Clinical and genetic spectrum of hereditary cardiac arrhythmia syndromes

Bhuiyan, Z.A.

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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*1, Zahurul A. Bhuiyan*2, David J. Tes-ter1, Nynke Hofman2, Hennie Bikker2, J Peter van Tintelen1, Marcel M.A.M Mannens2, Arthur A.M. Wilde2,4, Michael J. Ackerman1,5,6.

* AMD and ZAB are co-equal first authors

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

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ABSTRACT

Background:
Pathogenic mutations in the RYR2-encoded cardiac ryanodine receptor 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 105 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 QTc 428 ± 29 ms) with either an explicit 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). 200 controls individuals were examined to assess allelic frequency for all non-synonymous variants detected.

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 approximately 47% 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 mutation discovery may afford a more cost-effective approach to CPVT genetic testing.

Key Words: 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. 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 and can easily elude diagnosis. 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.

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

The cardiac ryanodine receptor (RyR2) is one of the largest ion channel proteins, is encoded by the 105-exon-containing \textit{RYR2} gene and comprises 4967 amino-acids; localizes to the sarcoplasmic reticulum, and controls intracellular calcium release and cardiac contraction. Since the sentinel discovery of a CPVT-causing \textit{RYR2} mutation, a cluster distribution involving three discrete protein regions has been reported. Based in a potential physiological role for these “hot-spots”, they have been termed “domains” I, II and III (Figure 1). Similar mutation clustering is observed in the \textit{RYRI} gene which encodes the skeletal
muscle RyR1 and is linked to malignant hyperthermia and central core disease. However, since the majority of CPVT cases haven’t been taken through the entire RYR2 scan, the prevalence of mutations residing outside these three canonical domains remains unknown (~61 exons that encode for 2570 amino-acids).

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 cases referred to 2 different institutions for exertional syncope and, using a combined analysis of the previous reported mutations and the novel mutations found on 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 the Windland Smith Rice Sudden Death Genomics Laboratory at Mayo Clinic, Rochester, MN and 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 exertional syncope, stress test induced ventricular ectopy but absence of 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 Review Board 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. 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. 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.
The demographic characteristics of the 155 unrelated patients included on this study are shown in Table 1. 96% were Caucasians, 49% were females, age at symptoms was $20\pm15$ yrs, and average QTc was $428 \pm 29$ msecs. The mean age of onset of symptoms was significantly lower in RYR2 mutation positive subjects compared to those with a negative genetic test ($16.7 \pm 12.317$ vs $23.8 \pm 16.6$ respectively, $p<0.004$).

Overall, 77 mutations (63 unique, 34 novel), disease causing mutations were identified in 73 cases (47%). (Table 2, Figure 2). 41/73 mutation positives (56%) were females. Each mutation was absent in 400 references alleles and was highly conserved across species.

### Table 1: Demographics Characteristics of the Cohort

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<td>RYR2 Positives (%)</td>
<td>n=47 (60.2%)</td>
<td>n=12 (37.5%)</td>
<td>n=14 (31.1%)</td>
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**RESULTS**

The demographic characteristics of the 155 unrelated patients included on this study are shown in Table 1. 96% were Caucasians, 49% were females, age at symptoms was $20\pm15$ yrs, and average QTc was $428 \pm 29$ msecs. The mean age of onset of symptoms was significantly lower in RYR2 mutation positive subjects compared to those with a negative genetic test ($16.7 \pm 12.317$ vs $23.8 \pm 16.6$ respectively, $p<0.004$).

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Clinical and Genetic Spectrum of Hereditary Cardiac Arrhythmia Syndromes
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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 “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%). Ten mutations found in 11 cases resided outside the three canonical domains, specifically, between domain I and II; 8 of them exhibiting a strong CPVT phenotype. Three large genomic rearrangements comprising exon 3 were detected in three unrelated cases involving a 3.6kb deletion in one and an 1.1kb 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-American controls.

<table>
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Table 2: Compendium of RYR2 mutations and polymorphisms reported to date

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

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.
icans; followed by G18865S with a prevalence of 6%, 4% and 2% respectively. 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)

**DISCUSSION**

**Exertional Syncope: LQTS or CPVT?**

Phenotypically, CPVT closely mimics LQTS, particularly “concealed LQT1”. 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 CPVT1-causing RyR2 mutation14. Here, we included only referral cases of “atypical/possible LQTS” with a phenotype of exertional syncope and QTc < 480msec. Herein, the yield of RYR2 mutations on this selected cohort was 31%; indicating the importance of differentiating between a clinical diagnosis of CPVT and LQTS. CPVT-related arrhythmias can be easily reproduced during an exercise stress test, isoproterenol infusion or by other forms of adrenergic stimulation15. 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 function16-18 and a defective inter-molecular interaction may be crucial in disease phenotypes. Interestingly, in this study 11/64 (17%) putative mutations
localize outside the three canonical domains, specifically, close to the domain I (between domain I and II). The region between domain II and III (exons 49 to 82) remains as a very large “mutation free” area where no disease-causing mutation have been described to date in CPVT cases. In fact, we found only one common polymorphism in this region, RyR2-Q2958R in exon 61. Interestingly, a mutation in exon 75 (RyR2-R3570W) was reported by Marjamaa et al. in 2 sudden unexplained death syndrome victims with dilated hearts, none of the living mutation carriers showed exercise-induced ventricular arrhythmias. This same group reported recently a mutational analysis conducted in 33 Finnish cases with CPVT, interestingly, they only found mutations between the canonical domains I and II and represents the only group worldwide reporting this atypical mutation distribution in RYR2. Thus, the majority of CPVT cohorts exhibit a RYR2 “hot spot” region that localize more precisely to exons 3-28, 37-50 and 83-105 (63 exons). 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, 83-105 and 75). 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 couldn’t be detected by regular genetic screening using DHPLC or direct DNA sequencing. Validating this observation, exon 3

Table 2: Summary of the 127 putative mutations known in RYR2

<table>
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<th>Exons Containing Mutations</th>
<th>Exons With No Reported Mutations</th>
</tr>
</thead>
<tbody>
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<tr>
<td>2nd Tier: 10,12,16,37,41,44,56,83,94,95,99,102,105,195 (exons with 2 mutations)</td>
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<tr>
<td>3rd Tier: 13,17,19,21,28,27,28,40,42,43,48,76,88,87,89,91,104 (exons with 1 mutation)</td>
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<tr>
<td>Exons With No Reported Mutations</td>
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</tr>
</tbody>
</table>

Figure 4: Tiered Strategy
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 build 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.
deletion was also reported recently in a different cohort where 2 unrelated cases out of 33, hosted a 1.1kb deletion, including exon 3. So far, cases with exon 3 deletion exhibit a strong CPVT phenotype, however, the precise physiological consequences of exon 3 inframe deletion remain to be determined.

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. This approach was developed considering the number of mutations in each exon reported to date in the literature, excluding exons containing only polymorphisms. As such; 127 distinct mutations were analyzed, including those found within this cohort. The first tier comprises those exons (N=16) reporting > 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 so far hosting only a single mutation.

If we applied this strategy to our cohort, 64.5% of the mutations would have been detected after the first tier, 21% after the second tier, and only 14.5% after the third tier (Figure 5).

In case of negative results, analysis of large genomic rearrangements involving exon 3 is encouraged followed by of calsequestrin 2 (CASQ2) and perhaps Kir2.1 (KCNJ2) mutational analysis. Finally, in selected “strong CPVT phenotype” cases where ~ 30 – 40% will remain gene-negative, analysis of the remaining 60 exons lying outside this tiered strategy might be warranted, beginning with those exons that localize within the 3 canonical domains and adjacent to the hot spot exons. With analysis of only 16 of RYR2’s 105 exons, this tiered strategy is predicted to detect more than 60% of the RYR2 positive cases. However, it is important to consider that cases with compound heterozygosity might be missed by this method. For example, in this study 4/74 RYR2 positives (5.5%) had double mutations in RYR2. This 3-tiered approach would have missed the second mutation in all these 4 compound heterozygous/multiple mutation individuals if the scan would have been stopped after the first tiered where the first mutations resided.
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 nonsynonymous 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 an amino acid substitution that is just there, just rare, just because.

We have learned that common polymorphisms in other ion channels have the potential to modify the clinical phenotype; 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 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. In the clinical setting, compound heterozygosity involving these two polymorphisms has been associated with right ventricular dysplasia. The potential functional effects of the 6 novel polymorphisms identified in this study are unknown.

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.
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 to date combined with a meta-analysis of all published CPVT1-associated mutations indicate that only 45 of RYR2’s 105 translated exons host a putative CPVT1-associated mutation. Moreover, an initial targeting of only 16 exons will yield the vast majority of mutations 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 of “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”.

ACKNOWLEDGEMENTS

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