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Dominant or recessive mutations in the *RYR1* gene causing central core myopathy in Brazilian patients

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Conflict of interest

The Authors declare no conflict of interest

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Abstract

Central Core Disease (CCD) is an inherited neuromuscular disorder characterized by the presence of cores in muscle biopsy. CCD is caused by mutations in the RYR1 gene. This gene encodes the ryanodine receptor 1, which is an intracellular calcium release channel from the sarcoplasmic reticulum to the cytosol in response to depolarization of the plasma membrane. Mutations in this gene are also associated with susceptibility to Malignant Hyperthermia (MHS).

In this study, we evaluated 20 families with clinical and histological characteristics of CCD to identify primary mutations in patients, for diagnosis and genetic counseling of the families.

We identified variants in the RYR1 gene in 19/20 families. The molecular pathogenicity was confirmed in 16 of them. Most of these variants (22/23) are missense and unique in the families. Two variants were recurrent in two different families. We identified six families with biallelic mutations, five compound heterozygotes with no consanguinity, and one homozygous, with consanguineous parents,

resulting in 30% of cases with possible autosomal recessive inheritance. We identified seven novel variants, four of them classified as pathogenic. In one family, we identified two mutations in exon 102, segregating in cis, suggesting an additive effect of two mutations in the same allele.

This work highlights the importance of using Next-Generation Sequencing technology for the molecular diagnosis of genetic diseases when a very large gene is involved, associated to a broad distribution of the mutations along it. These data also influence the prevention through adequate genetic counseling for the families and cautions against malignant hyperthermia susceptibility.

Key words: central core disease, *RYR1*, Next Generation Sequencing

Introduction

Central core disease (CCD) is one of the most common genetic congenital myopathies, characterized by muscle weakness, atrophy, hypotonia, hyporeflexia, and delayed motor development, starting commonly in the perinatal period. Muscle weakness is usually proximal and symmetrical, stable or slightly progressive [1].

The probable incidence of congenital myopathies has been estimated in ~1:25,000, and has been reported to account for 14% of all cases of neonatal hypotonia, or one out of ten of all cases of neuromuscular disorders [1].

The classification of congenital myopathies is under constant review, as more genes and forms are identified and associated with various phenotypic and muscle histological alterations. In structural forms, the classification is based on the characteristics observed on muscle biopsy. The histopathological hallmark of CCD is the presence of cores, areas with reduced oxidative activity, observed in muscle fibers under the reaction for oxidative enzymes (NADH or SDH). The genetic variants causing core myopathies primarily affect proteins involved in skeletal-muscle excitation-contraction coupling (ECC) by altering calcium ion (Ca2+) transits between the sarcoplasmic reticulum (SR) and sarcoplasm. Ineffective ECC causes muscle weakness and is also associated with the formation of mitochondria-depleted core lesions. However, the processes governing core formation are far from completely understood [2].

The *RYR1* gene encodes the major sarcoplasmic reticulum calcium release channel of the skeletal muscle [3], and mutations in this gene cause CCD and also lead to several other types of myopathy subtypes, such as Multiminicore Disease (MmD), Centronuclear myopathy (CNM) and Malignant Hyperthermia susceptibility (MHS, MIM# 145600) [3]. Malignant hyperthermia is a pharmacogenetic disorder of skeletal muscle, triggered by exposure to volatile anesthetic gases like halothane and depolarizing muscle relaxants such as succinylcholine [4]. Patients with CCD usually also present Malignant hyperthermia susceptibility.

RYR1 is located at 19q13.2 and contains 106 exons. The protein product of *RYR1* is composed by 5037 amino-acids and 535 kDa. The combination of four of these subunits, together with a number of accessory proteins, forms the major calcium channel in the skeletal muscle. RYR1 combined molecules are embedded in the membranes of sarco/endoplasmic reticula (SR/ER) and regulate the rapid intracellular release of Ca2+ following transverse tubule depolarization. RyR isoforms also contribute to maintaining cellular Ca2+ homeostasis under resting conditions [5]. Over 450 variants were identified in the *RYR1* gene causing CCD and MH, and these mutations were mainly located in three hotspots of the gene. The hotspots, also referred to as regions 1-3 (D1, D2, and D3), include N-terminal residues 1-614 (sarcoplasm), central region residues 2163-2458 (sarcoplasm), and C-terminal residues 4136-4973 (Pore-forming, SR lumen, and membrane). MH causing disease are predominantly located in D1 and D2, and mutations causing CCD are predominant in the C-terminal D3 region [3].

For many years, due to the large size of the *RYR1* gene and the broad distribution of the mutations along the gene, screening for mutations in candidate patients was done predominantly in the hotspot regions, restricting the effectiveness of the molecular diagnosis of the patients. In our days, a significant improvement has started with the introduction of sequencing using next-generation sequencing methodologies, which became a more economical and efficient way to study a large number of genes and regions simultaneously. Custom panels can be designed to include several hundred genes of interest, or ready to use panels, such as the Illumina Trusight panels, that are available with more than 6,700 genes for Mendelian diseases [6].

Here, we studied 20 families with CCD, aiming the molecular characterization of the patients, and evaluation of the frequency of mono versus biallelic mutations in the *RYR1* gene. The results have important implication for the study of physiopathological mechanisms involved in the disease, and for the prevention through genetic counseling in the Brazilian families.

Patients and methods

Patients

The ethics committee of the Biosciences Institute of the University of Sao Paulo approved this work, and the DNA samples are stored in the biobank repository of the Human Genome and Stem Cells Research Center of IB-USP. All patients agreed in participating in this study and signed an appropriated informed consent.

The patients included in this study have been followed in the last 20 years in the Myopathies Laboratory of clinic for neuromuscular diseases at the Human Genome and Stem Cells Research Center, Institute of Biosciences, University of Sao Paulo, Brazil. Patients were also referred from other hospitals in Sao Paulo and medical centers from Belo Horizonte, Brazil, where a complete clinical and neurological evaluation was also performed. The inclusion criteria was patients of any age and sex with clinical diagnosis of congenital myopathy, and a muscle biopsy with histopathological findings including cores in oxidative enzymes reaction in muscle fibers.

Molecular analysis

The DNA of the selected patients was extracted from peripheral blood lymphocytes using routine methodology. Parents were also studied, when available, for segregation analysis.

The genetic investigation was carried out by Next-Generation Sequencing, using first a customized panel including *RYR1* and additional 95 genes associated with neuromuscular diseases (NMD) genes. After, in order to expand our investigation, we begin to use the Illumina TruSight One Expanded panel, which targets more than 6700 genes and exonic regions that were associated to a described clinical phenotype

The SureSelect QXT library preparation kits and the SureSelect Human all exons and V6 capture kit (Agilent, United States) were used. The Hiseq2500 equipment (Illumina, United States) performed the sequencing. The data were aligned according to the reference version GRCh37/hg19 of the human genome.

Variants were filtered and compared to control populations of 1000 Genomes, NIH, gnomAD, 6500 Exome Sequencing Project (Washington University), and the recently created Online Archive of Brazilian Mutations – AbraOM (http://www.abraom.ib.usp.br). Rare variants were checked in the *RYR1* gene (OMIM#180901), and analyzed using bioinformatic tools. Pathogenic variants already described

were checked in Gene Mutations Databases HGMD, LOVD, and Clinvar. The pathogenicity of de novo variants was analyzed in prediction sites including: Mutation taster, Predict SNP1, CADD, DANN, FATHMM, FunSeq2, GWAVA, VEP, SIFT, Polyphen2 and Human splicing finder3.0.

Sanger sequencing of specific exons was done to confirm the mutation and screen other affected patients in the family, or to study the segregation of the mutation within the family.

The classification of the variants was carried out according to the American College of Medical Genetics and Genomics (ACMG) pathogenicity classification guidelines [7]. For this, we relied on the help of the Intervar software (http://wintervar.wglab.org)

Results

Patients characterization

Twenty-five patients, belonging to 20 unrelated families with at least one CCD affected patient were studied. Four families presented more than one patient, three with autosomal dominant (P9, P16 and P18 - Index patient from each family is identified as P#) and one with autosomal recessive inheritance (P8- two affected sibs), and 16 patients were isolated cases. Consanguinity among parents was present in only one family (P11).

Molecular analysis

Molecular screening for variants included the application of several filters of frequency and genes selection. 23 different variants in the *RYR1* gene were identified: 22 of them were missense, and one, a frameshift mutation. In only one patient (P20), no mutation in the *RYR1* gene was identified. Therefore, it was possible to molecularly characterize 19 of the 20 families. The majority of the variants, 21 of them, were unique, each family present a different variant. However, two variants were present in two families: p.Arg4861His was found in patients P12 and P13; variant p.Arg4861Cys, in patients P7 and P14. In addition, different variants were found in the same codon, such as p.Arg4861His (P13), p.Arg4861Cys (P14), p.Arg4914Met (P18), p.Arg4914Thr (P19).

Among the 23 variants, 16 were previously described in patients with CCD, HM or congenital myopathies, while seven variants are being described for the first time in this manuscript (P2, P3, P4, P7, P9, P10 and P18). Among them, four were classified as pathogenic or likely pathogenic (P2, P9, P10, P18), two were classified as variant of uncertain significance (VUS) (P4, P7), and one was considered as likely benign (P3). Therefore, molecular diagnosis with pathogenic mutations in the *RYR1* gene could confirm the diagnosis in 16 of the families. It is important to note that two among the three VUS were accompanied by a pathogenic mutation in the other allele.

The distribution of the variants along the coding sequence of the *RYR1* gene showed a predominance of CCD patients with mutations in the C-terminal domain in exons 94-102: P2, P4, P7, P8, P9, P10, P11, P12, P13, P14, P15, P16, P17, P18 and P19. One variant was localized in the N-terminal region in exon 2 (P1), and three in the central region of the gene, in exons 35 (P3), exon 66 (P5) and exon 73 (P6).

Monoallelic heterozygous variants were found in 12 patients, while patients with mutations in both alleles were identified in seven cases: one (P11) was homozygous for the same mutation (and the parents are consanguineous), five patients (P1, P2, P4, P7, and P8) were compound heterozygous. In one family (P18) with autosomal dominant pattern of inheritance, the two mutations in exon 102 segregate together in cis in the same allele. Therefore, biallelic cases constitute 6/20 of our cohort, or 30% of the cases.

Segregation analysis confirmed parental segregation of the mutation/s in families 1, 2, 8, 11, 16 and 18.

Histological analysis were possible in nine of the patients (Tab. I), and all presented visible cores with a predominant frequency of affected fibers - 80% in 7/9 cases (Fig. 1). In addition, four patients presented a big, unique and structured core (P4, P7, P10 and P12), while five patients showed non-structured cores, both small, multiple and unique large cores inside muscle fibers (P1, P3, P5, P11 and P17) (Tab. II).

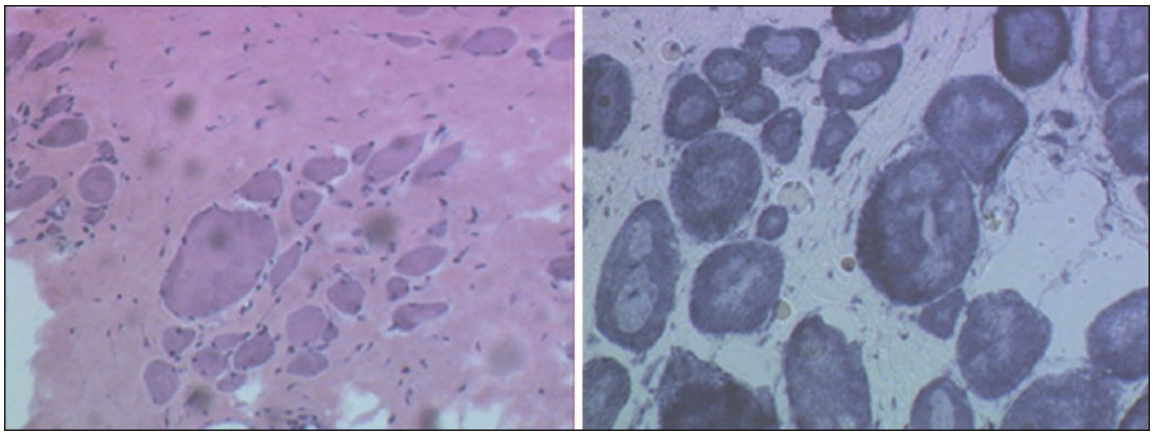

Figure 1.

Examples of type of cores observed in the patients: big and structured cores in almost all fibers in P4, and in less fibers in P10, few small and less structures cores in P11 and P3.

Table I.

Genetic data of the 20 studied families.

Fam	I / F	Consang	Exon	Mutation (segregation)	CADD/Phred	Classification	References
1	I	N	2	c.122 T > C:p.(Phe41Ser)	24.7	Likely pathogenic	Klein et al., 2011 [18]
			25	c.3362 C > G:p.(TyrY1121Cys) (maternal)	25.5	Likely pathogenic	Wilmshurst et al., 2010 [19]
2	I	N	**17**	c.C1840T:p.(Arg614Cys)	28.8	Pathogenic	Gillard et al., 1991 [8]
			104	c.14938_14939del:p.(Thr4980Ala*fs) (maternal)	-	Pathogenic	this MS
3	I	N	**35**	c.5723 A > G:p.(Lys1908Arg)	22.3	Likely benign	this MS
4	I	N	**46**	c.7433 C > A:p.(Thr2478Asn)	18.66	Uncertain significance	this MS
			94	c.13703 T > C:p.(Leu4568Pro)	29.6	Pathogenic	Wu et al., 2006 [12]
5	I	N	**66**	c.9758 T > C:p.(Ile3253Thr)	23.8	Uncertain significance	Böhm et al 2013 [22]
6	I	N	**73**	c.10747 G > C:p.(GluE3583Gln)	18.39	Uncertain significance	Robinson et al., 2006 [23]
7	I	N	**79**	c.11321 C > T:p.(Ala3774Val)	25.2	Uncertain significance	this MS
			101	c.14581 C > T:p.(ArgR4861Cys)	32	Pathogenic	Davis et al., 2003 [9]
8	F - AR	N	94	c.13673 G > A:p.(Arg4558Gln) (maternal)	32	Pathogenic	Kossugue et al., 2007 [4]
			101	c.14537 C > T:p.(Ala4846Val) (paternal)	25.8	Likely pathogenic	Gambelli et al., 2007 [21]
9	F- AD	N	**95**	c.13952 A > G:p.(His4651Arg)	25	Likely pathogenic	this MS
10	I	N	**100**	c.14411 A > C:p.(His4804Pro)	26.9	Likely	this MS

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Including inheritance (I- isolated case, F – familial case, AD – autosomal dominant inheritance, AR – autosomal recessive inheritance, Consanguinity in the parents- Y-yes, N-no, Exon with the mutation, description of the mutation using the NM_000540 transcript, the CADD (Combined Annotation Dependent Depletion) score for variation, the classification of the mutation according to the ACMG guidelines, and the references for the mutations previously described

Table II.

Data on muscle biopsies: type of cores, proportion, and distribution inside the muscle fiber.

Patient	Exon mutation	% Fibers with cores	Number of cores	Type of core	Position of cores
P1	2/25	96	Few small	Less structured	Central
P3	35	86	Few small	Less structured	Peripheral
P4	46/94	99	Big unique	Structured	Central
P5	66	52	Big unique	Less structured	Central
P7	19/101	99	Big unique	Structured	Central
P10	100	84	Big unique	Structured	Peripheral
P11	101/101	41	Few small	Less structured	Peripheral
P12	101	100	Big unique	Structured	Central
P17	102	100	Few small	Less structured	Peripheral

In one patient (P7), a severe clinical phenotype was associated to biallelic mutation in the *RYR1* gene, and a histopathological pattern on muscle biopsy showing very severe muscle degeneration and connective tissue replacement. However, in the remaining muscle fibers, big unique or multiple cores could be observed (Fig. 2).

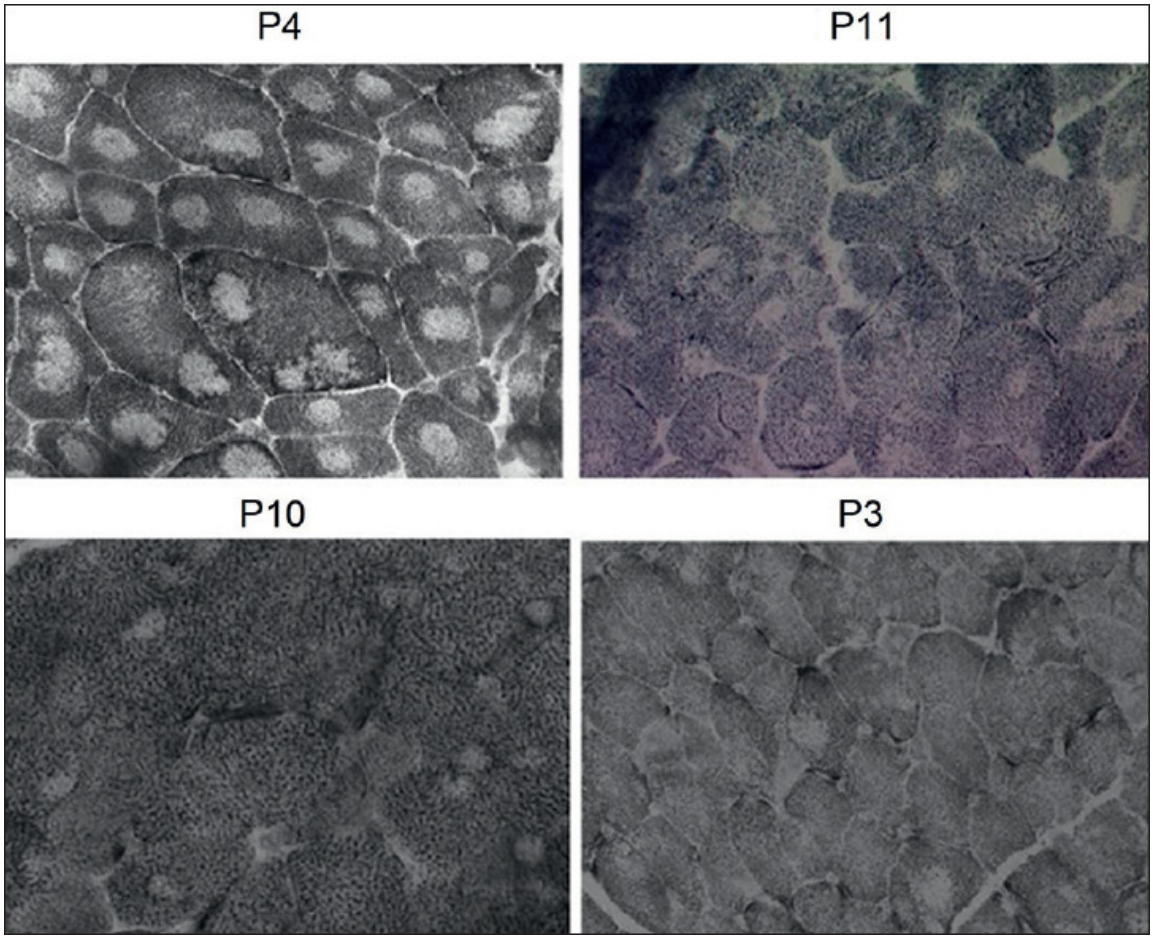

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Figure 2.

Histological characterization of patients P7: A) HE staining illustration massive muscle degeneration with a few variable muscle fibers in a massive connective tissue replacement; B) NADH staining showing the presence of unique or multiple large and structured-like cores in the remaining muscle fibers. Amplification: X 400.

Discussion

Central Core Myopathy (CCD) is caused predominantly by mutations in the *RYR1* gene, which is a huge gene composed by 106 exons. More than 450 different mutation causing disease were identified along the coding sequence of the gene, which makes the molecular screening through Sanger Sequencing methodology difficult, expensive and time consuming. For this reason, for many years, the screening was done focusing on three enriched hotspot regions: N-terminal region 1, amino acids 35-614; central region 2, amino acids 2163-2458; and C-terminal region 3, amino acids 4550-4940. Region 1 and 2 variants are predominantly associated with the MH susceptibility phenotype and region 3 variants with the classic CCD phenotype [5].

RYR1-related congenital myopathies present a significant genetic heterogeneity and the increasing utility of next generation sequencing (NGS) approaches to variant identification, coupled with reduction in sequencing cost, has enlarged the access to this methodology worldwide. Therefore, to

screen patients for pathogenic variants using NGS sequencing of the entire *RYR1* gene rather than only the three hotspots is now considered the best practice. In fact, using this new approach, we were able to identify mutations in the *RYR1* gene in 19 of the 20 tested families, confirming the utility of this powerful molecular tool.

Allelic heterogeneity

The large number of variants identified in the *RYR1* gene in patients with CCD has shown the occurrence of significant molecular variability, constituting the vast majority of particular mutations for each family, with only 10% of the variants in *RYR1* being functionally characterized [5]. This fact was also confirmed in our patients by the number of different variants found: a total of 23 different variants in 19 families. 20 of these variants were particular mutations. In addition, seven novel variants were identified in our patients, suggesting that the number of variants with possible clinical significance in this gene may increase with more studies using new molecular technologies.

Some mutations have been frequently described in different populations, such as p.Arg4861Cys (Davis et al., 2003 - LOVD: 12 reports), p.Arg4861His (Monnier et al., 2001 - LOVD: 16 reports), and p.Arg614Cys (Gillard et al. [8] - LOVD: 43 reports). In Brazilian patients, we also identified these variants, which were even present in more than one unrelated patient. We identified three recurrent mutations, each one in two unrelated families: p.Arg4861Cys in families P7 and P14, p.Arg4861His in families P12 and P13, and p.Arg4914Met or Thr in families P18 and P19. These mutations were located in hotspot of exon 101 or 102 of the *RYR1* gene, and also already described in other families [9-11].

The distribution of variants in the *RYR1* gene showed that fifteen variants localized in the C-terminal region (exons 94-104); two were located in the N-terminal region (exons 2 and 17); one was found in the central region of the protein (exon 46); and five variants were distributed between the N-terminal and central region of the *RYR1* sequence which is not in the typically studied regions (Tab. I). This illustrates how the extension of the screening is improving the identification of more mutations in this gene. But, the predominance of mutations in the C-terminal region continues higher [9,10,12].

As summarized by Fusto [2], two main pathological mechanisms are triggered by *RYR1* defects according to which protein domains are affected. Mutations in hotspots one and two lead to channel hyperactivity and an early release of Ca2+ from the sarcoplasmic reticulum [13]. Another consequence is the reduction of the threshold required for channel activation. All these effects together result in lack of a fine control of the channel, as it can be activated easier and longer than it is supposed to be. Patients harboring mutations in these hotspots are under susceptibility of Malignat hyperthermia syndrome [5]. The second pathological mechanism is associated with mutations in hotspot three that causes a defective coupling between membrane depolarization and calcium release from the SR [13]. Further evidence and the identification of novel *RYR1* mutations along the gene are showing that the pathological mechanism is not dependent only on mutation positioning. A study by Dirksen and Avila [14] reported the effect of mutations in hotspot two of the gene causing a channel that is both hypersensitive to agonist and voltage activation and has its basal activity increased.

Type of mutation

Of the approximately 460 already identified mutations in *RYR1* (HGMD) 399 or ~86% were point mutations (missense/nonsense); 23 were mutations in splicing sites; 26 were small deletions; 9 were small inserts; 4 were small indels; 3 were large deletions; and 2 were major insertions and duplications. Accordingly, we also identified in our patients a predominance of missensse variants, constituting 22 of the 23 variants found in the Brazilian patients with CCD. One of our patients presented a frameshift

mutation, but it was associated to a second previously described p.Arg614Cys mutation [8], in the other alelle (P2). In a study by Wu et al., (2006) [12], missensse mutations were found in 25 of the 27 studied patients with CCD. In fact, loss of function mutations, which lead to the absence of *RYR1* expression in the muscle, are incompatible with life, as demonstrated by Takeshima et al. [15], in a mouse knockout model for the *RYR1* gene. In the absence of the protein, the mouse presents perinatal death with gross muscle abnormalities. This study also showed that *RYR1* is essential both for muscle maturation and for E-C coupling to occur since homologous proteins to RYR1 like RYR2, (which is also expressed in muscle) do not have the ability to replace the effect caused by the absence of protein, leading to defects in the release of intracellular Ca2+ ions. The function of the RYR1 protein would therefore be essential in skeletal muscle during E-C coupling and could not be replaced by other receptor subtypes. This fact could explain the predominance of *RYR1* missense mutations causing disease.

Biallelic mutations

The classic form of CCD has been classified as autosomal dominant inherited disease. However, there are several reports in the literature of patients with autosomal recessive inheritance pattern [4,12,16,17]. In the present study, patients with biallelic mutations constitute 30% of the cases, compatible with the finding of 12/47 or 25% of the cases described by Todd et al. [11], illustrating how the recent introduction of studies using NSG approaches for the *RYR1* gene sequencing can help to identify more cases with biallelic mutations. Therefore, the autosomal recessive form of CCD would be more common than expected, as already proposed in a previous study by our group [4].

It is also possible that other mutations, previously found in individuals and considered non-pathogenic, are responsible for muscle weakness if present in compound heterozygosis or in homozygosis, as observed in P11, a 9-year-old affected girl with delayed neuropsychomotor development and difficulties to run and climb stairs. Molecular analysis identified the homozygous mutation p.Val4849Ile in exon 101 of the *RYR1* gene. The consanguineous non-affected parents were both heterozygous for this mutation. Muscle biopsy of P11 showed non-structured cores, both small and large inside about 40% of muscle fibers. In fact, this mutation was already described [16] in homozygous in a patient with MmD and a similar pattern of cores in the muscle biopsy.

Regarding the other five patients with biallelic mutataions, four presented at least one of the variants localized in the C-terminal hotspot domain of *RYR1* gene.

Interestingly, only two among the six cases showed a severe phenotype of CCD. In addition to P11, already discussed, the other patients are described bellow:

- **Patient P1**, a 4-year-old girl, an isolated case in the family, was diagnosed in early childhood, with hypotonia, and late deambulation, at 3 years of age. On muscle biopsy, she presented small multiple unstructured cores. Molecular analysis identified two variants in the *RYR1* gene, previously described as pathogenic, p.Phe41Ser and p.Try1121Cys, in exons 2 and 25 (this one, inherited from the mother). Interestingly, these two variants have been previously reported [18,19] causing the CCD phenotype also in compound heterozygosis: p.Phe41Ser was combined with p.Thr3933Cys variant [18] and p.Tyr1121Cys variant was in combination with p.Leu2689Ala variant [19]. These two variants are not in the C-terminal domain, usually involved in CCD, which could explain the need for both variants for the occurrence of the phenotype;
- **P2**, an 11-year-old girl, also has two distinct variants, in compound heterozygosis. One variant, p.Arg614Cys, was previously described [8] related to HM. The second is a novel frameshift deletion in exon 104, p.(Thr4980Ala*fs), in the C-terminal region of the protein, inherited from the mother, is predicted to be pathogenic. The patient has a severe clinical picture of CCD, requiring ventilatory support at birth, delayed neuropsychomotor development in the first two

years of life, and acquisition of gait after the age of 2 years old. At the age of 11, she presents myopathic facies, severe hypotonia and scoliosis. The pathogenic variant p.(Thr4980Ala*fs) alone could justify a clinical picture of CCD, as it is located in the C-terminal region of the protein and removes it from the reading frame. However, the mother - non affected – was carrier of the same mutation. On the other hand, the p.Arg614Cys variant was also described as pathogenic (HM susceptibility). Further studies would be necessary to assess whether the combination of the two variants present in the patient would have an additive effect and, in turn, to clarify the presentation of a more severe clinical picture;

- **Patient P4** has two variants, p.Thr2478Asn and p.Leu4568Pro in compound heterozygosis. The p.Leu4568Pro variant was previously described in a CCD patient [12]. The novel p.Thr2478Asn variant is in the central region of the protein and leads to an exchange in the threonine residue that is moderately conserved. This variant is present in low frequency in population databases (rs141298868, gnomAD MAF: 0.00002), but was not found in the literature in patients with RYR1-related disease. Algorithms of prediction are conflicting about the potential impact of this variant, ranging from benign (PolyPhen-2) to Disease causing (Mutation taster), and was classified as VUS. The clinical comparison between the patient described by Wu et al. [12] with our patient with biallelic mutations showed that the presence of the second variant does not seem to aggravate the clinical condition of our patients, since currently at 35 years of age, despite her weakness with difficulties to perform tasks like climbing stairs, she has a normal life and can even drive a car. Her muscle biopsy, on the other hand, is typical of CCD with large unique and structured cores in all muscle fibers;

- **P7** is a severely affected girl, which was unable to walk up to 5 years of age. She was a very hypotonic baby, requiring mechanical respiration support after birth. On muscle biopsy, she presented a very atypical dystrophic pattern of muscle degeneration, with scarce fibers surrounded by connective tissue. The isolated scarce fibers showed large and structured cores inside. She also presented two variants, p.Ala3774Val and p.Arg4861Cys in compound heterozygosis. The second variant was described [9] related to CCD in heterozygous state. The p.Ala3774Val still was not found in the literature in patients with RYR1-related disease, and showed very low frequency in the normal population. It was predicted to be potentially damaging. However, the presence of the first described mutation would be sufficient to cause the phenotype on this patient, since other patients with the same mutations presented also a severe phenotype with neonatal hypotonia, lordosis, severe weakness with inability to walk unassisted at the age of 11years old [20];

- **P8** is a 44-year-old female, with a 42-year-old affected brother, with clinically normal non-consanguineous parents. Both presented clinical history of slowly progressive weakness with frequent falls. In this family, two previously described pathogenic variants were found, involving the C-terminal region of the RYR1: p.Ala4558Gln in exon 94 [4] and p.Ala4846Val on exon 101 [21], inherited from the mother and father, respectively. These results corroborate the recessive biallelic pattern in this family. Therefore, according to the criteria used, both variants were classified as probably pathogenic.

Histopathological alterations

We could observe certain variability in our histopathological findings regarding the core pattern in the Brazilian CCD patients. Although a typical pattern of unique and well-structured core in practically all fibers was only found in patients with at least one mutation in the C-terminal region of the protein (P4, P7, P10 and P12), two patients with mutations in this region presented a pattern of few small and non-structured cores (P11 and P17), suggesting that it is not a mandatory pattern.

In summary, 20 families of patients with CCD were evaluated using NGS methods, and we identified 23 variants (7 novel) in the *RYR1* gene in 19 of them, confirming the pathogenicity in 16 cases. Most of these variants (22/23) were missense mutations and 20 of them were unique in families. Two variants were recurrent in two families. We also identified six families (five non-consanguineous) with biallelic variants resulting in 30% of the cases with a possible pattern of AR inheritance. In one family with AD inheritance, we identified two mutations in exon 102, segregating in cis, suggesting an additive effect of two pathogenic variants in the same allele.

This work highlights the importance of using Next-Generation Sequencing technology for the molecular diagnosis of genetic diseases when a very large gene is involved, associated to a broad distribution of the mutations along it. These data also influence the prevention through adequate genetic counseling for the families and cautions against malignant hyperthermia susceptibility.

Figures and tables

Acknowledgments

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