Title: Genetic screening for malignant hyperthermia and comparison of clinical symptoms in Japan

Running title: Genetic screening and clinical symptoms of MH

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Submission type: Article

Title Character count: 78

Number of References: 32

Number of Tables: 1

Number of Figures:3

Number of Supplemental data: 3

Word count of Abstract: 246

Word count of Paper: 3173

Abstract

Malignant hyperthermia (MH) is an anaesthetic complication that causes an abnormal hypermetabolic state. *RYR1* encoding ryanodine receptors of the sarcoplasmic reticulum and *CACNA1S* encoding α subunits of dihydropyridine receptors are known as the genes associated with MH pathogenicity. We performed genetic screening using a next-generation sequencer to evaluate the prevalence of genes associated with MH pathogenicity and clinical symptoms. This was a retrospective cohort study. Next-generation sequencing of 77 families diagnosed with MH predisposition by calcium-induced calcium release (CICR) tests from 1995 to 2019 was used to search for the *RYR1* and *CACNA1S* variants. Further, clinical symptoms and predisposition tests in participants with *RYR1* and *CACNA1S* variants were compared. In 77 families, 44.2%, 7.8%, and 48.1% had *RYR1*, *CACNA1S*, and no pathogenic variants, respectively. Clinically significant differences were found in the maximum body temperature, maximum elevated body temperature for 15 min, creatinine kinase, CICR rate, and half maximal effective concentration of caffeine and 4-chloro-m-cresol which showed ex vivo myotube sensitivity to ryanodine stimulants between *the RYR1* and *CACNA1S* groups. The rate of pathogenic *CACNA1S* variants seems to be prominent in Japan. The severity of clinical symptoms and the CICR rate were greater in individuals with *RYR1* variants than in those with *CACNA1S*, likely due to more direct regulation of calcium levels by ryanodine receptors than dihydropyridine receptors. Genetic analysis of MH in future studies may identify more genes associated with MH, which will further clarify the relationship between genotypes and symptoms and contribute to safer anaesthesia.

Keypoints: Malignant Hyperthermia, NGS, *RYR1*, *CACNA1S*

Introduction

Malignant hyperthermia (MH) is a rare anaesthetic complication (1 in 10,000 to 250,000[1]) caused by the administration of inducing agents, such as volatile anaesthetics and depolarizing muscle relaxants, to MH-susceptible patients. Patients may have clinical symptoms such as abnormal temperature rise, tachycardia/arrhythmia, muscle rigidity, and skeletal muscle breakdown, which may be fatal without effective treatment.[2] MH develops as a potentially fatal hypermetabolic crisis associated with a rapid and uncontrollable increase of calcium (Ca^{2+}) in skeletal muscle cells.[3]

The mechanism of MH is mainly the abnormality in Ca^{2+} metabolism caused by pathogenic variants in *RYR1* (gene encoding the ryanodine receptor 1 in the sarcoplasmic reticulum) and *CACNA1S* (gene encoding the alpha subunit of the dihydropyridine receptor (DHPR) in the plasma membrane of skeletal muscle cells). It has also been reported that *STAC3* is involved in the development of MH.[4] Although STAC3 is known to interact with DHPR, its exact functional role in MH is still unknown. Many people (34%–86%) with genetic predisposition have pathogenic *RYR1* variants[5] as the gene associated with MH, and about 1% of people have pathogenic *CACNA1S* variants.[6] *CACNA1S* is a rare gene associated with MH, and few studies have examined the clinical characteristics of MH caused by *CANCA1S* variants.[7] However, *CACNA1S* is the second most common gene associated with MH, and it is important to examine the clinical differences from MH caused by other genes such as *RYR1*. In this study, we performed genetic screening using a next-generation sequencer to evaluate the prevalence of genes associated with MH (*RYR1* and *CACNA1S*) and clinical symptoms of MH in Japan. We excluded *STAC3* in this study because no reports have demonstrated *STAC3* as the gene associated with MH in Asia.

Methods

Patients and DNA extraction

This was a retrospective cohort study. This study was approved by the Ethics Committee of Hiroshima University in Japan (Ethical approved #151). DNA was extracted from the blood, muscle tissues, and cells of 49 MH patients and 41 family members (Supplementary Table 1) with MH predisposition, as determined by a calcium-induced calcium release (CICR) test at Hiroshima University Hospital from 1995 to 2019. Seventy-seven independent families were included in this study. The automated DNA separation system Quick Gene-610L and Quick Gene DNA whole blood kit L (KURABO, Kurashiki, Japan) were used for DNA extraction from the blood, and DNeasy Blood and Tissue Kits (QIAGEN, Hilden, Germany) were used for DNA extraction from muscles and cells.

Genetic screening using next-generation sequencer

DNA samples of the participants were screened for pathogenic variants in *RYR1* (NM_000540) and *CACNA1S* (NM_000069) using a next-generation sequencer (Ion Personal Genome Machine (PGM), Life Technologies, Carlsbad, CA, USA). In total, 231 primer pairs for exonic regions of *RYR1* and *CACNA1S*

were designed using the Ion AmpliSeq designer (https://www.ampliseq.com/) as two subgroups: primer pool 1 for 116 amplicons and primer pool 2 for 115 amplicons (GRCh37/hg19) (Supplementary Table 2). Eight regions were not covered by the originally designed primers (Supplementary Table 3). Therefore, the uncovered regions were amplified by polymerase chain reaction (PCR) and sequenced directly by the Sanger method using an Applied Biosystems 3130 DNA sequencer (Life Technologies).

Ion PGM sequencing was performed using the Ion AmpliSeq Library Kit 2.0 (Life Technologies) and the Ion PGM Hi-Q sequencing kit (Life Technologies), according to the manufacturer's protocols. Variants were identified using TorrentSuite Software 5.0.5 (Thermo Fisher Scientific, Waltham, MA, USA).

We filtered variants based on the criteria of combined annotation dependent depletion (CADD, https://cadd.gs.washington.edu/) score ≥16, REVEL (Rare Exome Variant Ensemble Learner, https://sites.google.com/site/revelgenomics/downloads) score > 0.5, and minor allele frequency (MAF) < 0.1% in the Genome Aggregation Database (gnomAD v2.1.1, https://gnomad.broadinstitute.org/). These variants were confirmed by standard PCR-based amplification, followed by Sanger sequencing.

We evaluated the confirmed variants based on the EMHG scoring matrix for the classification of genetic variants in malignant hyperthermia susceptibility (https://www.emhg.org/genetic-scoring-matrix). To be evaluated as "Pathogenic" and "Likely pathogenic" according to EMHG scoring matrix, it is necessary to satisfy the conditions of "Pathogenic Strong" or "Pathogenic Moderate". Pathogenic Strong requires a diagnostic mutation in EMHG. To be pathogenic moderate, the prevalence of the variant was significantly increased ($P < 1 \times 10^{-7}$) compared with the prevalence of a relevant low-risk population. In previous studies, CICR-positive variants were also found to be positive for IVCT[8]. Therefore, we evaluated CICR-positive cases, all cases in this study, as "Pathogenic supporting": Association with a clinical reaction consistent with malignant hyperthermia under anaesthesia and confirmed by a positive IVCT in the EMHG scoring matrix. We chose "Pathogenic" and "Likely pathogenic" variants on EMHG scoring matrix as pathogenic variants, and included "Pathogenic" and "Likely pathogenic" variant for following analysis. For reference, CICR-negative samples from 17 independent MH patients and their families not included in this study were also genetically screened.

Evaluation of association between variants and clinical symptoms

Clinical symptoms, including maximum body temperature (Max BT), maximum elevated body temperature for 15 min (°C /15 min), maximum creatine kinase value (Max CK), clinical grading scale (CGS),[9] maximum serum K concentration, maximum $PaCO_2$, and minimum pH were extracted from anaesthesia charts and medical records. Statistical analysis was performed between the *RYR1* and *CACNA1S* groups to clarify differences in clinical symptoms using the Mann–Whitney U test. For comparison with normal controls, we used samples that were diagnosed as MHN by IVCT and CHCT in the previous study as the normal group. [8]

Calcium-induced calcium release (CICR) test

Ninety patients and their family members underwent skeletal muscle biopsies and were examined by a CICR test.[8,10]The CICR test was performed according to the method described by Endo and colleagues[11] with chemically skinned fibres. CICR rates were assessed by measuring induced calcium release from the sarcoplasmic reticulum at five different calcium concentrations (0, 0.3, 1.0, 3.0, and 10 μM). The CICR rate was considered to increase when the CICR values were two standard deviations (SD) above the average values of the normal at two or more calcium concentrations. Normal CICR values were derived from 12 MH-negative (MHN) individuals previously excluded by in vitro contracture test (IVCT).[12]

Data analysis

Data from the CICR test, myotube sensitivity to ryanodine stimulants, and clinical symptoms were analysed using PRISM 7.0 and EZR in the R software.[13] $p < 0.05$ was considered significant as calculated by the Mann–Whitney U test and Spearman's coefficient test.

Results

Screening of *RYR1* and *CACNA1S* variants

Thirty-two variants with CADD score of 16 or more, REVEL score of 0.5 or more, and MAF in gnomAD of less than 0.1% were selected, and all variants were confirmed by Sanger sequence. As a result of evaluating these variants based on EMHG scoring matrix, 28 *RYR1* variants and four *CACNA1S* variants which were classified as "Pathogenic" and "Likely pathogenic" were found in 90 subjects (49 MH patients, 41 MH family members) from 77 families (Table1). Among them, nine *RYR1* and one *CACNA1S* variant have been reported in the European Malignant Hyperthermia Group (EMHG, https://www.emhg.org/diagnostic-mutations). Thirteen *RYR1* variants have been reported in other studies.[10,14] Six *RYR1* and three *CACNA1S* variants were novel (Table 1, Supplementary Table 1). Thirty-four families had *RYR1* variants (41 individuals, 28 variants; *RYR1* group), six families had *CACNA1S* variants (seven individuals, four variants; *CACNA1S* group), and 37 families did not have *RYR1* and *CACNA1S* variants (42 individuals, Unknown group; Fig. 1, Supplementary Table 1). Two individuals in *the RYR1* group had two *RYR1* variants (Supplementary Table 1, M51, 60). The prevalence of variants was 44.2% in families with *RYR1*, 7.8% with *CACNA1S*, and 48.1% of families without *RYR1* and *CACNA1S* variants (Fig. 1). Although variants associated with MH pathogenicity are abundant in the *RYR1* hot spot region, variants have also been frequently recognised outside the hot spot (hot spot region I, p.M1-p.R614; region II, p.R2163-p.R2458; region III, p.R4136-P4973). For reference, we screened 17 CICR-negative MH patients and their families and found no likely pathogenic variants.

Evaluation of clinical symptoms

Max BT was recorded in 36 cases (18 cases with pathogenic variants in *RYR1*, 4 cases in *CACNA1S*, and

14cases in unknown group), and there was a significant difference between the *RYR1* and *CACNA1S* groups (p =0.0003) (Fig. 2A, Supplementary Table 1). °C /15 min was recorded in 32 cases (16 cases in *RYR1* group, 4 cases in *CACNA1S* group, and 12 cases in unknown group), and there was a significant difference between the *RYR1* and *CACNA1S* groups (p = 0.0239) (Fig. 2B, Supplementary Table 1). Max CK was recorded in 30 cases (15 cases in *RYR1* group, 3 cases in *CACNA1S* group, and 12 cases in unknown group), showing a significant difference between the *RYR1* and *CANCA1S* groups (p = 0.0098) (Fig. 2C, Supplementary Table 1). There was no significant difference in CGS (18 cases in *RYR1* group, 5 cases in *CACNA1S* group, and 15 cases in unknown group) (p = 0.1140) (Fig. 2D, Supplementary Table 1).

Comparison of CICR rate between pathogenic variants

CICR rates at five calcium concentrations were examined in all 90 participants. In Ca-free conditions, samples generally did not react, even in the presence of MH predisposition, and no significant difference was found between the *RYR1* and *CACNA1S* groups using the Mann–Whitney U test (Fig. 3A). However, significant differences were observed between the two groups at other four calcium concentrations (0.3 μM, p = 0.0240; 1.0 μM, p = 0.0284; 3.0 μM, p = 0.0016; 10 μM, p = 0.0011). Figure 3B shows a representative graph at calcium 3.0 μM.

Discussion

We screened the most common genes associated with MH pathogenicity in the Japanese population and analysed the frequency of the genes and their correlation with clinical symptoms. Comparing the frequency of genes associated with MH pathogenicity with that in other countries, pathogenic *CACNA1S* variants showed a high prevalence in Japan. Patients with pathogenic *RYR1* variants were more severe than those with pathogenic *CACNA1S* variants in clinical symptoms and predisposition tests.

The prevalence of variants was 44.2% in families with *RYR1*, 7.8% with *CACNA1S*, and 48.1% of families with neither *RYR1* nor *CACNA1S* variants. The prevalence of *RYR1* variants was less than that reported previously in the Japanese population.[10] Previous studies have reported that most MH patients possessed pathogenic variants in *RYR1*. In those study, the prevalence of *RYR1* variants in MH patients varied, specifically 76% in the UK (546 out of 722 families),[15]52% in the USA (62 out of 120 families),[16] 34% in Australia (33 out of 96 patients),[7,14] 57% in Japan (33 of 58 patients),[10] 72% in Italy (31 of 43 patients),[17] 86% in Canada (31 of 36 patients),[18] and 50% in Sweden (7 of 14 patients).[19] We used REVEL score, CADD score, and MAF in gnomAD to assess the pathogenicity of the variants. As such, the rate of pathogenic *RYR1* variants appears to be lower than that previously reported by simply excluding variants with high MAF. It may also be affected by the difference between CICR and IVCT/CHCT. However, few studies have examined the frequency of pathogenic *CACNA1S* variants, and a previous study reported that approximately 1% of MH patients had *CACNA1S* variants.[6] There have been no studies on the prevalence of genes associated with MH in Asia, except Japan; thus, the proportion of pathogenic *CACNA1S* variants

throughout Asia is unknown. In this study, the prevalence of *CACNA1S* variants was high, suggesting that *CACNA1S* variants may be more frequent in Japanese populations than in Western countries. The prevalence of families without variants in either *RYR1* or *CACNA1S* was also higher than that reported in many previous studies. However, it is difficult to make a simple comparison based solely on the frequency because the criteria for determining the genes associated with MH pathogenicity and the method for selecting cases varied. The prevalence of pathogenic variants and their statistics will change depending on the definition of pathogenic variants.

Since some *CACNA1S* variants were detected, we compared the clinical symptoms and predisposition tests for each group. These results suggest that patients with *CACNA1S* variants had mild symptoms in Max BT, °C /15 min, and Max CK. While a previous study showed that patients with *RYR1* variants were likely to exhibit high CGS,[7] our study did not show any significant difference in CGS, probably because of the type of anaesthetic and the presence or timing of therapeutic interventions. Thus, additional studies involving more cases are required. A previous study compared the IVCT of MH patients with *RYR1* and *CACNA1S* variants *in vitro* rather than clinical symptoms.[7] The study showed that *CACNA1S* variants trended towards lower responses to both caffeine and halothane in IVCT. Our study also revealed that the *CACNA1S* group showed milder abnormalities than the *RYR1* group in CICR and EC_{50} of caffeine and 4-CmC, as well as the clinical symptoms described above. DHPR encoded by *CACNA1S* does not directly participate in calcium dynamics, but acts indirectly through RYR1. Consequently, *CACNA1S* variants may not directly alter RYR1 activity compared to *RYR1* variants.

In a previous study, the phenotype of MH caused by *RYR1* variants varied depending on the position of the variants.[20] Our results also showed that the CICR data and clinical symptoms in the *RYR1* group were widely distributed, which may be due to the various locations of *RYR1* variants (Fig. 2, Fig.3). In the unknown group, the CICR data and clinical symptoms were also widely distributed, probably because multiple novel genes associated with MH pathogenicity and various locations of variants were involved (Fig. 2, Fig.3).

The penetrance of genes most commonly associated with MH pathogenicity was low, and recent studies have shown that penetrance of the *RYR1* gene variants is 40.6%, and the probability of developing MH upon exposure to triggers is only 25% in predisposed individuals.[21] Patient age (young), sex (male), and use of succinyl choline also affected the onset of MH. Due to the low penetrance and various conditions, patients may be susceptible to MH, even if they did not develop MH under general anaesthesia. In addition, not all individuals with pathogenic variants are present for general anaesthesia. In this case, they were not aware of the predisposition to MH. Genetic analysis can be performed without significant invasiveness compared to tests that require muscle biopsy (CICR, IVCT, and CHCT), which can clarify the genes associated with MH pathogenicity in more than 50% of predisposed individuals. In addition to genetic diagnosis and bioinformatics analysis, it is desirable to perform a combination of predisposition tests (CICR, IVCT, and CHCT), MAF evaluation, and functional analysis depending on the situation.[22] As the investigation for new

genes associated with MH pathogenicity progresses and genetic diagnosis becomes easier, predisposition to MH can be diagnosed before surgery, enabling safer anaesthesia.

This study revealed that MH with *RYR1* variants (MH-RYR1) or *CACNA1S* variants (MH-CACNA1S) could have different phenotypes of clinical symptoms. Specifically, we focused on the different properties of the symptoms of MH-RYR and MH-CACNA1S to further clarify the differences in each pathological condition. The differences in pathogenesis due to the genes associated with MH pathogenicity can also be useful in determining clinical treatment. Conversely, by clarifying features not according to the genes associated with MH pathogenicity, common characteristics of MH can be understood and applied as more useful diagnostic criteria. Additional investigations are needed to examine MH-RYR1 and MH-CACNA1S in more detail. As the correlation between the genotype and phenotype of MH becomes more apparent, genetic diagnosis can be used to determine treatment strategies and predict the severity and prognosis of MH.

Conclusions

This study investigated the frequency of genetic predisposition of MH using target sequencing. Pathogenic *CACNA1S* variants seems to be prominent in Japan than in Western countries. The clinical symptoms and predisposition test (CICR) were more severe for MH-RYR1 than for MH-CACNA1S. This is probably because the ryanodine receptor encoded by *RYR1* has a more direct effect on the regulation of Ca^{2+} than the DHPR encoded by *CACNA1S*. Further studies using larger samples can reveal more details about the relationship between genotypes and clinical symptoms of MH. Moreover, advances in genetic analysis, including the identification of novel genes responsible for MH, will improve the diagnostic rate of genetic screening and contribute to safer anaesthesia.

Details of author's contributions

Study design: R.K., H.M., K.M., H.K.

Data collection: R.K., K.M., S.O.

Data analysis: R.K., H.M., K.M.

Writing paper: R.K., Y.N., T.K.

Revising paper: all authors

Acknowledgments

We thank the families involved in this research and Ms. Eiko Nakajima for technical assistance.

Declaration of interest

The authors declare that they have no conflict of interest.

Funding

This study was supported by a Grant-in-Aid for Young Scientists (grant number: 17K16733 to Y.N. and 20K17783 to R.K.) from the Japan Society for the Promotion of Science, and by the Takeda Science Foundation (H.K.).

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Table 1. Variant List

We selected variants with CADD ≥16, REVEL >0.5, and MAF in gnomAD (East Asia) <0.1% in 90 subjects from 77 families. These variants were evaluated using the EMHG scoring matrix and extracted “Pathogeni ”and ”Likely Pathogenic” variants. Evaluation by three algorithms (SIFT; Mutation Taster, MTPred; PolyPhen-2, PP2Pred), and MAF with the Tohoku Medical Megabank Organization (ToMMo, https://jmorp.megabank.tohoku.ac.jp/), a genomic study using a large Japanese cohort evaluation is shown for reference. The underlines are for the same family.

Abbreviations: EMHG, Listed as mutation in EMHG; Reported, reported in other papers, but not listed as mutation in EMHG; N.S., not scored; B, benign; D, disease-causing

Position	Sample	No. of families	rsID	AA change	report	REVEL	CADD	gnomAD MAF(east Asia)	gnomAD(East Asia) p-value	EMHG scoring matrix	SIFT	MTPred	PP2Pred	ToMMo MAF
RYR1 chr19:38934851C>T	M80	1	rs118192161	p.R163C	EMHG[10]	0.959	25.7		<1×10^{-7}	Pathogenic	N.S.	D	D	
RYR1 chr19:38939352G>A	M68	1	rs121918592	p.G341R	EMHG[18]	0.864	26.0		<1×10^{-7}	Pathogenic	N.S.	D	D	
RYR1 chr19:38939352G>C	M35	1	rs121918592	p.G341R	EMHG[10]	0.876	25.7		<1×10^{-7}	Pathogenic	N.S.	D	D	
RYR1 chr19:38946112G>A	M60	1	rs144336148	p.R533H	EMHG[10]	0.824	24.2	0	<1×10^{-7}	Pathogenic	N.S.	D	D	
RYR1 chr19:38948186G>T	M21, 25, 27, 69	4	rs193922772	p.R614L	EMHG[23]	0.931	28.4		<1×10^{-7}	Pathogenic	N.S.	D	D	
RYR1 chr19:38989863G>A	M43	1	rs112563513	p.R2336H	EMHG[5]	0.903	25.4		<1×10^{-7}	Pathogenic	N.S.	D	D	
RYR1 chr19:38991539G>A	M46	1	rs193922818	p.R2508H	EMHG[24]	0.898	25.7		<1×10^{-7}	Pathogenic	N.S.	D	D	
RYR1 chr19:39002961G>A	M32, 33	2	rs193922832	p.E3104K	EMHG[5]	0.868	26.0	0	<1×10^{-7}	Pathogenic	N.S.	D	D	
RYR1 chr19:39071010C>G	M11, 12, 13, 14, 15(Family8), 16, 17, 19(Family9)	2	rs193922878	p.L4838V	EMHG[10]	0.882	28.4		<1×10^{-7}	Pathogenic	N.S.	D	D	
CACNA1S chr1:201061121G>A	M6, 7(Family5), 24, 75	3	rs772226819	p.R174W	EMHG[25]	0.91	29.6	0.000109	<1×10^{-7}	Pathogenic	D	D	D	0.0012
RYR1 chr19:38934827C>A	M44, 71	2	rs193922750	p.Q155K	Reported[10]	0.94	25.7		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38934892G>T	M23	1	rs1568436081	p.E176D	Reported[26]	0.504	20.9		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38935311G>A	M78	1	rs771058055	p.E209K	Reported[18]	0.711	23.6	5.46E-05	<1×10^{-7}	Likely pathogenic	N.S.	B	D	
RYR1 chr19:38939431G>A	M38	1	rs113332073	p.R367Q	Reported[17]	0.651	24.6	0	<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38973997C>T	M60, 84	2	rs193922777	p.P1592L	Reported[10]	0.927	27.7	0	<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38987541G>A	M51	1	rs193922797	p.V2280I	Reported[27]	0.641	25.3	0	<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38990281G>C	M74	1		p.S2345T	Reported[28]	0.744	24.7		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38990282C>A	M42	1	rs1448949048	p.S2345R	Reported[29]	0.747	22.9		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38990285delGGA	M40	1	rs193922800	p.E2347del	Reported[30]		21.3		<1×10^{-7}	Likely pathogenic	D	D		
RYR1 chr19:38990344C>G	M82	1	rs193922806	p.P2366R	Reported[5]	0.88	23.7		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38993557C>T	M51	1	rs377686237	p.R2625C	Reported[5]	0.785	29.1	0.000109	<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:39009935A>G	M3,37	1	rs118192126	p.K3367R	Reported[5]	0.632	18.9		<1×10^{-7}	Likely pathogenic	N.S.	D	B	
RYR1 chr19:39034034A>G	M34	1		p.N3913D	Reported[32]	0.924	26.9		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38934864A>G	M39	1		p.D167G	–	0.905	25.5		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38948155T>C	M88	1	rs901536375	p.S604P	–	0.951	28.1		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38990320T>C	M22	1		p.I2358T	–	0.912	25.1		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38990626C>A	M90	1		p.D2431E	–	0.743	23.1		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:38991282C>G	M29	1		p.R2454G	–	0.893	26.1		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
RYR1 chr19:39078017C>G	M70	1		p.A5025G	–	0.605	31.00		<1×10^{-7}	Likely pathogenic	N.S.	D	D	
CACNA1S chr1:201019613T>A	M66	1		p.D1382V	–	0.971	28.4		<1×10^{-7}	Likely pathogenic	D	D	D	0.0006
CACNA1S chr1:201028359G>C	M30	1		p.F1161L	–	0.923	26.7		<1×10^{-7}	Likely pathogenic	D	D	D	
CACNA1S chr1:201046197C>T	M89	1	rs763794604	p.A560T	–	0.876	27.7	0.0001	<1×10^{-7}	Likely pathogenic	D	D	D	0.0003

Figure Legends

Fig. 1. Percentage of genes associated with pathogenicity in MH patients and families. Screening results of genes associated with MH pathogenicity in 77 families, including MH patients and families. Of these, 34 families possessed variants in *RYR1* and six families in *CACNA1S*. In the remaining 37 families, there were no variants in *RYR1* and *CACNA1*S (unknown group).

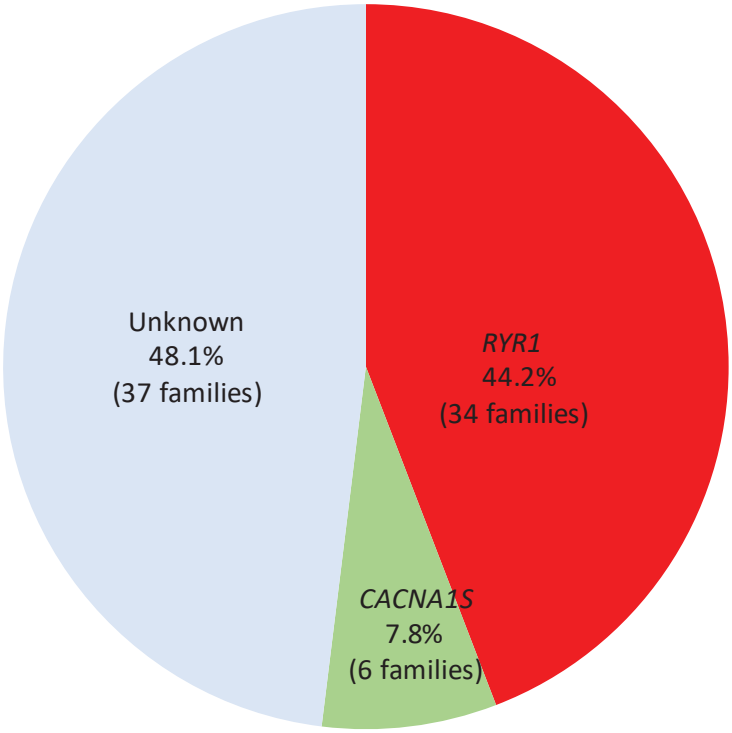


Fig.2. Comparison of clinical symptoms for each gene associated with MH pathogenicity. (A–D) Comparison of clinical symptoms for each gene associated with MH pathogenicity (Max BT, °C /15 min, Max CK, CGS) is shown. The value of each item is represented by a dot, and the interquartile range is represented by a line. The Mann–Whitney test was conducted for groups with *RYR* and *CACNA1S* variants. *p < 0.05. Abbreviations: Max BT, maximum body temperature; ˚C/15 min, elevated body temperature for 15 min; Max CK, maximum creatine kinase; CGS, Clinical Grading Scale

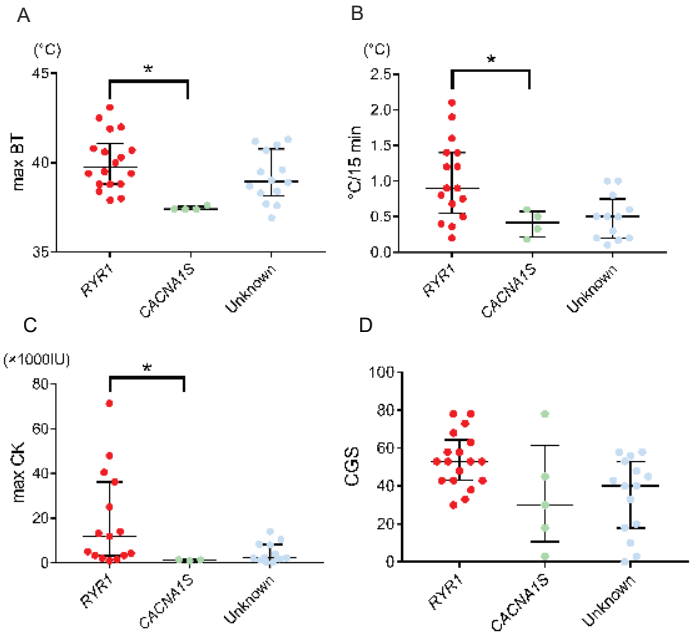
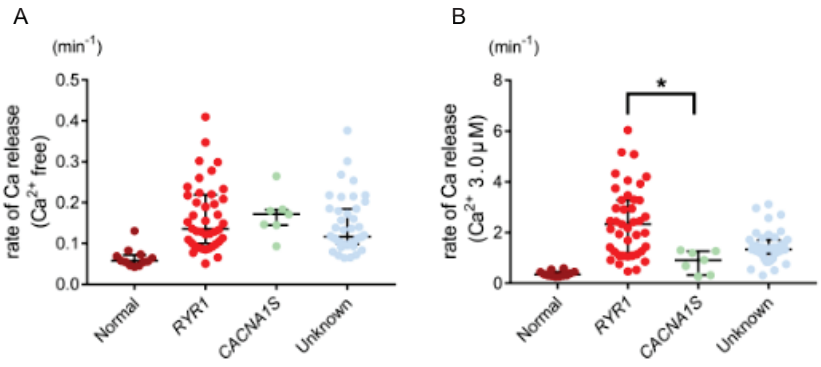


Fig. 3. Comparison of CICR and EC_{50} for each gene associated with MH pathogenicity and CICR-˚C/15 min correlation.

Comparison of CICR and EC_{50} (caffeine, 4-CmC) for each causative gene is shown. CICR was examined at each of the five Ca concentrations (0, 0.3, 1.0, 3.0, and 10 μM).

(A) Rate of Ca release for each gene associated with MH pathogenicity in Ca 0 μM (Ca-free, there is no difference related to MH status in Ca 0 μM).

(B) In the other four Ca concentrations, there was a significant difference between *the RYR1* and *CACNA1S* groups. Ca 3.0 μM is shown as representative.



Supplementary Table 1. Sample List

Variants ("Pathogenic" and "Likely pathogenic" based on the EMHG scoring matrix in Table1) and clinical symptoms of 90 people from 77 families are listed.

Abbreviations: AA change, amino acid change; MAX BT, maximum body temperature; °C /15 min, maximum elevated body temperature in 15 min; Max CK, maximum creatine kinase.

No	family	Sex	Age (y.o.)	MH or family member	AA change	Max BT(°C)	℃/15 (°C)	Max CK (IU)	CGS
M1	1	F	39	family member					
M2	1	F	65	family member					
M3	2	F	16	patient	*RYR1* p.K3367R	40.3	1.4	935	63
M4	3	F	41	patient					
M5	4	M	73	patient		41.0	1	2179	58
M6	5	M	52	patient	*CACNA1S* p.R174W	37.6	0.5	907	78
M7	5	M	47	family member	*CACNA1S* p.R174W				
M8	5	M	72	family member					
M9	6	F	25	patient		37.6	0.2	206	33
M10	7	M	18	patient					
M11	8	F	77	family member	*RYR1* p.L4838V				
M12	8	M	49	family member	*RYR1* p.L4838V				
M13	8	F	52	family member	*RYR1* p.L4838V				
M14	8	M	27	family member	*RYR1* p.L4838V				
M15	8	F	25	family member	*RYR1* p.L4838V				
M16	9	M	11	family member	*RYR1* p.L4838V				
M17	9	M	44	family member	*RYR1* p.L4838V				
M18	9	F	41	family member					
M19	9	F	15	family member	*RYR1* p.L4838V				
M20	9	M	13	family member					
M21	10	M	31	patient	*RYR1* p.R614L	38.8	0.4	13200	43
M22	11	F	40	family member	*RYR1* p.I2358T				
M23	12	F	39	family member	*RYR1* p.E176D				
M24	13	M	9	patient	*CACNA1S* p.R174W	37.4	0.18	1301	18
M25	14	M	61	patient	*RYR1* p.R614L	39.5	0.68	4290	33
M26	15	M	52	patient		38.4	0.3	2169	18
M27	16	M	7	patient	*RYR1* p.R614L	42.5	1.9	1603	53
M28	17	M	18	patient		38.7	0.2	8280	3
M29	18	M	25	patient	*RYR1* p.R2454G	43.1	2.1	11884	78
M30	19	M	35	patient	*CACNA1S* p.F1161L	37.4	0.6	1443	45
M31	20	M	23	patient		37.7	0.1	3940	0
M32	21	M	38	patient	*RYR1* p.E3104K	38.8	0.5		58
M33	21	F	10	family member	*RYR1* p.E3104K				

M34	22	F	40	family member	*RYR1* p.N3913D				
M35	23	M	33	patient	*RYR1* p.G341R				
M36	24	M	34	family member					
M37	25	M	48	family member	*RYR1* p.K3367R				
M38	26	M	34	family member	*RYR1* p.R367Q				
M39	27	M	31	patient	*RYR1* p.D167G	38.8	0.36	71310	43
M40	28	M	60	family member	*RYR1* p.V2346V,E2347del				
M41	29	F	21	patient		38.3	1	10525	40
M42	30	M	6	patient	*RYR1* p.S2345R	38.4	1.4	14000	68
M43	31	M	47	family member	RYR1 p.R2336H				
M44	32	F	22	family member	*RYR1* p.Q155K				
M45	33	M	11	family member					
M46	34	M	14	family member	*RYR1* p.R2508H				
M47	35	F	56	family member					
M48	36	M	45	family member					
M49	37	F	40	family member					
M50	38	M	71	family member					
M51	39	M	18	patient	*RYR1* p.R2625C, *RYR1* p.V2280I	39.4	1.2		48
M52	40	F	1	patient					
M53	41	M	5	patient		40.7	0.5	2392	43
M54	42	M	89	family member					
M55	43	F	60	patient		41.3	0.5	1270	53
M56	44	M	49	patient					
M57	45	F	15	patient					
M58	46	M	55	patient					
M59	47	M	8	patient		38.9	0.5		45
M60	48	M	30	patient	*RYR1* p.R533H, *RYR1* p.P1592L	37.9		40640	73
M61	49	M	20	family member					
M62	50	M	67	patient		39.5	0.8	386	48
M63	51	M	50	family member					
M64	52	M	42	family member					
M65	53	F	36	patient		36.9		14066	10
M66	54	M	52	patient	*CACNA1S* p.D1382V				
M67	55	M	33	patient		41.2	0.17		58
M68	56	M	34	patient	*RYR1* p.G341R				
M69	57	F	56	patient	*RYR1* p.R614L				
M70	58	M	52	patient	*RYR1* p.A5025G	38.0		48000	30
M71	59	F	73	family member	*RYR1* p.Q155K				
M72	60	M	40	family member					
M73	60	M	37	patient					
M74	61	M	42	patient	*RYR1* p.S2345T	40.0	0.9	25024	53
M75	62	F	32	patient	*CACNA1S* p.R174W				30
M76	63	M	37	patient					40
M77	64	F	34	family member					
M78	65	M	34	patient	*RYR1* p.E209K	39.4	0.2	3304	38
M79	66	M	20	patient		39.0		8000	20

M80	67	F	44	patient	*RYR1* p.R163C	40.6	0.9	3332	53
M81	68	M	56	patient		39.6	0.6	1803	56
M82	69	M	19	patient	*RYR1* p.P2366R	41.9	0.75	36258	58
M83	70	M	55	family member					
M84	71	M	18	patient	*RYR1* p.P1592L	40.7	0.8	2194	53
M85	72	F	51	family member					
M86	73	M	62	family member					
M87	74	F	1	family member	*RYR1p*.R163C				
M88	75	F	53	patient	*RYR1* p.S604P	42.0	1.2		43
M89	76	M	12	patient	*CACNA1S* p.A560T	37.4	0.33		3
M90	77	M	15	patient	*RYR1* p.D2431E	40.8	1.6	4984	78

Supplementary Table 2. Primer set designed by AmpliSeq designer

Excel file of AmpliSeq amplicons.

Supplementary Table 3. Eight areas not covered by AmpliSeq designer

1) *RYR* ex34 chr19:38976792-38976812
2) *RYR1* ex41 chr19:38987132-38987182
3) *RYR1* ex48 chr19:38993338-38993368
4) *RYR1* ex74 chr19:39019246-39019273
5) *RYR1* ex85 chr19:39034172-39034300
6) *RYR1* ex90 chr19:39055699-39056399
7) *RYR1* ex99 chr19:39070745-39070768
8) *CACNA1S* ex43 chr1:201009359-201009506