Clinical and genetic spectrum of hereditary cardiac arrhythmia syndromes

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Citation for published version (APA):
The Expanding Spectrum of Human RYR2-related Disease: New Electrocardiographic, Structural and Genetic Features

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Circulation 2007; 116:1569-1576
Clinical and Genetic Spectrum of Hereditary Cardiac Arrhythmia Syndromes
Abstract
Background—Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) is a disease characterized by ventricular arrhythmias elicited exclusively under adrenergic stress. Additional features include baseline bradycardia and, in some patients, right ventricular fatty displacement. The clinical spectrum is expanded by the two families described here.

Methods and Results—Sixteen members from two separate families have been clinically evaluated and followed over the last 15 years. In addition to exercise-related ventricular arrhythmias, they showed abnormalities in sino-atrial node (SAN) function, as well as AV-nodal function, atrial fibrillation (AF) and atrial standstill (ASS). Left ventricular dysfunction and dilatation was present in several affected individuals.

Linkage analysis mapped the disease phenotype to a 4 cM region on chromosome 1q42-q43. Conventional PCR-based screening did not reveal a mutation in either RYR2 or ACTN2, the most plausible candidate genes in the region of interest. Multiplex ligation-dependent probe amplification and long-range PCR identified a genomic deletion involving RYR2 exon-3, segregated in all the affected family members (n=16) in these two unlinked families. Further investigation revealed that the genomic deletion occurred in both families due to Alu repeat-mediated polymerase slippage.

Conclusions—This is the first report on a large genomic deletion in RYR2, which leads to extended clinical phenotypes, e.g. SAN- and AV-node dysfunction, AF, ASS and dilated cardiomyopathy. These features have not previously been linked to RYR2.

Key Words: genetics • cardiomyopathy • conduction • arrhythmia • alu repeats• replication slippage

Introduction
Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an arrhythmogenic disorder of the heart characterized by a reproducible form of polymorphic ventricular tachycardia, induced by physical activity, stress or catecholamine infusion, which can deteriorate into ventricular fibrillation. Patients present with recurrent syncope, seizures or sudden death following physical activity or emotional stress. CPVT can be inherited as an autosomal dominant or recessive trait. Typically, clinical cardiological examinations, including baseline ECG and echocardiography, reveal mostly normal findings, and post mortem examinations, when carried out, have not disclosed any significant morphological alterations in the fine structure of the heart, with the exception of mild fatty myocardial infiltration in a few patients. The hallmark of the disease comprises ventricular arrhythmias of varying morphology not present under resting conditions, but that appear only with physical exercise, excitement or catecholamine administration. These arrhythmias are first seen as
ventricular premature complexes, later in bigeminy, followed by bidirectional or polymorphic ventricular tachycardia, eventually leading to ventricular fibrillation. Clinical penetrance in this disease ranges from 25–100%, with an average of 70–80%. Syncope appears to be the first symptom in more than half of the patients. When untreated, the mortality from CPVT is high, reaching 30–50% by the age of 30 years.11 β-blockers without sympathomimetic activity are clinically effective in reducing syncope,2,5 but implantation of an automatic internal defibrillator is occasionally needed in these patients.3 The gene locus corresponding to autosomal dominantly inherited CPVT was mapped to chromosome 1q42-43 in two large Finnish families, and thereafter several groups reported various missense mutations in the RYR2 gene located in this locus.3-5,11 Clinical features of the probands and families examined by various groups were compatible with the CPVT phenotype,2-5 while those reported by Tiso et al.8 were judged to have arrhythmogenic right ventricular dysplasia features (ARVC2). It is a matter of debate whether CPVT and ARVC2 represent two separate entities or partly overlapping forms of the same disease, in particular as the causative mutations cluster in the same regions of the RYR2 gene.

In the present study, detailed clinical and genetic investigations were performed in two families with CPVT combined with additional features of dilated cardiomyopathy (DCM), progressive AV-block, sino-atrial node (SAN) dysfunction, atrial fibrillation (AF) and atrial standstill (ASS). Clinical evaluation of 16 family members has been conducted over a period of 15 years. We have shown a genomic RYR2 deletion causal to the extended clinical phenotype. This is the first deletion reported for RYR2, and it occurs due to polymerase slippage during replication. Analysis of the nucleotide sequences surrounding the deleted region suggested that the Alu-repeat sequences were involved in the deletion event, which was recapitulated in our in vitro study.

**Materials and Methods**

**Clinical Evaluation**

Family-1 came to our attention in 1987 when a 13-year-old girl (III:5; Figure 1a) was referred after near-drowning. She reported a similar episode one year earlier. Subsequent investigation revealed severe sinus bradycardia (30 beats/min) and ventricular arrhythmias (bigeminy, couplets), particularly during exercise-testing. An AAIR pacemaker was implanted and sotalol was added (with good effect). A 13-year-old nephew (III:13; Figure 1a) also experienced loss of consciousness with near-drowning and displayed a sick sinus syndrome-like disorder with sinus bradycardia and chronotropic incompetence, compounded by disturbed AV-node function (Wenckebach point 120 beats/min) and ventricular ectopy
during exercise (bigeminy, non-sustained ventricular tachycardia). He received a VVIR pacemaker and treatment with a beta-blocker. Screening of some family members was initiated right away (in the late 1980s) and others were referred in subsequent years (up to 2006), either for presymptomatic cascade screening or because of symptoms. Subjects were evaluated by medical history, physical examination, standard 12-lead ECG, ergometry, ambulatory 24-hour ECG (Holter) monitoring and echocardiography. Rhythm and conduction abnormalities were defined using established criteria. Specifically, SAN dysfunction was considered if one of the following conditions was recorded during one or more occasions.

Figures 1a and 1b: Pedigree structure and clinical features of the two families with the RYR2 deletion mutation. Microsatellite repeats used for haplotype analyses are shown at the top. *Genotype not performed.
when inappropriate for the physiologic circumstances: sinus bradycardia, sinus arrest, or SA exit block. Chronotropic incompetence, defined as a maximum heart rate during exercise testing < 85% that predicted for age and gender, was also taken to indicate SAN dysfunction. Atrial standstill (ASS) was defined as the absence of any discernible atrial activity on the ECG (no p-waves, no f-waves) or during pacemaker (PM) implant. Using ergometry and Holter-recording, special attention was given to the occurrence of arrhythmias during physical or mental stress. DCM was diagnosed in the case of unexplained left ventricular (LV) systolic dysfunction (as evidenced by ejection fraction < .45 and/or fractional shortening < .25) in combination with LV dilatation (as evidenced by end-diastolic dimension > 117% of the predicted value corrected for age and body surface area). LV function was considered “depressed” in the case of decreased systolic function and/or dilatation that did not qualify for DCM. After they gave written informed consent, blood was drawn from all eligible family members for genetic analysis.

The second family (Figure 1b) was identified in 2002, when a brother of the proband died suddenly while giving a speech at 50 years of age. Detailed clinical investigations were also performed in this family.

**Linkage Analysis**

A genome-wide scan was performed to map the causative gene using microsatellite repeat polymorphic markers from ABI-Prism for chromosomes 1 to 22 (ABI Prism Linkage Mapping Set). Fine mapping was performed using additional markers (D1S103, D1S179, D1S163, D1S2850, D1S2678 and D1S102). Phenotype and genotype data, and pedigree information were used for pairwise linkage analysis with the Fastlink software package. Two-point linkage analysis was performed assuming an autosomal dominant pattern of inheritance, a disease-allele frequency of 0.001, and a penetrance of 0 for non-carriers and 0.99 for heterozygous affected individuals. Gene frequency was assumed to be equal between males and females.

**RYR2 and ACTN2 Mutational Analysis**

Genomic DNA was isolated from peripheral blood lymphocytes (Gentra Systems, Minneapolis, MN 55441). The entire RYR2 coding exons (exons 1-105), the ACTN2 coding region (exons 1-21), and their exon-intron junctions were screened for mutations. Primer sequences and PCR conditions are available on request. Mutational analysis of the amplimers was performed by sequencing bi-directionally on an ABI 377 sequencer. RNA or protein analysis could not be performed due to limited access to materials.
Multiplex Ligation-Dependent Probe Amplification (MLPA)
Probes for MLPA analysis of RYR2 exons 3, 97 and 105 (SALSA MLPA Kit P168) were purchased from MRC Holland (Amsterdam, the Netherlands). MLPA was performed according to the manufacturer’s instructions. For fine mapping the deletion region in RYR2, we designed several MLPA probes specific for exons 1, 2, 4, 5, 6, 7, 8, 10, 20, 36 (Supplementary Table 1) and also several intronic probes in intron 2 and intron 3 (Supplementary Table 1).

Confirmation and Analysis of the Exonic Deletion in RYR2 by PCR
To confirm the deletion in RYR2, estimate the size of the deleted fragment and locate its boundaries, we performed PCR with the following primer combination: 5’-CACAGAAA-CAGGACCAAGTTAGAGGC-3’ (forward), located in intron 2, and 5’-CATTACCTTC-CTGACACACTTCATCCTAG-3’ (reverse), located in intron 3. Sequence primers used for precise deletion mapping were: 5’-AATCCATAAATACAATAGA-3’, 5’-GAGGAGATC-CAGAAAATTCTA-3’, 5’-ATAAGCTGCATGACACT-3’, 5’-GTGGCAGGAGCCTG-TAGTCC-3’ and 5’-CACTATGTTGGGCCAGGCT-3’.
Histology
Cardiac tissue samples (Family 1: III:4; Figure 1a) obtained on autopsy were fixed in formalin and embedded in paraffin. Four-micron thick sections were stained with hematoxylin and eosin and a Masson trichrome stain.
The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Clinical Features of Family 1
The pedigree and the clinical characteristics of the investigated family members are shown in Figure 1a. The clinical course in the proband from family 1 (III:5; Figure 1a) was complicated by progressive AV-block, paroxysmal AF (age 20) and later ASS (Figure 2a). During exercise, she developed arrhythmias with typical CPVT characteristics (Figure 2b). Furthermore, serial echocardiograms showed gradual development of depressed LV function, for which treatment with enalapril was instituted. When she was age 28 years, her sister (III:4; Figure 1a) died suddenly, after which the proband had an ICD implanted. A few months later a rapid, asymptomatic, non-sustained ventricular tachycardia was recorded not particularly associated with exercise/stress (Figure 3). The clinical course in her 13-year-old nephew (III:13; Figure 1a) was also characterized by development of paroxysmal AF and depressed LV function. As part of the initial screening, nine family members were investigated between 1987 and 1989. In two subjects (II:9, III:8; Figure 1a) no abnormalities were found, whereas five subjects (II:2, II:5, III:1, III:3, III:9; Figure 1a) showed mild abnormalities, ranging from isolated atrial or ventricular premature beats to short bouts of atrial tachycardia (AT) stable over the years. In two subjects, clinically significant abnormalities were found: a 45-year-old male (II:3; Figure 1a) with chronotropic incompetence, ventricular arrhythmias and mental stress-related paroxysmal AF and his 14-year-old daughter (III:4; Figure 1a) who developed a similar clinical phenotype, including ASS. At the age of 26 years, six months after delivery of her first child, she reported progressive dyspnea on exertion. Echocardiography showed DCM (end diastolic LV dimension 56 mm) with poor systolic function (ejection fraction ). She stabilized on medication (enalapril, furosemide, metoprolol, digoxin), without further deterioration of LV function. However, at the age of 30 years in 2002, she suddenly collapsed and died (no adrenergic trigger). At necropsy heart weight was 395 gm and microscopy showed mild myocyte hypertrophy and interstitial fibrosis, but there were no other abnormalities (although the right ventricle was not investigated at autopsy). In addition to the above initial cohort, other family members have
meanwhile presented, either because of symptoms or to be screened. These included several subjects without abnormalities (III:7, III:11, III:12, III:14; Figure 1a), whereas others were found to have significant abnormalities (I-1, II:7, III:2, III:10; Figure 1a). All affected subjects in the family are being treated with a β-blocker, titrated to the highest tolerated dose.

**Clinical features of Family-2**
The proband of the second family was evaluated at age 45 years (II:2; Figure 1b). Only atrial premature beats were found. Later, after the sudden death of one of her brothers (he was age 50, II:3; Figure 1b), she still had a normal baseline ECG but developed ventricular arrhythmias during exercise testing. Echocardiography revealed normal left and right ventricular function. Her father (I:1; Figure 1b) needed a pacemaker but refused to have one; he died at the age of 78 years. No additional data were available.

Her oldest brother (age 48, II:1; Figure 1b) suffered from a sick sinus syndrome, paroxysmal AF (for which he received a pacemaker) and ultimately permanent AF with virtually indiscernible atrial activity. Over the years left ventricular dimensions increased (LVEDD and ESD 59 mm and 47 mm, respectively) and LVEF decreased. Her younger brother (II:3, Figure 1b), was known to have a non Q-wave myocardial infarction, AV block, ASS and depressed LV function; he was given a pacemaker at age 49, but died suddenly at age 50 (II:3; Figure 1b).

**Histology**
Light microscopy of the RV endomyocardial biopsy obtained from III:4 (Family 1; Figure 1a) showed no inflammatory changes and no fibrolipomatous changes indicative of ARVC (figure not shown). Myocardial biopsy of the left interventricular septum showed enlarged...
irregular hyperchromatic nuclei indicative of myocyte hypertrophy as well as a slight increase in fibrillar collagen between myocytes indicative of interstitial fibrosis (Figure 4).

**Genotype analysis**

Linkage analysis in family-1 identified a single disease locus on chromosome 1, flanked distally by marker D1S2785 and proximally by D1S2850, which showed cosegregation with the disease phenotype (2-point log-of-the-odds [LOD] score of 4.5). In one patient (III:10) there is a recombination at marker D1S2785. Screening of all the coding exons of

![Figure 4: Light microscopy of left ventricular myocardium (see text for description).](image)

Ryr2 and Actn2 in this locus revealed no mutation.

MLPA analysis revealed an aberrant exon copy number in the proband. After quantification, the copy number of Ryr2 exon 3 turned out to be 1.0 (Figure 5 and Supplementary Figure 1), indicating that exon 3 of one of the alleles was deleted. PCR amplification using the intron 2 and intron 3 primers confirmed this finding and revealed that the region deleted was approximately 1.1 kb (Figure 6a). Sequencing of the deleted fragment showed the deletion comprises exon 3, part of intron 2 and also part of intron 3 (c.169-198_273+823del). This deletion of the complete exon 3 (c.161 to c.272) is expected to make an Ryr2 protein with an in frame deletion of 35 amino-acids p.Asn57_Gly91 (NM_001035).
Structural Characteristics of the Genomic Deletion

In order to delineate the extent of the putative deletion mutation, fine mapping MLPA analyses were done with the probes spread over the intron 2, exon 3 and intron 3 (Supplementary Table 1). MLPA probes int2fr6, int3fr1 (Figure 6c and Supplementary Table 1) were located upstream and downstream, respectively, to Alu-1. MLPA probe int3fr1 is just upstream of Alu-2. The findings suggest that the deletion which clearly affects exon 3, does not extend beyond the Alu-1 and Alu-2 sequences (Figures 6b and 6c). This genomic deletion involves part of intron 2 (198 bp upstream of exon 3), exon 3 and part of intron 3 (819 bp downstream of exon 3). This deletion was found in all the affected genotyped patients in both families. An intragenic nucleotide variation (SNP) and also an independent genealogy study was performed to see whether this deletion mutation was due to a founder effect or had occurred in two independent events. Both studies showed that the two families are not linked (data not shown).
Analysis of the deletion mechanism

We observed that long-range PCR for precise delineation of the breakpoint region also resulted in a faint band in the controls (Figure 6a), similar in size to the patients, where MLPA showed no aberration. Sequencing of this faint PCR product (* marked in Figure 6a) revealed that this faint/shadow band comprised the same breakpoint that was detected in the patients. This deletion is a Taq polymerase-mediated artefact which recapitulates the polymerase slippage in vivo in an ancestor of the patients.

Discussion

Here we report two families with typical CPVT in conjunction with additional features of progressive AV-block, SAN dysfunction, AF and ASS. In addition LV function was depressed in several subjects, including a female with DCM. The causal genetic defect is a large in-frame deletion in the N-terminus region of \( \text{RYR2} \).

The majority of previous investigators have linked RYR2 mutations to an electrical phenotype, i.e. catecholamine-induced (supra) ventricular arrhythmias in a structurally normal heart. A single group has proposed that \( \text{RYR2} \) is also the gene for ARVC2.8 Our results are significantly different from previously published reports.2-5 Firstly, our patients have a large genomic deletion, whereas previous reports were all of missense mutations. Secondly, the clinical presentations of our patients who carry this deletion expand the CPVT phenotypes, with progressive AV-block and SAN dysfunction, AF, ASS and depressed left ventricular function to DCM. We also observed apparently non-stress related arrhythmias (Figure 3). Several patients are being successfully treated with β-blocker and PM therapy. Later, after the sudden death at age 30 years of an affected female patient with severe LV dysfunction (III:4), prophylactic ICD therapy was installed in III:5 and III:13.
While most patients with missense \textit{Ryr2} mutations respond well to β-blocker therapy, some of our patients thus required more aggressive intervention (PM and/or ICD). Actually, bi-chamber ICD treatment might be the preferred treatment in affected individuals. At the moment, there is a wide debate about the regulatory mechanism of \textit{Ryr2} in calcium ion handling.\textsuperscript{18-20} It centers around the question whether a mutation in \textit{Ryr2} alters the affinity for the stabilizing molecule FKBp12.6.\textsuperscript{18-20} Despite this debate about the role of FKBp12.6 in \textit{Ryr2} handling, it is commonly agreed from various functional studies that \textit{Ryr2} mutations lead to a “gain of function” that sensitizes the \textit{Ryr2} to a premature release of calcium from the intracellular stores.\textsuperscript{18-20} All the previous functional studies were performed with various missense mutations in which drastic changes in the conformation of \textit{Ryr2} were not expected. This conformation is more likely to be distorted when a large chunk of \textit{Ryr2} is deleted, as found in our study. Functional studies with our reported \textit{Ryr2} deletion might provide clear insight into the role of the FKBp12.6 in \textit{Ryr2} handling. Interestingly, despite divergence in clinical phenotypes, CPVT is the common phenotype between all the described phenotypes. An intriguing question is how this deletion of 35-peptides in \textit{Ryr2} leads to the pathogenesis of extra features like DCM, SAN, AF and ASS. Is it only a calcium ion handling defect or does it involve any other interacting partners in the complex to elicit the divergence in clinical phenotypes? Alu-repeat mediated genomic deletion causing different diseases has already been described.\textsuperscript{21-22} However, no such deletion has been reported in any ion channel genes, which could well be possible as Alu’s are interspersed elements in the whole genome. Our in vitro experiment elucidated that the short 35-nucleotides direct repeats at the end of the upstream and downstream Alu’s caused polymerase slippage during chromosomal replication, which deleted the region in between, in this case \textit{Ryr2}-exon3. Our finding is novel in elucidating and recapitulating the Alu mediated deletion that occurred in real life in an ancestor of the affected individuals.

In conclusion, an N-terminal in-frame deletion of \textit{Ryr2} elicits a divergence in phenotype including DCM, SAN, AF, and ASS combined with CPVT. Further, MLPA testing in \textit{Ryr2} is currently not routine practice in a diagnostic setting, but the importance of identifying the causative mutation in individual patients and their relatives pleads for including MLPA analysis in the routine genetic testing of those individuals in whom a mutation has been excluded by the current exon-scanning methods.

**Acknowledgements**

We would like to thank Dr. Cathal J. McElgunn for designing the MLPA probes used in this study, Wilma van der Roest, genetic counsellor, for her help in informing family members, and Eric Hennekam for the genealogical investigation. We would also like to thank Jackie
Senior for reviewing the manuscript.

**Sources of Funding**
Dr. Arthur A. M. Wilde and Dr. M. P. van den Berg are supported by The Interuniversity Cardiology Institute of the Netherlands (ICIN) project 27.

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Supplementary Figure 1: MLPA based deletion/duplication analysis of the RYR2. The column heights represent the dosage of the respective segments in the genomic DNA with two alleles (value of approx. 1 corresponds to two alleles). The two-allele dosage for RYR2 exon 3 (marked by arrow) was found in the range of 0.5. The control fragments and rest of the fragments from Desmoplakin, Plakophilin-2, RYR2, TGFβ3 and Plakoglobin are near the value of approx. 1, which corresponds to two alleles.
| Exon 1-5 probe | 5’-CAGTCTAAAGGGCTGGAGGAGCAAGCTATGTCTACTGCA-3’ | S-phenylenepropane |
| Exon 3-2 probe | 5’-ACGACTACGGGCAACCGCTCGCCTTCGATCTGCTGAC-3’ | S-phenylenepropane |
| Exon 3-2 probe | 5’-CAGGCTCTAAAGGGCTGGAGGAGCAAGCTATGTCTACTGCA-3’ | S-phenylenepropane |
| Exon 1-5 probe | 5’-CAGTCTAAAGGGCTGGAGGAGCAAGCTATGTCTACTGCA-3’ | S-phenylenepropane |
| Exon 1-5 probe | 5’-CAGTCTAAAGGGCTGGAGGAGCAAGCTATGTCTACTGCA-3’ | S-phenylenepropane |
| Exon 1-5 probe | 5’-CAGTCTAAAGGGCTGGAGGAGCAAGCTATGTCTACTGCA-3’ | S-phenylenepropane |
| Exon 1-5 probe | 5’-CAGTCTAAAGGGCTGGAGGAGCAAGCTATGTCTACTGCA-3’ | S-phenylenepropane |
| Exon 1-5 probe | 5’-CAGTCTAAAGGGCTGGAGGAGCAAGCTATGTCTACTGCA-3’ | S-phenylenepropane |
| Exon 1-5 probe | 5’-CAGTCTAAAGGGCTGGAGGAGCAAGCTATGTCTACTGCA-3’ | S-phenylenepropane |

**Supplementary Table 1:** List of the MLPA probes used to delineate the deletion.