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Functional analysis of ryanodine receptor type 1 p.R2508C mutation in exon 47

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Abstract
Purpose. Malignant hyperthermia (MH) is a pharmacogenetic disorder of intracellular calcium homeostasis with an autosomal dominant inheritance. Most of the reported mutations in exon 47 were identified in Asian patients. However, no functional analysis of p.R2508C has been performed. We therefore conducted a functional analysis of the mutation by altering calcium homeostasis in human embryonic kidney (HEK) 293 cells transfected with the p.R2508C mutation in exon 47 of the ryanodine receptor 1 (*RYR1*).
Methods. The entire *RYR1* coding region from genomic DNA, which was extracted from the biopsied muscle specimens of two patients, was sequenced. The p.R2508C mutation was introduced into rabbit *RYR1* cDNA, and wild-type or p.R2508C mutant cDNAs were transfected into HEK-293 cells. Using the calcium-sensitive probe Fura 2, we utilized the 340/380 nm ratio to analyze alterations in calcium homeostasis following treatment with caffeine and 4-chloro-*m*-cresol (4CmC).
Results. Genetic analysis revealed a C→T point mutation of *RYR1* exon 47 at position 7522, resulting in an amino acid exchange of arginine for cysteine at amino acid 2508. The half-maximal activation concentrations (EC_{50}) of caffeine and 4CmC for HEK-293 cells transfected with the p.R2508C mutation were 1.86 ± 0.23 mM and 73.14 ± 19.44 μM, while those for wild-type *RYR1* were 2.62 ± 0.23 mM and 179.31 ± 35.23 μM, respectively.
Conclusion. We demonstrated that the transfected *RYR1* mutant was more sensitive to caffeine and 4CmC than wild-type *RYR1*. These findings suggest that the p.R2508C mutation may be pathogenetic for susceptibility to MH.

Key words Malignant hyperthermia · Ryanodine receptor 1 · HEK-293 cell · Calcium

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Introduction

Malignant hyperthermia (MH), a pharmacogenetic disorder, results from excessive calcium release from the sarcoplasmic reticulum (SR) in skeletal muscle following exposure to triggering agents, such as depolarizing muscle relaxants and/or volatile anesthetics. More than 200 MH- and Central Core Disease (CCD)-associated mutations have been identified in the ryanodine receptor 1 (*RYR1*) gene expressed in skeletal muscle [1]. *RYR1* mutations linked to MH and CCD are clustered in three regions of the protein, "hot spot"; the N-terminal, central region, and the C-terminal [1–3].

The entire *RYR1* coding sequence of genomic DNA from Japanese with susceptibility to MH was recently identified. A Japanese mutation index was then reported, showing the first documentation of a mutation in exon 47 [4], which is located outside the "hot spot" central region (exons 39–46). Including two cases in the present study, most of the mutations in exon 47 previously reported were found in Asian patients (Table 1) [3,4–6]. However, no functional analysis of p.R2508C in exon 47 has been performed. The European Malignant Hyperthermia Group (EMHG) has defined criteria for mutations in the *RYR1* gene associated with MH susceptibility. Each mutation should be characterized at the genetic level and assayed by recombinant in vitro expression on a defined genetic background or assay of *RYR1* function in ex vivo tissues [7]. In this study, we conducted a genetic and a functional analysis of the p.R2508C *RYR1* mutation.

Patients and methods

Case 1

A 63-year-old woman with scoliosis had an episode of MH (clinical grading scale, 43; MH rank, 5) when anes-

Table 1. Reported missense changes in exon 47 of *RYR1*

Exon	Nucleotide change	Protein change	Population (*n*)
47	c.7487C > T	p.Pro2496Leu	Japanese (1)
47	c.7522C > A	p.Arg2508Gly	Japanese (1)
47	c.7522C > T	p.Arg2508Cys	Japanese (4), Korean (1)
47	c.7523G > A	p.Arg2508His	Japanese (2)

thetized with sevoflurane in nitrous oxide/oxygen. She had a serum creatine kinase (CK) level of 628 $U \cdot l^{-1}$ at rest. After surgery, she underwent a muscle biopsy, and then genetic analysis and muscle pathological analysis were performed at the National Center of Neurology and Psychiatry (NCNP). The pathological diagnosis was myopathic changes with many fibers exhibiting core structures and type 1 fiber predominance. To diagnose her susceptibility to MH, the calcium-induced calcium release (CICR) rate test was performed [8,9], and it showed acceleration in comparison to the standard rate, which was described previously [10]. The CICR values were increased at four different calcium concentrations, meeting the criteria for diagnosing susceptibility to MH.

Case 2

A 13-year-old girl with congenital arthrogryposis and scoliosis underwent corrective surgery for scoliosis under propofol general anesthesia. She had an increased CK level of 561 $U \cdot l^{-1}$ preoperatively at rest. Prior to surgery, muscle biopsy was carried out to diagnose her susceptibility to MH. Pathological examination revealed myopathic changes with some fibers exhibiting cores or core-like structures, type 1 fiber predominance, and type 2B fiber deficiency (Fig. 1). The CICR rates were increased at four different calcium concentrations, meeting the criteria for assessing accelerated CICR function.

Her father had had an episode of MH under general anesthesia, and her sister had congenital myopathy with scoliosis and a myopathic countenance. They have never undergone molecular genetic analysis.

Genetic analysis

Genomic DNA was extracted from the biopsied muscle specimen and peripheral blood lymphocytes. We amplified the all 106 *RYR1* exons by polymerase chain reaction (PCR). Purified PCR products were directly sequenced in both directions according to the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems Japan, Tokyo, Japan) protocol, using an automated ABI 3130xl Genetic Analyzer (Applied Biosystems Japan). Mutations were detected by detailed eye-inspection of the DNA sequencing data.

Written informed consents for the genetic analysis were obtained from the patients and their families at the NCNP.

Functional analysis

Mutagenesis of ryanodine receptor 1 (RYR1)

Full-length rabbit skeletal muscle RYR1 cDNA was a kind gift from David H. MacLenann (University of Toronto). The standard system, introduced by Dr. MacLenann's group, used the expression of a rabbit *RYR1* cDNA construct in HEK-293 cells [11]. The short fragment of *RYR1* cDNA was removed from full-length *RYR1* cDNA and ligated into pBluescript II KS (+) (Stratagene, Santa Clara, CA, USA). A QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to mutate the *RYR1* cDNA insert. The integrity of the mutated segment of the cDNA insert was checked by sequencing the entire insert with an ABI 3100 DNA sequencer (Applied Biosystems Japan).

Cell culture

Human embryonic kidney 293 (HEK-293) cells, which were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 units of penicillin$\cdot ml^{-1}$ (Sigma, St. Louis, MO, USA) and 100 mg streptomycin$\cdot ml^{-1}$ (Sigma), at 37 °C under 5% CO_2.

DNA transfection

DNA transfection was carried out with Fugene HD Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA); 2 µg plasmid DNA was used to transfect 1×10^5 HEK-293 cells in a 35-mm poly-L-lysine-coated glass-bottomed dish. Control cells were treated similarly, but with expression vector DNA. For the measurement of Ca^{2+} release, cells were plated on 35-mm glass-bottomed microwell dishes (MatTek, Ashland, MA, USA), and measurement was carried out 72 h after transfection.

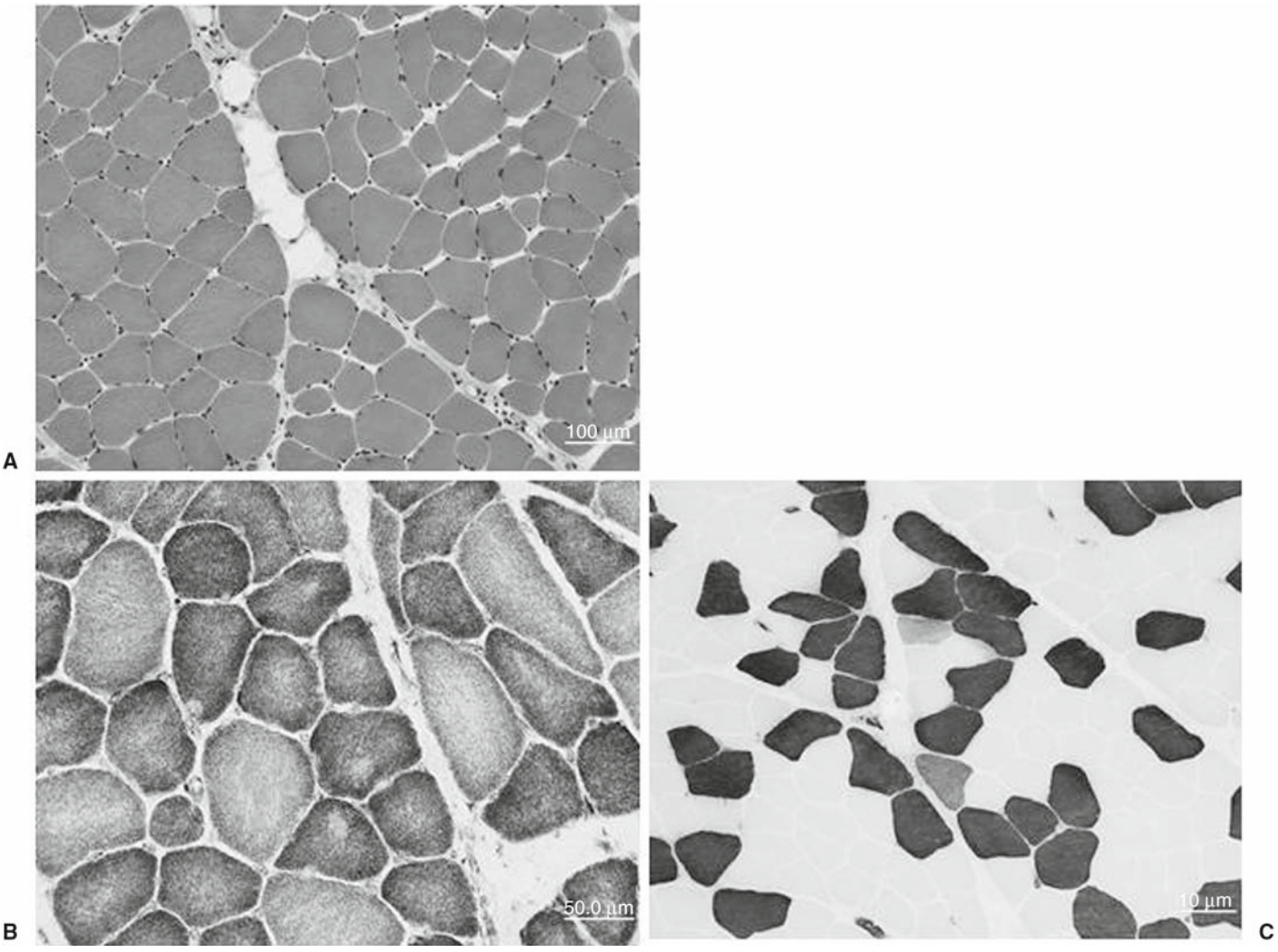

Fig. 1A-C. Muscle biopsy of case 2. **A** On hematoxylin and eosin (H&E) staining, we identified a moderate variation in fiber size with minimal endomysial fibrosis. **B** On nicotinamide adnine dinucleotide tetrazolium reductase (NADH-TR) staining, we observed disorganized intermyofibrillar networks in some fibers showing a core or core-like structures lacking clear rimming. **C** On ATPase analysis, there was a predominance of type 1 fibers that were decreased in size

Ca^{2+} fluorescence measurements

HEK-293 cells transfected with wild-type or mutant p.R2508C *RYR1* DNA were washed in hydroxyethylpiperazine ethanesulfonic acid (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 5.0 µM Fura-2 AM (Dojindo, Tokyo, Japan) in HBSS for 1 h at 37 °C and washed with HBSS. The cells were then excited alternately at 340 nm and 380 nm. Fluorescence emission at 510 nm was measured using a fluorescence microscope (Nikon, Tokyo, Japan). Images were acquired using a cooled high-speed digital video camera (ORCA-AG; Hamamatsu Photonics, Hamamatsu, Japan). HBSS was perfused into the sample dishes at a rate of 1.2 ml·min^{-1} at 37 °C. Only the cells that reacted to 10 mM caffeine (Wako, Osaka, Japan) were used for experiments. Caffeine-induced changes in Fura-2 AM fluorescence were measured using various caffeine concentrations; 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 mM, and the 340/380 nm signal ratio was calculated using a Ca^{2+} imaging system (Aquacosmos 2.5; Hamamatsu Photonics) within 60 min after washing away the excess Fura-2 AM. The dish was rinsed with HBSS for 3 min before the next dose was given. Similarly, 4-chloro-*m*-cresol (4CmC)-induced changes in Fura-2 AM fluorescence were measured using various 4CmC concentrations: 3, 10, 30, 100, 300, 500, 1000, and 3000 µM. The two

fluorescence ratios were converted into Ca^{2+} concentrations using a calibration curve constructed with a calibration kit (Fura-2 Calcium Imaging Calibration Kit; Invitrogen). Control cells were measured similarly.

Data analysis

The changes in the ratios were calculated from the difference of the maximal response and the preceding baseline. To obtain dose-response curves, data for caffeine or 4CmC were normalized to the maximum response observed with 10 mM caffeine or 1000 μM 4CmC, respectively. Data analysis was performed using PRISM software (GraphPad Software, San Diego, CA, USA) with Excel-based templates (Microsoft, Redmond, WA, USA).

Results

Genetic analysis

In both cases, genetic analysis revealed a C→T point mutation of *RYR1* exon 47 at position 7522, resulting in an amino acid exchange of arginine for cysteine at amino acid 2508. There were no further base exchanges in the coding sequence.

Functional analysis

We generated a dose-response curve for caffeine by normalizing the rise in calcium concentrations at each concentration to the maximum response using 10 mM caffeine or 1000 μM 4CmC. The dose-response curve was shifted to the left from that of wild-type *RYR1* (Figs. 2, 3). The half-maximal activation concentration (EC_{50}) of caffeine in HEK 293 cells expressing p.R2508C was 1.86 ± 0.23 mM, while that for wild-type *RYR1* was 2.62 ± 0.23 mM, and there was a significant difference between them. The EC_{50} values of 4CmC for mutant *RYR1* and wild-type *RYR1* were 73.14 ± 19.44 μM and 179.31 ± 35.23 μM, respectively (Table 2), and there was a significant difference between them. Control cells did not respond to caffeine and 4CmC.

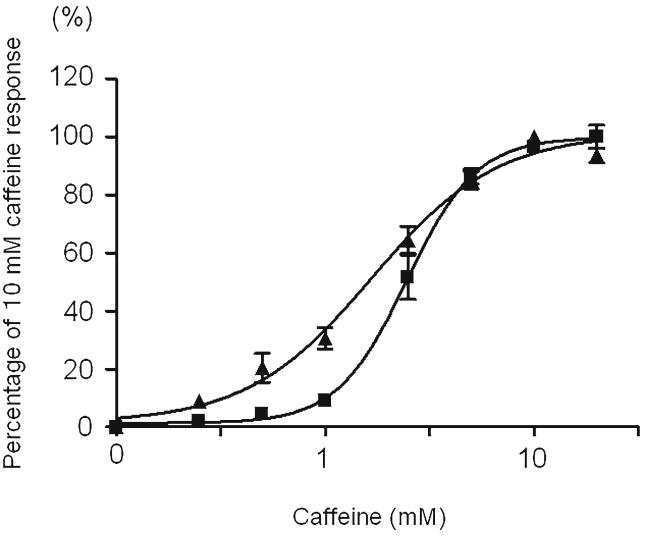

Fig. 2. Dose-response curve of the normalized calcium increases in response to caffeine stimulation. *Triangles*, p. R2508C; *squares*, wild-type. Dose-response curves were normalized to the maximal release response observed at 10 mM caffeine. Data represent the means ± SD

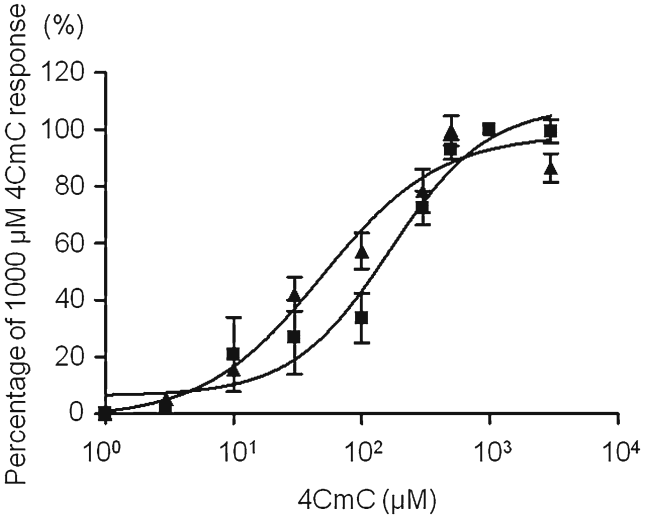

Fig. 3. Dose-response curve of the normalized calcium increases in response to 4-chloro-*m*-cresol (*4CmC*) stimulation. *Triangles*, p.R2508C; *squares*, wild-type. Dose-response curves were normalized to the maximal release response observed at 1000 μM 4CmC. Data represent the means ± SD

Table 2. EC_{50} for caffeine and 4CmC

	EC_{50} for caffeine (mM)	*n*	EC_{50} for 4CmC (μM)	*n*
Control	ND		ND	
wt *RYR1*	2.62 ± 0.23	8	179.31 ± 35.23	7
R2508C	1.86 ± 0.23*	11	73.14 ± 19.44*	5

* $P < 0.05$ vs wt *RYR1*
Values are expressed as means ± SEM
n, number of HEK 293 cells; ND, not determined

Discussion

Exon 47 is located in close proximity to, but not within the central "hot spot" of genetic variability (exons 39–46). Most of the previously documented mutations in exon 47 have been identified in Japanese and Korean people [3,4–6]; however, no functional analysis of p. R2508C was performed in these reports. This is the first report of the pathogenic analysis of the *RYR1* p.R2508C mutation in exon 47.

The p.R2508C mutation was reported in five independent pedigrees. At the NCNP laboratory 300 healthy chromosomes from a Japanese population have been analyzed and no missense mutations were found. Moreover, the affected residues (2508) among *RYR1* from different species were well conserved [5]. It was suggested that p.R2508C is not a common variant. We investigated this mutation from two independent pedigrees, and accelerations of the CICR rate in three independent pedigrees have been reported [4,5]. These results meet EMHG criteria for gene mutations to be used in the genetic testing of susceptibility to malignant hyperthermia [7].

Myotubes, microsomal SR preparations, and lymphoblasts derived from patients carrying mutations were used to investigate transient calcium currents in response to ryanodine receptor activators such as caffeine, 4CmC, and halothane, and the role of *RYR1* mutations in the pathogenesis of MH [12–15]. However, to perform functional analysis using human primary myotubes, several independent pedigrees of the mutation are needed. Therefore, HEK-293 cells [16,17] bearing *RYR1* mutations have been used. The EC_{50} values of *RYR1* activators in the mutant *RYR1* were reported to be half those of the wild-type of *RYR1* [14]. In the present study, the EC_{50} values of caffeine and 4CmC for HEK-293 cells expressing p.R2508C were 1.86 mM and 73.14 μM, and those for wild-type were 2.62 mM and 179.31 μM, respectively. Taken together, the thresholds of *RYR1* activator responses for HEK-293 cells expressing p.R2508C observed in this study are similar to those in the previous reports. We used the standard system introduced by Dr. MacLenann' s group, which meets the EMHG criteria for functional characterization. Consequently, p.R2508C was presumed to be pathologic for MH.

In Japan, the CICR rate test is performed to diagnose susceptibility to MH, by detecting an acceleration of CICR function in skeletal muscle. Individuals with MH-associated mutations show acceleration in the CICR rate test, because the *RYR1* mutations cause a functional abnormality in calcium release. The CICR rate test was performed In five patients with the p.R2508C mutation, four Japanese patients and one Korean patient, and all showed acceleration of the CICR rate [5]. In a previous genetic study in the Japanese individuals with CICR rate acceleration, the *RYR1* mutation detection rate was 56.9% [4].

Most CCD mutations are clustered in the *RYR1* C-terminal, the pore-forming region of the channel that is involved in channel function and regulation, which is associated with muscle weakness. In contrast, CCD patients with mutations outside the C-terminal had mild musculoskeletal abnormalities. The two patients presented in the present study were diagnosed as having MH with some fibers exhibiting cores in the muscle pathological analysis. The p.R2508C mutation identified in these cases was located outside the C-terminal near the central region. Of note, a CCD mutation in the non-C-terminal region is reported to predispose a patient to MH [4,5]. Our case 1 had had an episode of MH and case 2 was a member of an MH family, and both cases showed acceleration in the CICR rate test. We therefore assume that exon 47 is an important exon for MH.

Distribution of the mutation varies depending on ethnic groups. EMHG screens for a subset of mutations different from those screened by the North America Malignant Hyperthermia Group (NAMHG) [18,19]. The establishment of screens for Asians is needed.

In conclusion, we demonstrated increased sensitivity to caffeine and 4CmC in HEK-293 cells transfected with the *RYR1* mutation in exon 47. The *RYR1* p. R2508C mutation was associated with altered calcium homeostasis. These findings suggested that the p. R2508C mutation may be pathogenetic for susceptibility to MH and that exon 47 may be a "hot spot," at least for Asians.

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References

1. Stowell KM. Malignant hyperthermia: a pharmacogenetic disorder. Pharmacogenomics. 2008;9:1657–72.
2. Monnier N, Kozak-Ribbens G, Krivosic-Horber R, Nivoche Y, Qi D, Kraev N, Loke J, Sharma P, Tegazzin V, Figarella-Branger D, Roméro N, Mezin P, Bendahan D, Payen JF, Depret T, Maclennan DH, Lunardi J. Correlations between genotype and pharmacological, histological, functional, and clinical phenotypes in malignant hyperthermia susceptibility. Hum Mutat. 2005;26: 413–25.
3. Robinson R, Carpenter D, Shaw MA, Halsall J, Hopkins PM. Mutations in RYR1 in malignant hyperthermia and central core disease. Hum Mutat. 2006;27:977–89.
4. Ibarra MCA, Wu S, Murayama K, Minami N, Ichihara Y, Kikuchi H, Noguchi S, Hayashi TK, Ochiai R, Nishino I. Malignant hyperthermia in Japan. Anesthesiology. 2006;104:1146–54.
5. Wu S, Ibarra CAM, Malicdan MCV, Murayama K, Ichihara Y, Kikuchi H, Nosaka I, Noguchi S, Hayashi YK, Nishino I. Central

core disease is due to RYR1 mutations in more than 90% of patients. Brain. 2006;129:1470–80.
6. Kim DC, Kim DS. Mutation screening of the entire ryanodine receptor gene (RYR1) in Korean malignant hyperthermia families. J Anesth. 2008;22S:148.
7. http://www.emhg.org/index.php?option=com_content&task=view&id=60&Itemid=86. Accessed Dec 4 2008.
8. Endo M, Iino M. Measurement of Ca release in skinned fibers from skeletal muscle. Methods Enzymol. 1988;157:12–26.
9. Migita T, Mukaid K, Kawamoto M, Kobayashi M, Yuge O. Fulminant-type malignant hyperthermia in Japan: cumulative analysis of 383 cases. J Anesth. 2007;21:285–8.
10. Oku S, Mukaida K, Nosaka S, Sai Y, Maehara Y, Yuge O. Comparison of the in vitro caffeine-holothane contracture test with the Ca-induced Ca release rate test in patients suspected of having malignant hyperthermia susceptibility. J Anesth. 2000;14:6–13.
11. Tong J, Oyamada H, Demaurex N, Grinstein S, McCarthy TV, MacLennan DHJ. Caffeine and halothane sensitivity of intracellular Ca^{2+} release is altered by 15 calcium release channel (ryanodine receptor) mutations associated with malignant hyperthermia and/or central core disease. Biol Chem. 1997;272:26 332–9.
12. Wehner M, Rueffert H, Koening F, Neuhaus J, Olthoff D. Increased sensitivity to 4-chloro-*m*-cresol and caffeine in primary myotubes from malignant hyperthermia susceptible individuals carrying the ryanodine receptor 1 Thr2206Met (C6617T) mutation. Clin Genet. 2002;62:135–46.
13. Weigl LG, Ludwig-Papst C, Kress HG. 4-Chloro-m-cresol cannot detect malignant hyperthermia equivocal cells in an alternative minimally invasive diagnostic test of malignant hyperthermia susceptibility. Anesth Analg. 2004;99:103–7.
14. Wehner M, Rueffert H, Koening F, Meinecke CD, Olthoff D. The Ile2453Thr mutation in the ryanodine receptor gene 1 is associated with facilitated calcium release from sarcoplasmic reticulum by 4-chloro-*m*-cresol in human myotubes. Cell Calcium. 2003; 34:163–8.
15. Kaufmann A, Kraft B, Michalek-Sauberer A, Weigl LG. Novel ryanodine receptor mutation that may cause malignant hyperthermia. Anesthesiology. 2008;109:457–64.
16. Tong J, McCarthy TV, McLennan DH. Measurement of resting cytosolic Ca^{2+} concentration and Ca^{2+} store size in HEK-293 cells transfected with malignant hyperthermia or central core disease mutant Ca^{2+} release channels. J Biol Chem. 1999;274:693–702.
17. Monnier N, Romero NB, Lerale J, Nivoche Y, Qi D, MacLennan DH, Fardeau M, Lunardi J. An autosomal dominant congenital myopathy with cores and rods is associated with a neomutation in the *RYR1* gene encoding the skeletal muscle ryanodine receptor. Hum Mol Genet. 2000;9:2599–608.
18. Urwyler A, Deufel T, McCarthy T, West S for the European Malignant Hyperthermia Group. Guidelines for molecular genetic detection of susceptibility to malignant hyperthermia. Br J Anaesth. 2001;86:283–7.
19. Sambuughin N, Holley H, Muldoon S, Brandom BW, de Bantel AM, Tobin JR, Nelson TE, Goldfarb LG. Screening of the entire ryanodine receptor type 1 coding region for sequence variants associated with malignant hyperthermia susceptibility in the North American population. Anesthesiology. 2005;102:515–21.