New Compound Heterozygous Splice Site Mutations of the Skeletal Muscle Ryanodine Receptor (*RYR1*) Gene Manifest Fetal Akinesia: A Linkage with Congenital Myopathies

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Established Facts

- The skeletal muscle ryanodine receptor (*RYR1*) gene has been linked to malignant hyperthermia susceptibility, central core disease, and minicore myopathy with external ophthalmoplegia. *RYR1* is an intracellular calcium release channel and plays a crucial role in the sarcoplasmic reticulum and transverse tubule connection.
- To date, the mutation c.10347+1G>A in the *RYR1* gene is characterized as pathogenic, and it has been associated with myopathies.

Novel Insights

- Since the mutation c.10456–2A>G has never been associated with myopathy phenotypes, we reveal new findings of the disease spectrum of *RYR1*.
- In the present study, next-generation sequencing (NGS) analysis revealed a new compound heterozygous state for *RYR1* splice site mutations, thus expanding the disease spectrum of *RYR1*.
- We propose the involvement of *RYR1* mutations as the major hallmark for the most common myopathies such as central core disease.

Keywords

Compound heterozygous mutations · Fetal akinesia · Myopathies · Next-generation sequencing · Skeletal muscle ryanodine receptor

Abstract

Mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene have been linked to malignant hyperthermia susceptibility, central core disease, and minicore myopathy with ex-

ternal ophthalmoplegia. *RYR1* is an intracellular calcium release channel and plays a crucial role in the sarcoplasmic reticulum and transverse tubule connection. Here, we report 2 fetuses from the same parents with compound heterozygous mutations in the *RYR1* gene (c.10347+1G>A and c.10456–2A>G) who presented with fetal akinesia and polyhydramnios at 27 and 19 weeks of gestation with intrauterine growth restriction in the third pregnancy. The prospective parents of the fetuses were heterozygous carriers for c.10456–2A>G (mother) and c.10347+1G>A (father). Both mutations affect splice sites resulting in dysfunctional protein forms probably missing crucial domains of the C-terminus. Our findings reveal a new *RYR1* splice site mutation (c.10456–2A>G) that may be associated with the clinical features of myopathies, expanding the *RYR1* spectrum related to these pathologies.

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The extracellular and intracellular maintenance of proper calcium levels is crucial for muscle and bone development and function [Bronner, 2001]. The skeletal muscle ryanodine receptor (*RYR1*) which is located in the sarcoplasmic reticulum has a key role in calcium release [Cheng et al., 1993]. *RYR1* is located in chromosome 19q13.2 (38,433,700–38,587,564 [GRCh38/hg38]) and consists of 106 exons with 2 functional domains. The N-terminal domain has regulatory sites that interact with the dihydropyridine receptor, while the C-terminal domain (together with the central region of the protein) forms the channel pore [Zorzato et al., 1990].

Both dominant and recessive mutations in the *RYR1* gene have been linked to several congenital myopathies including central core disease (CCD), multi-minicore disease, minicore myopathy with external ophthalmoplegia, malignant hyperthermia, centronuclear myopathy, and congenital fiber-type disproportion [Jungbluth, 2007a; Clarke et al., 2010; Wilmschurst et al., 2010]. Specifically, the majority of these mutations refer to dominant mutations in the functional regions of the protein; especially for the CCD and multicore disease [Monnier et al., 2000, 2001; Tilgen et al., 2001; Davis et al., 2003].

The main feature of these pathologies is congenital hypotonia (for both dominant and recessive mutations). Specifically, *RYR1*-associated myopathy displays characteristics such as decreased fetal movement, polyhydramnios, arthrogryposis, kyphoscoliosis, or respiratory distress [Jungbluth et al., 2003; Romero et al., 2003; Goebel, 2005; Jungbluth, 2007b]. In this study, we report 2 cases of fetal akinesia presenting with polyhydramnios at 27 and 19 weeks of gestation. Next-generation sequencing (NGS)

Fig. 1. Family tree of patients and carriers of mutations in the *RYR1* gene. The father (I-1) is a heterozygous carrier for mutation c.10347+1G>A and the mother (I-2) is a heterozygous carrier for mutation c.10456–2A>G in the *RYR1* gene. II-1: asymptomatic male child. II-2: affected female neonate that died soon after birth and was found as compound heterozygous for both parental mutations in *RYR1*. II-3: affected fetus with a c.10347+1G>A and a c.10456–2A>G mutation in heterozygous state in the *RYR1* gene. II-IV: asymptomatic female child, heterozygous carrier only for the father's mutation in *RYR1* (c.10347+1G>A).

analysis revealed a new compound heterozygous state for *RYR1* splice site mutations, thus expanding the disease spectrum of the gene.

Clinical Report

A 30-year-old woman, gravida 4, para 3, was referred to our center following 2 consecutive pregnancies that were complicated by fetal akinesia. The couple was of Greek origin, healthy, and non-consanguineous (Fig. 1).

First and Fourth Pregnancy

Prenatal ultrasound assessment was normal. Anomaly scan of both pregnancies was normal. An asymptomatic male (Fig. 1, II-1) and an asymptomatic female (Fig. 1, II-4) neonate were born, respectively.

Second Pregnancy

Fetal ultrasound examination at 12 weeks showed increased nuchal translucency (NT: 3.3 mm) and consequently, an increased risk of Down syndrome. After genetic counseling, the parents opted for fetal karyotyping, and chorionic villus sampling was performed. Standard G-banding revealed a normal female karyotype, and chromosomal microarray analysis showed no pathogenic copy number variations. The ultrasound assessment at 21 weeks of gestation detected no other abnormalities, except for bilateral talipes. However, the growth scan at 27 weeks, due to bilateral talipes and reported absent fetal movements, revealed fetal akinesia with amniotic fluid volume towards the upper limit of normal for the gestational age. Following counseling by maternal-fetal medicine specialists regarding the guarded outcome, the parents opted to continue the pregnancy. At 32 weeks, the pregnancy presented with polyhydramnios. The neonate was born prematurely at 34 weeks of gestation, 2,200

Fig. 2. IGV screenshot of II-3 for intronic mutations in *RYR1* after NGS analysis. **A** Mutation c.10347+1G>A in *RYR1* in heterozygous state (inherited from the father). **B** Mutation c.10456-2A>G in *RYR1* in heterozygous state (inherited from the mother).

g, and died soon after birth because of complete generalized hypotonia. Bilateral talipes and mild micrognathia were observed. The parents decided against pathology assessment (Fig. 1, II-2).

Third Pregnancy

First-trimester ultrasound examination at 12 weeks of gestation revealed an increased risk for Down syndrome due to increased nuchal translucency measurement (NT: 3.1 mm). After counseling, the parents opted for fetal karyotyping, and chorionic villus sampling was performed. Standard G-banding revealed a normal karyotype, and chromosomal microarray analysis showed no pathogenic copy number variations. The anomaly scan at 19 weeks showed normal fetal growth with 358 g and no other obvious fetal defects. However, there was severe polyhydramnios and absent fetal movement. Following counseling by maternal-fetal medicine specialists and based on the poor outcome, the parents decided to terminate the pregnancy. Additionally, the parents opted for NGS analysis, but not for pathology assessment (Fig. 1, II-3).

Material and Methods

Molecular Karyotyping

Chromosomal microarray was performed with an aCGH platform of 60,000 oligonucleotides (Agilent Technologies). Briefly, DNA was extracted from the chorionic villus sample with Promega Maxwell 16, and it was hybridized with the human reference DNA of the same gender (Promega Biotech). The statistical test used as a parameter to estimate the number of copies was ADM-2 (provided by the DNA analytics software, Agilent Technologies) with a window of 0.5 Mb and a threshold of 6. Only those copy number changes that affected at least 5 consecutive probes with identically oriented change were considered as copy number variations. Consequently, for most of the genome, the average resolution of this analysis was 200 kb.

Next-Generation Sequencing

DNA isolation from the fetus II-3 was performed. Following, oligonucleotide-based target capture analysis and nucleotide se-

Fig. 3. Sanger sequencing data. **A** Mutation c.10347+1G>A in the *RYR1* gene in heterozygous state (father). **B** Mutation c.10456-2A>G in the *RYR1* gene in heterozygous state (mother).

quencing was performed using TruSight One Kit (Illumina) and NextSeq (Illumina) examining 226 disease-causing genes that were related to the ultrasound findings of the second and third pregnancies and the phenotype of the neonate (second pregnancy). Genes are available from the authors upon request. The software SOPHiA DDM® (SOPHiA GENETICS) was used for the data analysis (reference genome UCSC hg19 and reference database Human Gene Mutation Database [HGMD v.2017.1]). In particular, for bioinformatic analysis, the following applications were used: MaxEnt, NNSPLICE, HSF, SIFT, PROVEAN, MutationTaster, and PolyPhen-2. The mean depth coverage is 20× in 97% of analyzed regions (98%, >12×) and the mean depth coverage is 116.2× (96.7%, >20×).

Targeted Sequencing

Targeted sequencing was performed on parental blood samples as well as on the samples from the fetuses II-2 and II-IV after NGS analysis in fetus II-3 detected mutations. Sanger analysis was performed using primers *RYR1_F* (5'-GCCTGAAGTGTAGAGT-CAGCAA-3') and *RYR1_R* (5'-TGGGCAGCATCTTCTTCA-GTG-3') for mutation c.10456-2A>G in the *RYR1* gene, *RYR1B_F* (5'-AGGAGCTGTTTCAGGATGGTG-3') and *RYR1B_R* (5'-GT-GCTCCTCCCAGAACCAAG-3') for mutation c.10347+1G>A in *RYR1*, and primers *DMPK_F* (5'-CTCCAACCTTATGGAGGG-AGCAT-3') and *DMPK_R* (5'-CATTCAGCGTTGCTGT-GTC-3') for mutation c.1040C>G (p.Pro347Arg) in the *DMPK* gene. Reactions were performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), and analysis was performed using Sequencing Analysis (v5.3.1) software on ABI 3130xl genetic analyzer (Applied Biosystems).

Results

Chromosomal microarray results were normal for the 3 fetuses (Fig. 1, II-2, II-3, and II-4). NGS analysis of fetus II-3 showed a heterozygous state for splice site mutations in *RYR1* (c.10347+1G>A and c.10456-2A>G) and also a

heterozygous mutation c.1040C>G (p.Pro.347Arg) of the DM1 protein kinase (*DMPK*) gene (Fig. 2). The parents were tested by targeted sequencing for these mutations and were found to be heterozygous carriers for c.10456-2A>G and c.10347+1G>A, respectively (Fig. 3), indicating that they are located in *trans* in fetus II-3. Moreover, the mother is a heterozygous carrier for the mutation c.1040C>G (p.Pro347Arg) in *DMPK*. Subsequently, targeted sequencing of the fetus II-2 showed a compound heterozygous state for both mutations in the *RYR1* gene and a heterozygous state for the father's mutation in the fetus II-IV (data not shown). Standard G-banding revealed a normal karyotype, and chromosomal microarray analysis showed no pathogenic copy number variations in fetus II-IV.

Discussion

The proper function of skeletal muscle is directly linked to the maintenance of Ca²⁺ ion homeostasis. *RYR1* is a homotetrametric Ca²⁺ ion channel that is located in the sarcoplasmic reticulum membrane of skeletal muscle. The Ca²⁺ ion homeostasis is also important for several cellular processes such as protein synthesis, protein degradation, and mitochondrial respiration [Gehlert et al., 2015]. Dominant and recessive mutations in the functional regions of *RYR1* have been mainly associated with myopathies and especially with CCD and multicore disease [Monnier et al., 2000, 2001; Tilgen et al., 2001; Davis et al., 2003]. Myopathies are mainly characterized by the presence of specific structural and histochemical features on muscle biopsy and congenital hypotonia and muscle

weakness [Taratuto, 2002]. Our case displayed fetal akinesia and polyhydramnios at 27 weeks of gestation, and NGS analysis revealed inherited compound heterozygous mutations in the *RYR1* gene (c.10347+1G>A and c.10456–2A>G) and also an inherited heterozygous mutation c.1040C>G (p.Pro.347Arg) in the *DMPK* gene. Because of the fact that the mutation in *DMPK* is inherited, and mutations in *DMPK* have not been associated with clinical features of myopathies, the discussion of the possible role of this variant on the phenotype of the fetuses is only speculative. Despite the fact that our case lacks the histological analysis, the clinical features of the fetus and the NGS analysis seem to validate the pathological pattern of a myopathy.

The regulation of *RYR1* can be performed by post-translational modifications on several residues and/or by binding with molecules such as calstabin1 and Ca^{2+} ions. The regulation of *RYR1* by Ca^{2+} has positive and negative effects through its binding to the activating or inhibitory binding site of *RYR1*, respectively [Gehlert et al., 2015]. Also, the normal functionality of *RYR1* includes its interaction with the dihydropyridine receptor and the calsequestrin [Gehlert et al., 2015]. Moreover, S-nitrosylation of cysteine residues on *RYR1* correlated with muscular dystrophy [Bellinger et al., 2009]. As we know, the post-translational modifications in *RYR1* along with the regulatory molecules can modulate the skeletal muscle contractility. Based on this fact, it is not surprising how detrimental a mutation in “functional” nucleotide(s) of *RYR1* can be for muscle function and development. In our case, the compound heterozygous state of *RYR1* mutations (c.10347+1G>A and c.10456–2A>G) affect splice sites. This can result from the misregulation of splicing and alternative splicing through mutations in *cis* regulatory elements within the affected gene or from mutations that affect the activities of trans-acting factors that are

components of the splicing machinery [Ward and Cooper, 2010]. Thus, both mutations can lead to dysfunctional *RYR1* forms missing crucial domains or amino acids of the C-terminus that possess a regulatory role. To date, although the mutation c.10347+1G>A is characterized as pathogenic and it has been associated with myopathies [Farwell et al., 2015; Fattori et al., 2015; Alkhunaizi et al., 2019], the mutation c.10456–2A>G has never been associated with these phenotypes (fetal akinesia and polyhydramnios).

In conclusion, even though the diagnosis of most common myopathies such as CCD includes both histological analysis and molecular techniques for the identification of *RYR1* mutations, we propose the involvement of *RYR1* mutations as the most major hallmark for these pathologies. Finally, since the mutation c.10456–2A>G has never been associated with myopathy phenotypes, we reveal new findings expanding the disease spectrum of *RYR1* gene mutations.

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Statement of Ethics

The study was approved by the Ethics Committee of “P&A Kyriakou” Children’s Hospital, Athens, Greece, and performed with respect to the ethical standards of the Declaration of Helsinki, as revised in 2008.

Disclosure Statement

The authors declare no conflicts of interest.

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