RYANODINE RECEPTOR MUTATIONS: IN SICKNESS AND IN HEALTH

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ABSTRACT

Ryanodine Receptors (RyR) are large ion channels necessary for Ca^{2+} release from intracellular stores in many cell types. Since RyR was discovered nearly 30 years ago, a fruitful research field has devoted to understanding the physiological regulation of the three protein isoforms in health, and the mechanisms underlying their dysfunction in sickness. This minireview discusses the structural characteristics of RyR2, the cardiac isoform, its regulation during cardiac function and the clinical significance of specific mutations. While hundreds of RyR2 mutations are associated with cardiac disease with different levels of confidence, close to 1500 variants appear in the general population without inducing harmful phenotypes. Hence, studying RyR2 mutations variants may shed light on overall protein regulation and the mechanisms that compensate for constitutive changes in RyR2 function.

Keywords: excitation-contraction coupling, ryanodine receptor, calcium, cardiac arrhythmia.

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Introduction

The Ryanodine Receptor (RyR) is the largest ion channel known in nature. The name is derived from the alkaloid ryanodine, a high-affinity ligand that was fundamental for the initial characterization of the channel [1]. RyRs are localized on the sarco/endoplasmic reticulum of many cell types, where they control Ca^{2+} release from intracellular stores. Four identical subunits of nearly 5000 amino acids form a functional RyR with a total molecular mass over 2 million Daltons. Mammals express three isoforms, each encoded by separate genes located in different chromosomes; yet, they share ~65% sequence identity. RyR1 and RyR2 are mostly expressed in skeletal and cardiac muscle, respectively, while RyR3, the least known of the three isoforms, is expressed in several tissues. RyR2 is often called the "cardiac isoform," which is technically correct but may be misleading: while it is indeed the predominant isoform expressed in the heart, it is also the major isoform of the brain [2]. In the heart, RyR2 serves as the major Ca^{2+} release channel and is a component of the excitation-contraction (e-c) coupling machinery; therefore, it is fundamental for cardiac function. This minireview discusses the general characteristics of RyR2 and its potential involvement in different forms of heart disease (heart failure, cardiac arrhythmia and beyond) as a primary or secondary cause, topics that have captivated investigators for nearly 30 years.

RyR2 Structure

RyR2 is well-conserved among species. The human RyR2, for example, shares 97% sequence identity with the mouse RyR2 (the main model organism used in biomedical research), 99% with the rabbit RyR2 (a larger mammal commonly used in heart failure studies), and >99% with the porcine RyR2 (the species used for some recent structural studies by Peng et al. [3]). RyR2 folds into a mushroom-like structure with the "stem" embedded in the sarcoplasmic reticulum (SR) membrane and the "cap" located in the cytoplasm, spanning most of the gap between the SR and the sarcolemma (Figure 1A,B). This large cytosolic region contains anchoring points for a variety modulators, many of which have a stable association with RyR2 while others bind in a Ca^{2+}-dependent manner [4]. Hence, RyR2 is considered an "allosteric giant" [5], not only because of its size, but for its role as a scaffold of a macromolecular complex.

Elucidating the molecular architecture of RyR is a daunting task in part because of the sheer size of the channel. So far, the most informative approach for studying the organization of the protein has been cryo-electron microscopy (cryo-EM), which initially yielded maps of RyR1 with a resolution down to 9.6 Å [6]. Later, discrete domains were crystallized, solved at resolutions below 2 Å [7] and docked into known cryo-EM maps, providing a general idea of domain arrangement and interaction. Since then, a combination of enhanced computer power, clever purification steps and improved detector capabilities allowed the refinement of new cryo-EM structures to nearly atomic resolution. Elegant studies lately described RyR1 at an average resolution of 3.8 Å and in different states of activation by ligands such as Ca^{2+}, ryanodine, ATP, and caffeine [8]. RyR2 lags closely behind with recent structures in the closed and open states at resolutions of 4.1 Å and 4.2 Å, respectively [3]. Unfortunately, docking solved individual domains into improved cryo-EM structures remains problematic, particularly in the periphery of the cytosolic cap where the resolution of the cryo-EM maps is still poor [5]. Therefore, the central tower of the channel — formed by the N-terminal domain (NTD), and the central (CD) and pore-forming (PFD) domains on the C-terminal end — offers the most detail. A second issue with interpreting these new structures is the evident differences between isoforms. The most detailed studies often use RyR1 obtained from rabbit skeletal muscle [8], prompting informed speculation about the structural features of RyR2. Some of these differences have been addressed by crystalizing

discrete domains with the amino acid sequence of both RyR1 and RyR2 [9]. Nonetheless, since important structural features locate to relatively conserved regions of the protein, such as the binding sites for Ca^{2+}, ATP and ryanodine, the structure of RyR1 is expected to be consistent among the three isoforms. Then, the different biophysical properties of each type of RyR likely stem from the divergent regions and their interaction with conserved domains. All efforts devoted to determining the atomic architecture of RyR2 and the intricate domain re-arrangement that occur during the transition to the open state, whether directly on RyR2 or through isoform homology, are extremely valuable because they provide structural foundations to biochemical and physiological studies. Thus, it is not at all surprising, but certainly expected, that RyR2 researchers rush to search for specific residues whenever an improved structure of the channel is published. Whether it is the putative luminal Ca^{2+} sensor, specific phosphorylation sites, disease-causing mutations or "unzipping" domains, remains a matter of preference.

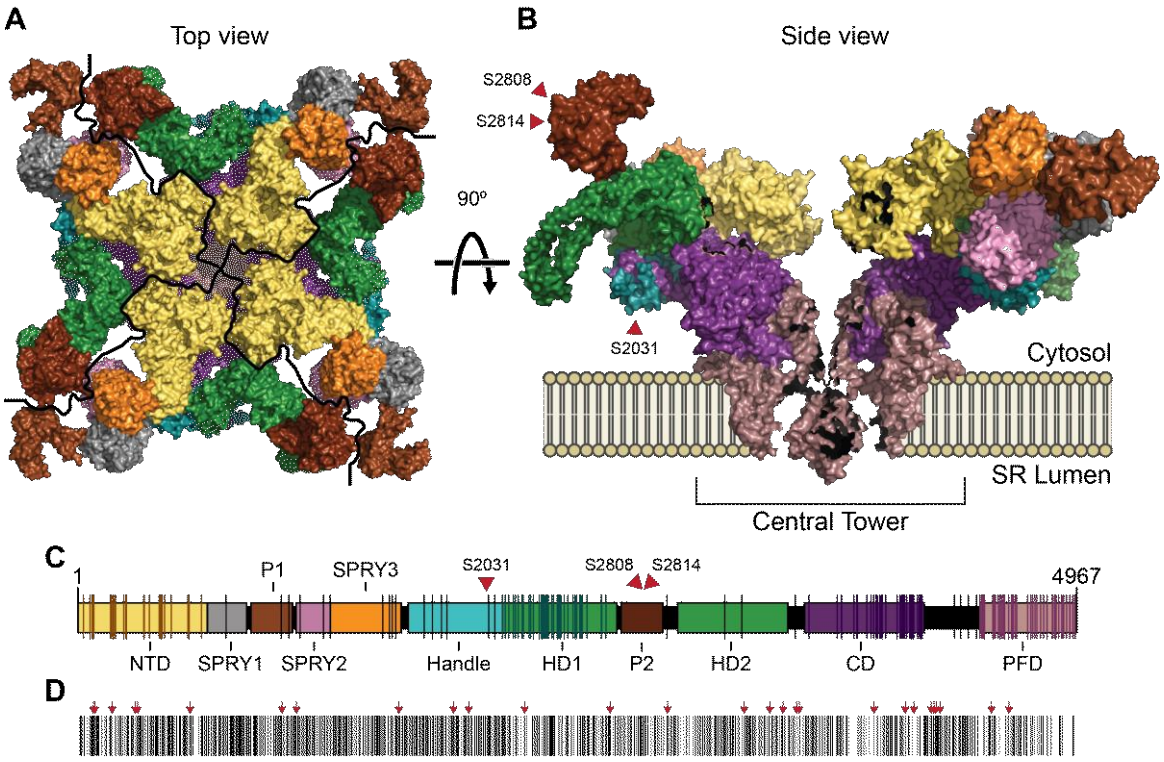

Figure 1. Ryanodine Receptor Structure. Human RyR2 domains were determined based on the structure of the porcine RyR2 reported by Peng et al. [3] (PDB ID 5GO9). Domains are color-coded in all panels. **A.** Single RyR2 channel observed from cytosolic side. Black lines delimit the four subunits. **B.** Side view of a RyR2 channel showing only two opposite subunits. The central tower of the channel, composed of NTD, CD and PFD is highlighted. Red arrowheads indicate the approximate location of the three known phosphorylation sites. **C.** Linear representation of a single RyR2 subunit. Individual domains are labeled. Vertical lines indicate the location of ~200 disease-causing mutations. **D.** Location of 1422 RyR2 residues susceptible to missense variants, drawn at the same scale as panel **C**. Arrows indicate the position of 29 variants that truncate the protein.

Cardiac Excitation-Contraction Coupling

RyR has a crucial role in e-c coupling, the process that converts electrical signals in the form of action potentials (AP) into mechanical contraction (figure 2A). In the heart, RyR2 is *activated* by Ca^{2+} entering the cell through L-type Ca^{2+} channels (LTCC), and *releases* Ca^{2+} from the SR; thus, RyR2 couples with the external membrane through Ca^{2+}-induced Ca^{2+} release (CICR). In contrast, LTCC physically triggers RyR1 opening in the skeletal muscle through voltage-gated Ca^{2+} release. CICR terminates shortly after RyR2 activation in healthy cells, and relaxation occurs as Ca^{2+} is removed from the cytosol by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), replenishing the SR with Ca^{2+}, and the electrogenic Na^+/Ca^{2+} exchanger (NCX), extruding Ca^{2+} from the cell. Other mechanisms, such as the plasma membrane Ca^{2+} ATPase (PMCA) and the mitochondrial Ca^{2+} exchange contribute to a much lower extent. To allow for a dynamic cardiac function that satisfies the metabolic demands of the organism, many components of e-c coupling are regulated by the sympathetic nervous system via β_1-adrenergic receptor (β_1-AR) signaling (figure 2B), with the goal of allowing faster and stronger contraction. In the ventricles, the ultimate effect of β_1-AR activation is to increase in the amount and speed of Ca^{2+} release, decrease the affinity of the myofilaments for Ca^{2+} and accelerate cytosolic Ca^{2+} removal. These changes are accomplished through direct phosphorylation of e-c coupling components (such as LTCC) or their partners (such as phospholamban [PLB], which regulates SERCA2a).

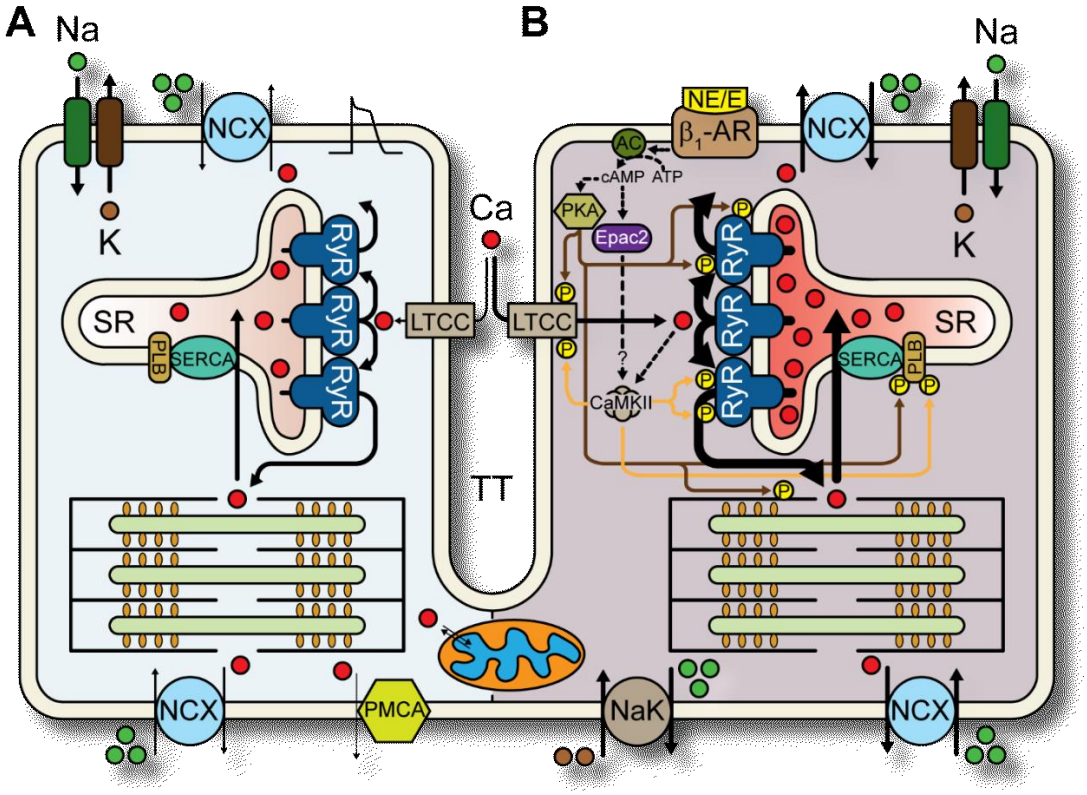

Figure 2. Cardiac Excitation-Contraction Coupling. A. Cardiac excitation-contraction coupling in basal conditions. Ca^{2+} enters the cell through LTCC and activates RyR2. Through CICR, RyR2 releases Ca^{2+} from the SR. Ca^{2+} is then removed from the cell through NCX and reuptaken into the SR by SERCA. **B.** Stimulation of β1-AR activates adenylyl cyclase (AC), which converts ATP into cAMP. PKA activated by cAMP then phosphorylates (P) several protein targets including LTCC, RyR2, PLB, and troponin I. Non-canonical β1-AR signaling that leads to activation of CaMKII via Epac2 is indicated. CaMKII is also activated by enhanced Ca^{2+} cycling. These changes lead to increased entry to the cell and release from the SR, and positive inotropy and lusitropy.

Each RyR2 subunit contains 353 serine and 222 threonine residues, many of which may be susceptible to phosphorylation based on computational analysis [10]. Nevertheless, only three sites have been extensively studied in the context of β_1-AR activation: S2031, S2808, and S2814 (figure 1, recently reviewed in [11]). Despite the solid experimental evidence suggesting that RyR2 is phosphorylated downstream of β_1-AR activation, it is still unclear whether RyR2 phosphorylation is *required* to regulate e-c coupling. The characterization of these three phosphorylation sites may suggest that phosphorylation of RyR2 results in increased channel activity rendering it "leaky" — susceptible to release Ca^{2+} spontaneously. However, all three expected functional outcomes of phosphorylation on channel activity appear in the literature: increase [12], decrease [13], and no effect [14]. Dissecting the role of RyR2 phosphorylation in the context of e-c coupling is complex because other factors that regulate RyR2 function, such as I_{CaL} and SR Ca^{2+} load, are modulated simultaneously by β_1-AR activation. Also, many of the studies that have addressed this issue looked at the overall phosphorylation state of the channel, while more recently the focus is on single residues. This reductionist approach, in which phosphorylation sites are ablated individually, has proven helpful and necessary to parse this issue. From the size of the channel and the multitude of possible phosphorylation sites, it is apparent that more comprehensive studies will be needed because phosphorylation sites may work in concert, rather than independently, to modulate channel function. Moreover, other post-translational modifications, such as oxidation and nitrosylation, may also control any effects phosphorylation has on the channel [15].

RyR2 Dysfunction and Heart Disease

The first indication of RyR2 dysfunction in human disease was in Heart Failure (HF), the advanced manifestation of other underlying conditions that deteriorate cardiac function, such as myocardial infarction and cardiomyopathy [16]. Interestingly, most reports dealing with RyR2 regulation in HF point to "hyperphosphorylation" of the channel as the basis of aberrant function. Phosphorylation of S2814 by the Ca^{2+}/calmodulin-dependent kinase (CaMKII) is generally considered the critical post-translational modification, while there has been disagreement with the involvement of S2808 phosphorylation (figure 2B) [11]. In any case, RyR2 dysfunction in HF is likely a consequence rather than the primary cause of the syndrome.

In 2001, Priori et al. reported the first RyR2 mutations in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) [17], a condition involving cardiac arrhythmia induced by stress in patients with structurally normal hearts [18]. This observation, together with subsequent studies that showed increased susceptibility to arrhythmia in mice harboring one of those mutations [19], demonstrated that RyR2 dysfunction may be the *primary* cause of heart disease. Nearly 20 years later, the number of mutations associated with CPVT exceeds 200 (figure 1C) and there is enough evidence in the literature to assert beyond doubt that *some* RyR2 mutations indeed produce CPVT. Several hypotheses have been proposed to explain CPVT in the context of RyR2 dysfunction (reviewed in [20]), but there is a common cellular feature connecting all: untimely release of Ca^{2+} mediated by mutant channels produces activation of NCX, which depolarizes the sarcolemma and induces extemporaneous action potentials and arrhythmia. Interestingly, the same mechanism seems to apply to both gain-of-function and loss-of-function RyR2 mutations, the difference being the timing of spontaneous Ca^{2+} release. Gain-of-function mutations generate diastolic Ca^{2+} waves and trigger *delayed* afterdepolarizations [21], while loss-of-function mutations promote Ca^{2+} release during an action potential and trigger *early* afterdepolarizations [22].

Clinical Significance of RyR2 Mutations

RyR2 mutations associated with CPVT cluster mainly in four domains of the channel (figure 1C). Together, these four domains contain around 93% of the mutations; hence, there seems to be a correlation between the region affected by a mutation and its potential pathogenicity. Remarkably, three of those domains form the central column of the protein (NTD, CD and PFD). HD1, on the other hand, is one of the peripheral domains but is touted as a region of inter-domain interactions and putative anchoring point for accessory proteins.

As the number of CPVT-associated mutations continues to grow, so does the spectrum of phenotypes attributed to primary RyR2 dysfunction. There are reports in the literature of RyR2 mutations possibly associated with long-QT syndrome, Brugada syndrome, arrhythmogenic right ventricular cardiomyopathy (ARVC), and even colorectal cancer and intellectual disability [23]. This information is stimulating for basic science researchers because of the great potential to unveil new pathogenic mechanisms, but it deserves careful consideration. A recent study reported that 7.5% of individuals in a whole-exome sequencing (WES) cohort carry variants in *RYR2*, the gene encoding for RyR2, but only 1.2% of those variants were likely pathogenic based on clinical information. This suggests that RyR2 is highly tolerant to variability [24]. Indeed, a larger cohort of WES and whole-genome sequencing information reports 1422 RyR2 residues susceptible to 1751 different amino acid changes [25]. Since these variants are distributed randomly throughout the protein (figure 1D), it further suggests that variation outside the canonical mutation "hot-spots" of CPVT is largely innocuous. Remarkably, the 31 specific mutations summarized in table 1 appear in both clinical reports and cohorts of healthy patients. Then, what is the clinical significance of novel RyR2 mutations? If a patient carries any given RyR2 mutation and shows an abnormal phenotype, should this be labeled as *the* disease-causing mutation?

This issue was addressed in a recent commentary, where it was argued that if the clinical diagnosis is inconclusive, any rare mutation identified in a patient is likely a false-positive — i.e. a variant of unknown significance (VUS) [26]. Indeed, new RyR2 mutations require careful analysis because even with a robust phenotype, a given variant could be merely coincidental. Classifying a mutation as pathogenic therefore requires a multi-level approach, where biophysical, biochemical and physiological studies support a robust clinical case; but it is impractical, and nearly impossible to apply these standards to all mutations. Hence, a strong clinical case is paramount to identify candidate mutations for in-depth molecular studies. Unfortunately, many clinical reports in the literature lack critical information, as summarized in table 1. In some cases, the original description did not provide details of the patients' phenotype, considered a mutation as "pathogenic" even when the patient carried variants in other relevant genes or provided a diagnosis without the appropriate tests. For other mutations, this issue is compounded by their high incidence in large cohorts. Such is the case of T1107M, reported in cases of CPVT but with higher prevalence than CPVT itself (~4 in 10,000 alleles) [20]. Therefore, this mutation is unlikely disease-causing, and other epigenetic or environmental factors probably synergized to produce a disease phenotype. This residue is also subject to inter-species variation, and the functional and structural characterization was performed using A1107M, the mouse analog [9,27].

Ultimately, the large variability in RyR2 should not discourage the study of rare mutations, even from patients with phenotypes other than CPVT. T1107M, for example, raised considerable interest in the field because it was the first RyR2 mutation associated with hypertrophic cardiomyopathy (HCM), a disorder that, unlike CPVT, involves severe structural remodeling of the heart. RyR2 is mainly a Ca^{2+} channel, and this ion modulates at least two signaling pathways involved in cardiac hypertrophy; consequently, it is easy to hypothesize a pathway linking primary RyR2 dysfunction and cardiac structural remodeling. Later identification of T1107M in patients with CPVT and its high prevalence in the general

population detached from an abnormal phenotype show the elusiveness of such link. As additional novel mutations are identified, one that finally allows to fill these gaps will most likely be identified. Furthermore, novel mechanisms may be unveiled from the study of other mutations associated with syndromes hitherto unrelated to intracellular Ca^{2+} handling, such as long-QT syndrome.

Perspectives

RyR2 is a remarkable protein with an intricate structure and subject to a myriad of regulatory mechanisms. A single mutation in RyR2 can destabilize the entire e-c coupling apparatus producing severe disease, and yet, thousands of mutations appear in the general population without apparent damage. While these seemingly harmless variants are often regarded as non-clinically relevant, an intriguing hypothesis is that they may act as genetic modifiers, affecting the severity of heart disease caused by other etiologies [28]. Additionally, at least 29 variants found in healthy individuals produce premature termination of protein synthesis (figure 1D), and comparable genetic mutations in mice [29] and rabbits [30] generate a ~50% decrease in RyR2 expression without an obvious abnormal phenotype. Hence, even more astonishing than RyR complexity is the capacity of the organism to compensate for such drastic physiological changes. Researchers working with animal models understand that in some cases it is more revealing to study the adaptation to a genetic manipulation than the genetic manipulation itself. It is clear, though, that the study of RyR2 and its sister isoforms in sickness and in health will continue fueling exciting research for years to come.

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Table 1. RyR2 Mutations Reported in both Clinical Cases and Large-Scale Sequencing Cohorts.

Substitution	Allelic Frequency	Clinical Presentation	Pathogenic Potential[A]		Reference
			Frequency	Clinical	
L62F	$4.06x10^{-6}$	CPVT	++	+	[31]
L73V	$4.06x10^{-6}$	SCD[B]	++	-	[32]
V186M	$2.44x10^{-5}$	CPVT	+	+	[31]
H240R	$1.08x10^{-5}$	CPVT	+	+	[31]
S406L	$2.17x10^{-5}$	CPVT	+	+	[33]
R414C	$8.13x10^{-6}$	SCD	++	+	[34]
P466A[C]	$8.68x10^{-5}$	ACA	+	+	[35]
L555V	$4.07x10^{-6}$	diLTQS[B]	++	-	[36]
R739H	$4.08x10^{-6}$	CPVT	++	+	[31]
R1013Q[C]	$4.76x10^{-4}$	CPVT	-	+	[31]
T1107M	$4.26x10^{-4}$	CPVT?, HCM	-	+	[31,37]
A1136V	$7.06x10^{-3}$	CPVT[B]	-	-	[38]
T1223A	$4.07x10^{-6}$	Syncope	++	-	[39]
P1256T[C]	$1.63x10^{-5}$	Syncope	+	-	[39]
N1551S	$2.75x10^{-4}$	CPVT	+	+	[40]
S1765C[C]	$1.73x10^{-4}$	?	+	-	[41]
V1810L	$8.14x10^{-5}$	CPVT[B]	+	-	[42]
E1837K	$2.04x10^{-5}$	CPVT	+	+	[31]
I2075T	$8.15x10^{-6}$	iVF[B]	++	-	[43]
V2113M	$3.97x10^{-4}$	CPVT	+	+	[31]
R2267H	$2.85x10^{-5}$	SID	++	+	[44]
R2359Q	$3.25x10^{-5}$	CPVT?[B]	+	-	[45]
Y2392C	$4.07x10^{-6}$	CPVT, SCD	++	+	[46]
A2439T	$1.62x10^{-5}$	?	+	-	[41]
A2498V[C]	$4.82x10^{-5}$	CPVT?	+	-	[47]
K4392R	$1.64x10^{-5}$	ACA, CPVT	+	+	[48]
G4471R	$3.23x10^{-5}$	Family History of SCD	+	-	[49]
H4552R	$1.63x10^{-5}$	SUD?	+	-	[50]
A4556T	$3.98x10^{-5}$	?	+	-	[35]
R4790Q	$4.06\ x10^{-6}$	CPVT	++	+	[31]

ACA: aborted cardiac arrest; CPVT: catecholaminergic polymorphic ventricular tachycardia; diLQTS: drug-induced long QT syndrome; iVF: idiopathic ventricular fibrillation; SCD: sudden cardiac death; SID: sudden infant death; SUD: sudden unexplained death. ?: Report does not give specific details of the patient.

A: Pathogenic potential based on variant frequency (arbitrarily defined as < $1x10^{-5}$: ++ and < $4x10^{-4}$: +) and the clinical report. -: indicates inconclusive diagnosis or insufficient clinical information.

B: Mutation identified with other variants in RyR2 or other genes such as *PKP2*, *MYBPC3* and *MYH7*.

C: More variants reported for this residue. Only the one indicated appears in HGMD.

Table compiled from the literature and the following databases: variants and allele frequency from the Genome Association Database (gnomAD) [25]; disease-associated mutations from the Human Gene Mutation Database (HGMD) [23].

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