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Functional characterization of the *RYR1* mutation p.Arg4737Trp associated with susceptibility to malignant hyperthermia

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Abstract

Aside from the in vitro contracture test, genetic screening for causative *RYR1* mutations is the established procedure to diagnose susceptibility to malignant hyperthermia (MH). However, currently only 34 out of more than 300 known *RYR1* mutations have been confirmed to be causative for MH by experimental studies addressing their functional impact on intracellular calcium homeostasis. The *RYR1* mutation p.Arg4737Trp has been recently detected in a German MH family. To evaluate the effects of that mutation on intracellular calcium handling, the response after stimulation with the RYR1 agonist 4-chloro-m-cresol was investigated in immortalized B lymphocytes containing the p.Arg4737Trp mutation and compared to the response of wild type RYR1 from unaffected family members and unrelated controls. Intracellular resting calcium was slightly but significantly elevated in mutation positive cells. Calcium release following stimulation with 4-chloro-m-cresol was significantly increased in B lymphocytes carrying the p.Arg4737Trp mutation compared to mutation negative controls. Hence, the functional properties of the *RYR1* mutation p.Arg4737Trp are consistent with susceptibility to MH. Together with previously published data, the mutation has now been reported in three independent MH positive families.

Keywords: Malignant hyperthermia; In vitro contracture test; Ryanodine receptor; Mutation; Calcium release, 4-chloro-m-cresol

1. Introduction

Malignant hyperthermia (MH) is a rare but life-threatening pharmacogenetic disorder of excitation contraction coupling and calcium homeostasis in skeletal muscle [1,2]. Exposure to halogenated volatile anesthetics as well as to depolarizing muscle relaxants may induce a hypermetabolic syndrome characterized by uncontrolled calcium release from the sarcoplasmic reticulum via functionally altered ryanodine receptors 1 (RYR1) or dihydropyridine receptors. Cardiac arrhythmia, hypercapnia, muscle rigidity, hyperkalemia and hyperthermia may result and are some of the typical symptoms of an acute MH reaction. Presymptomatic identification of individuals with susceptibility to MH is an important task in order to prevent this dangerous anesthetic complication [3].

According to the guidelines issued by the European and the North American MH Groups, the in vitro contracture test (IVCT) and the caffeine halothane contracture test (CHCT), respectively, are gold standard for identification of malignant hyperthermia susceptible (MHS) individuals. This invasive technique requires a surgical muscle biopsy usually taken from the quadriceps femoris muscle [4–6]. DNA analysis and searching for mutations is less invasive and has led to the identification of more than 300 different mutations in *RYR1* so far [7,8]. However, the pathogenic impact of most of these mutations is yet to be determined and so far only 34 mutations have been acknowledged to be causative for MH after functional evaluation [9]. Further expansion of this list by evaluation of every mutation is important in order to increase the number of patients and families who could be offered genetic rather than surgical testing [10].

In the present study we characterize a novel *RYR1* mutation that was identified in a German family. The index patient survived an acute MH reaction in the 1960s at the age of 10 years during general anesthesia for eye surgery. After inhalational induction with halothane he received 1 mg/kg succinylcholine

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for intubation. Immediately afterward masseter spasm as well as generalized muscular rigidity was observed. Ten minutes later he developed tachycardia (170 bpm), rapid temperature increase to 39.1 °C and hypercarbia which required repeated exchange of the CO_2 absorber in short time intervals. Although not all necessary items have been recorded, the Larach score for this episode reached at least 48 points, indicating an MH event to be "very likely" [11]. The incident was published as a case report in a regional medical journal at that time, when knowledge about the underlying pathomechanism of the so-called anesthesia associated hyperpyrexia was still very limited [12]. Many years later, the predisposition of the index patient to MH was confirmed by the IVCT, which showed a clearly positive result. Screening of the patient's *RYR1* for the most common causative mutations was negative but sequencing of the patient's entire *RYR1* gene identified the heterozygous variant c.14209C>T (p.Arg4737Trp) in exon 98.

Epstein–Barr virus immortalized human B lymphocytes have been shown to express the skeletal muscle isoform of RYR1 and have been successfully used to examine the functional effect of several *RYR1* mutations [13,14]. Here, we present the results of IVCT, the genetic segregation of the p.Arg4737Trp mutation in the pedigree and measurements of intracellular calcium levels in human B lymphocytes derived from family members and controls with and without the mutation in order to characterize the functional effect of the mutation.

2. Patients and methods

The study was undertaken with approval of the local ethics committee (University of Wuerzburg, no. 96/14). IVCT, blood samples and genetic screening were carried out as part of the standard diagnostic procedure following written and oral informed patient consent. The patients additionally consented in written form to the evaluation and publication of the results as well as to the functional characterization of calcium homeostasis in B lymphocytes; consent forms are available for review by the editor.

2.1. In vitro contracture test

The in vitro contracture test was performed according to the guidelines of the European Malignant Hyperthermia Group (EMHG) [4,15]. In brief, after surgical removal of a muscle bundle, specimens were incubated with increasing concentrations of caffeine (0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 32 mM) or halothane (0.5, 1.0, 2.0 and 3.0 vol%). If developing muscular contractures exceed the set threshold of 2 mN at caffeine ≤ 2.0 mM and halothane ≤ 2.0 vol%, the patients are classified as MH susceptible (MHS) while the absence of significant contractures leads to the diagnosis MH nonsusceptible (MHN).

2.2. Genetic testing

DNA was extracted from whole blood samples collected in EDTA tubes by standard procedures and used as a template for PCR and Sanger sequencing of all coding sequences of the *RYR1* gene plus 20 bp flanking intronic regions as described [16]. Sequences were aligned to the GenBank entry number NM_000540.2 (GRCh37, hg19) as the reference sequence.

2.3. Establishment of Epstein–Barr virus immortalized B lymphoblastoid cell lines and functional tests

Whole blood was collected in heparin-treated tubes at local primary care physicians and shipped to the Basel laboratory by courier. Within 48 hours after collection, B lymphocytes were isolated and immortalized using Epstein–Barr virus (EBV) and changes in the intracellular free calcium levels were assessed as previously described [13,14,17,18]. Briefly, 1×10^7 cells/ml were loaded with the fluorescent Ca^{2+} indicator fura-2/AM (5 μM final concentration) in Krebs Ringer solution containing 2 mM $CaCl_2$ for 60 minutes at 37 °C after which they were rinsed and resuspended in fresh Krebs Ringer (+2 mM Ca^{2+}). Experiments were carried out on cell populations (1×10^6 cells/ml) in a thermostatic LS-50 Perkin Elmer spectrofluorometer equipped with a magnetic stirrer. Cell populations from each individual were examined on three different occasions. Initially, resting calcium was determined for each cell population. Afterward peak increase of the Ca^{2+} transient obtained after addition of a given concentration of the RyR1 agonist 4-chloro-m-cresol [19] on the linear part of the fura-2 calcium sensitive curve was expressed as percentage of the peak Ca^{2+} released by maximal concentrations (1000 μM) of 4-chloro-m-cresol which was the same as that obtained by adding 400 nM thapsigargin. The latter represents the total amount of Ca^{2+} present in rapidly releasable intracellular Ca^{2+} stores.

2.4. Statistical analysis

Statistical analysis was performed using Mann–Whitney U test for differences in resting calcium levels between the two groups with and without the mutation p.Arg4737Trp. 2-way ANOVA was utilized to compare the response to different concentrations of 4-chloro-m-cresol in the groups with and without mutation; Bonferroni's post hoc test was applied for multiple comparisons. GraphPad Prism version 6.0f for Mac OS X (GraphPad Software, La Jolla, California, USA) was used for statistical analysis. Results are expressed as mean value (±SEM) of n results, where n indicates the number of measurements.

3. Results

The pedigree of the investigated family is shown in Fig. 1. Each of the two sisters and the two brothers (patient nos. 3, 5, 7 and 9) as well as their descendants (10, 11, 12, 13) underwent genetic testing, while only two siblings (3 and 5) were tested by IVCT. Muscle biopsy and IVCT to confirm the MHN phenotype in patients 9, 12 and 13 without the *RYR1* mutation is still pending.

3.1. In vitro contracture test

Individuals 3 and 5 underwent IVCT testing. In patient 3 the maximum contracture response was 5.5 mN after halothane 2.0 %vol and 9 mN after caffeine 2 mM; in patient 5 the resting tension increased to a maximum of 17.2 mN after halothane 2.0 %vol and 4.6 mN after caffeine 2 mM. Hence, the skeletal muscular contractions clearly exceeded the diagnostic