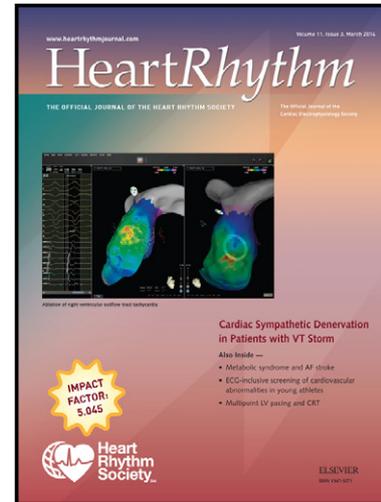


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Clinical and molecular characterization of a cardiac ryanodine receptor founder mutation causing catecholaminergic polymorphic ventricular tachycardia

SHORT TITLE: RyR2 founder mutation causing CPVT

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CONTRIBUTIONS: FW and CBC contributed equally to this work. FW, PB, PMRH, and JB, performed clinical investigation, study design, and clinical data collection and interpretation. CP performed genealogy investigation. CBC, OC, CA, and AI performed genetic testing and genetic data analysis. CBC, PBA, GJP and FSS, performed functional experimental design, data collection,

analysis and interpretation. CBC, PBA, and GJP drafted the manuscript. GJP and RB supervised the entire study and wrote the final version of the manuscript.

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ABSTRACT

Background. Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a difficult-to-diagnose cause of sudden cardiac death (SCD). We identified a family of 1400 individuals with multiple cases of CPVT, including 36 SCDs during youth.

Objectives. We sought to identify the genetic cause of CPVT in this family, to preventively treat and clinically characterize the mutation-positive individuals, and to functionally characterize the pathogenic mechanisms of the mutation.

Methods. Genetic testing was performed for 1,404 relatives. Mutation-positive subjects were preventively treated with beta-blockers and clinically characterized with a serial exercise treadmill test (ETT) and Holter monitoring. In vitro functional studies included caffeine sensitivity and store overload-induced calcium release activity (SOICR) of the mutant channel in HEK 293 cells.

Results. We identified the p.G357S_RyR2 mutation, in the cardiac ryanodine receptor, in 179 family members and in 6 SCD victims. No SCD was observed among treated mutation-positive subjects over a median 37-month follow-up; however, three relatives who had refused genetic testing (confirmed mutation-positive subjects) experienced SCD. Holter monitoring did not provide relevant information for CPVT diagnosis. One single ETT was unable to detect complex cardiac arrhythmias in 72% of mutation-positive subjects, though serial ETT improved the accuracy. Functional studies showed that the G357S mutation increased caffeine sensitivity and SOICR activity under conditions that mimic catecholaminergic stress.

Conclusion. Our study supports the use of genetic testing to identify individuals at risk of SCD to undertake prophylactic interventions. We also show that the pathogenic mechanisms of p.G357S_RyR2 appear to depend on beta-adrenergic stimulation.

Key words: Arrhythmias, genetic testing, CPVT, RyR2, SOICR, sudden cardiac death.

Glossary of abbreviations:

CPVT: Catecholaminergic polymorphic ventricular tachycardia

CVA: Complex ventricular arrhythmias

ETT: Exercise treadmill test

ICD: Implantable cardioverter defibrillator

PKA: cAMP-dependent protein kinase

PVC: Premature ventricular contractions

SCD: Sudden cardiac death

SOICR: Store overload-induced calcium release

TMHR: Theoretical maximum heart rate

VA: Ventricular arrhythmia

INTRODUCTION

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is one of the most difficult sudden cardiac death (SCD)-associated diseases to manage^{1,2}; indeed, CPVT risk stratification remains unclear.³ Initially described by Coumel et al.,⁴ CPVT is an inherited disease characterized by the presence of adrenergic-induced bidirectional or polymorphic ventricular tachycardia in individuals

with a normal basal electrocardiogram and structurally normal heart. CPVT patients can present syncope and SCD triggered by physical or emotional stress at young ages, and SCD can be the first manifestation in up to 30% of cases.⁵⁻⁷ Symptom onset can be very early (between 7-9 years of age).^{1,5,8} As these patients display normal ECG and no structural cardiac abnormalities, there is usually a delay in the diagnosis of the disease.⁵ The main tools used in the diagnosis of CPVT are exercise treadmill test (ETT) and ECG Holter monitoring.^{9,10}

Genetic links to CPVT were established in 1999 for the type 2 ryanodine receptor (*RyR2*)¹¹ and in 2001 for calsequestrin (*CASQ2*).¹² However, heterogeneity in phenotypic expression of these genetic mutations was soon demonstrated, with an average disease penetrance of 54%.^{3,13} The variable phenotype resulting from a single mutation among members of an affected family, suggests that additional factors might play a role in the phenotypic expression of *RyR2* mutations, which highlights the need for a mechanistic understanding of the disease.

Only 22 of the 176 mutations in *RyR2* that have been associated with CPVT have been functionally characterized in cellular models.¹⁴ However, of those, 14 mutations result in a propensity for store overload-induced Ca^{2+} release (SOICR).¹⁵⁻¹⁹ SOICR is proposed to elicit Ca-activated inward currents through the activation of the sodium calcium exchanger in the plasma membrane. This can alter the surface membrane potential and generate early and delayed afterdepolarizations, which in turn can lead to triggered arrhythmia (for review see Priori & Chen).²⁰

We present here a family affected by SCD with more than 1400 living members. These individuals share a common ancestor born in 1749. Within this family, SCD has occurred at young ages during exercise and stress but in individuals with a normal basal electrocardiogram, therefore we suspected CPVT. To determine the genetic cause of SCD in this cohort, we performed genetic analysis. After identifying a potentially causal mutation in the *RyR2* gene in one individual with SCD, we performed in vitro functional studies to determine the underlying mechanism in the mutant channel. Additionally, we used genetic screening of 1,404 living family members to identify individuals in the family at risk and begin treatment to prevent SCD. To date, this approach has averted four fatal outcomes. The

findings of this study underscore the utility of genetic screening for ascertaining individuals at risk of SCD.

METHODS

Detailed methods are provided in the Supplementary Material.

RESULTS

Family history

Between 1994 and 2007, four apparently non-related families from Gran Canaria Island (Spain) were investigated due to several episodes of SCD in young individuals (Figure 1A). In one of these families (Family 1, Figure 1A) CPVT was suspected because three of the four daughters had experienced SCD during emotional or physical stress. After ascertaining the four separate families, we suspected a high degree of kinship because all individuals were inhabitants of Gran Canaria Island, a relatively isolated population. Indeed, a genealogic study between these families allowed us to identify a single, large family with more than 1,400 living family members and a common ancestor who was born in 1749 (Figure 1B). There were 30 more SCD cases in the family whose deaths were suggestive of CPVT.

Genetic Analysis

Because mutations in *RyR2* and *CASQ* have been correlated with CPVT, the coding regions of these two genes were selected for an initial genetic analysis. Genetic analysis of the index case, a deceased member of family 1, (Figure 1A) revealed a *missense* mutation in exon 13 of the *RyR2* gene, in which adenine was substituted for guanine at base position 1069 of the coding sequence (Chr1: 237604682 G>A -Hg19-); this resulted in a glycine to serine substitution at residue 357 of the RyR2 protein (p.G357S_RyR2, NP_001026.2) (Figure 2). To identify possible additional mutations in ion channels, we also analyzed 55 genes previously associated with SCD using next-generation sequencing in the index case; only the p.G357S_RyR2 mutation was detected. The mutation was absent in 580 control alleles from the healthy population and was not found in the general population according to the locus-specific databases dbSNP, Exome Variant Server, and the 1000 Genomes database. In silico analyses

showed that the p.G357S_RyR2 variation was predicted to be “probably damaging” by PolyPhen-2, “deleterious” by Condel, Provean, and PhDSNP, and “pathological” by Pmut. A previous report about the p.G357S_RyR2 mutation showed that G357 residue in RyR2 was conserved among species, but its pathogenicity was unclear because no data about the co-segregation had been previously described.²¹ Familial genetic analysis in patients with documented ventricular arrhythmias (VA) and in six SCD victims with available DNA revealed that all of them had the p.G357S_RyR2 mutation. A cascade screening performed in 1,404 family members identified 179 living p.G357S_RyR2 mutation-positive subjects.

Functional characterization of the G357S mutation in basal conditions

To functionally characterize the effect of the G357S mutation, stable and inducible HEK293 cell lines expressing RyR2 WT or G357S were generated. Both cell lines showed RyR2 expression after 48 h of tetracycline induction, and no RyR2 expression was observed in the absence of tetracycline (Supplementary Figure 1). Calcium imaging experiments were used to evaluate the activity of the RyR2 channels in terms of caffeine sensitivity and SOICR activity as a function of extracellular Ca^{2+} . The evaluation of RyR2 caffeine sensitivity showed no significant differences between the RyR2 WT and RyR2 G357S variant ($\text{EC}_{50_{\text{WT}}} = 0.16 \pm 0.08 \text{ mM}$ vs $\text{EC}_{50_{\text{G357S}}} = 0.21 \pm 0.04 \text{ mM}$) (Figure 3A and B). Additionally, the SOICR analysis showed no shift between the SOICR curves and no significant differences in the EC_{50} for extracellular Ca^{2+} ($\text{EC}_{50_{\text{WT}}} = 0.74 \pm 0.03 \text{ mM}$ vs. $\text{EC}_{50_{\text{G357S}}} = 0.71 \pm 0.03 \text{ mM}$) (Figure 3C and D).

Functional characterization of the G357S mutation in conditions that mimic catecholaminergic stress

Increased sympathetic activity is a typical physiologic response to stress, and is thought to be the basis of arrhythmias in CPVT. Activation of the adrenergic signaling pathway generates increased cAMP levels and activates protein kinase A (PKA). In the heart, activated PKA can phosphorylate RYR2 at two residues, S2030 and S2808. The phosphorylation of these serines increases the resting open

probability of RyR2 promoting a Ca^{2+} leak from the sarcoplasmic reticulum.^{22,23} To evaluate the activity of the RyR2 channel in conditions that mimic catecholaminergic stress, calcium-imaging experiments were performed using forskolin to increase intracellular levels of cAMP. Caffeine sensitivity of the G357S channel was higher than that of the WT channel in the presence of forskolin (Figure 4A and B). We observed a significant shift to the left for the G357S caffeine curve, which produced significant differences between the EC50 under conditions that mimic catecholaminergic stress ($\text{EC50}_{\text{WT}} = 0.095 \pm 0.01$ mM and $\text{EC50}_{\text{G357S}} = 0.069 \pm 0.01$ mM, $p < 0.0001$, $n = 7$; Figure 4B). Further, in the presence of forskolin, SOICR activity was significantly higher in cells expressing RyR2 G357S than in cells expressing RyR2 WT, e.g., at 500 μM Ca^{2+} the oscillation per minute was 1.21 ± 0.04 for WT cells and 1.55 ± 0.05 for G357S cells ($p < 0.0001$, $n_{\text{WT}} = 234$, $n_{\text{G357S}} = 298$, Figure 4C and D). Additionally, forskolin modulated spontaneous release activity in a concentration-dependent fashion (Figure 4E), and RyR2 G357S cells had a greater response to increasing concentrations of forskolin. At 5 μM forskolin, the number of oscillations was 1.25 ± 0.02 for WT cells and 1.43 ± 0.02 for G357S cells ($p < 0.001$, $n=6$).

Analysis of RyR2 phosphorylation

Due to the differential response of cells harboring WT or mutant RyR2 channels to elevated cAMP levels, we hypothesized that the introduction of S357 may create a new PKA phosphorylation site, which could be phosphorylated in the presence of forskolin. We first immunopurified G357S RyR2 from forskolin-treated cells, and subjected it to proteomic analysis. Although protein coverage was relatively high (~40%) for such a large membrane protein (Supplementary Figure 2), the peptide containing S357 (tryptic peptide spanning residues Y355 to K380) was not detected by our mass spectrometric analysis. In an attempt to increase sequence coverage, we digested the same sample with chymotrypsin, but no peptide containing S357 was observed after mass spectrometric analysis.

We then decided to perform PKA phosphorylation assays in vitro, using synthetic peptides containing either G357 (WT) or S357 (mutant). However, we did not observe phosphorylation of S357 (not shown).

Baseline characteristics of SCD members and living mutation-positive subjects

Thirty-six SCDs had occurred in the family, and at a young age (< 42 years old, Table 1); most were in the last 20 years. Of all SCD events, 69.4% occurred in males, and 77.4% of deaths were associated with exercise or emotional stress. In the SCD group, the presence of previous symptoms, especially syncope (73% of cases), was common. In 22% of SCD victims with reliable data, SCD was the first manifestation of the disease. In the group of living mutation-positive individuals, 25.9% had presented symptoms before p.G357S_RyR2 was detected (Table 1 and Supplementary Table 3). The basal mean heart rate, without treatment, measured by 24-hour Holter was significantly lower ($p=0.011$) in mutation-positive subjects (71.0 ± 10.6 bpm, $n=139$) than in control subjects (76.3 ± 10.2 bpm, $n=32$). Sinus bradycardia, measured by basal ECG, occurred in 18.2% of mutation-positive individuals and in 3.1% of controls ($p=0.009$), adjusted by age in children under 16.²⁴ Given the malignancy of CPVT and the high frequency of SCD in the family, we decided to preventively treat all mutation-positive individuals with beta-blockers. One patient was additionally treated with flecainide because of paroxysmal atrial fibrillation and another was treated with calcium channel blockers due to high blood pressure. No sympathectomy was performed.

Clinical investigation in mutation-positive subjects

Basal ETTs were performed in 150 mutation-positive subjects under no pharmacological treatment as soon as genetic diagnosis was confirmed. Disease penetrance and expression in these individuals were heterogeneous. During basal ETT, 28% of mutation-positive individuals exhibited complex VA (CVA) (1.4% in controls); the proportion grew to 46.3% (10% in controls) if VA was considered. The accuracy of basal ETT to diagnose mutation-positive subjects was 0.504 for CVA and 0.586 for VA (Table 2). Mutation-positive individuals treated with beta-blockers ($n=138$) were clinically investigated with serial ETT (Table 3). With a mean/median of 6.5/7 ETT per patient, 1121 ETT were performed: 149 without treatment, 549 on Bisoprolol [mean maximum dose (MMD) = 7.5 ± 5 mg], 242 on Propranolol (MMD = 110.6 ± 65 mg), 47 on Metoprolol, 38 on Atenolol, and 51 on other beta-

blockers. Bisoprolol was preferentially used because it is taken once daily and, although it seems that Nanodol provides superiority of protection in CPVT,² it was not used in our protocol because it is scarcely available in Spain. We analyzed the two most used beta-blockers in our study in a series of 43 patients who took both treatments at different times. This series shows that the mean quantitative arrhythmia score (AS), considering ventricular ectopic beats (AS-1), was 64.73 for Bisoprolol and 29.43 for Propranolol ($p < 0.001$) and, considering CVA (AS-2), was 52.40 and 12.28, respectively ($p < 0.001$).

Serial ETT proved to be effective in uncovering at least one VA-positive test in mutation-positive subjects, despite beta-blockers. After 10 ETT, 74% of p.G357S_RyR2-positive subjects showed CVA, and if VA was considered, this proportion reached 91% (Table 3). A total of 750 Holter monitorings were performed during the follow-up (mean 4.3 Holter/ mutation-positive subjects); its accuracy (0.360) to diagnose CPVT in mutation-positive individuals was inferior to the ETT accuracy (Table 2).

Clinical events at follow-up

Implantable cardioverter defibrillators (ICD) were implanted in 22% of mutation-positive subjects ($n = 40$); 6 of them were implanted before the beginning of the protocol. The reasons for implantation were presyncope ($n = 4$, 10%), syncope ($n = 20$, 50%), and complex VA (CVA; $n = 16$, 40%) despite beta-blocker treatment. After more than five years of ICD implantation, five ICD events have been recorded in four patients: two non-sustained ventricular tachycardia (VT) (287 & 250 bpm) and three ICD discharges (VT-FV, 303 ± 21 bpm). Patients who received discharges were off beta-blockers for personal reasons. We only recorded 1 inappropriate discharge (inappropriate shock rate: 0.32 per year). The cumulative incidence of symptoms (dizziness or syncope) in mutation-positive subjects under protocol was 11%, and no SCD has been reported in this group since our intervention (Figure 5). Another 53 family members were potential mutation-positive individuals but refused genetic testing and clinical evaluation. These individuals were asymptomatic but considered at risk because they were either first-degree relatives of SCD victims or of G357S-positive subjects. During the follow-up, three SCD, one aborted, occurred in this group of patients. These three SCD occurred in a

16-year-old boy who died suddenly while playing at a music hall, a 31-year-old man who died while exercising, and a 38-year-old woman who experienced an aborted SCD. All of them were subsequently identified as p.G357S_RyR2-positive subjects .

DISCUSSION

We report the association of the p.G357S_RyR2 variation with CPVT. Our experimental evidence supports the pathogenic role of this founder mutation and a plausible mechanism that helps explain the phenotype. We actively performed cascade screening, involving genealogical studies, to identify individuals at risk. We present the clinical work-up of 179 mutation-positive subjects, all members of a large family from the Canary Islands. This identical mutation series constitutes the largest CPVT series studied so far. The reliability for the screening of CPVT using the currently recommended clinical diagnostic approach is controversial, and seems to have very low sensitivity, especially with a single test.

Functional characterization of G357S RyR2 channel

The majority of functional studies of RyR2 mutations associated with CPVT performed to date show an enhanced propensity for SOICR activity in cells expressing mutant channels¹⁵⁻¹⁹ and higher sensitivity to caffeine.²⁵ This is not the case for p.G357S_RyR2, which, in basal conditions, does not produce an increase in the SOICR activity nor an increase in the sensitivity to caffeine of the channel. However, SOICR of G357S cells and their sensitivity to caffeine was much greater in conditions that mimic beta-adrenergic stimulations. Loaiza et al.²⁶ showed that the SOICR activity of RyR2 was not modified by the V2475F RyR2 mutation; however, beta-adrenergic stimulated ventricular myocytes from V2475F +/- mice showed an increased propensity for spontaneous Ca²⁺ release events, which is very reminiscent of our observations. In the present study, in vitro experiments indicate that the mutated site, S357, itself is unlikely to be a target for PKA phosphorylation. Moreover, the recently published structure of RyR1²⁷ strongly suggests that large molecules like kinases would be sterically hindered to access the serine at position 357. Taken together, our findings suggest that p.G357S_RyR2

pathogenicity might depend on beta-adrenergic stimulation, which may underlie the variable phenotypic expression and incomplete penetrance of the disease in this family. Nevertheless, the description of the precise mechanism(s) that underlie pathogenicity of RyR2 G357S exceeds the scope of the present manuscript.

Clinical evaluation

Although ETT and Holter monitoring are currently recommended methods for CPVT diagnosis⁹, our clinical investigation, based on these methods, would not have diagnosed a significant proportion of mutation-positive subjects in this family. Holter monitoring for 24 hours did not provide relevant information for the diagnosis of CPVT. Notably, the performance of only one ETT misdiagnosed 54% of mutation-positive individuals (72% if CVA is used as threshold). Our serial ETT protocol notably improved sensitivity, and we strongly recommend the performance of several ETTs if CPVT is suspected in individuals with unavailable genetic testing. The protocol we used, based on 3 months of serial ETT and beta-blocker titration to achieve ≤ 80 % theoretical maximum heart rate (TMHR) and no VA, was very effective. Our results also suggest that Propranolol was more effective than Bisoprolol to prevent quantity and severity of ventricular arrhythmias in the stress test. No fatal events occurred in patients under our protocol. However three SCDs (one aborted) occurred in the group of family members that refused genetic testing and were not treated. These results reinforce the current clinical guidelines, which suggest that mutation-positive family members could receive beta-blockers even if they do not manifest signs of the disease during clinical evaluations⁹.

CONCLUSIONS

The present study provides important mechanistic insight to establish likely disease-causative links for this mutation. Our findings suggest that cAMP elevation unmasks a concealed pathogenic behavior of the G357S RyR2 channel. Our study also supports recent claims that advocate for the use of genetic testing to identify individuals at risk of SCD to undertake prophylactic interventions.²⁸ We demonstrated that beta-blockers and a serial ETT-based protocol were useful and effective in

management of CPVT and should be recommended to all CPVT mutation-positive subjects in the absence of reliable risk stratification tools.

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CLINICAL PERSPECTIVES

Our study provides evidence that genetic testing can become the first screening tool for primary prevention of SCD in large families, especially in those diseases in which there are effective, noninvasive therapies available. We demonstrate the efficacy of a protocol based on beta-blockers and serial ETT in management of CPVT. We recommend this close follow-up (every 3 months) to all CPVT mutation-positive individuals. Additional research is needed to determine whether p.G357S_RyR2-positive subjects experience a varied degree of arrhythmogenic events depending on the level of sympathetic activation. This may provide a rationale for the incomplete penetrance of the disease in this family.

FIGURE LEGENDS

Figure 1. Family pedigrees. **A.** Pedigrees of the 4 related families from Gran Canaria. Circles represent female and squares represent male subjects. Points indicate mutation-positive subjects. Painted symbols indicate family members diagnosed with CPVT. Labeled symbols represent family members who experienced SCD. Interrogation mark indicates unknown genotype. The index case is indicated by an arrow. **B.** Pedigree of the large family from the Canary Islands with a common ancestor and more than 1,400 living family members. It includes the four families from Figure 1A.

Figure 2. Electropherogram of the G357S RyR2 mutation. Sequencing analysis of genomic DNA revealed a missense mutation at position 1069 of the coding sequence of the *RyR2* gene, corresponding to exon 13 (Chr1: 237604682 G>A -Hg19-). This nucleotide change results in a glycine to serine substitution in the 357th residue of the protein sequence (p.G357S_RyR2, NP_001026.2).

Figure 3. Functional characterization of the RyR2 WT and mutant channel under basal conditions. **A.** Representative fluorescence over time traces, from multiple cells, showing caffeine-induced transients. **B.** Normalized amplitudes of Ca^{2+} transients are plotted as a function of caffeine concentration. No differences were found in sensitivity to caffeine between WT and G357S RyR2 expressing cells. **C.** Representative fluorescence over time traces, from 30 cells, showing SOICR activity of WT and G357S cells in 0.3 mM Ca^{2+} and in 1 mM Ca^{2+} , each color represents one cell. **D.** Oscillations per oscillating cells as a function of increasing extracellular Ca^{2+} . No differences were found in SOICR activity between cells expressing RyR2 WT and the G357S variant.

Figure 4. Functional characterization of the RyR2 WT and mutant channel in conditions that mimic catecholaminergic stress. **A.** Representative fluorescence over time traces, from multiple

cells, showing caffeine-induced transients in the presence of forskolin. **B.** Normalized amplitudes of Ca^{2+} transients are plotted as a function of caffeine concentration with forskolin. The G357S RyR2-expressing cells show higher caffeine sensitivity in the presence of forskolin than do WT RyR2-expressing cells. **C.** Representative traces of SOICR activity of cells expressing RyR2 WT or G357S in $200 \mu\text{M Ca}^{2+}$. Each trace represents individual cell fluorescence (54 cells imaged). Left panels show the frequency of oscillations in $200 \mu\text{M Ca}^{2+}$ for both cell types. Right panels show the increase in frequency and in number of oscillations with the forskolin treatment, with a greater change in activity in RyR2 G357S cells. **D.** Oscillations per minute as a function of Ca^{2+} , with or without $5 \mu\text{M}$ forskolin. SOICR activity is higher in cells expressing RyR2 G357S protein in the presence of forskolin. **E.** Number of oscillations per oscillating cells as a function of forskolin concentration. The G357S mutant channel-expressing cells show a higher SOICR activity in the presence of forskolin than WT RyR2 cells.

Figure 5. Prevalence of SCD during the follow-up. Incidence of SCD or cardiac arrest between 1994 and 2013, before and after the identification of the p.G357S variation in RyR2 in this large family from Gran Canaria.

Table 1. Baseline characteristics of studied subjects

| | SCD (n=36) | Mutation-positive (n=179) | SCD/Mutation-positive, P value |
|--|------------------|------------------------------|-----------------------------------|
| Age of death (mean \pm SD) | 18.1 \pm 8.8 * | - | - |
| Age at genetic diagnosis (mean \pm SD) | - | 38.5 \pm 20.8 | |
| Male gender (%) | 69.4 | 44.1 | 0.006 ^a |
| Symptoms (%) [†] | 75 | 25.9 | < 0.001 ^b |
| <i>Dizziness</i> (%) | 14.3 | 10.0 | 0.642 ^a |
| <i>Syncope</i> (%) | 73.7 | 17.9 | < 0.001 ^a |
| Syncope, age of onset (mean \pm SD) | 14.1 \pm 6.5 | 24.7 \pm 17.5 | 0.286 ^c |

^a Fisher Exact Test, ^b Chi-square. ^c Mann-Whitey test.

*Range [5-42]

Table 2. Evaluation of basal exercise treadmill test (ETT) and Holter monitoring

| Basal Test | VA-ETT (n = 220) | CVA-ETT (n = 220) | VA-Holter (n = 166) |
|-----------------|------------------|-------------------|---------------------|
| Sensitivity (%) | 46.0 | 28.0 | 21.6 |
| Specificity (%) | 85.7 | 98.6 | 84.4 |
| PPV* (%) | 87.3 | 97.7 | 85.3 |
| NPV *(%) | 42.5 | 39.0 | 20.5 |
| Accuracy | 0.586 | 0.504 | 0.360 |

*PPV: positive predictive value; NPV: negative predictive value

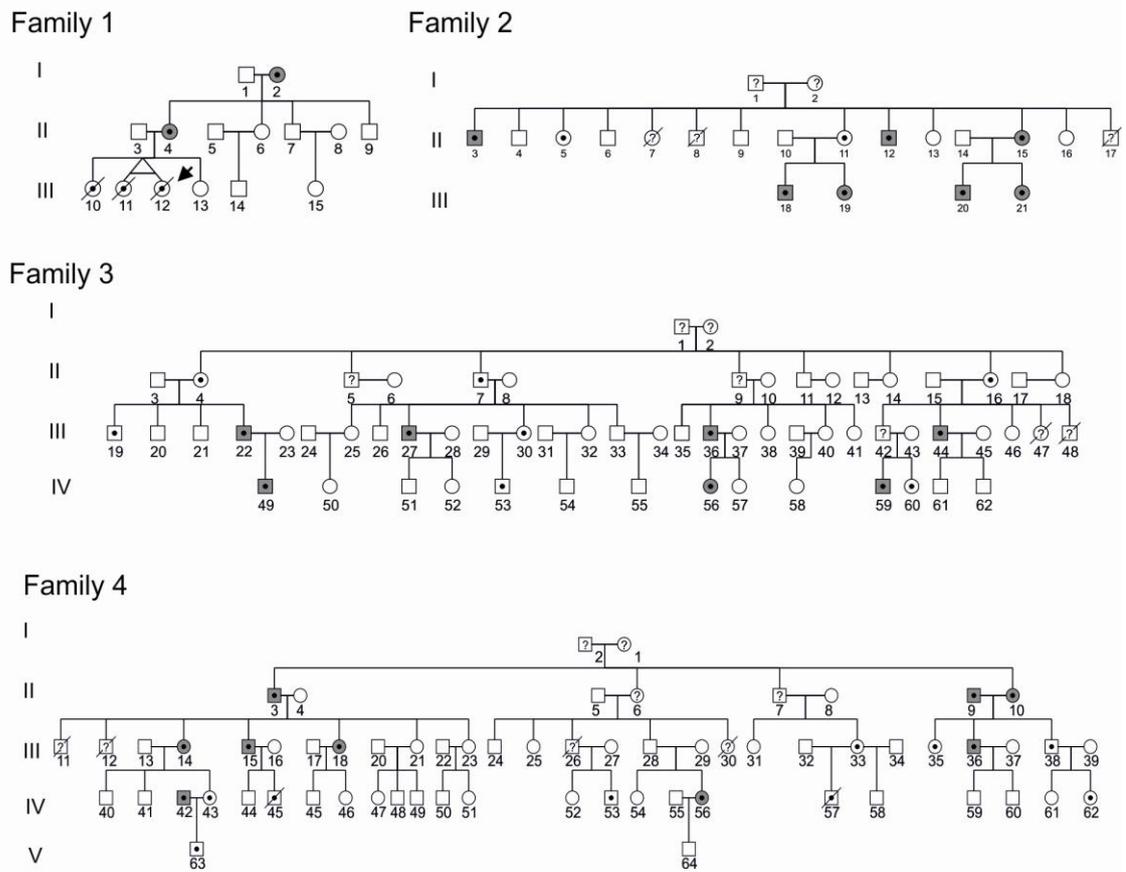
Table 3. Mutation-positive subjects with arrhythmias in consecutive exercise treadmill tests

| | Complex ventricular | | | | |
|-----------------------|-------------------------|-----|------|-------------|------|
| | Ventricular arrhythmias | | | arrhythmias | |
| | N | n | % | n | % |
| Arrhythmias ETT1 | 150 | 69 | 46.0 | 42 | 28.0 |
| Arrhythmias ETT1-pe2 | 141 | 78 | 55.3 | 53 | 37.5 |
| Arrhythmias ETT1-pe3 | 132 | 91 | 67.9 | 59 | 44.6 |
| Arrhythmias ETT1-pe4 | 131 | 96 | 72.7 | 63 | 48.1 |
| Arrhythmias ETT1-pe5 | 130 | 99 | 76.2 | 68 | 52.3 |
| Arrhythmias ETT1-pe6 | 127 | 101 | 79.5 | 70 | 55.1 |
| Arrhythmias ETT1-pe7 | 120 | 101 | 84.2 | 73 | 60.8 |
| Arrhythmias ETT1-pe8 | 116 | 102 | 87.9 | 79 | 68.1 |
| Arrhythmias ETT1-pe9 | 114 | 103 | 90.4 | 82 | 71.9 |
| Arrhythmias ETT1-pe10 | 114 | 104 | 91.2 | 85 | 74.5 |

N: patients available for analysis. n= Mutation-positive subjects with arrhythmias in the ETT

Figure 1

A



B

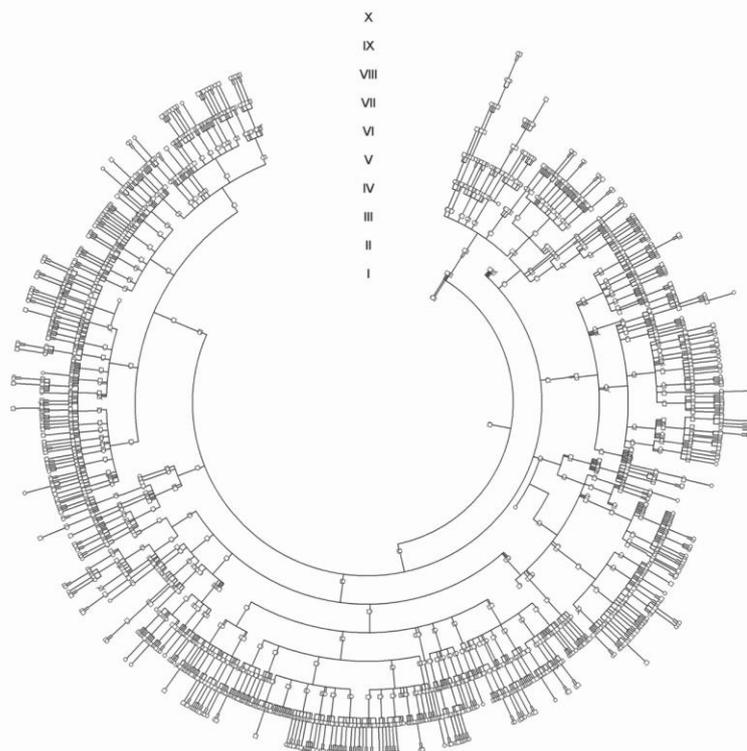


Figure 2

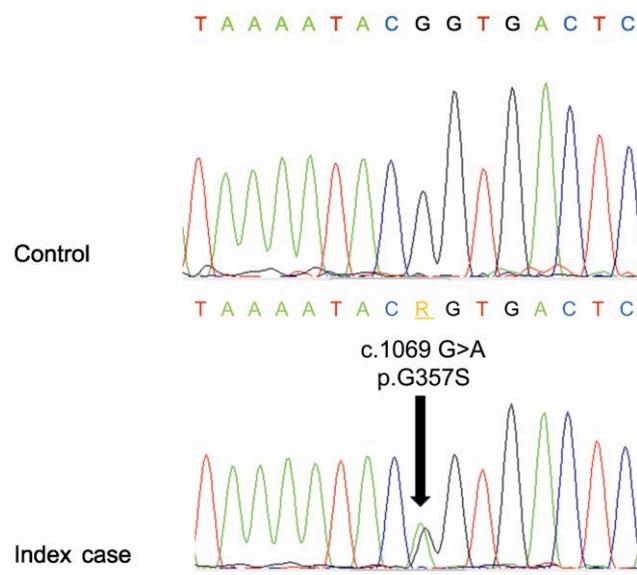
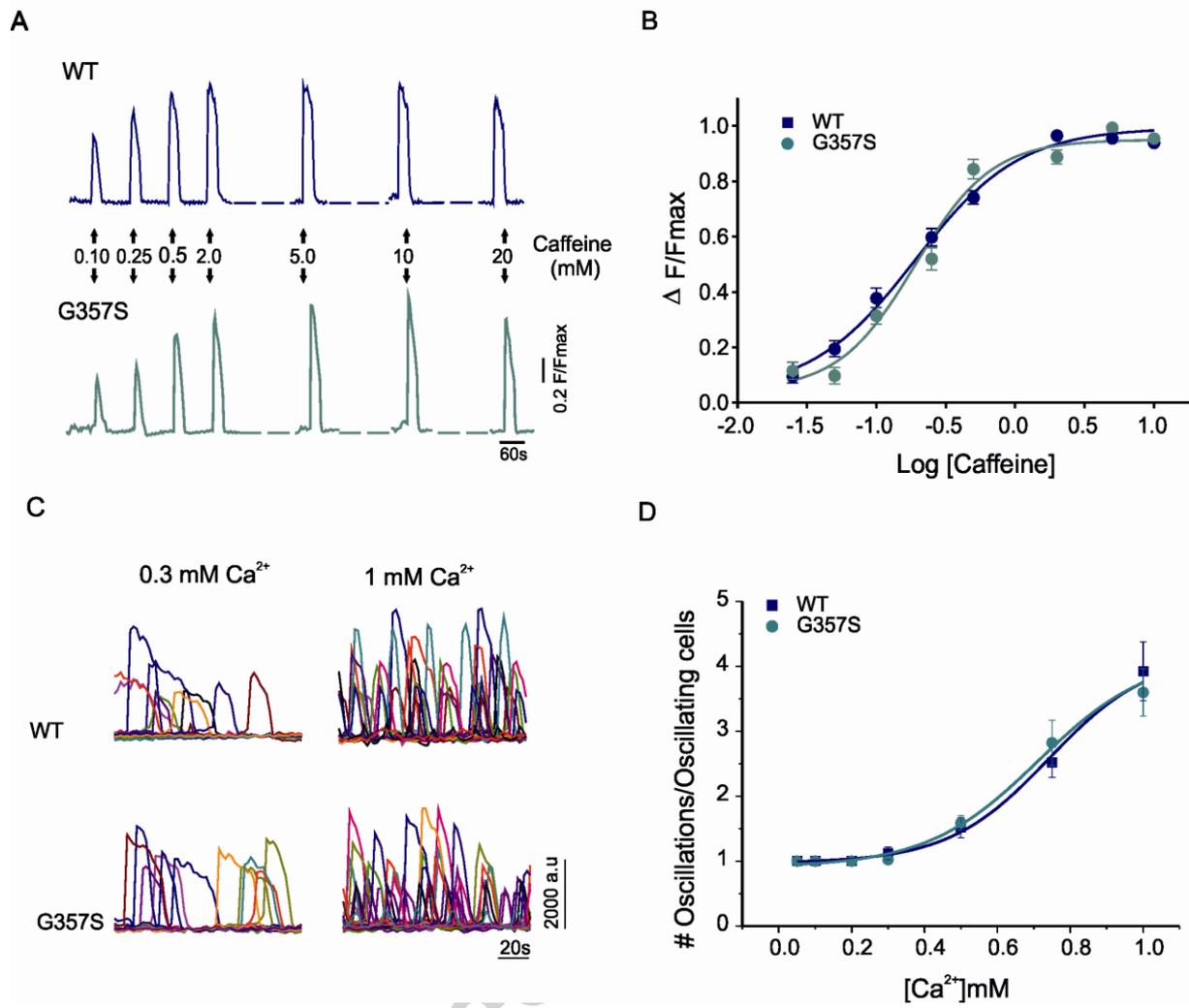


Figure 3



Accepted

Figure 4

