determined whether MH susceptibility in this Canadian MH pedigree is associated with a mutation in the *RYR1* gene by sequencing the *RYR1* transcript from the leukocytes of one MHS family member. Direct sequencing of the complete *RYR1* transcript revealed the presence of three polymorphisms: two synonymous G/A substitutions and one novel mutation—a heterozygous C982T transition resulting in the substitution of Arg328 with Trp (R328W) (table 2). As in two thirds of other known MH mutations in *RYR1*,[23] the C982T mutation occurs at a C residue in a CpG dinucleotide. Transcript heterozygosity for the C982T transition in this MHS patient (303) was confirmed by direct sequencing of a 395-bp PCR-amplified fragment spanning part of exon 11 and the adjacent part of intron 11 in genomic DNA (fig. 2A).

Table 2. Polymorphisms Found in the *RYR1* Transcript from Patient 303

Exon	Substitution	Amino Acid	Amino Acid Change
7	G594A	Leu198	Synonymous
11	C982T	Arg328Trp	Nonsynonymous
15	G1668A	Ser556	Synonymous

Direct sequence analysis of the corresponding *RYR1* region from genomic DNA of the proband (301) and the three family members diagnosed as MHS by CHCT (203, 204, 206) showed the presence of the C982T transition in a heterozygous state. The mutation was absent in the one family member (306) who tested MH negative by the CHCT.

To extend our study to additional members of the family, we developed a simple diagnostic assay based on the fact that the C982T base substitution in *RYR1* leads to the loss of an *Aci*I restriction site in the sequence of exon 11. In individuals with the normal allele, *Aci*I endonuclease digestion cleaves the 395-bp PCR fragment containing two constant *Aci*I restriction sites into three fragments of 173, 137, and 85 bp; the same fragment with the C982T base substitution is cleaved by AciI

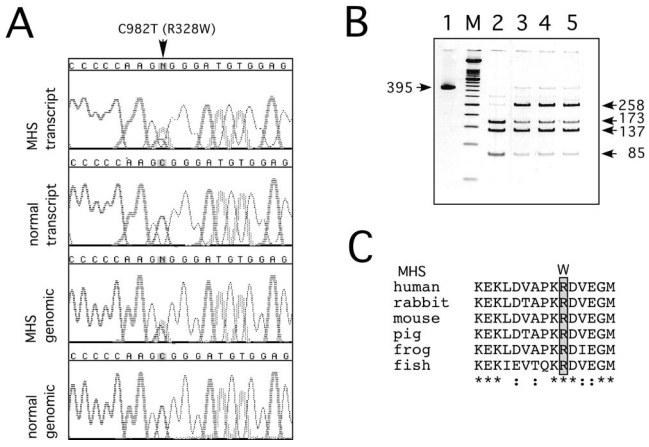

Fig. 2. Identification of the R328W mutation in transcript and genomic DNA derived from leukocytes. (*A*) The C982T transition was first identified by direct sequencing of the *RYR1* transcript and then of the corresponding fragment from genomic DNA. The four chromatographs, from *top* to *bottom*, correspond to malignant hyperthermia–susceptible (MHS) transcript, normal transcript, malignant hyperthermia–susceptible genomic DNA, and normal genomic DNA. The *arrow* indicates the position of the C982T heterozygous mutation. (*B*) The R328W mutation was detected by *Aci*I restriction endonuclease digestion of the 395-bp polymerase chain reaction–amplified fragment from genomic DNA, as described in the Materials and Methods section, from individuals within the pedigree shown in figure 1. *Lane 1*, before *Aci*I digestion; *lane 2*, *Aci*I digestion of the normal allele generates 173-, 137-, and 85-bp fragments; *lanes 3–5*, loss of one *Aci*I restriction site in one allele in malignant hyperthermia–susceptible individuals is detected by the appearance of a 258-bp fragment and a reduction in intensity of the 173- and 85-bp fragments; *lane M*, a 50-bp marker ladder. (*C*) Alignment of the human RyR1 protein sequence with those in rabbit, mouse, pig, bullfrog, and fish in the region flanking the mutated amino acid residue shows evolutionary conservation of Arg328.

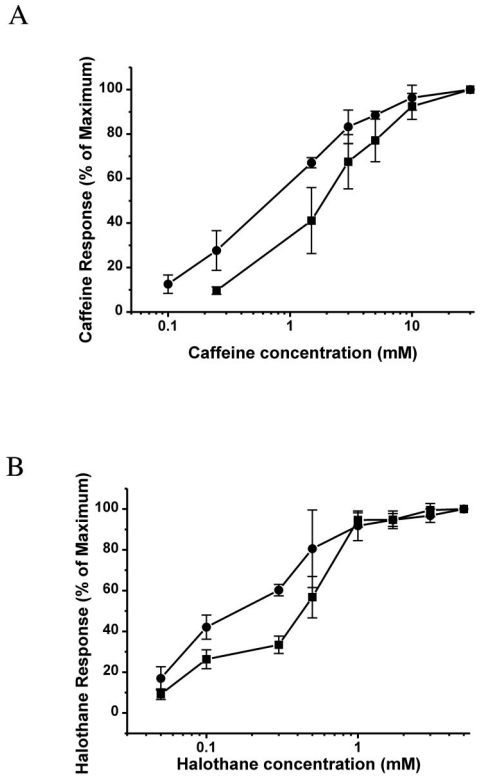

Fig. 3. Triggering of Ca^{2+} release by caffeine (*A*) and halothane (*B*) into the cytoplasm of human embryonic kidney line 293 cells transfected with wild-type (■) or mutant (●) *RYR1* complementary DNAs. For details, see the Materials and Methods section.

endonuclease into fragments of 258 and 137 bp. In the heterozygote, four bands of 258, 173, 137, and 85 bp form a characteristic pattern that is diagnostic (fig. 2B).

Screening for the presence of the R328W mutation within the four generations and 28 individuals that form the family (fig. 1) revealed six heterozygous individuals (102, 304, 401, 403, 406, 408) and16 homozygous normal family members (including three presumed normal spouses). The mutation was absent from 95 unrelated healthy individuals (190 normal chromosomes), indicating that the R328W mutation is not a benign polymorphism.

Arg328 is located in a region of RyR1 protein that is conserved across vertebrate species (fig. 2C). Further evidence for a causative role of the R328W mutation in MH was sought in functional studies. The R328W substitution was incorporated into the corresponding position in a rabbit *RYR1* cDNA. Wild-type and mutant cDNAs were expressed in HEK-293 cells, and intracellular Ca^{2+}release, induced by caffeine or halothane, was measured by photometry.[19] The R328W mutant Ca^{+2} release channel was significantly more sensitive to caffeine (ED_{50} = 0.80 mM, where ED_{50} refers to the concentration of caffeine or halothane required to reach the half-maximal Ca^{2+} release response) than wild-type RYR1 (ED_{50} = 1.40 mM) and more sensitive to halothane (ED_{50} = 0.16 mM) than wild-type RYR1 (ED_{50} = 0.4 mM) (fig. 3). The higher sensitivity of the expressed mutant protein to caffeine and halothane indicated the pathogenic potential of the R328W mutation.

The Arg328Trp mutation was not present in other unrelated Canadian individuals who had been diagnosed as MHS by the CHCT, nor is it listed among previously reported MH causative mutations.[8,24] On the basis of its association with MH and its functional characteristics, we conclude that the C982T transition is a private MH mutation, restricted, as yet, to this Canadian pedigree.

Discussion

In this study, we identified a novel mutation, R328W, in a large MH family by direct sequencing of the complete *RYR1* transcript amplified from mRNA obtained from peripheral blood leukocytes, thus demonstrating that blood can be used as an alternative source of *RYR1* mRNA for genetic studies. In this family, we were unable to demonstrate genetic linkage to a satisfactory level of statistical significance (lod score favoring linkage = 1.50) because the power of genetic linkage was limited by the small number of subjects phenotyped by CHCT and the small number of informative meioses. Phenotyping was offered to members of the family, but further testing was declined. Sensitivity analysis in this pedigree showed that odds favoring linkage might have been observed if phenotyping had been performed on eight members of the youngest generation in the pedigree. However, discordance between genotype and CHCT is also likely, given the known limitations in specificity of contracture testing to rule out MHS.[25,26] However, the perfect correlation between the presence of the R328W mutation and positive CHCT results within the family as well as the absence of the mutation in a control population of 190 normal chromosomes lends support to the causal nature of the mutation for MHS in this Canadian pedigree.

Further support to the causal nature of this mutation comes from functional studies that demonstrated that the expressed mutant protein was significantly more sensitive to caffeine and to halothane than wild-type RyR1. The effect of the R328W mutation on the sensitivity of RyR1 to caffeine and to halothane was comparable to the effect of several other RyR1 mutations, now recognized as causal of MH.[19]

The C982T transition is located within MH/CCD region 1, one of the three mutational hot spots in the *RYR1* gene.[27] Alignment of the amino acid sequence of the human RyR1 protein with sequences from rabbit, mouse, and pig, as well as with orthologs in bullfrog and fish, shows the conservation of Arg328 across vertebrate species (fig. 2C). Alignment of the same protein sequence with other ryanodine receptor isoforms shows

that Arg328 is highly conserved between RyR1 and RyR2 proteins. Arginine is replaced in RyR2 protein with lysine, but this is a conservative substitution. In our MH pedigree, the hydrophilic, positively charged residue, arginine, is replaced by a hydrophobic tryptophan residue, a substitution that is likely to have a critical effect on RyR1 function and, thus, be the cause of MH susceptibility within this pedigree. Accordingly, we believe that the location of the mutation in a mutational hot spot in *RYR1* previously linked to MHS, and the altered Ca^{2+} release channel function is sufficient evidence to recommend that the R328W mutation in *RYR1* be included in the European MH group list of *RYR1* mutations considered to be causal of MH sensitivity.[24]

The search for a noninvasive method for MH diagnosis has led to the proposal that alterations in Ca^{2+} homeostasis can be studied either in cultured skeletal muscle cells[28] or in lymphoblastoid cells naturally expressing *RYR1*.[29] Such diagnostic methods, however, do not identify causal mutations. The procedure described here is compatible with the isolation and sequencing of *RYR1* transcripts from cultures of small numbers of human cells. Selective sequencing of the 1- to 2-kb PCR fragments corresponding to three MH domains in the transcript offers an even more economical alternative to exon-by-exon scanning of genomic DNA. Regardless of the origin of the tissue, transcript analysis offers a cost-effective way for a complete mutation scan in the *RYR1* coding sequence. While this approach cannot identify mutations at the exon–intron junctions, it can readily detect splicing aberrations that result from such mutations. As yet, there is no indication that such mutations are associated with MH.

We believe that the search for MH-associated mutations within an MH family should be limited neither to known MH mutations nor to the MH/CCD regions of the RYR1 gene because several MH- or CCD-linked mutations have now been identified outside these regions.[30,31] Direct sequencing of the *RYRI* transcript offers the highest potential for finding all MH mutations. Until now, sequencing of the entire *RYR1* coding sequence has not been feasible because of the cost and the complexity of the gene, as well as the fact that muscle tissue is not always available. With the protocol that we have described, sequencing of the entire *RYR1* transcript from a sample of blood is now feasible and could be a method of choice for genetic testing that is compatible with one of the developing "noninvasive" methods of diagnosis of MH status.

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