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IDENTIFICATION OF A NOVEL MUTATION IN *RYR1* GENE IN MALIGNANT HYPERTHERMIA-LIKE PATIENT'S FAMILY MEMBERS

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Malignant hyperthermia (MH) is a rare pharmacogenetic disorder with an autosomal dominant inheritance that presents as a hypermetabolic response in skeletal muscle to volatile anaesthetic (halothane, isoflurane, desflurane, sevoflurane) and the depolarising muscle relaxant succinilcholine and rarely to stresses such as vigorous exercise and heat. We investigated the relatives of an individual with suspected MH and found a novel mutation in RYR1 *gene. The molecular analysis of* RYR1 *gene revealed a novel nucleotide substitution in exon 6 – G528T (Glu-176-Asp) in four family members of the patient. The* in vitro *contracture test (IVCT) according to the European Malignant Hyperthermia Group (EMHG) guidelines showed a MH susceptible phenotype in two tested family members.*

Key words: *malignant hyperthermia, mutation in* RYR1 *gene, IVCT, anaesthesia, complications.*

INTRODUCTION

Malignant hyperthermia (MH) is a rare pharmacogenetic disorder with an autosomal dominant inheritance that presents as a hypermetabolic response in skeletal muscle to volatile anaesthetics (halothane, isoflurane desflurane, sevoflurane) and the depolarising muscle relaxant succinilcholine and rarely to stresses such as vigorous exercise and heat. MH may occur at any time during anesthesia and in the early postoperative period. The earliest signs are tachycardia, rise in end-expired carbon dioxide concentration despite increased minute ventilation, accompanied by muscle rigidity, especially following succinilcholine administration. Body temperature elevation is a dramatic but often late sign of MH. Other signs include acidosis, tachypnea and hyperkalaemia. The progression of the syndrome may be rapid and dramatic, particularly if precipitated by succinylcholine, or slower and might not manifest until after several hours or after anaesthesia (Rosenberg *et al*., 2007). In 1960, it was reported for the first time as a potential hereditary disease (Denborough *et al*., 1960).

All volatile anaesthetics except nitrous oxide are triggers for MH. The muscle relaxant succinylcholine also is a trigger for MH.

Malignant hyperthermia was recognised for the first time as an autosomal inherited disease in 1960 by Denborogh and Lovell in Australia. Untreated MH is a potentially fatal disease. Due to significant progress in the clinical management, identification of MH susceptible persons and understanding of the pathophysiology mortality associated with MH has considerably decreased. The incidence of fulminant crisis reaches 6.5% of all cases of MH, although this number might be too high as mild or variant forms tend to be overlooked and do not enter into published reports. The underlying causes for the variability of the clinical picture have not yet been identified. Varying potency, concentration and duration of trigger exposure, as well as additional factors such as temperature, age and genetic variability, might explain the heterogeneity of clinical picture (Wappler, 2001). Current MH mortality is 2–3%, because of worldwide used calcium channel blocker dantrolene (the only one specific and effective treatment for MH) and therapy standards in comparison with 75–80% thirty years ago. The incidence of MH reactions ranges from 1:40 000 to 100 000 anaesthesias. The other method of analysis is the mutation frequency which ranges from 1:15 000 for children and adolescents to 150 000 for adults (Halsall *et al*., 1979; Denborough, 1998). In Latvia the situation is much

worse; the mortality is around 100%, because of the unavailability of dantrolene treatment and low activity from medical specialists in MH diagnostic. It is safe to use trigger-free anaesthesia in susceptible patients. Scala and colleagues have analysed data from 11 MHS patients who had received non-triggering agents and did not find any MH-related complications (Scala *et al*., 2006). However, many publications show that anaesthesia with triggers is safe for MH-negative (MHn) patients (Allen *et al*., 1990; Ording *et al*., 1991; Islander and Ranklev-Twetman, 1995). MH-susceptible (MHs) patients did not develop any clinical signs of MH when non-triggering anaesthesia was given without the use of prophylactic dantrolene. Routine use of prophylactic Dantrolene is not recommended because of its unpleasant side-effects, such as weakness, speech and visual disturbances, mental depression and headache (Krause *et al*., 2004).

The aim of our study was to investigate the association between a novel nucleotide substitution in exon 6 - G528T (Glu-176-Asp) and MH clinical manifestation.

MATERIALS AND METHODS

Patient's history. A 57-year-old male patient (AN II-2) underwent thyreoidectomia in 2003 in the State Hospital in Rīga and developed a syndrome suspected to be a MH crisis: end tidal CO_2 ($EtCO_2$) max 80 mmHg, heart rate till 150 beats/min, temperature max 40.8 ^{o}C, creatine cinase (CK) 1616 U/l after anaesthesia with sevoflurane, fentanyl and succinylholine. Dantrolen was not available in Latvia, so the treatment was ineffective. The patient died five hours after anaesthesia. Material for MH investigation was not taken. DNA analysis was performed for patient relatives.

***In vitro* contracture test (IVCT).** IVCT is the only method for presymptomatic diagnosis of MH. In our study it was performed according to the protocol of the European Malignant Hyperthermia Group (Ording *et al*., 1997) in the Leipzig MH unit.

To provide the IVCT test the patient needs to undergo a minor surgical procedure where a skeletal muscle specimen from the quadriceps femoris muscle is taken at an open biopsy.

The muscle sample is split into separate bundles and spread out between two electrodes. It is then stimulated using a supramaximal current and the resulting muscle twitches. After equilibration, halothane is introduced into the system using a vaporiser, in increasing concentrations. The same procedure is carried out on a second muscle bundle but using cumulative concentrations of caffeine instead of halothane. Both tests are repeated with fresh muscle samples.

If a pathological muscle contracture develops after administration of the test agents the diagnosis of MH susceptibility has been confirmed (MHs). Absent or insufficient contractures lead to the diagnosis of a MH negative result (MHn). Unfortunately, it is possible for contractures to develop after either caffeine or halothane exposure and not both. These individuals are classified as MH equivocal (MHe).

DNA analysis. The blood samples studied were obtained from seven relatives of the MH patient. DNA was extracted from peripheral blood leukocytes and exon 6 of *RYR1* gene was amplified by use of the primer pairs and conditions described elsewhere (Tamarro *et al*., 2003) and bidirectionally sequenced using the same PCR primers with fluorescent-dye terminators, on an ABI 310 automatic sequencer. PCR products were gel purified (Qiagen) prior to sequencing.

One hundred DNA samples of volunteers were investigated for the absence of the sequence change to exclude the population specific polymorphism.

Functional analysis of intracellular calcium release. Calcium homeostasis was investigated in a myotube assay. Surplus material of the muscle probe was used for culture of myotubes as described by Wehner *et al*. (2002). Intracellular calcium concentration in the cultured myotubes was determined with Fura 2-AM (Molecular probes, Eugene, USA) on a fluorescence imaging set (Polychrom IV by Till Photonics, Gräfelfing, Germany) coupled to an inverse microscope (Axioskop by Zeiss, Jena, Germany). The EC_{50} was determined by fitting of the dose response curve of a single myotube to the Hill equation.

The study was conducted in accordance with the Helsinki convention and approved by the Ethics Committee of Leipzig University Hospital and Latvian Physicians Society.

RESULTS

The family pedigree is shown on Figure 1. The Index-patient is II-2. Person (III-8) had a MH-like episode in childhood (high temperature during three days in intensive care unit (ICU) after appendectomia with general anaesthesia). His DNA sample is not available for molecular diagnostics at this moment.

The molecular analysis of *RYR1* gene revealed a novel nucleotide substitution in exon 6 – G528T (Glu-176-Asp) (Fig. 2) in four family members (II-3, III-4, III-7 and IV-4). Family members III-3, IV-2 and IV-3 did not show an abnormal pattern of the DNA sample.

IVCT test for family members II-3 and III-7 showed MH results (Fig. 3).

Calcium ligand binding measurements in myotubes was also provided. In MHN individuals the resting calcium concentration was 50.7 nmol/l (41.4; 68.8 nmol/l; 25th, 75th percentile), whereas in the carriers of the novel mutation the resting calcium concentration was 74.0 (64.0; 77.9) nmol/l and 76.5 (62.2; 111.3) nmol/l, respectively. The difference between MHN and mutation carriers was significant ($P < 0.001$, Mann-Whitney Test), a typical feature seen in myotubes of mutation carriers.

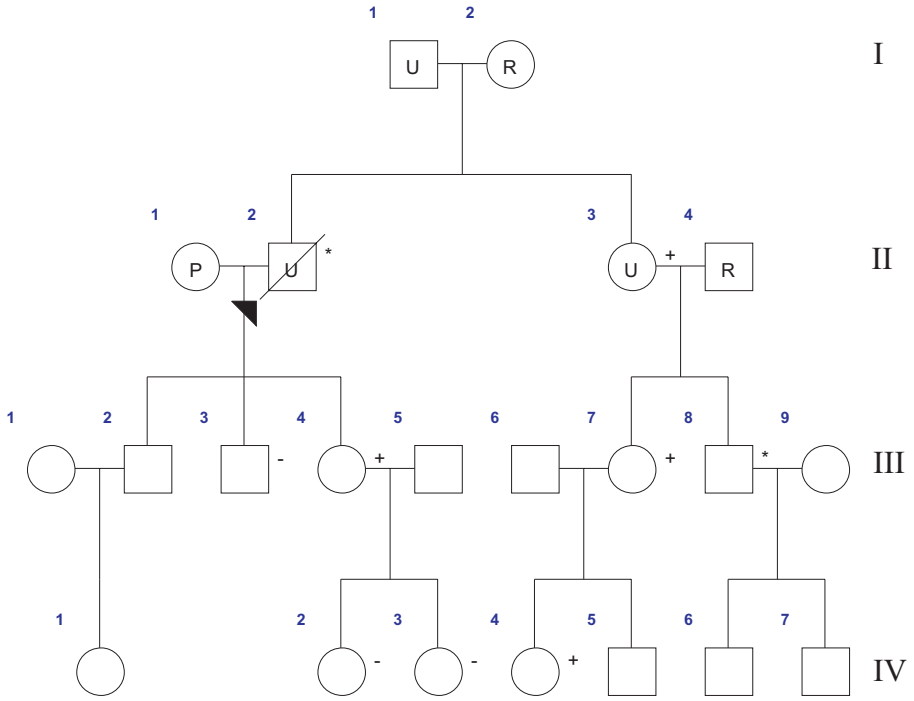
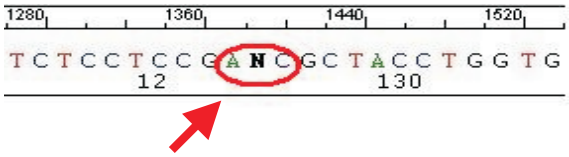


Fig. 1. MH patient family tree ("+" and "-" – persons analysed by DNA testing with positive (mutation in exon 6 – G528T (Glu-176-Asp)) or negative results accordingly; "*" – persons with MH-like clinical episodes; U, R and P – patient nationality: Ukrainian, Russian and Polish accordingly).



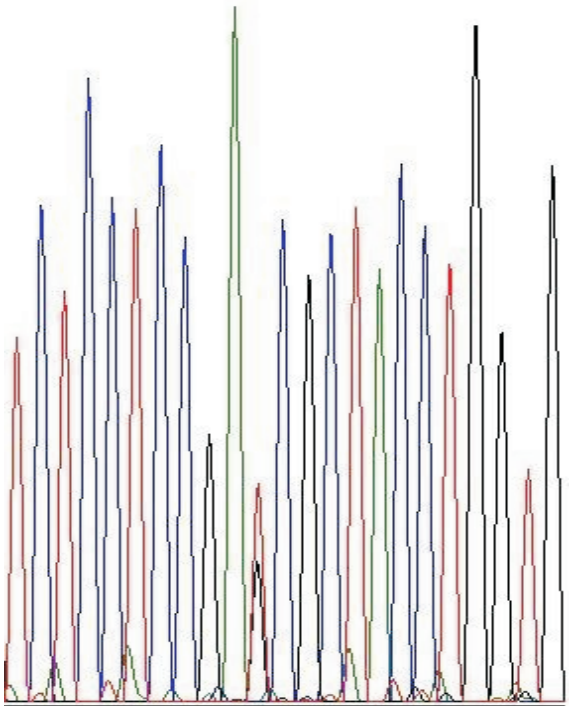


Fig. 2. DNA sequencing electropherogramme of a novel nucleotide substitution in exon 6 – G528T (Glu-176-Asp) of the *RYR1* gene. Substituted nucleotide is indicated by an arrow.

The half-maximal effect concentration (EC_{50}) of 4Chloro-m-cresol (4CmC) was determined by fitting of dose response in myotubes to the Hill equation. The EC_{50} is reduced in the myotubes of the mutation carriers as compared with the MHN myotubes. The reduction is statistical significant (Mann Whitney-test, $P < 0.05$; Table 1, Fig. 5).

DISCUSSION

Malignant hyperthermia is inherited in an autosomal dominant manner, caused by mutation in the ryanodine-receptor gene *RYR1*. The gene is located on the long arm of chromosome 19 (19q13.1). Ryanodine receptor type1 opens in response to increases in intracellular Ca2+ level mediated by L-type calcium channels, thereby resulting in a drastic increase in intracellular calcium levels and muscle contraction leading to hypermethabolic state with hyperthermia and metabolic and electrolyte disturbances with a risk of patient death. The *RYR1* gene is one of the largest genes (15.5 kb cDNA encodes a 5035 amino acid protein with molecular mass of 563.5 kD) with more than 90 known mutations, but only 29 considered as causative mutations for MH. There is no most common causative mutation in this gene, which makes the DNA analysis time- and labour-consuming. Mutations distribution is geographically determined.

In Latvia and Baltic countries, knowledge about the MH clinical picture and diagnostics is still insufficient. For the most physicians MH crisis is associated with patient death and mild and abortive forms are not recognised, therefore, there is no available statistical data about the presence of MH in Latvia.

In old medical files, a 57-year-old patient, who probably had a MH crisis in 2003, was recognised. He had tachycar-

exon 6 nucleotides: 425 - 537	_GA GAG GCT TGC TGG TGG ACC ATG CAC CCA GCC TCC AAG *C*AG A*G*G TCT GAA GGA GAA AAG GTC CGC GTT *G*GG *GA*T GAC ATC ATC CTT GTC AGT GTC TCC TCC GA*G* *C*GC TAC CTG
aa: 142 - 179	Gly Glu Ala Cys Trp Trp Thr Met His Pro Ala Ser Lys *Gln* *Arg* Ser Glu Gly *Glu* Lys Val Arg Val *Gly* *Asp* Asp Ile Ile Leu Val Ser Val Ser Ser Glu *Arg* *Tyr* Leu
marked variations	c.463C>A p.Gln155Lys c.467G>A p.Arg156Lys c.479A>G p.Glu160Gly c.487C>T p.Arg163Cys c.488G>T p.Arg163Leu c.488G>A p.Arg163Leu c.493G>A p.Gly165Arg c.496G>A p.Asp166Asn c.497A>G p.Asp166Gly c.528G>T p.Glu176Asp c.529C>T p.Arg177Cys c.533A>T p.Tyr178Cys

Fig. 3. Nucleotide sequence of exon 6 with the appropriate sequence of amino acids. Novel nucleotide substitution with appropriate amino acid substitution is shown in red.

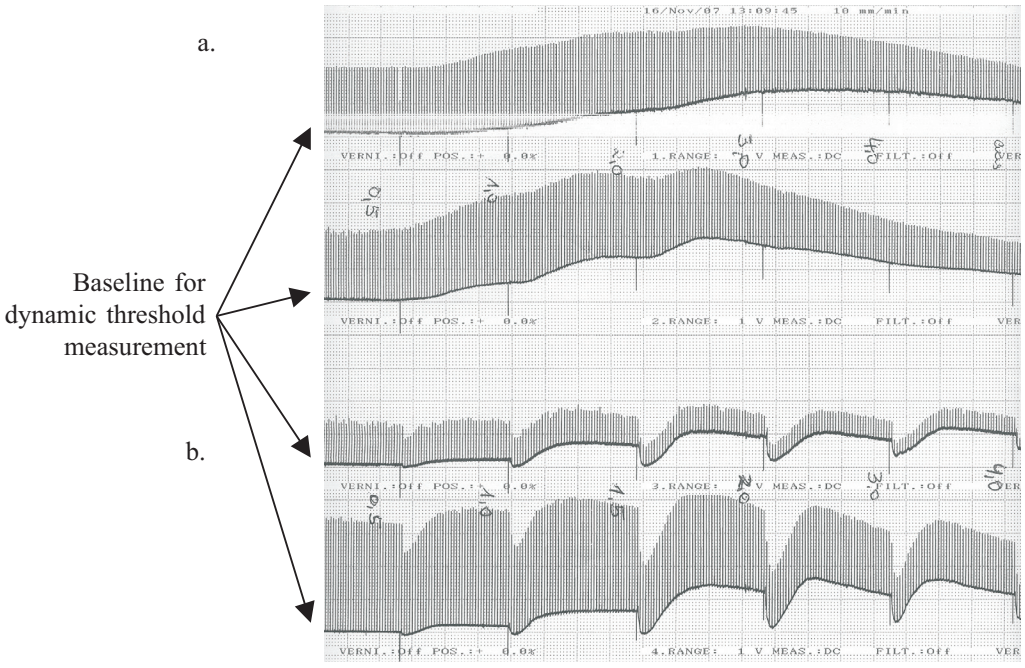


Fig. 4. Original tracings of IVCT results of an MH patient's four different skeletal muscle bundles in bath with halothane (a) and caffeine (b). Test showed muscle disability to relax to baseline after contracture confirming the diagnosis of malignant hyperthermia susceptibility.

dia, muscle rigidity, creatine kinase and end-tidal CO_2 elevation. Treatment with dantrolene was not available and the patient died within several hours after surgery. We investigated the relatives from his family for the presence of known causative mutations. The preliminary findings did not bring results and finally direct sequencing of full *RYR1* gene was performed for a few members. A novel mutation G528T in *RYR1* gene in for four family members was found. Mutation G528T is a nucleotide substitution mutation that results in a change of amino acid residue and affects the ryanodine receptor type 1 protein structure.

The guidelines for molecular genetic detection of susceptibility to MH clearly point out that the standard test to establish an individual's risk of susceptibility to MH is the *in vitro* contracture test (IVCT) (Urwyler *et al.,* 2001). For two family members of the MH-like patient's the IVCT test revealed positive results. The test showed muscle disability to relax to baseline after contracture with halothane and caffeine confirming the diagnosis of malignant hyperthermia susceptibility. The functional investigation of *RYR1* function in *ex vivo* tissues from MHs patients was performed by calcium ligand binding measurements in myotubes and a

Table 1

THE HALF-MAXIMAL EFFECT CONCENTRATION (EC_{50}) OF 4CmC IN μmol/l.

Column	Size	Median	225th percentile	75th percentile
MHn	308	391,125	279,870	538,044
MHs (mutation carriers)	34	300,432	184,760	392,252

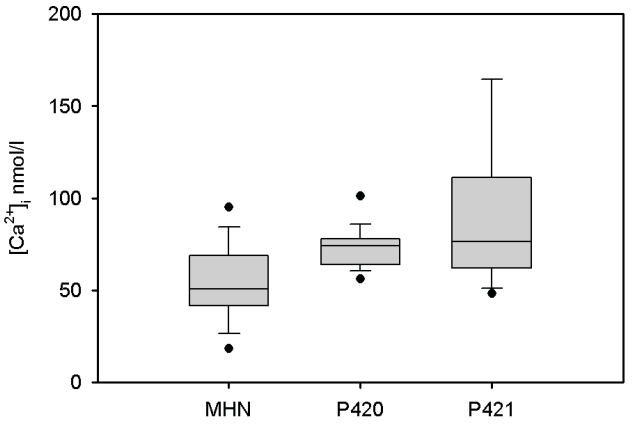


Fig. 5. Rest calcium concentrations difference in malignant hyperthermia patients and patients with a novel mutation in *RYR*1 gene.

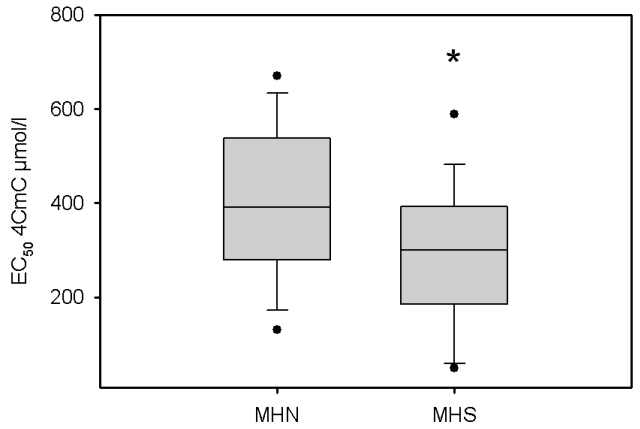


Fig. 6. The half-maximal action concentration with 4CmC (* $P < 0.05$ in comparison with the control group).

significant statistical difference in rest calcium concentrations in MHs and MHn myotubes was found. Also the half-maximal action's concentration with 4CmC was significantly lower in comparison with the control group ($P < 0.05$).

The mutation concordance with MH was verified according to EMHG guidelines. The results of our present investigation provide strong evidence for the occurrence of a spontaneous G528T mutation in the human *RYR1* gene in a family with an MH susceptible trait. The spontaneous nucleotide exchange at position 528 has not previously been reported. The described mutation was found in four family members and was not found in 100 unrelated controls (200 different chromosomes). To include the mutation G528T in the MH causative *RYR1* mutations list the other families with the same mutation are needed. Persons with identified *RYR1* gene mutation should consequently be regarded as susceptible to MH and in case of surgery volatile anesthetics and succinilcholine should not be used for general anaesthesia.

More active education about changes in the MH clinical picture is needed because of changes in routinely used medicaments in today's anaesthesiology practice. Analysis of Cardiac Arrests and Deaths Associated with Malignant Hyperthermia in North America from 1987 to 2006 showed results of 291 MH events, 8 (2.7%) resulted in cardiac arrests and 4 (1.4%) resulted in death (Green *et al*., 2008). This investigation revealed that more than 95% of MH cases are abortive and do not give a full picture of MH. Neuromuscular diseases also could be related to MH (Greenberg, 1999; Cohen and Kaplan, 2006). Anaesthesiologists have to be more careful to find those patients with abortive MH forms. Bonciu *et al.* (2007) describe a mild MH crisis with hearth rate until 95 beats per minute, end-tidal CO2 ($EtCO_2$) rise from 4.8 to 5.2 kPa and rectal temperature till 39.6 ^{o}C. Mild rise of serum creatine kinase (154 IU/l) and potassium (6.1mmol/l) were observed, but the later provided IVCT test was positive. This example shows that more attention has to be drawn to mild symptoms of MH.

The anaesthesiologists' knowledge about MH still is not enough. Sometimes anaesthesiologists do not give anaesthesia to MHs patients during procedures such as childbirth just because of threat of MH.

These examples show that we need to give more education about MH in the medical society. Further investigation of MH susceptible families in Latvia will be provided in the future.

In conclusion, we consider that this novel mutation the G528T should be included in the MH causative *RYR1* Mutations list, although the EMHG guidelines reclaim the necessity to find the same mutation in other unrelated families. Because of our MH patient parents come from different geographical regions—mother from the eastern part of Latvia and father from Ukraine, the mutation origin can not be detected. Unfortunately, we are not able to establish who of the MHs patient's parents was the carrier of mutation G528T. This means that our patient's genome could be influenced from Ukrainian, Russian and Polish populations. In these countries MH diagnostics are not performed and the *RYR1* gene mutation spectrum is not known.

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JAUNAS *RYR1* GĒNA MUTĀCIJAS NOTEIKŠANA ĻAUNDABĪGĀS HIPERTERMIJAS PACIENTA ĢIMENES LOCEKĻIEM

Ļaundabīgā (malignā) hipertermija (MH) ir reta autosomāli dominanta farmakoģenētiska slimība, kas izpaužas ar skeleta muskulatūras hipermetabolisku reakciju uz gaistošiem anestēzijas līdzekļiem (halotānu, izoflurānu, desflurānu un sevoflurānu) un depolarizējošo muskuļu relaksantu sukcinilholīnu. Tā reti manifestējas kā atbilde uz stresu, piemēram, lielu slodzi vai karstumu. Mēs izmeklējām pacienta ģimeni, kurš nomira pēc anestēzijas ar MH līdzīgiem simptomiem, un atradām jaunu mutāciju *RYR1* gēnā. Molekulārā analīze četriem viņa ģimenes locekļiem uzrādīja jaunu nukleotīdu aizvietojošu mutāciju 6. eksonā – G528T (Glu-176-Asp). Lai apstiprinātu mutāciju diviem ģimenes locekļiem tika veikts *in vitro* kontrakcijas tests un kalcija koncentrācijas mērījumi miotubuļos atbilstoši Eiropas Malignās hipertermijas grupas vadlīnijām. Lai izslēgtu populācijas specifisku polimorfismu, tika izmeklēti 100 brīvprātīgo asins paraugi, kuros šīs mutācijas esamību nekonstatēja.