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ORIGINAL ARTICLE

# Genetic and functional analysis of the RYR1 mutation p.Thr84Met revealed a susceptibility to malignant hyperthermia

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## Abstract

**Purpose** The aim of this study was to analyze the genetic and functional role of a novel RYR1 variant c.251 C > T (p.Thr84Met) identified in a patient with muscle weakness demonstrating MH susceptibility.

**Methods** DNA testing of family members was conducted for assessment of pathogenicity of the genetic variant. For functional analysis, $Ca^{2+}$ measurement using patient-derived myotubes and p.Thr84Met RYR1-transfected human embryonic kidney (HEK)-293 cells was performed to evaluate reactivity to RYR1 activators. The half-maximal effective concentration ($EC_{50}$) values of two RYR1 activators, caffeine and 4-chloro-*m*-cresol (4C*m*C), were calculated from the acquired dose–response curves. The $EC_{50}$ was compared between two groups: for myotubes, the control group and the patient, and for HEK-293 cells, WT and p.Thr84Met.

**Results** Dose–response curves for caffeine and 4C*m*C were shifted to the left in both myotubes and HEK-293 cells compared to controls. The 50% effective concentration values for caffeine and 4C*m*C were significantly lower in both myotubes and HEK-293 cells compared to controls ($P < 0.001$ for all comparisons).

**Conclusions** Our results of functional testing indicated RYR1 hypersensitivity to caffeine and 4C*m*C. We conclude that the genetic variant was associated with MH susceptibility.

**Keywords** Malignant hyperthermia · Ryanodine receptor · Mutation · Calcium release

## Introduction

Malignant hyperthermia (MH) is a pharmacogenetic disorder associated with mutations in the ryanodine receptor 1 (RYR1) gene encoding the skeletal muscle RYR1, and is triggered by exposure to halogenated volatile anesthetics and skeletal muscle depolarizing muscle relaxants [1]. RYR1 is an ion channel that governs $Ca^{2+}$ release from the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR); this release plays an important role in excitation–contraction coupling and $Ca^{2+}$ homeostasis of skeletal muscle. Genetic mutations of RYR1 in MH patients can cause life-threatening conditions such as abnormal hyperthermia, cardiac arrhythmias, lactic acidosis, hypoxia, and muscle rigidity as a result of hypermetabolism of skeletal muscle due to excessive calcium release from the SR [2].

Mutations in the RYR1 gene have been also associated with several RYR1-related congenital myopathies (RYR1-RMs) such as central core disease (CCD) [3]. The pathophysiological mechanism of RYR1-RMs is considered to be an increase of intracellular $Ca^{2+}$ concentration and mitochondrial damage due to dysfunction of RYR1, resulting in muscle weakness [4].

MH and MH susceptibility (MHS) closely resemble RYR1-RM in terms of clinical symptoms [1]; therefore,

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caution is needed when providing general anesthesia to patients with a suspected diagnosis of congenital myopathy.

## Materials and methods

A 9-year-old male having a congenital myopathy was scheduled to undergo surgical patellar stabilization under general anesthesia for recurrent bilateral patellar dislocation. The physical findings revealed delayed motor development, muscle weakness of the limbs, and high-arched palate. Motor developmental delay was also observed in the patient's sister; however, the symptoms were less severe. Blood examination showed elevated IgE antibodies to egg white. Anesthesia was induced and maintained with remifentanil, propofol, and rocuronium. The use of halogenated volatile anesthetics and depolarizing muscle relaxants was avoided. The operation was executed uneventfully; subsequently, diagnostic muscle biopsy and blood sampling for DNA testing were performed after obtaining written informed consent for the evaluation of possible congenital myopathies and MH.

Muscle biopsy from the left vastus lateralis muscle showed only mild fiber size variation with each fiber being distributed in a mosaic pattern. No structural abnormalities indicating congenital myopathy such as cores and nemaline bodies were seen. The $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) rate was tested to evaluate $Ca^{2+}$ release from the SR, and it was significantly higher than the standard value (described previously [5, 6]) at four different $Ca^{2+}$ concentrations (Fig. 1). This result indicated susceptibility to MH. DNA testing revealed the RYR1 genetic mutation c.251 C > T (p.Thr84Met), which has not been previously reported as a causative mutation for MH/MHS.

The European Malignant Hyperthermia Group (EMHG) has published the diagnostic criteria of MH/MHS [7]. The criteria specify that each mutation of the RYR1 gene must be characterized at the genetic level, and also assayed by recombinant in vitro expression on a defined genetic background as well as in ex vivo tissues, including myotubes. Given the CICR test results showing susceptibility to MH and the genetic variant of RYR1, we conducted genetic analysis of the patient's family members and functional analysis of p.Thr84Met RYR1 using patient-derived myotubes and kidney 293 HEK-293 cells. The in vitro test using human embryonic (HEK-293) cells transfected with the RYR1 gene is frequently employed to evaluate RYR1 function [8–10]. The study was approved by the local ethics committee (Hiroshima University).

### Genetic testing

DNA of the patient and his family members was extracted from the peripheral blood lymphocytes. All coding sequences of the RYR1 gene were amplified by polymerase chain reaction (PCR), and sequenced by the Sanger method. Sequences were aligned to the reference RYR1 sequences from GenBank accession NM_000540.2. We assessed the functional effects of the variant using algorithms from three different software tools: Mutation Taster [11], PolyPhen-2 [12], and SIFT [13].

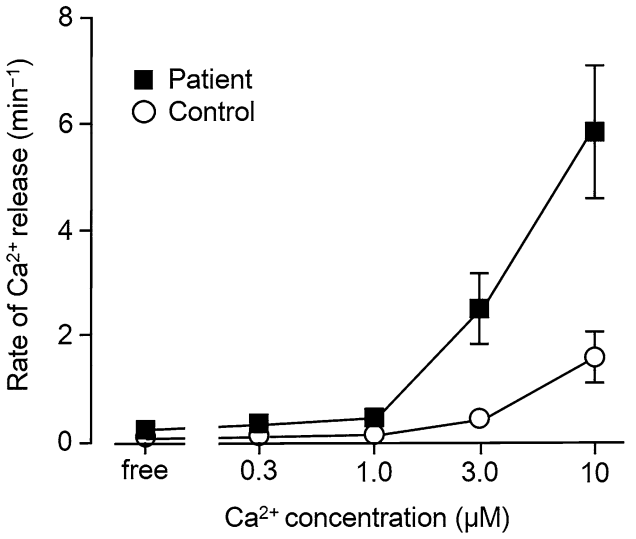


**Fig. 1** $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) results in the patient and in controls. Before measuring CICR rates, the skeletal muscle fibers were dissected and isolated in the relaxing solution. The skinned fiber membrane was chemically destroyed by saponin (50 μg/ml) for 30 min, and the CICR rates were assessed by measuring induced calcium release from the SR at five different calcium concentrations per subject. The CICR rates at four different $Ca^{2+}$ concentrations were higher in the patient than in the control group. The control group consisted of 12 non-MH individuals diagnosed by the IVCT. If the CICR values were more than two standard deviations from the mean of the control group, the CICR was defined as "accelerated." Data represent the mean ± SD

### Cell preparation

#### Myotubes

Myotubes were obtained according to Kobayashi's method [6]. Surplus muscle specimens were extracted from the patient after the CICR test was performed. Myoblasts were obtained from the muscle specimens and cultured in Dulbecco's modified Eagle medium and F12 medium (DMEM/F12; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated bovine calf serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). Proliferating myoblasts were removed and cultured in DMEM/F12 supplemented with 2% FBS, 1% kanamycin sulfate, ampicillin sodium salt, and 2.5 μg/ml amphotericin B. Myoblasts were fused to form multinuclear myotubes, and

myotubes were confirmed by indirect immunofluorescent staining of sarcomeric α-actinin and RYR1.

### HEK-293 cells transfected with the p.Thr84Met RYR1 gene

The HEK-293 Tet-off advanced cell line (Takara Bio, Kusatsu, Japan), restriction enzymes (Takara Bio), and other drugs for $Ca^{2+}$ measurement, including caffeine and 4-chloro-*m*-cresol (4C*m*C: Wako Pure Chemical Industries, Osaka, Japan), were purchased for this study.

Full-length rabbit skeletal muscle RYR1/pcDNA (rabbit-RYR1/pcDNA) was provided courtesy of David H. MacLennan (University of Toronto). After rabbit-RYR1 cDNA was removed from rabbit-RYR1/pcDNA by the restriction enzymes *Xba*I/*Hind*III, it was inserted into the pTRE-Tight-BI (pBI; Takara Bio)-*Aequorea coerulescens* green fluorescent protein (AcGFP) vector (RYR1/pBI) using the Takara DNA ligation kit (Takara Bio) to confirm expression of the RYR1 gene.

A short fragment of N-terminal RYR1 removed from RYR1/pBI using restriction enzymes was inserted into a pBluescript II KS(+) (Stratagene Cloning Systems, La Jolla, CA, USA) vector, and used as a template for mutagenesis of RYR1. We used a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) for mutagenesis. The primer used for mutagenesis was 5′-GATGCTGGCCAACATGGTGGA GGCTGGCGT-3′. After mutagenesis, the desired mutation contained in pBluescript II KS(+) was confirmed by sequencing with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and a BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems). The mutated fragment was removed from the pBluescript II KS(+) plasmid using restriction enzymes *Xba*I/*Kpn*I, and ligated into RYR1/pBI to construct the expression vector of the p.Thr84Met RYR1 gene.

HEK-293 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% Tet System-Approved FBS (Takara Bio), 100 U/ml of penicillin (Sigma-Aldrich), and 100 mg/ml of streptomycin (Sigma-Aldrich) at 37 °C under 5% $CO_2$. DNA transfection of the mutated RYR1 gene into HEK-293 cells was carried out after $1 \times 10^5$ HEK-293 cells were subcultured in 35-mm poly-L-lysine-coated glass-bottomed dishes (Matsunami Glass Ind., Osaka, Japan) for 24 h. Control cells were similarly transfected with RYR1/pBI [wild type (WT)]. The FuGENE HD Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) was used 72 h before $Ca^{2+}$ measurement.

### $Ca^{2+}$ fluorescence measurements

$Ca^{2+}$ measurement using myotubes and HEK-293 cells transfected with the mutated RYR1 gene activated by caffeine and 4C*m*C was performed according to previously described methods [6–9].

Prior to measurement, myotubes and HEK-293 cells transfected with the WT or mutated RYR1 gene were washed in HEPES-buffered salt solution (HBSS) containing 130 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.5 mM glucose at pH 7.4. The cells were loaded with 5 μM $Ca^{2+}$-sensitive fluorescent dye (Fura-2 AM; Dojindo Molecular Technologies, Tokyo, Japan) in HBSS for 1 h at 37 °C. Measurements were performed after perfusion with HBSS for 30 min (for myotubes) or 15 min (for HEK-293 cells) at a rate of 1.2 ml/min at 37 °C. Only myotubes that reacted to 10 mM caffeine were used for experiments. Successful transfection of the RYR1 gene into HEK-293 cells was confirmed by fluorescence emission of AcGFP at 510 nm using a fluorescence microscope (Nikon, Tokyo, Japan). The myotubes and AcGFP-positive HEK-293 cells were alternately excited at 340 and 380 nm, and fluorescence emissions of Fura-2 AM at 510 nm were observed at 5-s intervals to evaluate intracellular $Ca^{2+}$ changes. Images were acquired by a cooled high-speed digital video camera (ORCA-AG; Hamamatsu Photonics, Hamamatsu, Japan). The Aquacosmos 2.5 $Ca^{2+}$ imaging system (Hamamatsu Photonics) was used to determine relative changes in $Ca^{2+}$ by calculating the 340/380 nm signal ratio of Fura-2 AM fluorescence intensity. The absolute concentration of $Ca^{2+}$ was also calculated by comparing the 340/380 nm ratio against a standard curve.

HBSS containing incremental levels of caffeine or 4C*m*C was perfused into one side of the culture dish and aspirated from the opposite side for 2 min at a rate of 1.2 ml/min at 37 °C. Each solution was washed out 2 min before adding the next incremental level of the drug. Caffeine was tested at 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 mM, and 4C*m*C was tested at 3, 10, 30, 100, 300, 500, and 1000 μM in myotubes. In HEK-293 cells, caffeine was tested at 0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, and 20.0 mM, and 4C*m*C was tested at 15.125, 31.25, 62.5, 125, 250, and 500 μM (Fig. 2).

### Data analysis

For $Ca^{2+}$ measurement in myotubes, we used 13 myotubes per RYR1 activator (caffeine and 4C*m*C). The current patient's data were compared with a control value defined by the mean data of 17 patients who underwent non-accelerated CICR testing as documented in a previous study [6]. Cellular measurement was performed in 120 different HEK-293 cells. The changes in the 340/380 nm ratios for caffeine and 4C*m*C in myotubes and HEK-293 cells were calculated from the

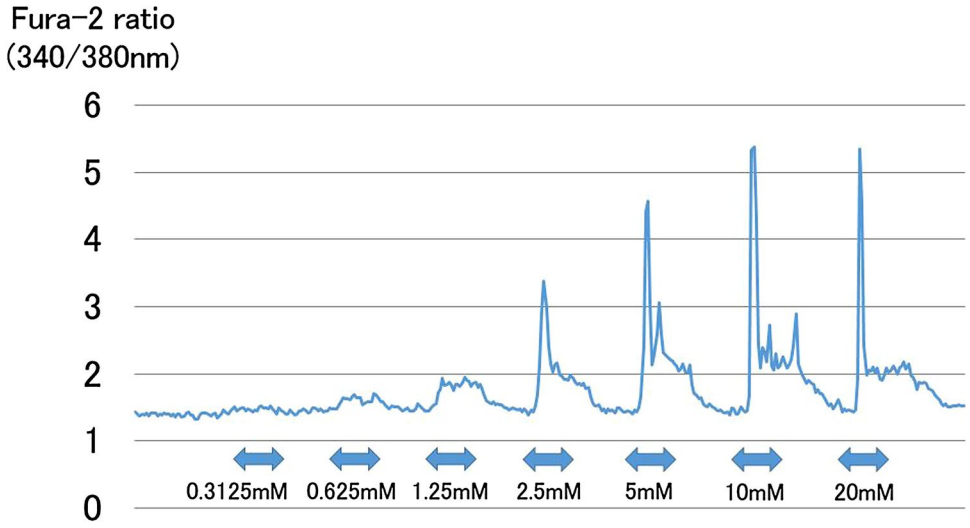


**Fig. 2** The representative recording of $Ca^{2+}$ fluorescence measurement in the HEK-293 cell for caffeine. The 340/380 nm signal ratio of Fura-2 AM fluorescence intensity was used to determine relative changes in $Ca^{2+}$ and the absolute concentration of $Ca^{2+}$ for incremental doses of caffeine

difference between the maximal response and the baseline. To trace dose–response curves, the data were normalized to the maximal response of each cell. The half-maximal effective concentrations ($EC_{50}$) of caffeine and 4C*m*C were calculated from the acquired dose–response curves. All data were analyzed using PRISM 6.0 software (GraphPad Software, San Diego, CA, USA). The $EC_{50}$ was measured in each cell and compared between two groups: for myotubes, the control group and the patient, and for HEK-293 cells, WT and p.Thr84Met. Statistical significance was determined using an unpaired 2-tailed *t*-test. *P* values of 0.01 were considered to be statistically significant.

## Results

### Genetic testing

Sequencing of the whole RYR1 gene in the patient's family members demonstrated the same variant c.251 C > T that resulted in the amino acid substitution p.Thr84Met (Fig. 3). The variant was considered pathogenetic based on the results of Mutation Taster and PolyPhen-2. The pedigree of the investigated family is shown in Fig. 4.

### Functional analysis

#### Myotubes

The dose–response curves for caffeine and 4C*m*C are shown in Fig. 5. The patient's curves are shifted to the left compared to the control. The $EC_{50}$ values for caffeine and 4C*m*C are shown in Tables 1 and 2. The $EC_{50}$ value of the patient for caffeine was 3.02 ± 0.88 mM, which was significantly lower than that of the control (5.08 ± 0.60 mM). For 4C*m*C, the patient's $EC_{50}$ value was 160.6 ± 92.8 µM, which was also significantly below the control value (277.2 ± 63.8 µM). There was no difference in the absolute concentration of $Ca^{2+}$ between the control and patient data at either baseline or with maximal response.

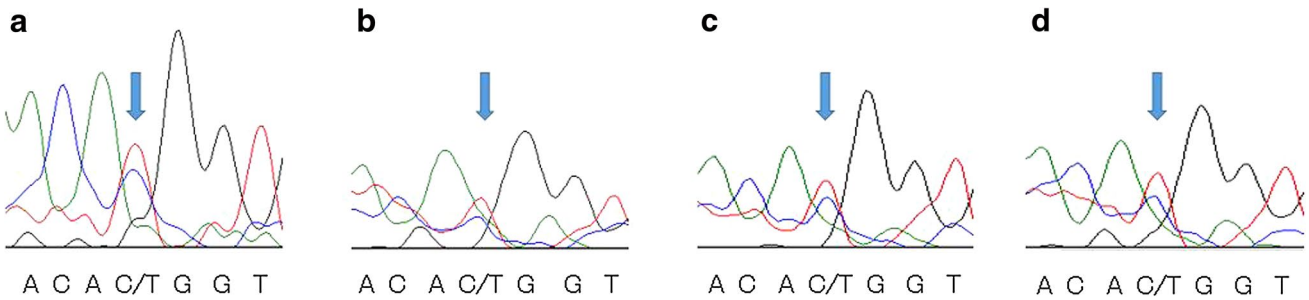


**Fig. 3** Sanger DNA sequencing results of the patient's family members indicating the RYR1 variant c.251 C > T (*arrows*). **a** The patient. **b** The patient's mother. **c** The patient's brother. **d** The patient's sister

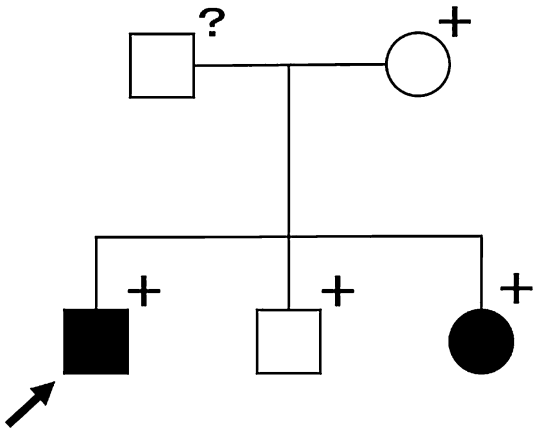


**Fig. 4** A family pedigree diagram. *Squares* represent males and *circles* represent females. A *black* symbol indicates a clinically affected individual with the p.Thr84Met variant of ryanodine receptor type 1. A *white* symbol indicates an individual whose genetic test could not be performed. The existence of the p.Thr84Met mutation is marked with *plus*. The *arrow* indicates the proband

### HEK-293 cells

The proportion of AcGFP-positive cells was around 50%, with no obvious difference in transfection efficiency between the WT- and p.Thr84Met RYR1-transfected cells. The dose–response curves for caffeine and 4C*m*C are shown in Fig. 6. The curves of the p.Thr84Met RYR1-transfected cells were shifted to the left compared with the WT-transfected cells (WT). The $EC_{50}$ values for caffeine and 4C*m*C are shown in Tables 3 and 4. The $EC_{50}$ value of the p.Thr84Met RYR1-transfected cells for caffeine was 1.81 ± 0.70 mM, which was significantly lower than that of WT (2.70 ± 0.57 mM). For 4C*m*C, the $EC_{50}$ value of the p.Thr84Met RYR1-transfected cells was 84.0 ± 31.3 μM, which was also significantly lower than that of WT (119.8 ± 30.1 μM). There was no difference in the absolute concentration of $Ca^{2+}$ between the WT- and p.Thr84Met RYR1-transfected cells at either baseline or with maximal response.

**Table 1** $EC_{50}$ values for caffeine in myotubes

| | $EC_{50}$ values (mM) | 95% CI (mM) | *P* value | *n* |
|---|---|---|---|---|
| Control | 5.08 ± 0.60 | 4.766–5.391 | | 17 |
| Patient | 3.02 ± 0.88 | 2.493–3.552 | < 0.0001 | 13 |

Values are mean ± SD. *P* values are versus the control group
*$EC_{50}$* 50% effective concentration, *95% CI* 95% confidence interval

**Table 2** $EC_{50}$ values for 4C*m*C in myotubes

| | $EC_{50}$ values (μM) | 95% CI (μM) | *P* value | *n* |
|---|---|---|---|---|
| Control | 277.2 ± 63.8 | 244.4–310.1 | | 17 |
| Patient | 160.6 ± 92.8 | 104.6–216.7 | 0.0003 | 13 |

Values are mean ± SD. *P* values are versus the control group
*4CmC* 4-chloro-*m*-cresol, *$EC_{50}$* 50% effective concentration, *95% CI* 95% confidence interval

## Discussion

In this study, we performed an analysis of the RYR1 mutation p.Thr84Met found in a patient who was suspected of having a congenital myopathy and MH. Our results

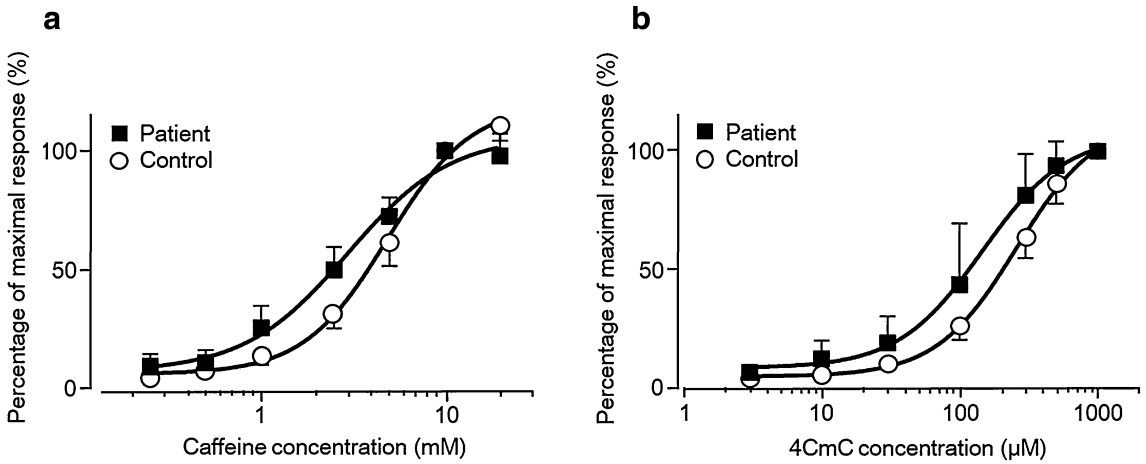


**Fig. 5** Dose–response curves of $Ca^{2+}$ release to caffeine or 4-chloro-*m*-cresol (4C*m*C) in myotubes. **a** Responses of myotubes from the patient (*filled squares*) and the control group (*open circles*) to caffeine. **b** Responses of myotubes from the patient (*filled squares*) and the control group (*open circles*) to 4C*m*C. Data represent the mean + SD (*upward*) in the patient, and the mean − SD (*downward*) in the control group

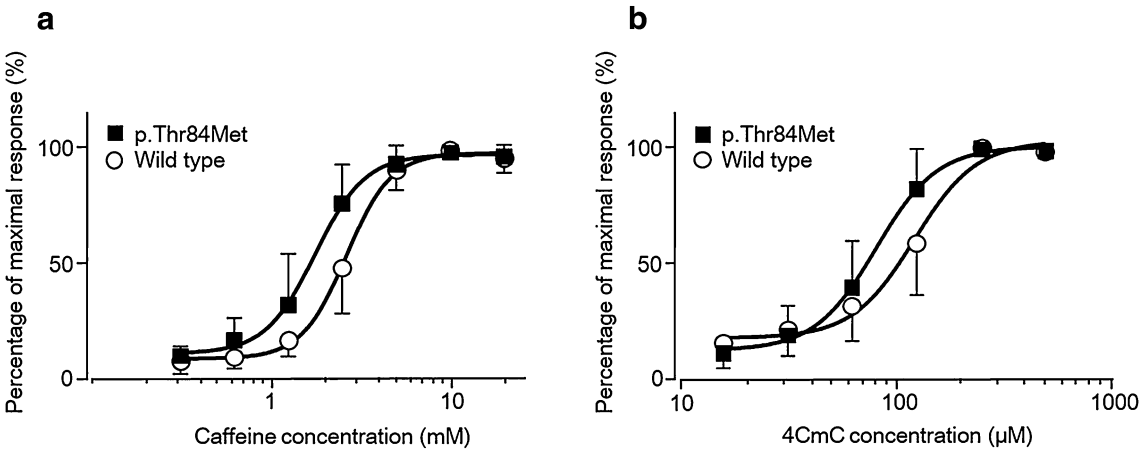


**Fig. 6** Dose–response curves of $Ca^{2+}$ release to caffeine or 4-chloro-*m*-cresol (4C*m*C) in human embryonic kidney (HEK)-293 cells. **a** Responses of p.Thr84Met ryanodine receptor (RYR)-1-transfected cells (*filled squares*) and wild-type (WT) RYR1-transfected cells (*open circles*) to caffeine. **b** Responses of p.Thr84Met RYR1-transfected cells (*filled squares*) and WT-RYR1-transfected cells (*open circles*) to 4C*m*C. Data represent the mean + SD (*upward*) in the p.Thr84Met cells and the mean − SD (*downward*) in the WT cells

**Table 3** $EC_{50}$ values for caffeine in HEK-293 cells

| | $EC_{50}$ values (mM) | 95% CI (mM) | *P* value | *n* |
|---|---|---|---|---|
| Wild type | 2.70 ± 0.57 | 2.485–2.907 | | 30 |
| p.Thr84Met | 1.81 ± 0.70 | 1.547–2.068 | < 0.0001 | 30 |

Values are mean ± SD. *P* values are versus wild type

*HEK-293 cells* human embryonic kidney-293 cells, *$EC_{50}$* 50% effective concentration, *95% CI* 95% confidence interval

**Table 4** $EC_{50}$ values for 4C*m*C in HEK-293 cells

| | $EC_{50}$ values (μM) | 95% CI (μM) | *P* value | *n* |
|---|---|---|---|---|
| Wild type | 119.8 ± 30.1 | 108.5–131.0 | | 30 |
| p.Thr84Met | 84.0 ± 31.3 | 72.34–95.74 | < 0.0001 | 30 |

Values are mean ± SD. *P* values are versus wild type

*4CmC* 4-chloro-*m*-cresol, *HEK-293 cells* human embryonic kidney-293 cells, *$EC_{50}$* 50% effective concentration, *95% CI* 95% confidence interval

demonstrated that this mutation was hereditable, and that patient-derived myotubes and p.Thr84Met RYR1-transfected HEK-293 cells were hypersensitive to caffeine and 4C*m*C.

The patient showed muscle weakness from birth, which is common in congenital myopathies, including RYR1-RMs such as CCD. According to guidelines for the diagnosis of congenital muscle disorders [14, 15], the clinical findings of the current patient were consistent with RYR1-RMs, but the histopathological findings were nonspecific. However, RYR1-RMs show various patterns of expression and may be non-penetrant [16]. Moreover, the characteristic core structure in RYR1-RMs may be absent in some muscle biopsies [17]. Therefore, it could not be ruled out that genetic mutation of the RYR1 gene was related to the muscle weakness in this patient.

RYR1-RMs overlap clinically and pathologically with MH, and the mutation of RYR1 in MH/RYR1-RM has three clinical phenotypes: (a) MH only, (b) RYR1-RM only, and (c) MH with variable penetrance of RYR1-RM [1]. The onset of MH is multifactorial, and various cell components other than RYR1 are related to the increase of intracellular $Ca^{2+}$ concentration leading to MH, including the SR/ER $Ca^{2+}$-ATPase; the plasma membrane ATPase; and the dihydropyridine receptor (DHPR), associated with excitation–contraction coupling, located in the L-type voltage-dependent $Ca^{2+}$ channel situated in the T-tubule [2]. The pathology of MH can sometimes be mild and nonspecific [18], which makes identifying patients with MH susceptibility difficult.

The diagnostic process for MH takes into account clinical findings, genetic testing, muscle biopsy, and functional testing; specifically the caffeine-halothane contracture test (CHCT) in North America and the in vitro contracture test (IVCT) in Europe [7, 19]. In the current patient, MH susceptibility was diagnosed based on the fact that CICR rates were accelerated at different $Ca^{2+}$ concentrations. The CICR test has been performed mostly in Japan, using one or more chemically skinned muscle fibers. Muscle fibers are treated with saponin before measuring the CICR rate. After saponin exposure, the sarcolemma and the T-tubules are no longer intact, resulting in deficient plasma membrane function. Thus, the CICR test can specifically detect SR abnormalities, eliminating other mechanisms related to the onset of MH such as excitation–contraction coupling or $Ca^{2+}$ homeostasis mediated by other $Ca^{2+}$ transporters. Although the results

of the accelerated CICR test are well correlated with abnormal results on the CHCT/IVCT [6], there are differences in the interpretations of these various tests. The CHCT and IVCT, which have been the gold standards for the diagnosis of MH, are based on the contracture of intact muscle fibers in the presence of halothane or caffeine as the RYR1 activator. Compared to the CICR test, which indicates only abnormalities in the SR, the CHCT/IVCT can detect various abnormalities such as those of DHPR in the skeletal muscle. In this study, we performed additional functional analysis using myotubes and HEK-293 cells to obtain more precise determination of abnormal $Ca^{2+}$ homeostasis in positive CICR muscle.

Our previous study demonstrated a good correlation between the CICR test and the response to RYR1 activators in human cultured myotubes [6]. Functional tests using myotubes can evaluate overall $Ca^{2+}$ homeostasis in muscle cells, and the results are associated with those of the IVCT [20]. In this study, the accelerated CICR rate and the increased hypersensitivity reaction of myotubes to caffeine and 4C*m*C indicated that $Ca^{2+}$ release from the SR increased in response to the RYR1 activators. Our functional test using mutated RYR1-transfected HEK-293 cells can detect only RYR1 functional abnormalities in $Ca^{2+}$ homeostasis, and the assay is approved for the molecular genetic detection of susceptibility to MH [8]. We demonstrated the increased sensitivity of mutated p.Thr84Met RYR1-transfected cells to caffeine and 4C*m*C. The results of these three functional tests (the CICR test, the test using myotubes, and the test using mutated RYR1-transfected HEK-293 cells) indicate that dysfunction of RYR1 due to the p.Thr84Met mutation was associated in this patient with increased $Ca^{2+}$ release from the SR in response to RYR1 activators.

The mechanisms of RYR1 dysfunction in MH/RYR1-RMs have been categorized into the following four types: hypersensitivity (MH), $Ca^{2+}$ leakage from the SR (CCD), excitation–contraction uncoupling between RYR1 and DHPR (CCD), and low expression of RYR1 (other recessive RYR1-RMs) [21, 22].

$Ca^{2+}$ leakage from the SR increases intracellular $Ca^{2+}$ concentrations. In our study, there was no difference in the absolute concentration of $Ca^{2+}$ between the control cells and mutant cells either at baseline or at maximal response (data not shown). The increase of $Ca^{2+}$ release does not occur if excitation–contraction coupling is inhibited [23]. The responses to $Ca^{2+}$ and RYR1 activators were elevated in this study, which means that excitation–contraction coupling was maintained. These results indicate that the p.Thr84Met RYR1 mutation is related to hypersensitivity of RYR1.

The RYR1 gene mutation sites associated with MH/CCD are mainly located in three "hotspots": domain 1, in the N-terminal region (p.Met1-p.Arg614); domain 2, in the central region (p.Arg2163-p.Arg2458); and domain 3, in the C-terminal region (p.Arg4136-p.Pro4973) [24]. Recent studies have shown that many mutations associated with MH susceptibility can occur outside these hotspots [25, 26], and the full details regarding the RYR1 mutations that cause MH are not clear.

Most mutations associated with MH are located in the N-terminal region or the central region, while CCD mutations are located in the C-terminal region [27]. Some mutations are related to both MH and CCD, and a previous study documented that five of the 15 mutations in the RYR1 N-terminal region that is considered causative for MH susceptibility were associated with CCD [28].

We found a novel gene mutation site leading to RYR1 hypersensitivity, located in the N-terminal region inside the hotspots. This gene mutation results in the amino acid substitution of hydrophilic threonine, which contains a hydroxyl group at the 84th position of the RYR1 gene, to hydrophobic methionine. This substitution changes the structure of RYR1, and we believe that it caused dysfunction of the receptor in our patient. RYR1 is a tetrameric channel in which the N-terminal region is separated into three domains that interact with each other through a hydrophilic interface and control channel opening. The mutation of the N-terminal region suppresses interdomain interaction and destabilizes the closed state of the channel, and consequently the channel tends to open to release $Ca^{2+}$ from the SR [29]. These findings indicate that the mutation of the N-terminal region of RYR1 increases the sensitivity of the receptor, which supports the results of our study.

There are several limitations to this study. First, since the patient did not present with symptoms of MH, the clinical effects of the hypersensitivity caused by the RYR1 mutation could not be evaluated. Second, further genetic tests of the family are required to clarify the details of the mutation's inheritance. The criteria of the EMHG for genetic testing of MH susceptibility require genetic characterization that includes co-segregation of the mutation with the disease in at least two pedigrees. Another report in a second family must be published to meet the EMHG criteria for confirming that this mutation causes MH susceptibility.

In conclusion, we performed genetic and functional analysis of the RYR1 mutation p.Thr84Met. The mutation was inherited, and RYR1 responsivity to caffeine and 4C*m*C was higher in myotubes derived from the patient and in p.Thr84Met RYR1-transfected HEK-293 cells than in control cells. This RYR1 hypersensitivity indicates that the mutation was associated with MH susceptibility.

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## Compliance with ethical standards

**Conflict of interest** This study was supported in part by a Grant-in-Aid (Grant number 16K20098) for Scientific Research from the Japan Society for the Promotion of Science, Japan.

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