

RESEARCH PAPER

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Functional analysis of *RYR1* variants linked to malignant hyperthermia

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ABSTRACT

Malignant hyperthermia manifests as a rapid and sustained rise in temperature in response to pharmacological triggering agents, e.g. inhalational anesthetics and the muscle relaxant suxamethonium. Other clinical signs include an increase in end-tidal CO₂, increased O₂ consumption, as well as tachycardia, and if untreated a malignant hyperthermia episode can result in death. The metabolic changes are caused by dysregulation of skeletal muscle Ca²⁺ homeostasis, resulting from a defective ryanodine receptor Ca²⁺ channel, which resides in the sarcoplasmic reticulum and controls the flux of Ca²⁺ ions from intracellular stores to the cytoplasm. Most genetic variants associated with susceptibility to malignant hyperthermia occur in the *RYR1* gene encoding the ryanodine receptor type 1. While malignant hyperthermia susceptibility can be diagnosed by *in vitro* contracture testing of skeletal muscle biopsy tissue, it is advantageous to use DNA testing. Currently only 35 of over 400 potential variants in *RYR1* have been classed as functionally causative of malignant hyperthermia and thus can be used for DNA diagnostic tests. Here we describe functional analysis of 2 *RYR1* variants (c. 7042_7044delCAG, p.ΔGlu2348 and c.641C>T, p. Thr214Met) that occur in the same malignant hyperthermia susceptible family. The p.Glu2348 deletion, causes hypersensitivity to ryanodine receptor agonists using *in vitro* analysis of cloned human *RYR1* cDNA expressed in HEK293T cells, while the Thr214Met substitution, does not appear to significantly alter sensitivity to agonist in the same system. We suggest that the c. 7042_7044delCAG, p.ΔGlu2348 *RYR1* variant could be added to the list of diagnostic mutations for susceptibility to malignant hyperthermia.

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Introduction

Malignant hyperthermia (MH) is an autosomal dominant pharmacogenetic disorder that predisposes susceptible individuals to an acute increase in cytosolic [Ca²⁺] during general anesthesia using volatile anesthetics or depolarizing muscle relaxants.^{1,2} In addition, several studies suggest that MH-susceptibility is commonly associated with elevated resting cytosolic [Ca²⁺].^{3–5} Acutely elevated cytosolic [Ca²⁺] results in a hypermetabolic state, characterized by an increase in end-tidal CO₂, increased O₂ consumption, and tachycardia. Hyperthermia results with core temperatures increasing by 1°C every 5 minutes.⁶ Uncontrolled hypermetabolism leads to respiratory and metabolic acidosis,

rhabdomyolysis, hyperkalemia, widespread organ failure and death if untreated by the drug dantrolene.⁷

The majority (50–70%) of individuals affected with MH have gain-of-function variants in the *RYR1* gene, encoding the skeletal muscle ryanodine receptor,^{8,9} while ~1% have variants in the $\alpha 1s$ subunit of the dihydropyridine receptor (DHPR) gene (*CACNA1S*).¹⁰ The DHPR is a multi-subunit voltage-dependent Ca²⁺ channel, which resides in the T-tubule membrane. Upon response to membrane depolarization it causes the RyR1 tetramer to open due to physical contact between the 2 channels. As a result Ca²⁺ is released into the cytoplasm from the sarcoplasmic reticulum (SR) stores, triggering muscle contraction as well as a range

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of other metabolic responses. This process is known as excitation-contraction coupling.¹¹ Ca^{2+} is returned to the sarcoplasmic reticulum by a Ca^{2+} -ATPase (SERCA).¹² This tightly controlled balance of intracellular Ca^{2+} concentrations is dysregulated in an MH crisis.

Because MH is a life-threatening disorder, susceptibility should ideally be determined prior to general anesthesia, such that non-triggering agents can be used. MH susceptibility in New Zealand is normally determined by *in vitro* contracture test (IVCT) using skeletal muscle biopsy tissue following the European Malignant Hyperthermia Group (EMHG) protocol.¹³ DNA-based diagnostic tests were introduced in New Zealand in 1998,¹⁴ to augment the IVCT and, while these are now becoming more accepted,^{15,16} they do not replace the IVCT for many individuals because of the genetic heterogeneity of MH.¹⁷⁻¹⁹ In addition, DNA-based diagnosis for MH susceptibility can be carried out only in families with known mutations that have been functionally characterized.¹⁸ This constraint, while appropriate, represents a major hurdle to being able to offer DNA testing on a more general basis. In addition, an MHN diagnosis cannot be made upon a DNA test alone, rather an IVCT is recommended where a DNA test is negative for a familial mutation.²⁰

Here we report functional analysis of 2 *RYR1* variants, c.641C>T, p.Thr214Met and c.7042_7044delCAG, p.ΔGlu2348 in the same family, both of which have been linked to MH previously.^{21,22} We constructed human *RYR1* cDNAs with either variant as well as a construct containing both. Functional analysis was carried out in transiently transfected HEK293T cells using 4-CmC as a specific RyR1 agonist and fura-2 as the fluorescent Ca^{2+} indicator. The c.641C>T, p.Thr214Met RyR1 variant did not appear to affect Ca^{2+} release in this system, but the c.7042_7044delCAG, p.ΔGlu2348 deletion resulted in a hypersensitive channel suggesting that it is likely to be causative of MH.

Results

In vitro contracture tests

Muscle biopsies and *in vitro* contracture tests were carried out as described in methods for the proband and his mother (II:1 and I:1 respectively in Fig. 1). The proband was diagnosed MHS and the mother MHN. Thus far the father (I:2, Fig. 1) has declined IVCT testing. The IVCT results are shown in Table 1.

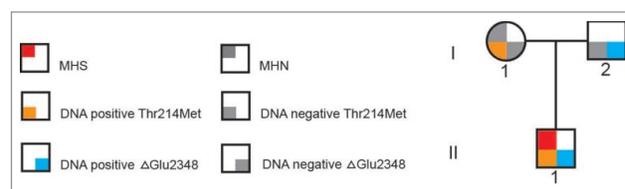


Figure 1. Pedigree diagram showing inheritance of RyR1 variants from each parent and MH status where known.

DNA sequencing and variant screening

The entire *RYR1* gene of the proband (Fig. 1, II:1) and his mother (Fig. 1, I:1) as well as the *CACNA1S* gene and 48 others with potential roles in skeletal muscle Ca^{2+} handling were screened using the HaloPlexTM target enrichment system followed by next generation sequencing as described in methods. All exons of the *CACNA1S* and *RYR1* genes were represented in the data set with an average coverage between 30 and 156 with the exception of *RYR1* exon 91, which was completed using Sanger sequencing. The proband carries both the c.641C>T, p.Thr214Met and c.7042_7044delCAG, p.ΔGlu2348 RyR1 variants. Neither variant is listed in the Exome Variant Server. The mother carries the c.641C>T, p. Thr214Met RyR1 variant only. No other rare (minor allele frequency of < 0.1%) variants in any other genes in this dataset were identified for either subject. The c.7042_7044delCAG, p.ΔGlu2348 RyR1 variant was detected in genomic DNA from the father (Fig. 1, I:2) using kinetic PCR followed by high resolution amplicon melting (HRM).²³ Neither variant was detected by HRM screening of a panel representing 150 MHN patients.

Expression of recombinant RyR1 in HEK293T cells

RyR1 expression in transiently transfected HEK293T cells was determined by western blotting to confirm approximately equal amounts of recombinant protein was produced for each construct (Fig. 2). Alpha-tubulin (~50 kDa) was used as a loading control. No RyR1

Table 1. IVCT values.

Patient	Contracture (g) at 2% halothane	Contracture (g) at 2 mM caffeine	MH Status
I:1	0.4	0.2	MHN
II:1	5.2	3.0	MHS

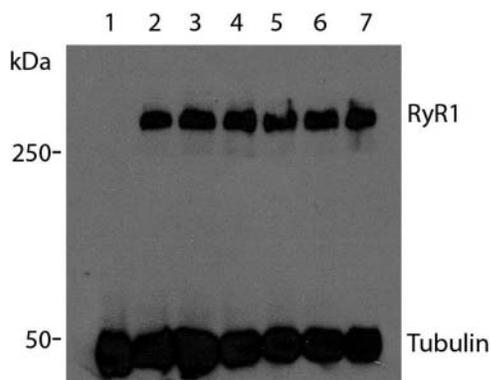


Figure 2. Immunoblot showing expression of RyR1 constructs. Immunoblot of HEK293T cells transiently transfected with RYR1 expression plasmids. Total protein (270 μ g) extracts were separated on a 7.5% SDS-PAGE gel and transferred onto a PVDF membrane. RyR1, > 250 kDa, and α -tubulin, 50 kDa, were detected using anti-RyR1 (34C) and anti- α -tubulin antibodies respectively. Lane 1, pcDNA3.1+ vector only; lane 2, pcRyR1 wildtype; lane 3, pcRyR1 Gly248Arg; lane 4, pcRyR1 Arg2452Trp; lane 5, pcRyR1 Thr214Met; lane 6, pcRyR1 Thr214Met/ Δ Glu2348; lane 7, pcRyR1 Δ Glu2348.

protein was detected in the vector-only (pcDNA3.1+) negative control.

Immunofluorescence was used to confirm correct co-localization of the recombinant RyR1 proteins with the endoplasmic reticulum (ER) marker protein disulfide isomerase, (PDI, Fig. 3). No RyR1 was detected in HEK293T cells transfected with empty pcDNA3.1+ vector, used as a negative control.

Ca²⁺ release assays

The activities of variant RyR1 channels expressed in the ER of HEK293T cells were compared with the activity of the wild-type (WT) RyR1 channels using Ca²⁺ release assays under identical conditions. The increase in cytoplasmic calcium was measured after the addition of incremental doses of the RyR1 agonist 4-CmC to the cells and then calculated as a percentage of total Ca²⁺ release (Fig. 4). The RyR1 variants c.742G>A, p.Gly248Arg and c.7354C>T, p.Arg2452Trp were used as positive controls as the former has been validated as an MH-causative mutation by the EMHG (<https://emhg.org/genetics/mutations-in-ryr1/>) and the latter was shown to be causative using our system.²⁴ Both had significantly increased sensitivities to 4-CmC compared to WT as expected (Table 2). There was no significant difference between the c.641C>T, p. Thr214Met RyR1 variant and WT, with

respect to stimulation by 4-CmC, although the concentration-response curve lay between WT and the positive controls (Fig. 4, yellow trace). The p. Δ Glu2348 RyR1 variant was hypersensitive compared to WT when stimulated with 4-CmC as with both agonists the curve was shifted to the left (Fig. 4, dark blue trace). A similar result was observed for the double RyR1 variant p.Thr214Met/ p. Δ Glu2348. EC50 values are presented in Table 2. Resting cytosolic calcium was significantly different to empty vector for the p.Arg2452Trp and p. Δ Glu2348 RyR1 constructs only. All other constructs including the double variant, p. Thr214Met/p. Δ Glu2348 were similar to the vector only control (Table 3).

Discussion

The objective of the current study was to determine the effect of 2 RyR1 variants on the function of the skeletal muscle Ca²⁺ channel using a heterologous system *in vitro*. Both variants occur in the proband, having been inherited from each parent (Fig. 1). As positive controls for hypersensitivity, the previously-characterized MH-causative c.742G>A, p.Gly248Arg²⁵ and c.7354C>T, p.Arg2452Trp²⁴ RyR1 mutations were also tested under the same conditions. Under our experimental conditions RyR1 containing the c.641C>T, p. Thr214Met variant cannot be functionally distinguished from the WT RyR1 channel, although the different shaped concentration response curves for 4-CmC suggest some abnormality may exist. While the mother who carries this variant was diagnosed as MHN by IVCT, 3 families in the United Kingdom show concordance between this variant and disease and/or IVCT phenotype.²¹ The IVCT results for the mother (Fig. 1, I:I and Table 1) are borderline between MHS and MHN so the MHN diagnosis could be a false negative given that the IVCT has been determined to have a specificity of only 93.6% and sensitivity of 99%.²⁶ False negative IVCT results could be due to the testing laboratory-defined cut-off values²⁷⁻²⁹ or to variable penetrance of mutations detected.⁹ In support of the MHN diagnosis however, a retrospective study of 329 patients diagnosed MHN in New Zealand, showed no evidence of any adverse reaction in subsequent anesthesia using triggering agents.²⁸ In addition it is important to note that the transfected HEK293T cells express only exogenous RyR1 with none of the other proteins known to be part of the skeletal muscle triad, the functional unit of excitation-

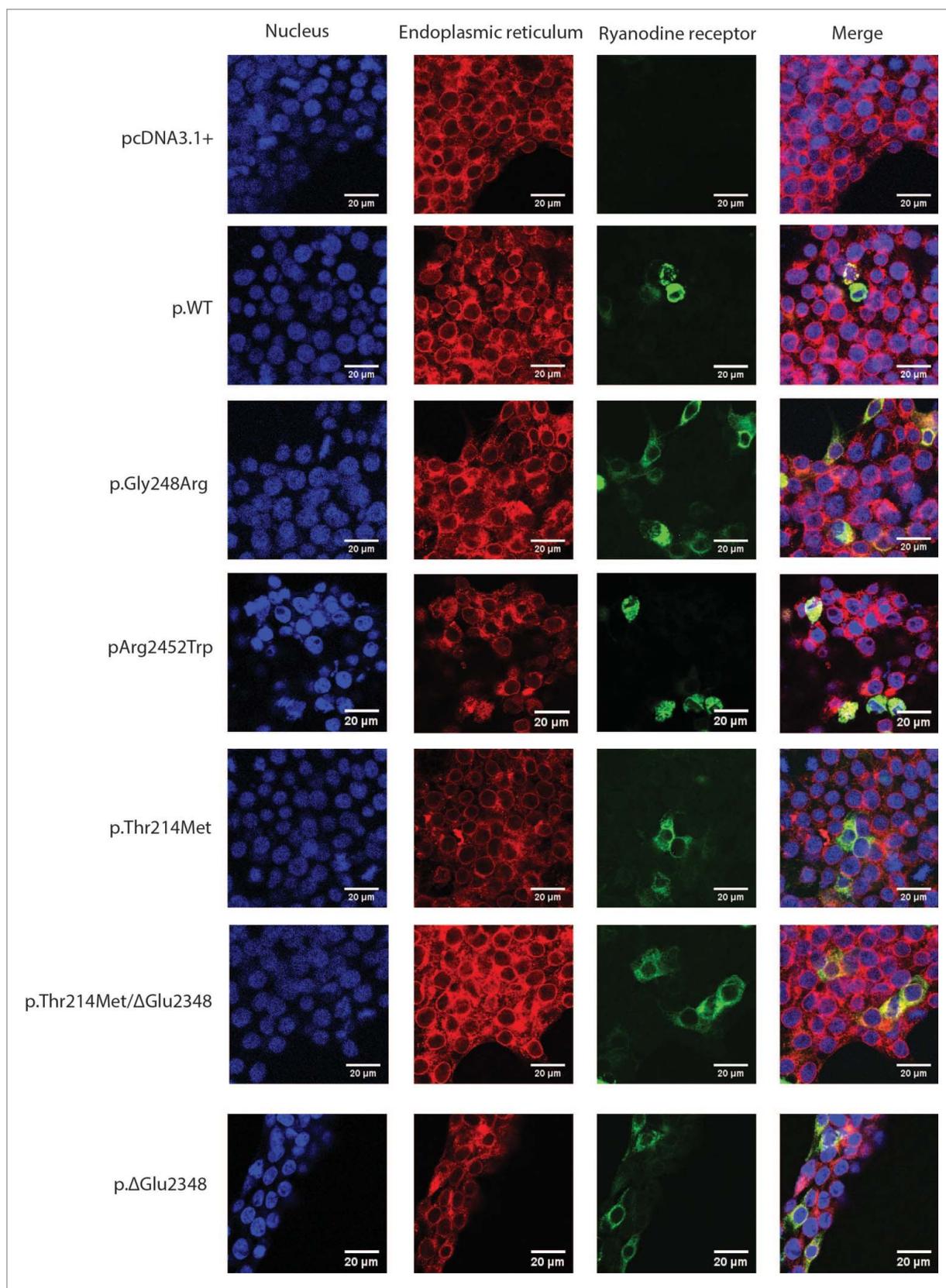


Figure 3. Co-localization of RyR1 with the endoplasmic reticulum. Immunofluorescence of transiently transfected HEK293T cells with RyR1 cDNA. Variants are indicated by their amino acid change. Primary antibodies that specifically recognize RyR1 (34C) and PDI as well as fluorescently-labeled secondary antibodies FITC (green) and TRITC (red) were used to visualize RyR1 and PDI respectively, while nuclei were visualised by staining the cells with DAPI (blue). Cells were examined by confocal fluorescence microscopy at a magnification of 1260 X; the scale bar in each merged image represents a length of 20 microns.

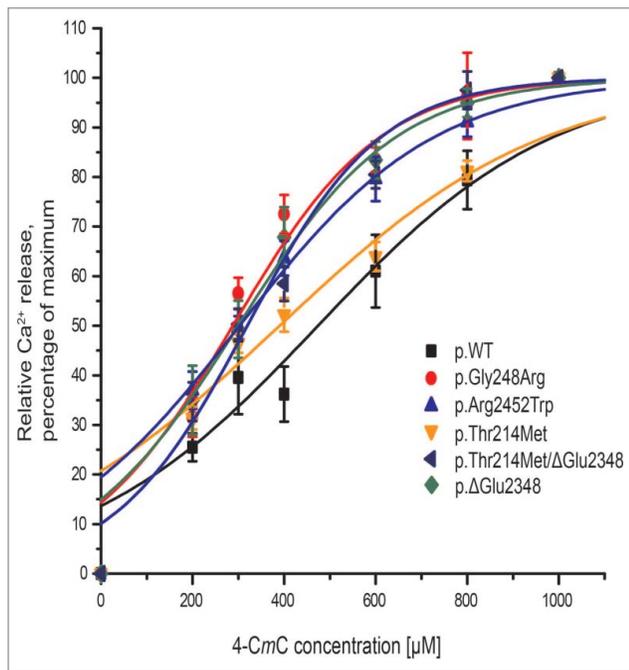


Figure 4. Ca^{2+} release assays in HEK293T cells. Ca^{2+} -release is illustrated in concentration-response curves for transiently transfected HEK293T cells. Ca^{2+} released was measured for 4-CmC concentrations between 0 and 1000 μM , values were normalized to Ca^{2+} released at 1000 μM 4-CmC and represented as mean \pm SEM ($n \geq 8$). Curves were plotted using OriginLab Origin 8 software.

contraction coupling, being present. This variant may therefore have an RyR1 function that cannot be investigated in our system due to the lack of protein-protein interactions with partners of the excitation-contraction system. The c.641C>T, p.Thr214Met residue is located within a β sheet in the β -trefoil B domain in the structurally-characterized N-terminal domain encompassing residues 1-559 of rabbit RyR1. This region forms 3 domains predicted to interact through a hydrophilic interface.³⁰ The Thr214 residue does not appear to be

Table 2. EC50 values 4-CmC.

construct	EC50 μM 4-CmC	SEM	p-value
WT	481	15.00	
Gly248Arg	270	6.00	* 8.21×10^{-4}
Arg2452Trp	341	7.70	* 6.6×10^{-4}
Thr214Met	403	15.00	0.250
Thr214Met/ Δ Glu2348	219	15.22	* 2.08×10^{-5}
Δ Glu2348	261	20.70	* 1.45×10^{-4}

The concentration of 4-CmC required to half maximally activate RyR1 from individual assays was determined using OriginLab Origin 8 software. Results are represented as mean \pm SEM. The Student's unpaired T test, followed by a Bonferroni correction, was used to determine the statistical difference between the EC50 value for cells transfected with WT *RYR1* cDNA and each variant.

Table 3. Resting cytosolic Ca^{2+} .

construct	Ratio 340/380	SEM	p-value
pcDNA3.1+ vector only	1.030348	0.006029	
WT	1.038354	0.002792	0.22291
Gly248Arg	1.030568	0.004414	0.13118
Arg2452Trp	1.061786	0.005017	* 2.99×10^{-5}
Thr214Met	1.038319	0.003382	0.99381
Thr214Met/ Δ Glu2348	1.049861	0.005694	0.05691
Δ Glu2348	1.088946	0.004969	* 7.98×10^{-16}

Ratio of fluorescence emission at 510 nm using excitation at 340 nm and 380 nm prior to the exposure of RyR1 agonists. Represented is the mean fluorescence emission ratio \pm SEM for each construct. The Student's unpaired T test, followed by a Bonferroni correction, was used to determine the statistical difference between cells transfected with the pcDNA3.1+ vector only, and each RyR1 construct.

part of the direct domain interaction but is located in a linker region between 2 domains, which is buried in the 3-dimensional structure. Methionine is an aliphatic residue however, as well as being larger than the hydrophilic threonine, which may lead to structural alteration of the folded protein or localized misfolding. Although our functional data suggest the c.641C>T, p. Thr214Met variant is unlikely to be a variant altering Ca^{2+} release from the SR/ER, the concordance between this variant and MHS in 3 other families, as well as the limitations of the HEK293T assay system indicate that currently we cannot rule out a functional consequence of this variant for RyR1.

On the other hand the c. 7042_7044delCAG, p. Δ Glu2348 variant results in a hypersensitive channel, which suggests that it is likely to cause MH. While the clinical signs of MH for the proband (Fig. 1, II:1) were minor, the IVCT data (Table 1) indicated very strong contractions and a clear MHS diagnosis. DNA testing has shown that the father (Fig. 1, I:2) carries the c. 7042_7044delCAG, p. Δ Glu2348 RyR1, and while MH status has not been confirmed by IVCT, he should be considered at high risk. This *RYR1* variant has been associated with MHS in at least one other unrelated family, also associated with very strong contracture tension.³¹ No predictions however, can be made about any mechanistic effect that this deletion would have on structure or function of the channel. While a near-atomic resolution cryo EM structure predicts this residue to be situated in a helical domain, the resolution was sufficient to trace the backbone structure only.³²

It has been suggested that MH can be caused by hyperactive RyR1 channels resulting in a higher than normal Ca^{2+} release under stimulating conditions

causing a crisis event.³³ Our results support this conclusion for the c. 7042_7044delCAG, p.ΔGlu2348 variant. As RyR1 with the p.Arg2452Trp and p.ΔGlu2348 variants demonstrated significantly higher resting calcium levels, these variants may result in a functionally “leaky” channel. The p.Arg2452 residue is thought to lie at a domain interface³⁴ in the cytoplasmic domain of the RyR1 tetramer and perturbation of predicted surface interactions by the introduction of the bulky hydrophobic tryptophan in place of the positively charged arginine residue could have functional consequences for channel stability in the unstimulated state. These observations support the “leaky” channel mechanism suggested as an alternative mechanism leading to an MH crisis.³⁵ They also support previous work with synthetic peptides representing this region, which indicate that interdomain interactions are likely to be responsible for opening and closing the tetrameric channel.^{36,37}

While calcium release was similar for the RyR1 p.ΔGlu2348 and the p.Thr214Met/p.ΔGlu2348 variants, the p.Thr214Met/p.ΔGlu2348 variant did not appear to result in a “leaky” channel. This suggests that the p.Thr214Met variant may in some way compensate for the p.ΔGlu2348 variant in the resting state. It should be noted however, that these 2 variants are in the same RyR1 monomer in this recombinant system, rather than in different monomers as would be the case in the proband. Even though co-transfection of different *RYR1* constructs has been performed in previous studies,^{33,38} the exact assembly of the tetrameric RyR1 channel with the different monomers cannot be predicted.

As the c. 7042_7044delCAG, p.ΔGlu2348 RyR1 variant has been identified in at least 2 unrelated families and is a conserved residue,³¹ as well as conferring hypersensitivity to a specific RyR1 agonist in a heterologous system, it should be classed as an MH causative mutation. A substitution at this position (c.7043 A>G, p.Glu2348Gly) also segregates with MHS in a UK family,³⁹ supporting the potential importance of this residue in RyR1 function.

Patients and methods

Case history

A 7 y old, 22 kg child with no family history presented for squint correction in 1999. He had a gas induction with Sevoflurane in oxygen, and was then intubated

with 15 mg Rocuronium. Once intubated, he was noted to have a high end-tidal CO₂ of 67 mmHg. This resolved with minute ventilation of 2.5 L/min bringing end-tidal CO₂ down to 42 mmHg. After induction, the volatile agent was changed to isoflurane for maintenance of anesthesia. Approximately 20 minutes into the case, the end-tidal CO₂ again rose from 42 to 58 mmHg, and he developed a mild tachycardia. A rectal temperature was taken which was 37°C and he felt warm to the touch. He was given analgesia with fentanyl 40 μg, and then morphine 2 mg, and the tachycardia resolved. He was given antiemetic prophylaxis with 2 mg of Ondansetron. The anesthetist was concerned that these subtle changes (tachycardia and raised end-tidal CO₂) may represent MH, so he stopped administering the volatile agent, went onto high fresh gas flows and maintained anesthesia with a propofol infusion. At the closure of surgery the child was extubated and taken to recovery. Creatine kinase was 221 U/L; the upper limit of normal at the laboratory was 220 U/L. His temperature never rose above 37°C degrees and he was not given dantrolene. He was observed overnight in the intensive care unit, and no other signs of MH were noted. He was referred for *in vitro* contracture testing at Palmerston North hospital and a diagnosis of MHS was confirmed.

Regulatory authority approvals

This study has been approved by the Central Regional Ethics Committee, Ministry of Health, Wellington, New Zealand (MWH/03/04/018) and the Massey University Genetic Technology Committee (GMO 05/MU/01 and GMO 00/MU/60) acting as an Institutional Biological Safety Committee for the Environmental Protection Authority, Wellington, New Zealand.

In vitro contracture tests

Muscle biopsies were obtained and *in vitro* contracture testing (IVCT) was performed at Palmerston North hospital using the standard diagnostic procedure for MH-susceptibility according to the European Malignant Hyperthermia group (EMHG) protocol and local laboratory control data.²⁶⁻²⁸ Briefly this involves exposure of muscle bundles to incremental amounts of halothane and caffeine, while measuring contracture tension in g. A diagnosis of MHS is

Table 4. Oligonucleotide primers.

Primer name	Sequence	Purpose
Thr214Met F_SDM	GAAGAGGGCTTCGTGATGGGAGGTCACGTCCTC	Site-directed mutagenesis
Thr214Met R_SDM	GAGGACGTGACCTCCCATCACGAAGCCCTCTTC	Site-directed mutagenesis
ΔGlu2348 F_SDM	GTC AACGGCGAGAGCGTGGAGAAGCCCAATGTGGTGGTGC GG	Site-directed mutagenesis
ΔGlu2348 R_SDM	CCGCACCACACATTGGCGTTCTCTCCACGCTCTCGCCGTTGAC	Site-directed mutagenesis
Thr214Met F_HRM	CAACTCCCTTGCTCT	HRM
Thr214Met R_HRM	CGCTGGTCATCACTGTCA	HRM
ΔGlu2348 F_HRM	CCAGGCGAGAGCGTGGAG	HRM
ΔGlu2348 R_HRM	CGGATGAGCAGCCGACC	HRM

made if the responses to both halothane and caffeine are above the threshold of 0.4 g using 2% halothane and 0.2 g using 2 mM caffeine. An MHN diagnosis is made if the response is below threshold for both agents. If the response is above threshold for only one of the agents the diagnosis is MHS(h) or MHS(c) for halothane and caffeine respectively. All patients tested MHS, MHS(h) or MHS(c) would be classed as susceptible to MH and be given non-triggering anesthesia.¹³

DNA sequencing

Genomic DNA was isolated from patient blood samples (3 mL) using the Promega WizardTM genomic DNA extraction kit according to the manufacturer's instructions. This was followed by quantification using the Quant-iTTM dsDNA high-sensitivity DNA assay kit (Life Technologies, Q-33120) following the manufacturer's instructions and diluted in TE buffer to a final concentration of 5 ng/μL. The HaloPlexTM Target Enrichment system (Agilent Technologies, G9901C) was used to prepare custom libraries from the genomic DNA representing 50 genes with known or potential association with MH. Purified amplified libraries were quantified using the High Sensitivity DNA kit (Agilent Technologies, 5067-4626) with the 2100 Bioanalyzer. Libraries were pooled in equimolar (400 pmol) amounts prior to massively parallel sequencing on the Illumina MiSEQ platform by the Next Generation Sequencing Facility, University of Leeds and the Leeds Teaching Hospitals NHS Trust, St James University Hospital Leeds, UK or New Zealand Genomic Limited at Massey University, Palmerston North, New Zealand. Sequences were aligned using Agilent SureCallTM software against hg19 using the default parameters. Exon 91 of *RYR1* was amplified in 3 segments using Dream TaqTM DNA polymerase (Fermentas, FMTEP0702), the

products purified using ExoSAP-IT[®] (Affymetrix, 78200) and then 10 ng of each PCR product was mixed with 4 pmol of the appropriate primer and Sanger sequencing was carried out by MacroGen (Korea). The PCR and sequencing primers are available on request. Sequences were aligned to the *RYR1* cDNA (NM_000540.2) using the web based tool needle through EMBOSS (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

High resolution amplicon melting

Primers (Table 4) for high resolution amplicon melting (HRM) assays were designed for the c.641C>T, p. Thr214Met and p.ΔGlu2348 RyR1 variants using the Light CyclerTM Primer Design software version 2.0 (Roche). cDNA and protein numbering are according to NM_000540.2 and NP_000531.2, respectively. Reactions contained 3 μM MgCl₂, 0.3 or 0.2 μM of each primer for the p. Thr214Met and p.ΔGlu2348 variants respectively and, 5 μL of 2x LC480 HRM master mix (Roche, 04909631001) and 25 ng of genomic DNA in a total volume of 10 μL. PCR amplification was carried out on a Roche Light Cycler 480 using a touch-down protocol as follows: 95°C for 10 minutes (ramp rate 4.40°C/sec), followed by 40 cycles of 95°C (ramp rate 4.40°C/sec) for 10 sec, 65°C (ramp rate 2.20°C/sec) with secondary target of 58°C (step size 0.5°C) for 10 sec and 72°C (ramp rate 4.40°C/min) for 4 sec. Single acquisition of data was at the 72°C amplification step. The melt program was as follows: 95°C for 1 min (ramp rate 4.40°C/sec), 40°C for 1 min (ramp rate 1.5°C/sec), 76°C for 1 sec (ramp rate 4.40°C/sec) followed by continuous acquisition at 92°C with a ramp rate of 0.02°C/sec and 25 acquisitions per °C. The final cooling cycle had a 40°C target, 30 sec hold and a ramp rate of 1.5°C/sec. Alleles were called using GeneScan software version 1.5.1.

Site directed mutagenesis and cloning

Mutagenesis was carried out by PCR-amplification of ~70 ng of plasmid DNA containing approximately 2.7 kb of the relevant subclones of human *RYR1* cDNA⁴⁰ (NM_000540.2) using the 2x Kapa HiFi HotStart Readymix (Kapa Biosystems, KR0370) diluted 4x. Complementary mutagenic primer pairs (Table 4) were sourced from Integrated DNA Technologies and used for QuikChangeTM (Stratagene) mutagenesis. Cycling conditions were 95°C for 5 min; 98°C for 2 minutes; 57°C for 30 seconds; 72°C at 1 min/kb; 72°C for 5 minutes; steps 2-3 were repeated 18 times. The PCR products were digested with 20 units of restriction endonuclease *DpnI* (New England BioLabs, R0176S) and buffer according to the manufacturer's instructions.

The full-length *RYR1* cDNA containing the specified variants was generated in 3 sequential cloning steps by standard methods.⁴⁰ Constructs were confirmed by Sanger DNA sequencing using a capillary ABI3730 Genetic Analyzer with BigDyeTM Terminator v3.1 chemistry at the Massey Genome Service, Palmerston North, New Zealand.

Cell culture and transient transfection

HEK293T cells for all applications were grown in DMEM (Dulbecco's Modified Eagle's Medium, Sigma D7777), 10% FBS (fetal bovine serum, Gibco 10091-148), 0.5% penicillin/streptomycin (Life Technologies, 15140-122) at 37°C, 5% CO₂ in a humidified atmosphere. For immunoblotting cells were grown to 90% confluence in T25 flasks using 8 mL of medium and for immunofluorescence, to 50% confluence in a 4 chambered slide containing 1 mL medium. For Ca²⁺ release assays, cells were grown to 80% confluence in UV transparent 96 well plates with each well containing 200 μ L medium. Medium was replaced one hour prior to transfection once the correct level of confluence was achieved.

For immunoblotting HEK293T cells were transiently transfected with *RYR1* cDNA or empty pcDNA3.1+ (Invitrogen, V790-20) plasmids, using 6 μ g plasmid DNA and 24 μ L FuGENE HD (Promega, E2311) to a total volume of 300 μ L with unsupplemented DMEM, which was added directly to the 8 mL of medium in T25 flasks. After 48 hours, the medium was replaced and growth continued for another 24 hours. Protein extracts were prepared after

washing cells with PBS and resuspending in 150 μ L of lysis buffer [0.1 M Tris HCl, pH 7.8, 0.5% triton X-100, 20 μ L 7x cComplete Mini EDTA-free protease inhibitor (Roche, 11836170001)]. Insoluble proteins were separated via centrifugation at 13,000 rcf at 4°C. Supernatant was stored at -80°C with limited freeze-thaw cycles.

For immunofluorescence, transient transfection was carried out using 1 μ g DNA, 3 μ L Fugene 6 (Roche, 11814443001) and DMEM without supplements, to a final volume of 50 μ L. Fresh complete medium was replaced after 48 hours and growth continued for another 24 hours prior to processing.

For Ca²⁺ release assays, HEK293T cells (with 100 μ L complete medium per well) were transfected with 100 ng DNA, 1.2 μ L Fugene HD in unsupplemented DMEM to a final volume of 14 μ L. Cells were incubated overnight prior to the addition of an extra 100 μ L complete DMEM. After 24 hours incubation, media was replaced with 200 μ L complete DMEM. After a further 24 hours incubation the transfected cells were used in calcium release assays.

Immunoblotting

Protein extracts (~270 μ g) were resolved by SDS-PAGE (4% stacking gel and 7.5% separating gel) for 2 hours at 120 V using a Mini PROTEAN electrophoresis system (Bio-Rad). Proteins were transferred to a PVDF membrane at 70 mA for 20 hours at 4°C. After transfer, the membrane was blocked in 5 mL 5% skim milk in TBST (0.05 M Tris HCl, 0.15 mM NaCl, 0.1% tween 20) for 3 hours at room temperature with gentle agitation. The membrane was cut to separate the 565 kDa RyR1 from the 50 kDa α tubulin loading control. The membrane containing higher molecular weight proteins was incubated overnight at 4°C in 5 mL mouse 34C primary antibody (Sigma, R129) diluted 1:1000 in 2.5% skim milk in TBST with gentle shaking. The membrane containing the lower molecular weight proteins was incubated overnight at 4°C in 5 mL mouse α -tubulin primary antibody (Sigma, T8203) diluted 1:5000 in 2.5% skim milk in TBST with gentle shaking. Both membranes were then incubated at room temperature for 20 minutes with shaking prior to 3 washes in TBST. Both membranes were incubated in 5 mL anti-mouse horse radish peroxidase conjugated secondary antibody (Sigma, A9044) diluted

1:5000 in 2.5% skim milk in TBST for one hour at room temperature. The membranes were washed 3 times in TBST prior to detection. Chemiluminescence blotting substrate (Roche, 11500694001) was prepared by mixing 3 mL luminescence substrate solution A with 30 μ L starting solution B. The substrate was applied to the membranes just prior to visualization of the proteins by the exposure to X-ray film.

Immunofluorescence

After an initial washing step in 500 μ L of PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 pH 7.2) cells were fixed in 500 μ L 2% paraformaldehyde in PBS for 15 minutes at room temperature and washed 3 times in PBS and then permeabilised using 0.1% triton X-100 in PBS for 5 minutes. After washing 3 times in PBS, the cells were blocked in 1 mL 5% bovine serum albumin, 0.5% Tween-20 in PBS with gentle shaking at room temperature for 5 minutes, then incubated overnight at 4°C with gentle shaking in 1 mL primary antibody solution [mouse 34C diluted 1:1000 or rabbit anti protein disulphide isomerase, PDI (Sigma, p7496) diluted 1:1000 in PBS]. The cells were then incubated at room temperature for 20 minutes, and washed 3 times in PBS. Finally, the cells were incubated at room temperature with gentle shaking for 1 hour in 500 μ L secondary antibody solution [fluorescein isothiocyanate (FITC) conjugated goat anti-mouse secondary antibody (Jackson immuno research, 15095003) diluted 1:200 in 500 μ L PBS, for RyR1 or tetramethylrhodamine (TRITC) conjugated goat anti-rabbit secondary antibody (Jackson immuno research, 11025003) diluted 1:200 in 500 μ L for PDI and then washed 3 times in PBS. A cover slip was mounted using 7 μ L ProLong Gold AntiFade mounting solution containing DAPI (Invitrogen, p-36931) to stain the nucleus. The slides were incubated over night before being visualised using a Leica SP5 DM6000B Scanning Confocal Microscope at 1260 \times magnification at the Massey Microscopy and Imaging Center.

Measurement of Ca^{2+} release

Transiently-transfected HEK293T cells were incubated in 100 μ L balanced salt solution, BSS (140 mM NaCl, 2.8 mM KCl, 1 mM MgCl_2 ,

10 mM glucose, 10 mM HEPES, pH 7.25) containing 2 mM CaCl_2 , 2 μ M fura2-AM (Molecular Probes, F-1221) and 0.01% pluronic F-127 (Sigma P2443) per well for one hour at 37°C in the dark. The cells were washed once with 100 μ L BSS plus Ca^{2+} and once in 100 μ L Ca^{2+} -free BSS buffer (containing 2 mM EGTA). Activation of the RyR1 using 4-CmC (Sigma, C55402) as the agonist was measured by the change in the fluorescence emission ratio at 510 nm, when excited at 340 nm and 380 nm, using an Olympus XI81 fluorescence microscope. Each well was assayed after establishing a fluorescence ratio baseline for using 100 μ L Ca^{2+} -free balanced salt solution before adding 100 μ L Ca^{2+} -free balanced salt solution containing 4-CmC. The baseline of the fluorescence ratio was used to estimate integrity of the ER membrane and potential leakage due the presence of RyR1 variant proteins. The final concentrations of 4-CmC used were: 200, 300, 400, 600, 800 and 1000 μ M.

Statistical analysis

The amount of Ca^{2+} release at each concentration of 4-CmC was normalized to account for any differences in cell density for each assay and calculated as a percentage of the total Ca^{2+} released with 1000 μ M 4-CmC. A minimum of 8 analytical replicates were carried out for each construct, the results were pooled and a sigmoidal curve fitted for each data set using OriginLab Origin 8 software. Results are presented as the mean \pm standard error of the mean (SEM) for each value of 4-CmC used. The concentration of agonist required for half maximal fluorescence change (EC50) was calculated from curves fitted to individual replicates and represented as mean \pm SEM. The statistical significance of each EC50 value was determined using the unpaired Student's t-test with OriginLab Origin 8 software and represented as a p-value with respect to WT and a Bonferroni correction was carried out to confirm statistical significance.

Abbreviations

BSS	Balanced salt solution
CACNA1S	Gene encoding the α 1s subunit of the dihydropyridine receptor
cDNA	complementary DNA
4-CmC	4-chloro- <i>m</i> -cresol

DAPI	4',6-diamidino-2-phenylindole
DHPR	Dihydropyridine receptor
DMEM	Dulbecco's Modified Eagle's Medium
EC50	Half maximal effective concentration
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EM	Electron Microscopy
EMBOSS	European Molecular Biology Open Software Suite
EMHG	European Malignant Hyperthermia Group
ER	Endoplasmic Reticulum
FBS	Fetal Bovine serum
FITC	Fluorescein isothiocyanate
HRM	High Resolution Amplicon Melting
IVCT	<i>In vitro</i> contracture test
MH	Malignant hyperthermia
MHN	Malignant hyperthermia negative
MHS(h)	Malignant hyperthermia susceptible with abnormal response to Halothane
MHS(c)	Malignant hyperthermia susceptible with abnormal response to caffeine
MHS	Malignant hyperthermia susceptible
PBS	Phosphate buffered saline
PDI	Protein disulphide isomerase
PVDF	polyvinylidene fluoride
RyR1	Ryanodine receptor protein type 1
<i>RYR1</i>	Gene encoding ryanodine receptor protein type 1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERCA	Sarco/endoplasmic reticulum ATPase
SEM	Standard error of the mean
SR	Sarcoplasmic reticulum
TBST	Tris buffered saline tween-20
TE	Tris-EDTA buffer
TRITC	Tetramethylrhodamine
UV	Ultraviolet
WT	Wildtype.

Disclosure of potential conflicts of interest

No potential conflicts of interest are disclosed.

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