Identification of a novel compound heterozygous mutation in RyR1 gene in an Indian family affected with congenital myopathy

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Abstract

Within the ryanodine receptor family (RyR), three genes (RyR1, RyR2, and RyR3) are involved in Ca\textsuperscript{2+} homeostasis, storage, and regulation. Mutations in RyR1 causes a wide range of clinical phenotypes, including several congenital myopathies (CM), central core disease (CCD), and hyperthermia susceptibility. RyR1-related CCDs usually show clinical heterogeneity and an early onset of disease pathogenesis. Here, we present a family that includes unaffected parents and three siblings who have been affected with muscle problems since childhood. The clinical features include lower proximal muscle weakness, difficulties in standing up and climbing, skeletal malformations and hypotonia. Clinical examinations (e.g., nerve conduction velocity, electromyography, and muscle magnetic resonance imaging) showed weak muscle intensity, activity, and muscle atrophy. Whole-exome sequencing was performed in two affected siblings along with unaffected mother in the family using Illumina NovaSeq2500. Bioinformatic analysis and filtering of multiple variants revealed a novel variant in RyR1. This compound heterozygous variant (c.A5096G: p.D1699G+c.C5097AA: p.D1699E; 13423\textsuperscript{13424}del:p.K4475Efs*106) has not been reported in public databases and in silico analysis predicted that the variant is damaging. Furthermore, this novel variant segregates within the family and in silico protein analysis showed putative changes in the protein activity between the wildtype versus mutant RyR1. The initial functional analysis showed changes in calcium channel activity, however, additional confirmational assays are required. Our study explains a genotype-phenotype correlation in the family. It expands the requisite prenatal diagnosis in the family and in the near future will provide a platform for therapeutics in RyR1-related diseases.

Introduction:

RyR1 gene has been involved in many disorders including malignant hyperthermia susceptibility (MHS), Malignant hyperthermia (MH), Central Core Disease (CCD), multi mini core disease (MmD), centronuclear myopathy (CNM) and congenital fiber type disproportion (CFTD) \cite{1}. CCD, a category of CM has been seen at the early age of onset \cite{2}. Previously, the \textit{RYR1} has been involved in both MH and CCD \cite{3}. Most of the mutations in the N-terminal of the \textit{RYR1} are present in MH while the majority of mutations in the C-terminal of the RyR1 gene are present in CCD \cite{4}. Therefore, a higher number of mutations present in the C-terminal of the \textit{RYR1} have been shown with an early age of onset of congenital myopathies and it is always suggesting a high degree of clinical prediction of CCD phenotypes \cite{5}. In addition, dominant and recessive mutations in the\textit{RYR1} are shown to be associated with typical CCD clusters in a mutational hotspot affecting the RyR1 C-terminus \cite{4}. Whilst those that are implicated in NHS and MMD are distributed throughout the\textit{RYR1} coding sequence. Typical features of CCD include mild proximal weakness, climbing issues, hip girdle musculature and motor developmental delay \cite{6}. CCD has an onset similar to congenital myopathies.
CCD associated with *RYR1* mutation is almost entirely caused by dominant mutations. The typical pediatric presentation for CCD is one of neonatal hypotonia, muscle hypotrophy, and extremity muscle weakness, often accompanied by significant skeletal abnormalities such as chest wall deformities, scoliosis, joint contractures, and hip dysplasia [7,8]. In addition, respiratory failure is also seen in some cases [9]. However, the course of the disease is typically quite stable and, although delayed, individuals often acquire all motor developmental milestones. It may be noted that there may be some mild facial muscle involvement, including ptosis and lower facial weakness, but ophthalmoparesis is rarely encountered [6]. There is also a milder CCD presentation that includes minimal weakness that may only be recognized in adulthood; there are also several cases of dominant mutations causing late-onset axial myopathy [10]. Rarely, heterozygous *de novo* mutations can present with extreme weakness in the perinatal period that results in death in infancy [11]. The mutations in *RYR1* that cause CCD are enriched in the C-terminal aspect of the gene.

**Materials and Methods**

The family was recruited at the Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi, India. All the family members provided their consent prior to starting this study. The ethical committee clearance was taken from the Institute of Science, BHU, following the Declaration of Helsinki. All the study subjects were explained about the study aims, nature of the study, follow-ups, and future consequences of the study.

**Clinical assessment**

This family belongs to the Northern part of India (Uttar Pradesh) and consists of unaffected parents and three siblings who were affected with muscle weakness and problems while climbing up after sitting (Figure 1A, Videos 1, 2, and 3). A detailed clinical analysis was performed which included nerve conduction velocity (NCV), electromyography (EMG) and muscle magnetic resonance imaging (MRI) examinations. Five to six mL of peripheral blood was taken from all available participating members in a heparinized centrifuge tube. Further, the genomic DNA was isolated using the standard salting-out protocol routinely used in the laboratory [12].

**Genetic analysis**

Whole exome sequencing (WES) was performed on two affected siblings (IV-4), (IV-5) and the unaffected mother (III-2) using ˜3 μg of DNA samples. The Agilent SureSelect Human All Exon V5 Kit (Agilent Technologies, Santa Clara, CA) was utilized as per the manufacturer’s protocol. The paired-end sequencing was carried out on the Illumina NovoSeq 2500 platform (Illumina, Medgenome, Kerala). The DNA libraries were prepared with at least 95-100X raw target depth using 100 bp paired-end sequencing protocol. All the steps were followed in accordance with the previously reported study (Kirola et al., 2016). The WES data revealed a mean depth of 126 bp and the coverage was ˜99% (Supplementary Table S1). The detailed WES data statistics, variants types, variants filtration, and interpretation have been shown in Supplementary Tables S1, S2, and S3). Considering an autosomal recessive mode of disease inheritance in the family, both homozygous as well as compound heterozygous variants were selected for the downstream analysis. These variants were filtered later on the basis of their types, position (exonic, intronic, splice sites, etc.), class (synonymous, nonsynonymous, etc.) and minor allele frequency (a frequency of less than or equal to 0.01 in 1000 Genome, ExAC database, etc.). All the information is provided in Supplementary Table S3.

**Validation and segregation analysis using Sanger sequencing**

The primers (Supplementary file T2) were designed by using primer 3 ([https://primer3.ut.ee/](https://primer3.ut.ee/)) and the variants (c.A5096G: p.D1699G+c.C5097AA: pD1699E; 13423_13424del:p.K4475Efs*106) present in the *RYR1* were analyzed using PCR-based Sanger sequencing. Both these variants were confirmed in the affected siblings first followed by segregation analysis on the other members of the family. This compound heterozygous variant (c.A5096G: p.D1699G+c.C5097AA: pD1699E; 13423_13424del:p.K4475Efs*106) was present in all three affected siblings in the family while one variant was contributed by father (III-1) and other variant was contributed from mother (III-2).
In silico variant prediction and protein modeling

Many online tools have observed the deleterious nature of the compound heterozygous variant in our study (Supplementary file T1). The impact of the compound heterozygous variant on amino acid sequences was also checked by modeling the reference sequence of the protein (NM_000540) using SwissProt (P21817). The PDB file (https://www.rcsb.org/structure/7T65) was downloaded from RCB for the prediction and modeling of the structural protein of RYR1. Multiple approaches were also exploited such as I-TASSER (https://zhanggroup.org/I-TASSER/), and Lomets (https://zhanggroup.org/LOMETS/). Both mutant and wild-type models were generated and checked by using Procheck (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) (Supplementary additional file1 and 2). These PDB files were uploaded to DynaMut software (https://biosig.lab.uq.edu.au/dynamut/) for stability, flexibility, and conformational changes in the RYR1 protein. The server determines the impact of the mutation on the destabilization energy and also calculates changes in atomic fluctuations, vibrational entropy and deformation energies (Figure 2 B, C).

Calculating the functional impact of mutation:

To predict the functional effects of the compound heterozygous variant present in RYR1 gene, we analyzed the in silico impact of these mutants using PredictSNP software (https://loschmidt.chemi.muni.cz/predictsnp/). Further, combined tools include other consensus classifiers such as PANTHER, MAPP, PhD-SNP, nsSNPAnalyzer, PolyPhen-1, PolyPhen-2, SNAP, and SIFT were also used. The results are shown in supplementary file T3.

Conservation analysis

The protein sequences were used for the multiple sequence alignment using Cobalt (https://www.ncbi.nlm.nih.gov/tools/ctcweb/ctc.cgi) of NCBI. Both these variants were present in the conserved amino acid residue in the vertebrates (Figure 1D).

Generation of stable HEK293 cells expressing RyR1 mutants

D1700 mutations (D1700G and D1700E) were introduced in rabbit RyR1 and stable cells were generated using Flp-In T-REx system (Life Technologies, CA, USA).

[3H] Ryanodine binding:

Microsomes from HEK293 cells expressing RyR1 were incubated for 2 h at 37°C with 5 nM [3H] ryanodine in reaction media (0.17 M NaCl, 20 mM MOPS, pH 7.0, 2 mM dithiothreitol, and 1 mM AMP-PCP). Free Ca2+ was adjusted with 10 mM EGTA using Webmaxc Standard (http://www.stanford.edu/cpatton/webmaxcS.htm) online software. The [3H] ryanodine binding data (B) were normalized to the maximum number of binding site (Bmax), which was separately determined by Scatchard plot analysis using various concentrations (3–20 nM) of [3H] ryanodine in a high-salt medium. The resultant B/Bmax represents the averaged activity of each mutant.

[K+]–induced Ca2+ release

Time lapse ER [Ca2+] measurements were performed using the FlexStation3 fluorometer (https://www.moleculardevices.com/). Stable R-CEPIA1er HEK293 cells were seeded on 96-well flat clear-bottom black microplates at a density of 3×104 cells/well in 100 μL culture medium. One day after seeding, 100 μL culture medium containing baculovirus solutions for RyR1 (WT or mutants), Cav1.1, β1a, JP2, Stac3, and Kir2.1 (2 μL each) were added to each well. After 24 h, the culture medium was replaced with 81 μL normal Krebs solution and the microplate was placed in a FlexStation3 fluorometer preincubated at 37°C. Signals from R-CEPIA1er, which was excited at 560 nm and emitted at 610 nm, were captured every 5 seconds for 150 seconds. Thirty seconds after starting, 54 μL of the high [K+] solution was applied to the cells. The fluorescence change was expressed as F/F0 in which averaged fluorescence intensity of the last 25 seconds (F) was normalized to that of the initial 25 seconds (F0). For drug testing, compounds were added to normal Krebs solution at the indicated concentrations.
Clinical history in the family:

This family belongs to the Northern state of India. The father belongs to Uttar Pradesh while the mother is from Haryana. They have three children (the elder son was 15 years old; the middle daughter was 14 years old and the third child was an 8-year-old boy) and all of them have similar phenotypes that include muscle weakness and joint contractures. The clinical features also include lower proximal muscle weakness, difficulties in standing up and climbing chairs in addition to some skeletal malformations and hypotonia at birth. Both the parents were normal, they didn’t show any muscular problems. Though the father had slight gait problem with his left leg while walking. The parents explained that their first child didn’t cry after his birth and also faced respiratory issues and hence was in the neonatal intensive care unit (NICU) for seven months after birth. His mobility was delayed and happened after 2 years and 6 months. Currently, he tops his class and likes to eat out, listen to music and takes part in regular physical activities. He experienced standing-up movement issues in addition to muscle weakness at the age of 11 years. The girl child started slowing in movements at 7-8yrs and her current age is 14yrs. Her delivery was normal; the delivery was vacuum based. Like her brother, she also didn’t cry after her birth for 5-10 minutes, and she was kept in NICU for up to 5 months. She started walking herself after 15-18 months. The mother explained she was completely doing well and normal up to the age of 7years. At the age of 7 years, she started moving slowly and was later had similar clinical phenotypes like her brother. She too did not have any problem with cognition. Their third child was also affected with muscle weakness, since in 2020 at the age of 7 years old. He also started similar phenotypes like slow mobility, issues climbing the chairs. The genetic history of both the parents is associated with muscular as well as psychological problems. Furthermore, the father’s brother’s daughter (cousin sibling) has seizures and hyperactivity. One of the mother’s uncles was diagnosed with similar slowness in movements and one son of the other uncle had a phobia, fear, and aversion. Thus, although overlapping clinical phenotypes were observed in the family but none had exactly similar phenotypes.

The creatine kinase (CK) levels were normal in all three children. Their NCV showed a normal pattern while their EMG was abnormal. The muscle MRI showed generalized atrophy of muscles of bilateral thighs and legs (Figure 3).

Results

We found one novel compound heterozygous variant (NM_000540:exon34:c.A5096G; p.D1699G+c.C5097AA: pD1699E; NM_000540:exon91:c.13423_13424del:p.K4475Efs*106) which was confirmed by Sanger sequencing in all three affected siblings along with parents. Both the parents were heterozygous for one variant and each variant was contributed by one parent (either mother or father not both). In addition, this variant was not found in any of the aforementioned publicly available databases. Interestingly, two heterozygous missense variants (NM_000540:exon34:c.A5096G; c.A5097E) were contributed from the mother while a frameshift deletion (NM_000540:exon91:c.13423_13424del:p.K4475Efs*106) was inherited from the father (Figure 1B). Sanger sequencing with forward and reverse primers were used for confirmation and segregation of this compound heterozygous variant in the family (Figure 1B). These variants (NM_000540, exon34:c.A5096G; c.A5097E) are novel and not reported in any publicly available databases (for example, dbSNP, NCBI, HapMap, 1000G, ExAC, NIBMRI, HGMD, etc). Multiple in silico tools (SIFT, Polyphen2, MutationTaster, LRT, and PhyloP, etc.) predicted that both the missense variants (NM_000540, exon34:c.A5096G; c.A5097E) were highly damaging (Supplementary file T1). The probable functional consequences of these missense variants were also analyzed using PredictSNP software (https://loschmidt.chemi.muni.cz/predictsnp1/) and the results are shown in supplementary file T3. Multiple sequence alignment-based evolutionary conservation analysis had shown an impact of these variants on evolutionarily conserved residue (Figure 1D). However, these variants on RYR1 gene explained the genotype-phenotype correlation that were seen in our patient. The missense changes (His138Arg) in the amino acid substitution were anticipated to affect significantly the protein structure, stability, and interactions (Figure 2 B, C, and Supplementary file T4). Many algorithms assessed that the impact of the altered protein structure would be deleterious (Supplementary file-T5-PredictSNP). Additionally, the in silico tools such as SIFT (https://sift.bii.a-star.edu.sg/), POLYPHEN
We describe a family with three siblings who have been affected by muscle weakness and developmental delay since their childhood. Their EMG details described significant changes in muscle activity and muscle MRI showed generalized atrophies of muscle thighs and legs. All three children have low or severe problems while standing up after sitting. In addition, they have difficulties climbing stairs.

Upon WES, we identified a compound heterozygous variant (NM_000540:exon34c.A5096G: p.D1699G+c.C5097AA: pD1699E; NM_000540:exon91:c.13424 del:p.K4475Efs*106) in RYR1 gene. Of note, the compound heterozygous variant in RYR1 was absent in public databases available to date and in silico tools predicted the missense variants (NM_000540, exon34c.A5096G: p.D1699G+c.C5097AA: pD1699E) were damaging. Further, protein modeling showed the missense variants in RYR1 have an impact on protein stability and function. This supports that these variants are extremely rare (supplementary file T1, T3) and the putative causal nature of these variants was carried out by multiple in silico tools (supplementary file T1, T3). To the best of our knowledge, this is the first report from India that explains the involvement of RYR1 mutations in three siblings with CCD.

The RYR1 gene encodes a Ca2+ release channel in human skeletal muscle that releases Ca++ ions in the sarcoplasmic reticulum [13]. The first time the RYR1 was cloned and its exon and intron boundaries were determined along with a 30 bp intronic region flanked by splice junctions [14]. The sarcoplasmic reticulum has an internal membrane network that helps in the regulation of skeletal muscle contraction and relaxation by controlling the Ca++ concentrations present in myoblast cells [15]. Because the Ca2+ release channel has a higher affinity towards plant alkaloids called ryanodine and it is also known as ryanodine receptor [16]. The RYR1 codes a 15000 bp cDNA that further encodes a ryanodine about 5035 amino acid proteins with a molecular mass of around 2,252,000 (563 kDa) [17]. It is tetrameric in nature and is one of the largest proteins. The Drosophila melanogaster has been shown to produce a 25.7 kb protein and it consists of 26 exons [14,18]. The RyR family codes mainly three RyR genes which are highly expressed in the brain in addition to a wide range of tissues [19]. The RYR1 has also been shown to be detected in muscle cells and mutations in this gene lead to affect muscle activity, especially muscle contraction [19]. RYR1 is crucial and required in skeletal muscle for excitation-contraction coupling [19]. The RyR2 plays a major role in cardiac muscle contraction, whereas, the RyR3 has been studied and plays a major role in memory and synaptic plasticity [20]. The RyR family is involved in the initial propagation of Ca++ signaling events from intracellular stores and release to the endoplasmic reticulum [19]. RyR1 encodes a ryanodine receptor (RyR), which, in vertebrates, is encoded by a family of three genes [19]. The regulation of the RyR channel is governed by a distinct group of proteins along with other molecules like Ca++, cyclic ADP ribose, FK506BP and Ca2+-calmodulin [21]. Most importantly, the role of the RyR family is linked to muscle contraction and it has been reported to be conserved among invertebrates [22]. For example, single gene RyR in C. elegans expresses solely in muscle and functions exclusively in muscle cells [22]. A null mutant of the unc-68 gene in C. elegans has demonstrated decreased movement-related functions, slow growth, lower muscle activity and function and low brood sizes [23]. A single Ryr gene in Drosophila has been shown with ~45% uniqueness with mammalian and expresses highly during the Drosophilaembryogenesis (for example, body wall development,
and head muscle growth) suggesting an important role in muscle contraction [24,25]. Recently, it has been shown that the RyR1 is involved in phototransduction [24,25].

RyR1 mutations cause a wide range of inherited muscle disorders that include MHS, MH (SA), CCD, CNM) and CFTD [9,26]. The clinical phenotype spectrum of RyR1 is variable and it ranges from mild, moderate and progressive, severe to fatal [26,27]. Most individuals with a dominant mutation in the Ry1 gene have been seen with mild symptoms of muscle weakness in the proximal lower limb while individuals with recessive mutations in RyR1 are linked with severe and variable phenotypes including generalized weakness and muscle wasting, extraocular muscle involvement along with respiratory issues [26–28]. The patients with RyR1 mutations have clinical symptoms since the neonatal stage, during infancy and early childhood [10].

To summarize, we have identified a compound heterozygous mutation (NM_000540,exon34c.A5096G:p.D1699G+c.C5097AA:p.D1699E;NM_000540:exon91:c.13423_13424del:p.K4475Efs*106) in RyR1 that is associated with CCD phenotypes seen in affected members of the family in this study. This is the first report from India. Such discoveries are required to understand the probable underlying disease causes in CCD and this can help in clinical profiling and management in addition to family counseling and early genetic testing and screening.

Figure 1
[A] Shows the pedigree of the study family; the affected siblings are shown with filled dark symbols and the unaffected parents are shown with open circled symbols. Consanguineous marriage is highlighted with double bars; Patients with ID, IV-3, 4, and 5 are affected siblings, and III-1 and 2 are unaffected parents, respectively; patient samples (IV-4 and 5) and the mother (III-2) have been exome sequenced. [B] Electropherogram shows Sanger sequencing confirmatory testing of both the mutations found in the family– the exon 34 (NM_000540: c.A5096G: p.D1699G; NM_000540:exon91:c.13423_13424del:p.K4475Efs*106) and the exon 91 (NM_000540, c.13423_13424del:p.K4475Efs*106 c.413A>G:p.His138Arg) by using bidirectional sequencing (shown is the forward primer): Both the parents were heterozygotes for these mutations (the mutation on exon 34 was contributed by mother whilst the father contributed the mutant on exon 91) whilst all three affected siblings showed the compound heterozygous condition suggesting the recessive mode of transmission. Segregation analysis was performed and mapped in the family. [C] IGV snapshots have shown both the mutations present in exon 34 and exon 9, respectively. [D] Multiple sequence alignment of RyR1 protein sequence across different species. The highlighted square box shows the conserved Aspartic acid and Lysine residue, respectively.

Figure 2
[A] Shows the wild-type protein and the positions of both the mutants on the RyR1 gene. [B] and [C] Showed the destabilization of the RyR1 protein due to Asp129Gly and Asp129Glu mutations.

Figure 3
Shows the effect of D1700 mutants on the function of the RyR1 channel: [A] CICR activity was performed in mutants D1700G (Human D1699G) and D1700E (Human D1699E). This exhibited increased [3H] ryanodine binding in D1700E (Human D1699E), indicating an enhanced CICR activity whilst D1700G (Human D1699G) was similar to wild-type. [B] Both these mutants were unchanged in DICR activity.

References
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