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Comprehensive Open Reading Frame Mutational Analysis of the *RYR2*-Encoded Ryanodine Receptor/Calcium Channel in Patients Diagnosed Previously with Either Catecholaminergic Polymorphic Ventricular Tachycardia or Genotype Negative, Exercise-Induced Long QT Syndrome

Argelia Medeiros-Domingo, MD, PhD[*,1], **Zahurul A. Bhuiyan, MD, PhD**[*,2], **David J. Tester, BS**[1], **Nynke Hofman, MSc**[2], **Hennie Bikker, PhD**[2], **J Peter van Tintelen, MD, PhD**[3], **Marcel M.A.M Mannens, PhD**[2], **Arthur A.M. Wilde, MD, PhD**[2,4], and **Michael J. Ackerman, MD, PhD**[1,5,6]

[1]Department of Molecular Pharmacology & Experimental Therapeutics, Mayo Clinic, Rochester MN USA [2]Department of Clinical Genetics, Academic Medical Center, University of Amsterdam, Netherlands [3]Department of Genetics, University Medical Center Groningen, University of Groningen, the Netherlands [4]Department of Cardiology and Heart Failure Research Centre, Academic Medical Center, University of Amsterdam, Netherlands [5]Department of Medicine/Division of Cardiovascular Diseases, Mayo Clinic, Rochester MN USA [6]Department of Pediatrics/Division of Pediatric Cardiology, Mayo Clinic, Rochester MN USA

Abstract

Objective—To determine the spectrum and prevalence of mutations in the *RYR2*-encoded the cardiac ryanodine receptor in cases with exertional syncope and normal QTc.

Background—Mutations in the *RYR2* cause type 1 catecholaminergic polymorphic ventricular tachycardia (CPVT1), a cardiac channelopathy with increased propensity for lethal ventricular dysrhythmias. Most *RYR2* mutational analyses target 3 canonical domains encoded by < 40% of the translated exons. The extent of CPVT1-associated mutations localizing outside of these domains remains unknown as *RYR2* has not been examined comprehensively in most patient cohorts.

Methods—Mutational analysis of all *RYR2* exons was performed using PCR, DHPLC, and DNA sequencing on 155 unrelated patients (49% females, 96% white, age at diagnosis 20 ± 15 years, mean

Reprints and correspondence: Michael J. Ackerman, MD, PhD, Director, Long QT Syndrome Clinic and the Mayo Clinic Windland Smith Rice Sudden Death, Genomics Laboratory, Mayo Clinic, Guggenheim 501, 200 First Street SW, Rochester, MN 55905, USA, Fax: 507.284.3757 Phone: 507.284.0101, ackerman.michael@mayo.edu .
*AMD and ZAB are co-equal first authors

DISCLOSURES

Dr. Ackerman is a consultant for PGxHealth and chairs their FAMILION Medical/Scientific Advisory Board (approved by Mayo Clinic's Medical-Industry Relations Office and Conflict of Interests Review Board). In addition, a license agreement pertaining to "mutations in the ryanodine receptor 2 gene and heart disease", resulting in consideration and royalty payments, was established between PGxHealth and Mayo Clinic Health Solutions in 2007.

QTc 428 ± 29 ms), with either clinical diagnosis of CPVT (n = 110) or an initial diagnosis of exercise-induced long QT syndrome (LQTS) but with QTc < 480 ms and a subsequent negative LQTS genetic test (n = 45).

Results—Sixty-three (34 novel) possible CPVT1-associated mutations, absent in 400 reference alleles, were detected in 73 unrelated patients (47%). Thirteen new mutation-containing exons were identified. Two thirds of the CPVT1-positive patients had mutations that localized to one of 16 exons.

Conclusions—Possible CPVT1 mutations in *RYR2* were identified in nearly half of this cohort. 45 of the 105 translated exons are now known to host possible mutations. Considering that ~65% of CPVT1-positive cases would be discovered by selective analysis of 16 exons, a tiered targeting strategy for CPVT genetic testing should be considered.

Keywords

Ryanodine Receptor; Catecholaminergic Polymorphic Ventricular Tachycardia; Sudden Cardiac Death; Exertional Syncope

INTRODUCTION

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a potentially lethal, heritable arrhythmia syndrome often manifesting as exercise-induced ventricular arrhythmias, syncope or sudden death.[1] With mortality rates of 30-50% by age 35 years, CPVT is one of the most malignant cardiac channelopathies expressed predominately in young patients with otherwise structurally normal hearts[2]. While the resting 12-lead electrocardiogram (ECG) is typically normal, the hallmark arrhythmia, bidirectional VT, is often present during exercise stress testing and has been considered pathognomonic for CPVT.[1,3]

CPVT stems from an alteration of intracellular calcium handling involving the critical calcium-induced calcium release mechanism of myocardial cells. At the molecular level, gain of function mutations in the cardiac ryanodine receptor encoded by *RYR2* account for at least 50% of CPVT cases and is annotated as type 1 CPVT (CPVT1).[3] Mutations in *CASQ2*-encoded calsequestrin are responsible for the very rare, autosomal recessive form known as type 2 CPVT (CPVT2).[2,4]

The cardiac ryanodine receptor (RyR2), encoded by the 105-exon-containing *RYR2* gene, is one of the largest ion channel proteins comprised of 4967 amino acids; localizes to the sarcoplasmic reticulum, and controls intracellular calcium release and cardiac contraction. Since the sentinel discovery of a CPVT-causing *RYR2* mutation[5], a cluster distribution involving three discrete protein regions has been reported. Based in a potential physiological role for these "hot-spots", these regions have been termed "domains" I, II and III (Figure 1)[6, 7]. Similar mutation clustering is observed in the *RYR1* gene which encodes the skeletal muscle RyR1 and is linked to malignant hyperthermia and central core disease[8-10]. However, since the majority of CPVT cases have not undergone the entire *RYR2* scan, the prevalence of mutations residing outside these three canonical domains (i.e. ~61 exons that encode for 2570 amino-acids) remains unknown.

Currently, among research laboratories and clinical diagnostic laboratories, there is no consensus or clear definition of the "*RYR2* targeted scan" resulting in enormous discrepancy in the number of exons studied by each research group or commercial company. This situation has an important impact in "gene-negative" definition, genotype-phenotype correlation and patient quality of care. In the present study, we sought to determine the prevalence of mutations throughout *RYR2's* entire open reading frame in a large cohort of unrelated cases referred to 2 different institutions for exertional syncope and, using a combined analysis of the previous

reported mutations and the novel mutations found in this cohort, we propose a novel, targeted "genetic approach" for CPVT1 genetic testing.

METHODS

Study Participants

We studied a cohort of 155 unrelated patients referred to either the Windland Smith Rice Sudden Death Genomics Laboratory at Mayo Clinic, Rochester, MN or the Department of Clinical Genetics, Academic Medical Center, University of Amsterdam, Netherlands for genetic testing between August 2001 and June 2008. A clinical diagnosis of CPVT was rendered in 110 patients by either one of the authors (MJA, AAMW) or the referring physician. Of these, 78 were classified as "strong CPVT phenotype" because of exertional syncope plus documentation of bidirectional or polymorphic ventricular tachycardia (BVT/PVT) while 32 were classified as "possible CPVT phenotype" based on the presence of exertional syncope and stress test induced ventricular ectopy but not BVT/PVT. In addition, 45 cases were referred as "possible/atypical long QT syndrome (LQTS)" because of exertional syncope and QTc values < 480 ms. All were genotype negative for the 12 known LQTS-susceptibility genes.

Following receipt of written consent for this Mayo Foundation Institutional Review Board and Amsterdam Academic Medical Center Medical Ethical Committee approved protocol, genomic DNA was extracted from peripheral blood lymphocytes using the Purgene DNA extraction kit (Gentra, Inc, Minneapolis, MN, USA). In cases with suspected mosaicism, additional DNA from saliva was isolated using the ORAgene kit (DNA Genotek, Ottawa, Ontario, Canada) and DNA from skin fibroblasts and hair-roots was isolated using the QIAamp DNA minikit (Qiagen, USA).

Mutational Analysis

Comprehensive open reading frame/splice site mutational analysis of all 105 *RYR2* exons was performed using polymerase chain reaction (PCR), denaturing high performance liquid chromatography (DHPLC), and DNA sequencing as described previously.[11] The flanking primers used for PCR were published previously or designed with Oligo software (Molecular Biology Insights, Inc., Cascade Colo.) and are available on request. We also searched for large genomic rearrangements affecting exon 3 as reported previously[12].

All putative pathogenic variants must have been absent in 400 reference alleles (100 healthy white and 100 healthy black) obtained from the Human Genetic Cell Repository sponsored by the National Institute of General Medical Sciences and the Coriell Institute for Medical Research (Camden, New Jersey) in order to be considered as potentially disease-related.

Statistical Analysis

We used the JMP Statistical Software (JMP 6.0, 2005; SAS Institute Inc, Cary, NC). All continuous variables are reported as mean ± SD. Differences between continuous variables were evaluated using unpaired Student *t* tests, and nominal variables were analyzed using chi-square analysis. Statistical significance was considered at $p < 0.05$.

RESULTS

The demographic characteristics of the 155 unrelated patients are shown in Table 1. 96% were Caucasians, 49% were females, age at symptoms was 20 ± 15 yrs, and average QTc was 428 ± 29 ms. The mean age of onset of symptoms was significantly lower in *RYR2* mutation positive subjects compared to those with a negative genetic test (16.7 ± 12.3 vs 23.8 ± 16.6 yrs respectively, $p<0.004$).

Overall, 77 (63 unique, 34 novel) putative disease causing mutations were identified in 73 cases (47%, Table 2, Figure 2). 41/73 mutation positive cases (56%) were females. Putative mutations were absent in 400 references alleles and most of the mutated residues exhibit highly conservation across species (Supplemental Table). The yield of the genetic test was significantly higher among the 78 cases classified clinically as "strong CPVT phenotype" compared to the 32 cases diagnosed as "possible CPVT phenotype" (60% vs 37.5%, $p < 0.04$). Notably, nearly one-third of the 45 "gene negative LQTS" cases had a rare missense mutation in *RYR2* (Table 1, Figure 3). Four out of the 73 *RYR2* mutation-positive cases hosted multiple mutations (5.5%). As expected, we observed a mutation clustering distribution across *RYR2*; nevertheless, ten mutations found in 11 cases resided outside the three canonical domains, specifically, between domain I and II; 8 of them exhibited a strong CPVT phenotype. Three large genomic rearrangements comprising exon 3 were detected in three unrelated cases involving a 3.6 kb deletion in one and a 1.1 kb deletion in two cases.

One proband had a maternally inherited Y4149S (tyrosine, Y, at position 4149 mutated to serine, S) missense mutation. Although the proband's mother was asymptomatic and had an unremarkable exercise ECG; germline mosaicism was suspected clinically because more than one offspring was affected. Accordingly, Y4149S mosaicism was detected in her being highest in the hair-roots (~25%), less in leucocytes (~20%) and in fibroblasts and buccal epithelium (~15-18%).

Twelve non-synonymous single nucleotide polymorphisms (6 novel) were also identified, 7 of them were seen only in controls and 5 in cases and controls (Table 2). Four novel polymorphisms localize between domain I and II. The most common polymorphism was Q2958R with an heterozygous prevalence of 34% in Caucasians and 10% in African-Americans; followed by G1886S with a prevalence of 20% (African Americans) and 9% (Caucasians). V377M was found only in African-Americans with a prevalence of 3%. Finally, Y2156C, E2183V, M2389L, V4010M, A4282V and G4315E are rare variants observed only once in different control subjects. Thus, within the exons hosting putative CPVT1-associated mutations, the background prevalence of rare amino acid substitutions among the 200 apparently healthy volunteers was 3% (3/100 Caucasians and 3/100 African Americans, Table 2).

We evaluated the number of mutations in each exon reported to date in the literature (Table 2), excluding exons containing only polymorphisms. As such; 127 unique mutations were analyzed, including those found within this cohort. Sixteen exons hosted > 3 distinct CPVT1-associated mutations; 13 exons had at least 2 mutations reported while an additional 16 exons had, so far, only a single mutation reported (Figure 4). This mutation clustering phenomena might facilitate a tiered strategy that may yield a more cost-effective approach for CPVT genetic testing. If we consider that the average charge for the current *RYR2* commercial tests available on the market is approximately $0.40 per coding nucleotide (http://pgxhealth.com, www.preventiongenetics.com), the estimated charge for the entire *RYR2* coding region scan would be approximately $6000 per patient, meaning that the commercial charge to analyze this 155 patient cohort in its entirety would have approached $1 million US dollars. In comparison, the total charge to scan only the 45 mutation-hosting exons that have been reported to date exon-containing mutations reported to date would be about 50% less. Further, a reflex tiered strategy would reduce the cost significantly. As modeled here, using a 3-tiered reflex genetic test strategy based on Figure 4, the genetic scan of the first tier of exons in our cohort would cost $190,960.00 (~$1200 per case) and would detect nearly two-thirds of those CPVT cases that are due to mutations in *RYR2*. The charge to reflex to the second tier genetic scan would add < $1000 per case and combined, nearly 90% of the RYR2-mutation positive cases (CPVT1) would be identified. Reflexing to the third tier would capture the remaining *RYR2*-positive cases and the charge to do so would be ~$123,225 US dlls ($795.00 US dlls/case, Figure 5).

DISCUSSION

Exertional Syncope: LQTS or CPVT?

It has been reported that nearly 30% of CPVT cases have been misdiagnosed as "LQTS with normal QT intervals" or "concealed LQTS".[13] Recently, we demonstrated that nearly 6% of 269 LQTS genotype negative patients hosted a putative CPVT1-causing RyR2 mutation[14]. Here, we included only referral cases of "atypical/possible LQTS" with a phenotype of exertional syncope and QTc < 480 ms. Herein, the yield of *RYR2* mutations for these 45 cases was 31%; indicating the critical importance of properly distinguishing between CPVT and LQTS. CPVT-related arrhythmias can be easily reproduced during an exercise stress test, isoproterenol infusion or by other forms of adrenergic stimulation[15,16]. The induction of polymorphic ventricular tachycardia or bidirectional VT, characterized by 180° alternating QRS axis on a beat-to-beat basis, sets CPVT apart from "concealed" or "borderline" LQTS.

RYR2 genetic approach: Targeted scan and tiered strategy

Our results confirm that mutation clustering exists. The functional significance of mutation clustering remains unclear. It has been suggested, however, that a domain-domain interaction is crucial for channel function[17-19] and a defective inter-molecular interaction may be crucial in disease phenotypes. Interestingly, in this study 11/64 (17%) of the putative mutations localize outside the considered canonical domains.

Based upon our results and after analyzing a large publicly available compendium of the 127 *RYR2* putative mutations known to date (Table 2), we propose an expanded genetic approach for research/investigational laboratories. A reasonable *RYR2* scan will include the analysis of at least 45 exons in total known to host all published mutations reported to date. Since some exons ([19]) imbibed in the hot-spot region remain free of mutations so far, a more ambitious and "comprehensive" *RYR2* genetic test would include these exons as well resulting in a 64-exon scan (exons 3-28, 37-50, 75 and 83-105).

The mutation clustering phenomena might facilitate a tiered strategy that may yield a more cost-effective approach for CPVT genetic testing. Figure 4 summarizes this proposed tiered strategy. The approach was developed considering the number of mutations in each exon reported to date in the literature. The first tier comprises those exons (N=16) now known to host > 3 unique CPVT-associated mutations. The second tier includes 13 exons with at least 2 mutations reported while the third tier consists of the final 16 exons where, so far, only a single mutation within that exon has been reported. Considering that ~65% of the *RYR2* mutation-positive cases might have a mutation in the first tier of 16 *RYR2* exons, the charge of the genetic analysis in this group could be reduced by approximately half (predicted $1232.00 US dlls/case for the first tier of 16 exons vs $3019.00 US dlls/case for the entire sequencing of exons-containing reported mutation).

In case of negative results, we suggest that the pseudo-comprehensive (64 exon) *RYR2* scan mentioned previously (exons 3-28, 37-50, 75 and 83-105) be performed. Additional "rare" although documented causes of CPVT should also be considered, like large *RYR2* genomic rearrangements involving exon 3 and mutations in calsequestrin 2 (*CASQ2*) and Kir2.1 (*KCNJ2*)[20]. The area surrounding exon 3 is highly susceptible to large *Alu*-repeat-mediated genomic rearrangements; we documented 3 unrelated cases hosting large heterozygous deletions involving exon 3 that could not be detected by regular genetic screening using DHPLC or direct DNA sequencing. Validating this observation, exon 3 deletion was also reported recently in a different cohort where 2 unrelated cases (out of 33), hosted a 1.1kb deletion, including exon 3[21].

Polymorphisms in RYR2, not that rare and with potential functional effect

It has been considered that *RYR2* is not a polymorphic gene. However, 15/142 (10.5%) missense variants reported to date were found in controls. We did not scan the entire *RYR2* gene in control subjects. Instead, since we focused on the exon-containing mutations, the rate of non-synonymous genetic variation throughout all of *RYR2* may be higher. Importantly however, among the exons now known to host possible CPVT1-associated missense mutations, similarly rare amino acid substitutions were found in only 6 of the 200 control subjects examined in this study. Although not a true case-control genetic epidemiologic study, if validated, this would suggest that among cases where CPVT is strongly suspected, there would be a 95% estimated probability that the identification of a rare missense mutation would likely represent the pathogenic basis for the patient's CPVT rather than merely being only a rare amino acid substitution.

We have learned that common polymorphisms in other ion channels have the potential to modify the clinical phenotype[22,23]; polymorphisms in *RYR2* may have the same potential. RyR2-Q2958R is the most common *RYR2* polymorphism; was described for the first time 9 years ago[24] and is particularly common in Caucasians (34%). The second most common polymorphism in *RYR2* is G1886S (20% African Americans, 9% Caucasians) followed by G1885E (6% Caucasians). Interestingly, in vitro studies in heterologous systems have demonstrated that both G1885E and G1886S polymorphisms caused a significant increase in the cellular Ca(2+) oscillation activity compared with RyR2 wild-type channels. Further, when both polymorphisms were introduced in the same RyR2 subunit, the store-overload-induced calcium release activity was nearly completely abolished[25]. The clinical consequences of this "RyR2 loss of function" in vitro phenotype is not clear, however, compound heterozygosity involving these two polymorphisms has been reported in right ventricular dysplasia[26]. The potential functional effects of the 6 novel polymorphisms identified in this study are unknown.

It is important to remark that none of the novel mutations detected on this study have been functionally characterized to further bolster the contention of pathogenicity. However, less than 15% of the mutations reported to date in *RYR2* have been studied in vitro, pathogenicity has been suspected based on co-segregation with the disease and absence in control subjects. Here, co-segregation with the disease data was not available for all cases. Instead, the prevalence of putative mutations amongst strong cases (~60%) was markedly higher than in controls (~3%) and all putative mutations were absent in 400 reference alleles. Thus, although the precise contribution of each discrete mutation to the phenotype remains to be determined, statistically, the estimated probability for pathogenicity for *RYR2* mutations found in strong cases is quite high (~95%).

Mosaicism in RYR2

This is the first report involving *RYR2* mosaicism which was transmitted to descendants, presumably causing sudden death in two children and full blown CPVT in one child from the age of 9 years. *RYR2* mutations, in many circumstances (~20% in our cohort) are *de novo* in origin, but it could also be present in a mosaic form in the asymptomatic parents, which requires attention during genetic counseling as well as during genetic screening.

Clinical Significance

This study represents the first analysis of *RYR2* mutation distribution in a large cohort of cases. Our results contribute to a better delineation of the "hot spot" region with important consequences in "gene negative" definition. The identification of novel common variants in control subjects will allow a better interpretation of the CPVT genetic test and the detection of *RYR2* mosaicism and confirmation of exon 3 deletion in different patients-cohort, provide novel genetic possibilities in the pathogenesis of CPVT. Moreover, the possibility of a tiered

strategy for *RYR2* genetic scan may enable a more cost-effective genetic approach to analyzing one of the largest genes in the human genome. Finally, we emphasize the critical importance of properly distinguishing between CPVT and LQTS (including Andersen-Tawil syndrome), two different diseases with a similar clinical presentation but different clinical outcomes and different responsiveness to pharmacotherapy.

CONCLUSION

Although intimidating as one of the largest genes in the human genome, results from this comprehensive open reading frame analysis involving one of the largest cohorts of unrelated patients examined, combined with a detailed analysis of all published CPVT1-associated mutations indicate that to date, only 45 of *RYR2's* 105 translated exons host a putative CPVT1-associated mutation thus far. Moreover, an initial targeting of only 16 exons would allow the identification of putative mutations in ~65% of the *RYR2*-mutation positive cases, though compound heterozygosity may be missed. Finally, given the present estimate of 3% frequency for rare missense mutations among controls, one must be cognizant of the possibility of a "false positive" especially as the pre-test probability of a CPVT diagnosis decreases. The ~33% yield that was observed among the "possible" cases of CPVT indicates that perhaps 90% of the mutations, identified among cases labeled as "possible CPVT" or so-called "atypical LQTS" with exercise-induced syncope and QTc < 480 ms, are pathogenic whereas 10% of those mutations may represent "false positives".

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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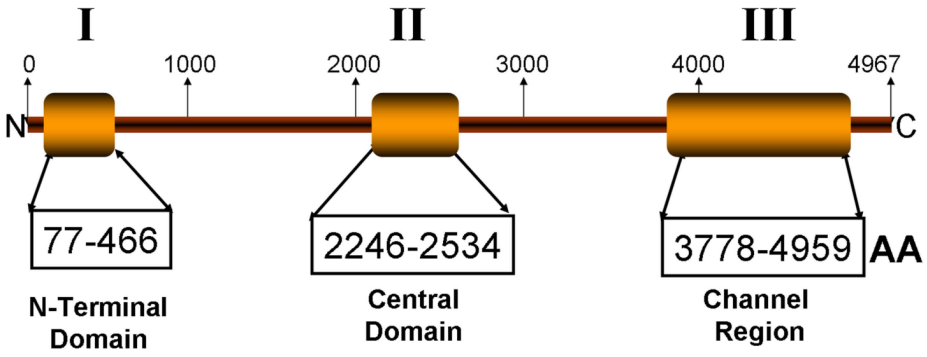

Figure 1. Mutation clustering in the cardiac ryanodine receptor (*RyR2*)

Mutations are distributed in three "hot-spots" regions, called domains I (N-terminal), II (central) and III (channel region)[6,7]. AA: amino-acid number estimated for each domain. Adapted from George CH, et.al[7]., and Yano M, et. al[6].

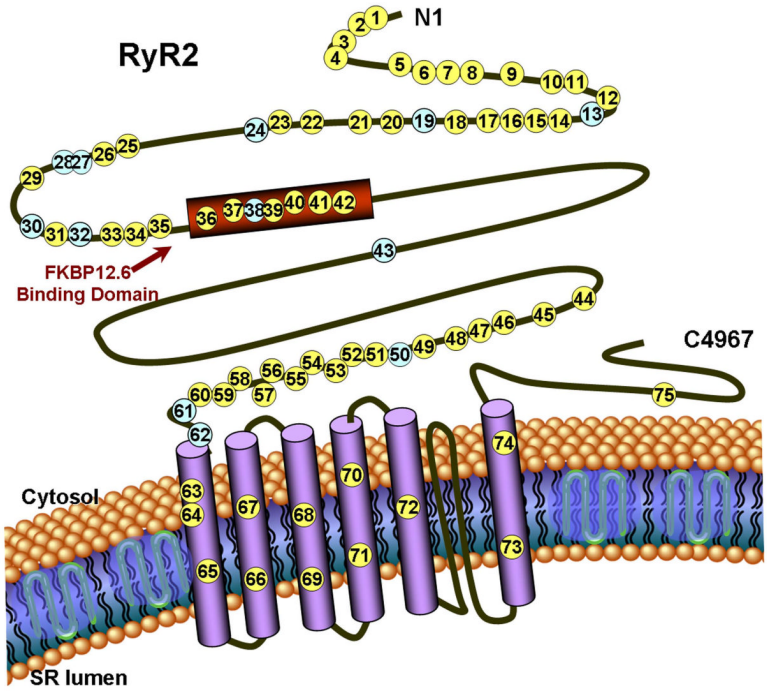

Figure 2. RyR2 channel topology and localization of mutations and polymorphisms

Linear topology of the cardiac ryanodine receptor (RyR2); putative pathogenic mutations (yellow circles) and polymorphisms (blue circles) found on this study-cohort are shown in the approximate location. The number within the circle corresponds to the mutation # on Table 1.

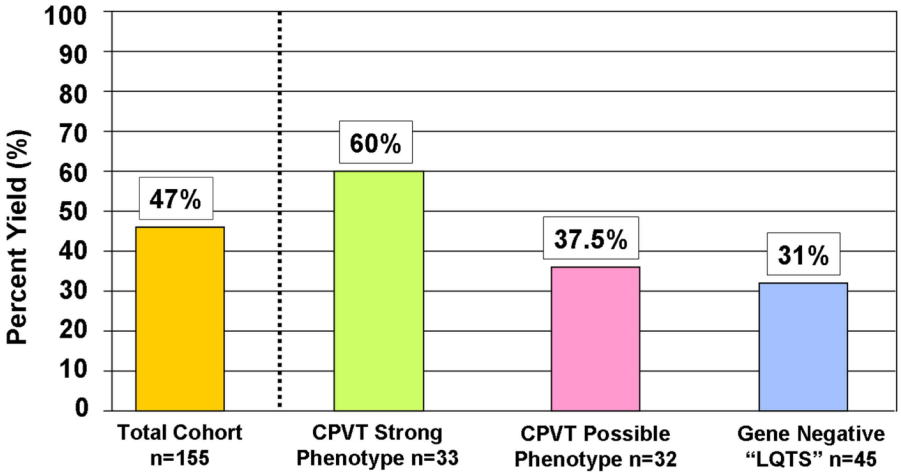

Figure 3. Prevalence of *RYR2* mutations by subgroups

The yield from the entire *RYR2* scan on this cohort is shown on the left. Bars on the right side show the sensitivity in the 3 different subgroups of this cohort.

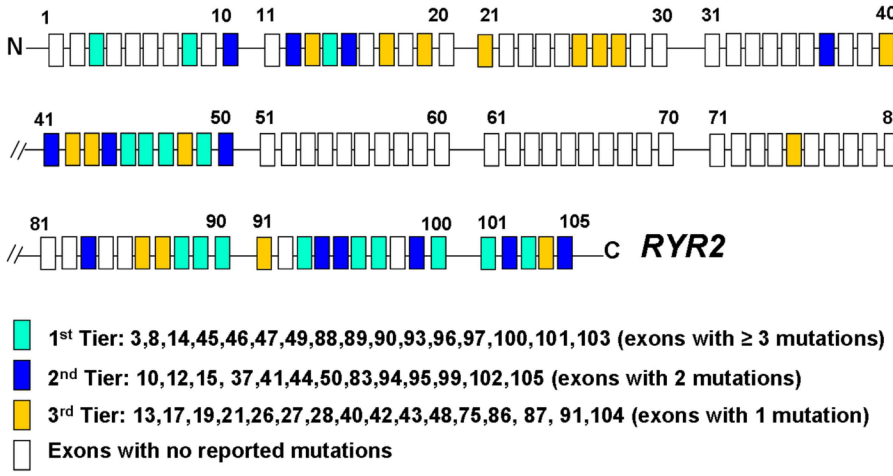

Figure 4. Possible tiered strategy for reflex genetic testing

Schematic representation of the 105 coding exons of the *RYR2* gene. Boxes in colors: all the exon-containing mutations reported to date. Boxes in white: exons free of reported mutations. The tiered strategy was built based on the number of mutations containing in each exon as shown by three different colors. The 1st tier included 16 exons, 2nd tier 13 exons and 3rd tier 16 exons. Exons containing control variants were not included.

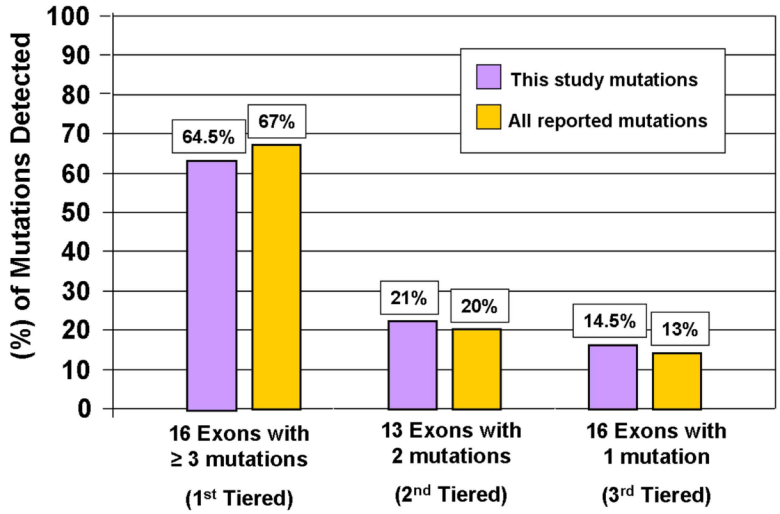

Figure 5. Yield from *RYR2* mutational analysis based on a tiered strategy

Retrospective analysis of the mutations detected in our cohort and in the world-wide compendium of mutations reported to date. The percentage of mutations that would be detected using the tired strategy is shown.

Table 1
Demographics Characteristics of the Cohort

	CPVT Strong Phenotype	CPVT Possible Phenotype	Gene Negative LQTS	Total
No. of Patients	78	32	45	155
Age (yrs) mean ± SD	20 ± 15	20 ± 16	22 ± 14	20 ± 15
QTc (ms) mean ± SD	415 ± 26	434 ± 30	434 ± 27	427 ± 29
%Female	47	44	57	49
RYR2 Positives (%)	n=47 (60.2%)	n=12 (37.5%)	n=14 (31.1%)	n=73 (47.1%)

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Table 2

Compendium of RYR2 mutations and polymorphisms reported to date

Putative mutations are indicated in red, n=129 (including 2 large genomic rearrangements involving exon 3, not detectable by regular genetic scan), polymorphisms in blue n=15.

No.	Mutation Number (Figure 1)	Exon	Base Position	Amino-acid Change	Location	Cases hosting the variant (n=108)	AA hosting the variant (n=100)	CC hostingthe variant (n=100)	Reference
1	1	3	1.1kb deletion*	Exon 3 del	NT	2			Bhuiyan[12]
2	2	3	3.6kb deletion*	Exon 3 del	NT	1			**Novel**
3	3	3	184 C>T	L62F	NT	1			**Novel**
4		3	230 C>T	A77V	NT				d'Amati G[27]
5	4	3	241 A>C	M81L	NT	1			**Novel**
6	5	8	493 C>T	P164S	NT	1			Choi[28]
7		8	506 G>A	R169Q	NT				Hsueh[29]
8	6	8	527 G>A	R176Q	NT	1			Tiso[24]
9	7	8	556 G>A	V186M	NT	1			Tiso[24]
10	8	8	567 A>T	E189D	NT	1			Davis[30]
11		10	6337 G>A	H240R	NT				Tester[31]
12	9	10	727 G>A	E243K	NT	1			**Novel**
13	10	12	985 T>C	F329L	NT	1			**Novel**
14	11	12	994C>T	R332W	NT	1			**Novel**
15	12	13	1072 G>A	G357S	NT	1			**Novel**
16	13	13	1129 G>A	V377M	NT		3		**Novel**
17		14	1240 C>T	R414C	NT				Tester[32]
18	14	14	1241 G>T	R414L	NT	1			Choi[28]
19	15	14	1244 C>G	T415R	NT	1			**Novel**
20		14	1255 A>T	I419F	NT				Choi[28]
21	16	14	1258 C>T	R420W	NT	3			Bauce[33]

No.	Mutation Number (Figure 1)	Exon	Base Position	Amino-acid Change	Location	Cases hosting the variant (n=108)	AA hosting the variant (n=100)	CC hostingthe variant (n=100)	Reference
22	17	14	1259 G>A	R420Q	NT	2			**Novel**
23		15	1298 T>C	L433P	NT				Tiso[24]
24	18	15	1396 C>G	P466A	NT	1			Tester[14]
25	19	16	1519 G>A	V507I	NT	5		4	**Novel**
26	20	17	1646 C>T	A549V	NT	1			**Novel**
27		19	Not Reported	S616L	CL				Marjmaa[21]
28	21	21	2216 G>A	R739H	CL	1			**Novel**
29	22	26	3038 G>A	R1013Q	CL	1			**Novel**
30		27	Not Reported	R1051P	CL				Marjmaa[21]
31	23	28	3320 C>T	T1107M	CL	1			**Novel**
32	24	28	3407 C>T	A1136V	CL	3		2	**Novel**
33	25	37	5170 G>A	E1724K	CL	2			Postma[34]
34	26	37	5509 G>A	E1837K	CL	1			**Novel**
35	27	37	5654 G>A	G1885E	CL	2	6		Milting[26]
36	28	37	5656 G>A	G1886S	CL	11	20	9	Milting[26]
37	29	40	6137 A>G	E2045G	CL	1			**Novel**
38		41	6337 G>A	V2113M	CL	1			Tester[31]
39		41	Not Reported	G2145R	CL				Marjmaa[35]
40	30	42	6467 A>G	Y2156C	CL			1	**Novel**
41	31	42	6504 C>G	H2168Q	CL	2			**Novel**
42	32	42	6548 A>T	E2183V	CL		1		**Novel**
43	33	43		D2216V	CL	1			**Novel**
44	34	44	6740 C>T	S2246L	CL	1			Priori[5]
45	35	44	6761 C>T	A2254V	CL	1			Postma[34]
46		45	6800 G>A	R2267H	CL				Tester[36]

No.	Mutation Number (Figure 1)	Exon	Base Position	Amino-acid Change	Location	Cases hosting the variant (n=108)	AA hosting the variant (n=100)	CC hostingthe variant (n=100)	Reference
47	36	45	6886 G>C	E2296Q	FKBP	1			**Novel**
48		45	6916 G>A	V2306I	FKBP				Laitinen[37]
49		45	6919 T>C	F2307L	FKBP				Berge[38]
50		46	6933 G>A	E2311D	FKBP				Priori[3]
51		46	6992 T>C	V2321M	FKBP				Nishio[39]
52		46	6982 C>T	P2328S	FKBP				Laitinen[37]
53		46	6992 T>C	F2331S	FKBP				Creighton[40]
54		46	7076 G>A	R2359Q	FKBP				Aizawa[41]
55		47	7157 A>T	N2386I	FKBP				Tiso[24]
56	37	47	7158 G>A	A2387T	FKBP	3			Tester[14]
57		47	7158 G>A	A2387P	FKBP				Bagattin[42]
58	38	47	7165 A>C	M2389L	FKBP		1		Tester[43]
59		47	7175 A>5	Y2392C	FKBP				Bauce[33]
60		47	7181 C>G	A2394G	FKBP				Postma[34]
61	39	47	7202 G>A	R2401H	FKBP	1			Aizawa[41]
62		47	7207 G>T	R2401L	FKBP				Creighton[40]
63	40	47	7207 G>A	A2403T	FKBP	1			Choi[28]
64	41	47	7210 C>A	R2404T	FKBP	1			Beckman[44]
65	42	48	7258 A>T	R2420W	FKBP	1			**Novel**
66		49	7422 G>C	P2474S	FKBP				Priori[3]
67		49	7423 G>T	V2475F	FKBP				Tester[32]
68		49	7511 C>T	T2504M	FKBP				Tiso[24]
69		49	Not Reported	L2487I	FKBP				Tester[43]

No.	Mutation Number (Figure 1)	Exon	Base Position	Amino-acid Change	Location	Cases hosting the variant (n=108)	AA hosting the variant (n=100)	CC hostingthe variant (n=100)	Reference
70		50	7528 T>C	T2510A	FKBP				Tester[31]
71		50	7599 C>G	L2534V	FKBP				Hasdemir[45]
72	43	61	8874 A>G	Q2958R	Cytosol	40	10	36	Tiso[24]
73		69	Not Reported	N3308S	Cytosol				Marjamaa[21]
74		75	Not Reported	R3570W	Cytosol				Marjamaa[35]
75		83	11332 C>T	L3778F	Cytosol				Priori[3]
76	44	83	11399 G>T	C3800F	Cytosol	1			Tester[14]
77		86	11636 T>C	L3879P	Cytosol				Tester[31]
78		87	11773 C>G	Q3925E	Cytosol				Tester[31]
79	45	88	11814 C>A	S3938R	Cytosol	1			Tester[14]
80		88	11836 G>A	G3946S	Cytosol				Priori[3]
81		88	Not Reported	G3946A	Cytosol				Davis[30]
82		88	11876 C>T	S3959L	Cytosol				Tester[31]
83	46	89	11916 G>T	M3972I	Cytosol	1			**Novel**
84	47	89	11917 G>C	D3973H	Cytosol	1			**Novel**
85	48	89	11921 T>A	L3974Q	Cytosol	1			**Novel**
86	49	90	11989 A>G	K3997E	Cytosol	1			**Novel**
87	50	90	12028 G>A	V4010M	Cytosol		1		Tester[43]
88		90	Not Reported	F4020L	Cytosol				Postma[34]
89	51	90	12226 A>G	E4076K	Cytosol	1			Postma[34]
90		90	12290 A>G	N4097S	Cytosol				Tester[46]
91		90	12311 A>T	N4104I	Cytosol				Postma[34]
92		90	12312 C>G	N4104K	Cytosol				Priori[3]
93		90	Not Reported	L4105F	Cytosol				Hasdemir[47]

No.	Mutation Number (Figure 1)	Exon	Base Position	Amino-acid Change	Location	Cases hosting the variant (n=108)	AA hosting the variant (n=100)	CC hostingthe variant (n=100)	Reference
94	52	90	12322 C>A	H4108N	Cytosol	1			Postma[34]
95		90	Not Reported	H4108Q	Cytosol				Postma[34]
96	53	90	12370 A>G	S4124G	Cytosol	1			**Novel**
97	54	90	12371 G>C	S4124T	Cytosol	1			Tester[14]
99		90	Not Reported	R4144C	Cytosol				Berge[38]
100		90	12436 G>A	E4146K	Cytosol				Tester[46]
101	55	90	12446 A>G	Y4149S[†]	Cytosol	1			**Novel**
102	56	90	12470 G>A	R4157Q	Cytosol	1			**Novel**
103		90	12472 A>C	T4158P	Cytosol				Tester[46]
104	57	90	12476 A>G	Q4159P	Cytosol	1			**Novel**
105	58	90	12533 A>G	N4178S	Cytosol	3			**Novel**
106	59	90	12559 G>C	E4187Q	Cytosol	1			**Novel**
107	60	90	12586 A>G	T4196A	Cytosol	1			Tester
108		90	12601 C>A	Q4201R	Cytosol				Laitinen[37]
109	61	90	12845 C>T	A4282V	Cytosol			1	Tester[43]
110		90	12919 C>T	R4307C	Cytosol				Callis[48]
111	62	90	12944 G>A	G4315E	Cytosol			1	**Novel**
112		91	13291 G>A	E4431K	Cytosol				Berge[38]
113		93	13489 C>T	R4497C	TMD				Priori[5]
114	63	93	13496 T>G	F4499C	TMD	1			Choi[28]
115		93	13512 G>A	M4504I	TMD				Bagattin[42]
116	64	93	13528 G>A	A4510T	TMD	2			Choi[28]
117		93	Not Reported	F4511L	TMD				Beckmann[44]
118	65	94	13666 G>A	A4556T	TMD	1			Tester[14]

No.	Mutation Number (Figure 1)	Exon	Base Position	Amino-acid Change	Location	Cases hosting the variant (n=108)	AA hosting the variant (n=100)	CC hostingthe variant (n=100)	Reference
119		94	13695 C>A	S4565R	TMD				Tester[36]
120		95	13819 G>C	A4607P	TMD				Bagattin[42]
121		95	13831 G>A	E4611K	TMD				Berge[38]
122		96	Not Reported	W4645R	TMD				Beery[49]
123	66	96	13948 A>G	K4650E	TMD	1			**Novel**
124		96	13957 A>G	V4653F	TMD				Laitinen[37]
125	67	97	13967-13972 Dup	4657-4658 ins EY	TMD	1			Tester[14]
126		97	Not Reported	G4662S	TMD				Postma[34]
127	68	97	14011 G>C	G4671R	TMD	1			Choi[28]
128	69	99	14205-14208 Del	N4736 Del	TMD	1			**Novel**
129		99	14285 A>C	H4762P	TMD				Postma[34]
130	70	100	14311 G>A	V4771I	TMD	2			Priori[3]
131	71	100	14369 G>A	R4790Q	TMD	1			**Novel**
132	72	100	14414 A>G	K4805R	TMD	1			**Novel**
133	73	101	14465 G>A	R4822H	TMD	1			**Novel**
134	74	101	14542 G>A	I4848V	TMD	2			Choi[28]
135		101	14552 T>G	F4851C	TMD				Aizawa[41]
136		101	14579 C>G	A4860G	TMD				Priori[3]
137		102	14601 T>G	I4867M	CT				Priori[3]
138		102	14639 T>C	V4880A	CT				Bagattin[42]
139		103	14683 A>G	N4895D	CT				Priori[3,5]
140		103	14705 C>T	P4902L	CT				Laitinen[37]
141		103	Not Reported	P4902S	CT				Postma[34]

No.	Mutation Number (Figure 1)	Exon	Base Position	Amino-acid Change	Location	Cases hosting the variant (n=108)	AA hosting the variant (n=100)	CC hostingthe variant (n=100)	Reference
142		104	14806 G>A	G4936R	CT				Tester[31]
143		105	14848 G>A	E4950K	CT				Priori[3]
144	75	105	14876 G>A	R4959Q	CT	2			Laitinen[37]

Predicted location: NT = Amino-Terminal, CL = Cytoplasmic Loop, FKBP = 12.6 (Calstabin) binding domain, TMD = Transmembrane domain, CT = C Terminal.

*Large genomic rearrangement comprising intron 2-3 and intron 2-4 resulted in inframe deletion of exon 3. AA: African Americans Controls, CC: Caucasians Controls.

†Mosaicism.