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ORIGINAL ARTICLE

Array comparative genomic hybridization identifies a heterozygous deletion of exon 3 of the *RYR2* gene

IVONE U. S. LEONG¹, JENNIFER SUCICH¹, DEBRA O. PROSSER¹, JONATHAN R. SKINNER^{2,3,4}, JACKIE R. CRAWFORD^{2,3}, COLLEEN HIGGINS⁵ & DONALD R. LOVE^{1,3}

¹Diagnostic Genetics, LabPLUS, Auckland City Hospital, PO Box 110031, Auckland 1142, New Zealand, ²Greenlane Paediatric and Congenital Cardiac Service, Starship Children's Hospital, Grafton Auckland, Private Bag 92024, New Zealand, ³Cardiac Inherited Disease Group, Auckland City Hospital, Auckland, New Zealand, ⁴Department of Child Health, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand, and ⁵School of Applied Sciences, Auckland University of Technology, Private Bag 92006, Auckland, New Zealand

Abstract

Background. Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a heritable cardiac disorder characterized by life-threatening ventricular tachycardia caused by exercise or acute emotional stress. The standard diagnostic screening involves Sanger-based sequencing of 45 of the 105 translated exons of the *RYR2* gene, and copy number changes of a limited number of exons that are detected using multiplex ligation-dependent probe amplification (MLPA).

Methods. In the current study, a previously validated bespoke array comparative genomic hybridization (aCGH) technique was used to detect copy number changes in the *RYR2* gene in a 43-year-old woman clinically diagnosed with CPVT.

Results. The CGH array detected a 1.1 kb deletion encompassing exon 3 of the *RYR2* gene. This is the first report using the aCGH technique to screen for mutations causing CPVT.

Conclusions. The aCGH method offers significant advantages over MLPA in genetic screening for heritable cardiac disorders.

Key words: aCGH, array comparative genomic hybridization, catecholaminergic polymorphic ventricular tachycardia, CPVT, ryanodine receptor 2 gene, *RYR2*

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited cardiac disorder characterized by life-threatening arrhythmias during adrenergic stimulation, such as during exercise or acute emotional stress (1). Those affected by CPVT have structurally normal hearts, and symptoms present at an early age (2); if left untreated the mortality rate of CPVT is between 30%–50% by 35 years of age (2). There are two main forms of the disease: CPVT1 and CPVT2. CPVT1 is an

autosomal dominant disorder caused by mutations in the *RYR2* gene, which encodes for the cardiac Ca²⁺ release channel (Ryanodine receptor isoform 2) (3,4). CPVT2 is an autosomal recessive form of the disease, which is caused by mutations in the *CASQ2* gene that encodes for a Ca²⁺ binding protein (Calsequestrin 2) (5,6). Both proteins are located in the cell's sarcoplasmic reticulum. Mutations in the *RYR2* and *CASQ2* genes account for 50% and 3%–5%, respectively, of all CPVT cases (1,7). A third (and minor) form of CPVT is caused by mutations in the *KCNJ2* gene (8).

Correspondence: Donald R. Love, Diagnostic Genetics, LabPLUS, Auckland City Hospital, PO Box 110031, Auckland 1142, New Zealand.
Fax: +64-9-3074939. E-mail: donald@adhb.govt.nz

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The *RYR2* gene contains 105 exons and encodes for one of the largest ion channel protein in the human body. CPVT-causing mutations in the *RYR2* gene have been found in 45 of the 105 translated exons (8,9); many of these mutations are substitution mutations. The main mutation screening method involves PCR-based amplification of 40–43 exons of the *RYR2* gene, and subsequent bidirectional Sanger-based sequencing (tier 1). If no mutations are found in this targeted group of exons then the remaining exons are sequenced (tier 2). In the absence of sequence-detectable mutations, screening methods then move on to those that can detect larger-scale events such as an exon 3 deletion (the deletion size ranges from 1.1 to 37.7 kb; 29 affected individuals) (9–13), a small deletion in exon 99 (one affected individual) (10), and duplication/insertion in exon 97 (one affected individual) (14). These larger mutations are usually detected using multiplex ligation-dependent probe amplification (MLPA), and this method is used to complement the Sanger-based screening; however, this method only screens for deletion and duplication events in a subset of exons of the *RYR2* gene (15). In contrast, array comparative genomic hybridization (aCGH) offers enhanced exon coverage to detect exonic duplications and deletions. We have implemented a custom-designed aCGH array in our routine diagnostic testing of cardiac referrals to examine copy number changes in the coding regions of 99 genes associated with inherited cardiac and neuromuscular disorders (15–18).

In the present study, we screened a 43-year-old woman clinically diagnosed with CPVT using an aCGH assay and detected a 1.1-kb deletion encompassing exon 3 of the *RYR2* gene. A custom-designed PCR-based assay was developed to screen for this deletion event in her children. The aCGH method has not been previously applied to CPVT screening (15–18).

Materials and methods

Genomic DNA (gDNA) was extracted from peripheral blood EDTA samples using the Gentra Puregene DNA Extraction kit (Qiagen inc., Germantown, Maryland, USA), according to the manufacturer's instructions.

A Roche NimbleGen 12x135K Custom CGH Array (Roche NimbleGen Inc., Madison, Wisconsin, USA) was used for copy number change analysis. The aCGH was designed to examine the coding regions of 99 genes associated with several cardiac and neuromuscular disorders, including genes responsible for long QT syndrome (LQTS), Brugada syndrome, hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), short QT syndrome (SQT), and CPVT (18). See Supplementary Materials for the full gene list. Details about exonic and intronic probe densities and

low-density 'backbone' probes that screen the human genome have previously been described (15).

A total of 500 ng of the proband's gDNA was processed according to the manufacturer's instructions (NimbleGen Array User's Guide: CGH and CGH/LOH Arrays v9.1) (19), and has been described previously (15,17). In brief, a patient's gDNA and Promega control DNA were fluorescently labeled with Cy3 (sample) and Cy5 (control) dyes, and subsequently purified via ethanol precipitation. The fluorescently labeled patient sample and the sex-matched control were combined in equimolar amounts and hybridized to one of 12 arrays on the aCGH slide for approximately 48 h using a Roche NimbleGen Hybridisation Chamber. Subsequently, the slides were washed and scanned using a NimbleGen MS 200 Microarray Scanner. The array image files (.tif) were imported into DEVA v1.2.1 (Roche NimbleGen Inc.) for analysis. The data were filtered using a log₂ratio threshold of less than -0.4 over six probes for a deletion event and a log₂ratio threshold of greater than 0.4 over 15 probes for a duplication event. All copy number changes that met these criteria were examined further using the UCSC Genome Browser, human genome assembly NCBI36/hg18 (released March 2006) (20), to determine the location and significance of the change.

All significant copy number changes were PCR-verified to determine the exact breakpoint of the deletion. Two sets of primers were designed to flank the deletion site detected by the aCGH approach (*RYR2_ex3_del_F* 5' GCGTATCAGAGTAAGCTGTGTC 3'; and *RYR2_ex3_del_R*, 5' AACTCTGTGACTTTGGAAAAGGAAT 3'). PCR was performed as follows: 1x FastStart PCR buffer, 2 mM magnesium chloride, 0.8 μM each of the forward and reverse primer, 0.4 mM dNTP, 0.04 U FastStart Taq DNA Polymerase (Roche), and 50 ng of gDNA. The following cycling conditions were used: 95°C for 4 min, 35 cycles of 94°C for 45 s, 60°C for 30 s, 72°C for 2 min 45 s, and a final extension at 72°C for 10 min.

PCR products were purified with ExoSAP-IT (Affymetrix Inc, Santa Clara, California, USA) prior to bi-directional DNA sequencing using BigDye Terminator v3.1 (Applied Biosystems by Life Technologies, Carlsbad, California, USA) to determine the exact deletion breakpoint. The sequenced products were purified using the BigDye X Terminator Purification Kit (Applied Biosystems Ltd) and were then subjected to capillary electrophoresis using the Applied Biosystems model 3130xl Genetic Analyzer. The analysis of sequence traces was performed using Geneious software (Biomatters Ltd, Auckland, New Zealand) (21).

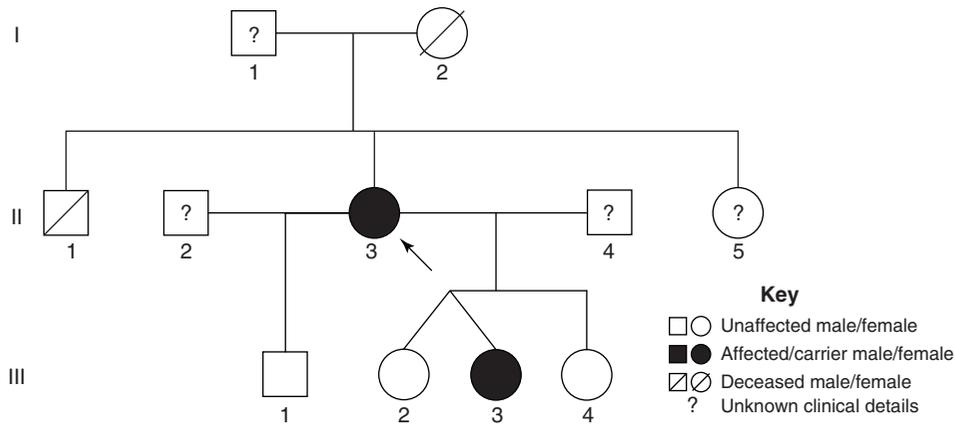


Figure 1. Pedigree of the *RYR2* exon 3 deletion carriers. The proband (II-3) is indicated by the black arrow. Family members carrying the *RYR2* exon 3 deletion are indicated in solid black. The members with unknown clinical and genetic background information are indicated by ?.

Results

Clinical history

The proband (II-3) was a 43-year-old woman who was suspected to have CPVT, and she was referred to the Cardiac Inherited Disease Group (CIDG) of Auckland City Hospital for further cardiac/genetic investigation (Figure 1). The proband had previously experienced syncope with exertion and multiple ventricular premature complexes (VPCs) whilst monitored (Figure 2A). An exercise tolerance test (ETT) showed runs of polymorphic ventricular tachycardia (VT) with minimal exertion, and echocardiography confirmed a structurally normal heart. She was managed with beta-blockade and an implantable cardioverter-defibrillator (ICD). To control further the VT episodes she later underwent a left thoracoscopic sympathectomy and more recently has responded well to flecainide.

The family history was significant for the sudden death of the proband's brother (II-1) at the age of 15 years. He had suffered repeated syncope with documented polymorphic VT, and was taking beta-blockers at the time of his death whilst running. The proband's mother (I-2) died suddenly at age 52 years, and reportedly had a myocardial infarction on autopsy. Clinical details were not available for II-2, II-4, and II-5. The proband's four children (III-1 to III-4) also underwent cardiac investigation due to her probable CPVT diagnosis (Figure 1). All had echocardiography Holters and exercise testing. None had evidence of structural inherited heart disease, but one of the symptomatic fraternal twins (III-3) at 7 years of age had ventricular ectopy during exercise both on ETT and Holters. She was managed with beta-blockers and left cardiac sympathetic denervation, and has had no syncope over the ensuing 6 years.

As well as probable CPVT, III-3 also exhibited symptoms of absence seizures. An electroencephalogram confirmed that II-3 suffered from primary generalized epilepsy with absence of seizures. Ethosuximide was administered for the absence seizures. She also exhibited learning difficulties and poor concentration, and was diagnosed with attention deficit hyperactivity disorder (ADHD). ETT (Bruce protocol, see Figure 2B) and Holter conducted eight months post-surgery showed a reduction in VPCs. Four beats of VT were recently documented on exercise, and flecainide has since been added. There may be early signs of sinus node dysfunction, with the resting heart rate having fallen on the same dose of nadolol over recent years; the repolarization pattern has begun to look normal. She is asymptomatic for the bradycardia at present, and there have been no prolonged pauses on Holter.

Molecular genetic analysis

The aCGH analysis of the proband identified a heterozygous deletion on chromosome 1 (1q43) that was approximately 816 bp in size (hg18 coordinates chr1:235,560,602-235,561,416) (Figure 3). This deletion encompasses the whole of exon 3 of the *RYR2* gene with flanking intronic regions (Figure 4). Sanger-based sequencing was used to confirm the aCGH results and also to determine the exact breakpoints of the deletion. Primers were designed to flank the region of interest (Figure 4A), and subsequent Sanger-based sequencing of the resulting smaller amplicon confirmed the aCGH results but with greater resolution such that the deletion proved to be 1,126 bp in size (Figure 4A). This mutation (c.169-198_c.273+823 del1126) has been previously reported by Ohno et al. (12) and Szentpali et al.

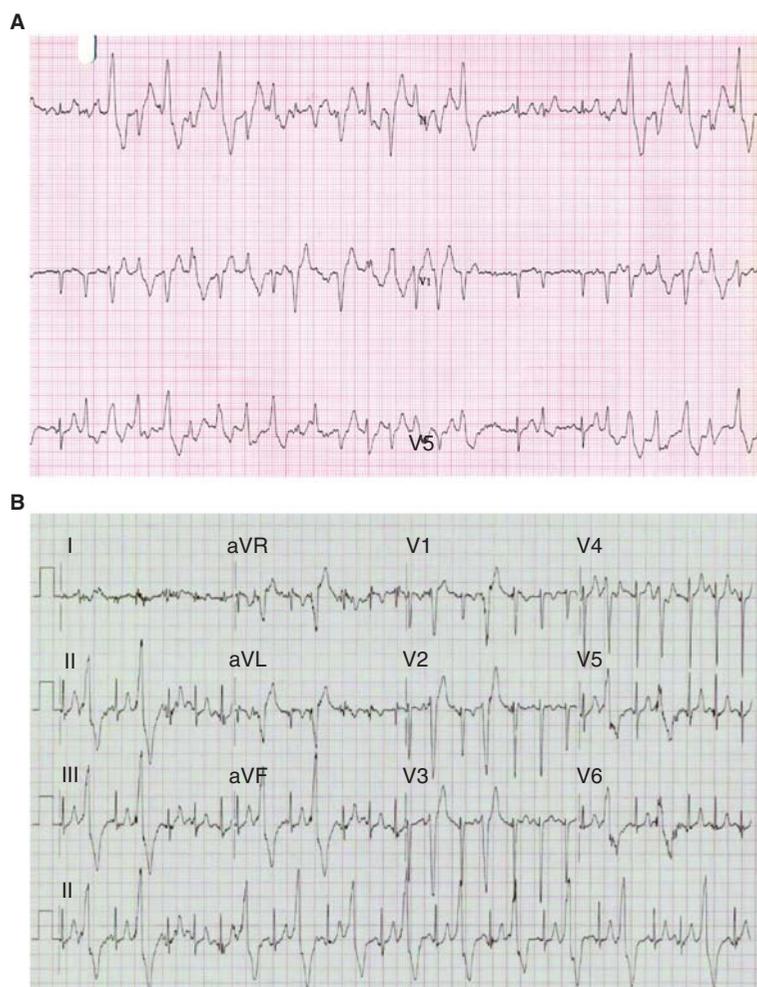


Figure 2. Rhythm strips of the proband and her daughter. A: Three lead rhythm strip (25 mm/s, 10 mm/mV) taken during exercise of the female proband (II-3), at age 29 years following presentation with exertional syncope. Two minutes into stage 1 of the Bruce protocol, there are runs of polymorphic ventricular ectopy and ventricular tachycardia interrupted with occasional sinus beats. B: 12 lead rhythm strip (25 mm/s, 10 mm/mV) taken during exercise of an asymptomatic 7-year-old daughter of the proband (III-3) in stage 3, 9 min into the Bruce protocol. Frequent left bundle branch, inferior axis monomorphic ventricular extra beats develop into bigeminy.

(Figure 5) (13). Exon 3 of the *RYR2* gene encodes for a highly conserved region of the *RYR2* protein, and the mutation is an in-frame deletion of 35 amino acids (p.Asn57-Gly91).

The proband's four children were also tested for the same mutation using a PCR-based screen, with only the daughter (II-3) proving to be a carrier of the deletion event; interestingly, this assay preferentially amplified only the deleted *RYR2* gene allele in carrier individuals (Figure 4B). The outcome of this mutation screen correlated with the clinical phenotypes of the proband's children.

Discussion

The custom-designed NimbleGen 12x135K aCGH confirmed the CPVT diagnosis of the proband, and the targeted PCR-based amplification assay confirmed

the CPVT diagnosis of her daughter. The microarray was able to detect the deletion of only one exon of the *RYR2* gene. The custom-designed aCGH described here has been used previously in screening for deletion/duplication events in a large number of cardiac genes, but the design criteria have also been more generally applied to allow screening for mutations in non-cardiac-related referrals (15-18).

Missense mutations are common within the *RYR2* gene, with many of the mutations located in 45 out of the 105 exons. While deletion and duplication mutations are not common in the *RYR2* gene, it is still important to check for copy number changes for this gene as deletion and duplication events have been detected (9-13). These large heterozygous deletions cannot be detected using standard genetic screening such as direct DNA sequencing and high-performance liquid chromatography (9). While

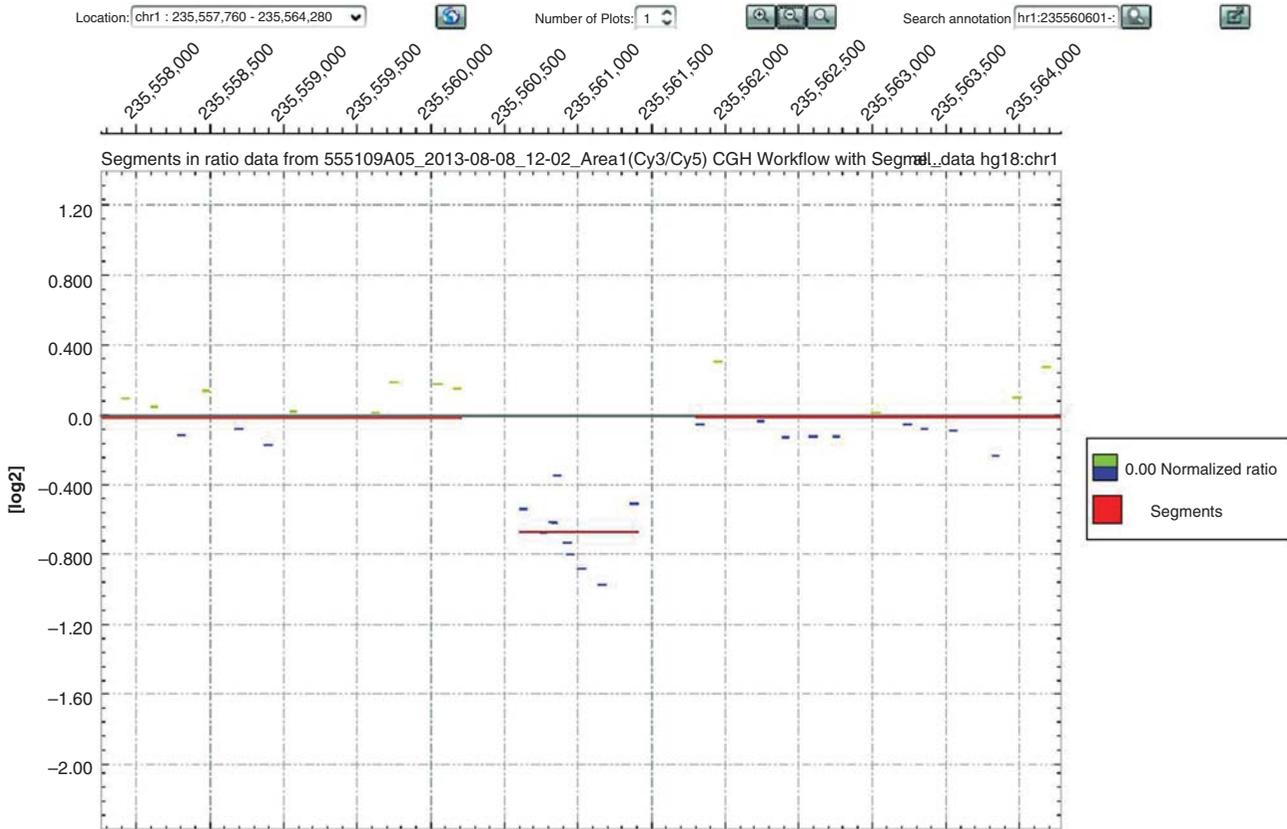


Figure 3. Copy number changes in chromosome 1q43 of the proband. DEVA software output showing a copy number change (deletion; 10 probes; log2ratio: -0.6726) localized to chromosome 1q43 (235,560,602-235,561,416; hg18 co-ordinates) for the proband (II-3).

MLPA is able to detect exonic deletions, the technique can only be applied to a small number of genes and a limited number of exons. The current aCGH method has made it more efficient in screening for dosage changes in several patient samples simultaneously across a wide range of suspected genes.

The deletion of exon 3 has been reported by five different groups (9-13), and 31 individuals carry this mutation (nine families, including this study). Of the 31 individuals, only three appear to have a deletion of the same location and extent as the one reported here (Figure 5, Table I) (12,13). It is tempting to suggest that the deletion cluster represents recombination between repetitive elements (Alu family of repeats) (Figure 5) that flank exon 3. Interestingly, the three individuals previously reported do not come from the same geographical region as our patients; the two unrelated cases reported by Ohno et al. (12) are ethnically different from our proband. Nineteen individuals have deletions of similar sizes in this region (Figure 5) (10,11), and two individuals have much larger deletions in this region (3.6 kb and 37.7 kb; these two mutations are not shown in Figure 5) (9,12). Approximately 80% of these cases suffer from ventricular tachycardia, and approximately

half experience atrial fibrillation and sino-atrial dysfunction. A quarter have received an ICD or pacemaker, and only the two subjects in the current study have received a left thoracoscopic cardiac sympathectomy. The symptoms exhibited by the patients in the study reported here match the other symptoms found in those with the same mutations (i.e. syncope, bradycardia, ventricular tachycardia, and VPCs); however, the two affected members in our study do not yet show clear signs of sino-atrial dysfunction and left ventricular non-compaction cardiomyopathy (LVNC) which were found in the two other studies (12,13).

The RYR2 protein is a major Ca^{2+} releasing channel of the sarcoplasmic reticulum (SR) in cardiac muscle and plays an essential role in excitation-contraction coupling and Ca^{2+} homeostasis in SR (22). All mutations found in the RYR2 gene to date have a gain-of-function effect, which causes a lowered threshold for either cytoplasmic Ca^{2+} or SR Ca^{2+} levels (23). This increase in cytoplasmic Ca^{2+} leads to delays after depolarization (DAD) (23). The exon 3 region of the RYR2 gene is highly susceptible to large Alu repeat-mediated genomic rearrangements (9). *In vitro* studies of the effect of RYR2 lacking the

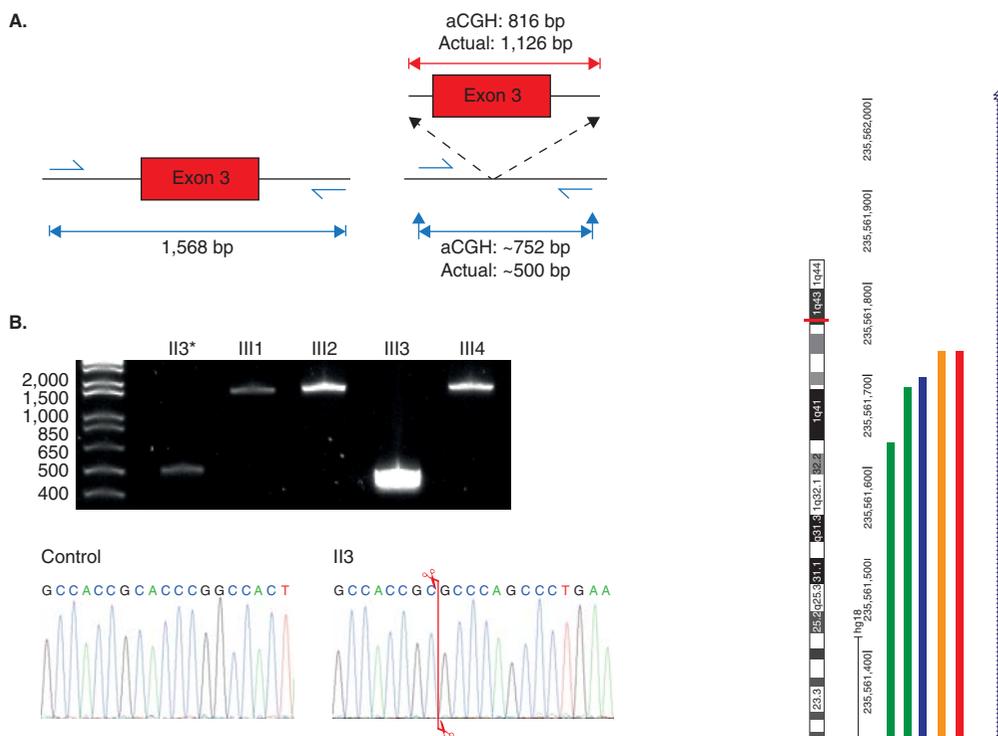


Figure 4. PCR amplification of the region encompassing exon 3 of the *RYR2* gene. A: Amplicon sizes of the expected PCR products using DNA from an unaffected individual carrying no deletion of exon 3 of the *RYR2* gene (left), and for DNA with the exon 3 deletion (right). The deletion size according to aCGH and the actual deletion size confirmed by Sanger-based sequencing are shown (above red lines). The expected PCR amplicon size of the deletion mutant according to the aCGH data and the actual product size are both shown. B: 2% agarose gel showing the results of PCR amplification of the genomic region encompassing exon 3 of the *RYR2* gene for the proband (II-3) and her four children (III-1 to III-4). Chromatogram of the control and the proband showing where the breakpoint is (indicated by the red line).

exon 3-encoded domain in HEK293 cells have shown increased Ca^{2+} release into the cytoplasm, which has been attributed to Ca^{2+} overload leading to DADs (24).

Of the five members of the family that were tested here, two members were affected by ADHD (one daughter was confirmed to have CPVT, the other was asymptomatic). As the *RYR2* gene mutation did not co-segregate with the appearance of ADHD, it could not be concluded that the gene is associated with the disorder. However, there have been two reported cases where CPVT patients (substitution mutations in the *RYR2* gene) suffer from ADHD (25,26). The proband's daughter (II-3) was also affected by generalized epilepsy with absence seizures, which could be caused by the *RYR2* exon 3 deletion. Previous reports have found that approximately half of children with CPVT also present with seizures (27), and half of *RYR2* gene mutation carriers

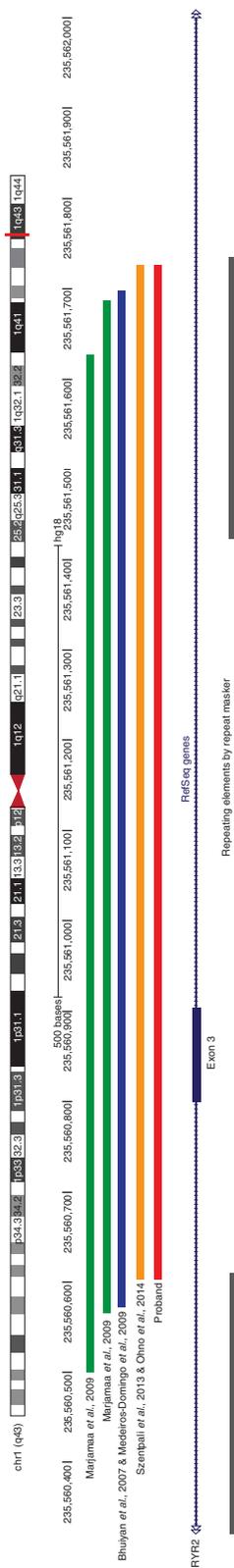


Figure 5. Reported deletions in the *RYR2* gene. Ideogram of chromosome 1 showing the location of reported *RYR2* gene deletions encompassing exon 3 (shown in green, blue, and yellow bars). The location of the proband's exon 3 deletion is shown in red, and the location and extent of Alu family repetitive sequences are shown at the bottom of the figure. These graphics were redrawn from the UCSC genome browser by accessing the NCBI36/hg18 assembly (<http://genome.ucsc.edu/>).

Table 1. Clinical summary of reported families with RYR2 exon 3 deletion. The rows shaded grey represent patients with the same deletion mutation as the current study.

Family	Deletion	n	Syn	Brady	AV block	VT	VPC	LVNC	AF	SA dysfunction	LV dysfunction	ICD	Pacemaker	Ref.
1	c.161-236_c.272+781del1126	11		3	11				10	7	4	1		(10)
2	c.161-236_c.272+781del1126	2		1	2				2	1	1		1	
3	3.6 kb del of exon 3	1												(9)
4	c.168-301_c.273+722del1128	4	1	2	1	4	1		2	2				(11)
5	c.168-228_c.273+793del1126	2	1		2	2	2	1	1	1				(13)
6	c.169-198_273+823del1126	1	1	1	1	1	1	1	1	1				(12)
7	c.169-22924_c.273+14653del137682	6	2	5	1	2	2	5	1	3		2	1	(12)
8	c.169-198_c.273+823del1126	2	2	2	1	1	1	2		1			1	Current study
9	c.169-198_c.273+823del1126	2	1	2	2	2	2	2				2		Current study
Total		31	8 (25.8%)	12 (38.7%)	7 (22.6%)	25 (80.6%)	9 (29.0%)	9 (29.0%)	16 (51.6%)	16 (51.6%)	5 (16.1%)	5 (16.1%)	3 (9.7%)	

AF = atrial fibrillation; AV = atrioventricular block; Brady = bradycardia; ICD = implantable cardioverter-defibrillator; LV = left ventricular; LVNC = left ventricular non-compaction cardiomyopathy; SA = sino-atrial; Syn = syncope; VPC = ventricular premature complex; VT = ventricular tachycardia.

in a large Dutch cohort suffered from seizures (28). Knock-in mouse studies conducted by Lehnart et al. (29) showed that a RYR2 gene missense mutation found in CPVT patients caused seizures and sudden death in mice, providing evidence that mutations in the RYR2 gene can cause seizures.

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