Expanding Spectrum of Human *RYR2*-Related Disease

New Electrocardiographic, Structural, and Genetic Features

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Background—Catecholaminergic polymorphic ventricular tachycardia 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 2 families described here.

Methods and Results—Sixteen members from 2 separate families have been clinically evaluated and followed over the last 15 years. In addition to exercise-related ventricular arrhythmias, they showed abnormalities in sinoatrial node function, as well as atrioventricular nodal function, atrial fibrillation, and atrial standstill. 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 polymerase chain reaction–based screening did not reveal a mutation in either the Ryanodine receptor 2 gene (*RYR2*) or *ACTN2*, the most plausible candidate genes in the region of interest. Multiplex ligation-dependent probe amplification and long-range polymerase chain reaction identified a genomic deletion that involved *RYR2* exon-3, segregated in all the affected family members (n=16) in these 2 unlinked families. Further investigation revealed that the genomic deletion occurred in both families as a result of *Alu* repeat–mediated polymerase slippage.

Conclusions—This is the first report on a large genomic deletion in *RYR2*, which leads to extended clinical phenotypes (eg, sinoatrial node and atrioventricular node dysfunction, atrial fibrillation, atrial standstill, and dilated cardiomyopathy). These features have not previously been linked to *RYR2*. **(*Circulation*. 2007;116:1569-1576.)**

Key Words: arrhythmia ■ cardiomyopathy ■ gene mapping ■ genetics

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 after physical activity or emotional stress.[1] CPVT can be inherited as an autosomal dominant or recessive trait.[2–7] Typically, clinical cardiological examinations, such as baseline ECG and echocardiography, reveal mostly normal findings, and postmortem 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.[8–10] The hallmark of the disease comprises ventricular arrhythmias of varying morphology not present under resting conditions but 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, which eventually leads to ventricular fibrillation.

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Clinical penetrance in this disease ranges from 25 to 100%, with an average of 70 to 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 to 50% by the age of 30 years.[11] β-Blockers without sympathomimetic activity are clinically effective in the reduction of syncope,[2,5] but implantation of an automatic internal defibrillator is occasionally needed in these patients.[3]

The gene locus that corresponds to CPVT inherited as an autosomal dominant trait was mapped to chromosome 1q42-

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q43 in 2 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] whereas those reported by Tiso et al[8] were judged to have arrhythmogenic right ventricular dysplasia features. It is a matter of debate whether CPVT and arrhythmogenic right ventricular dysplasia features represent 2 separate entities or are partly overlapping forms of the same disease, in particular because the causative mutations cluster in the same regions of the *RYR2* gene.

In the present study, detailed clinical and genetic investigations were performed in 2 families with CPVT combined with additional features of dilated cardiomyopathy (DCM), progressive atrioventricular (AV) block, sinoatrial node (SAN) dysfunction, atrial fibrillation (AF), and atrial standstill. Clinical evaluation of 16 family members was 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 as a result of polymerase slippage during replication. Analysis of the nucleotide sequences that surround the deleted region suggested that the *Alu*-repeat sequences were involved in the deletion event, which was recapitulated in the present in vitro study.

Methods

Clinical Evaluation

Family 1 came to our attention in 1987 when a 13-year-old girl (patient III:5) (Figure 1A) was referred after nearly drowning. She reported a similar episode 1 year earlier. Subsequent investigation revealed severe sinus bradycardia (30 bpm) and ventricular arrhythmias (bigeminy, couplets), particularly during exercise-testing. An atrial inhibited, rate-modulated pacemaker was implanted and sotalol was added (with good effect). A 13-year-old nephew (patient III:13) (Figure 1A) also experienced loss of consciousness while nearly drowning and displayed a sick sinus syndrome-like disorder with sinus bradycardia and chronotropic incompetence, compounded by disturbed AV-node function (Wenckebach point, 120 bpm) and ventricular ectopy during exercise (bigeminy, nonsustained ventricular tachycardia). He received a ventricle-sensed, inhibited, rate-responsive pacemaker and treatment with a β-blocker. Screening of some family members was initiated immediately (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 according to medical history, physical examination, standard 12-lead ECG, ergometry, ambulatory 24-hour ECG (Holter) monitoring, and echocardiography. Rhythm and conduction abnormalities were defined with established criteria.[12] Specifically, SAN dysfunction was considered if one of the following conditions was recorded during $\geq$1 occasions when inappropriate for the physiological circumstances: sinus bradycardia, sinus arrest, or sinoatrial exit block. Chronotropic incompetence, defined as a maximum heart rate $<$85% during exercise testing that predicted for age and gender, was also taken to indicate SAN dysfunction. Atrial standstill was defined as the absence of any discernible atrial activity on the ECG (no p-waves, no f-waves) or during pacemaker implant. With the use of 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 $<$0.45 and/or fractional shortening $<$0.25) in combination with LV dilatation (as evidenced by end-diastolic dimension $>$117% of the predicted value corrected for age and body surface area).[13] 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 with microsatellite repeat polymorphic markers from ABI-Prism for chromosomes 1 to 22 (ABI Prism Linkage Mapping Set; Applied Biosystems, Foster City, Calif). Fine mapping was performed with additional markers (D1S103, D1S179, D1S163, D1S2850, D1S2678, and D1S102). Phenotype and genotype data as well as pedigree information were used for pair-wise linkage analysis with the Fastlink software package (Fastlink Software, Anthem, Ariz). Two-point linkage analysis was performed with the assumption of an autosomal dominant pattern of inheritance, a disease-allele frequency of 0.001, and a penetrance of 0 for noncarriers 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, Minn). The entire *RYR2* coding region (exons 1 to 105), the *ACTN2* coding region (exons 1 to 21), and their exon-intron junctions were screened for mutations. Primer sequences and polymerase chain reaction (PCR) conditions are available on request. Mutational analysis of the amplimers was performed by bidirectional sequencing on an ABI 377 sequencer (Perkin Elmer, Foster City, Calif). RNA or protein analysis could not be performed because of limited access to materials.

Multiplex Ligation-Dependent Probe Amplification

Probes for multiplex ligation-dependent probe amplification (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 instructions. For fine mapping of the deletion region in *RYR2*, we designed several MLPA probes specific for exons 1, 2, 4 to 8, 10, 20, 36 (Table I, online-only Data Supplement) and also several intronic probes in intron 2 and intron 3 (Supplementary Table I, online-only Data Supplement).

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′-CACAGAACAGGACCAAGTTAGAGGC-3′ (forward), located in intron 2, and 5′- CATTACCTTCCTGACACACTTCATCCTAG-3′ (reverse), located in intron 3. Sequence primers used for precise deletion mapping were 5′-AATCCATAAATACAATAGA-3′, 5′-GAGGAGATCCAGAAATTCTA-3′, 5′-ATAAGCTGCATGACACT-3′, 5′-GTGGCAGGAGCCTGTAGTCC-3′, and 5′-CACTATGTTGGCCAGGCT-3′.

Histology

Cardiac tissue samples (family 1: III:4) (Figure 1A) obtained on autopsy were fixed in formalin and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin & 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

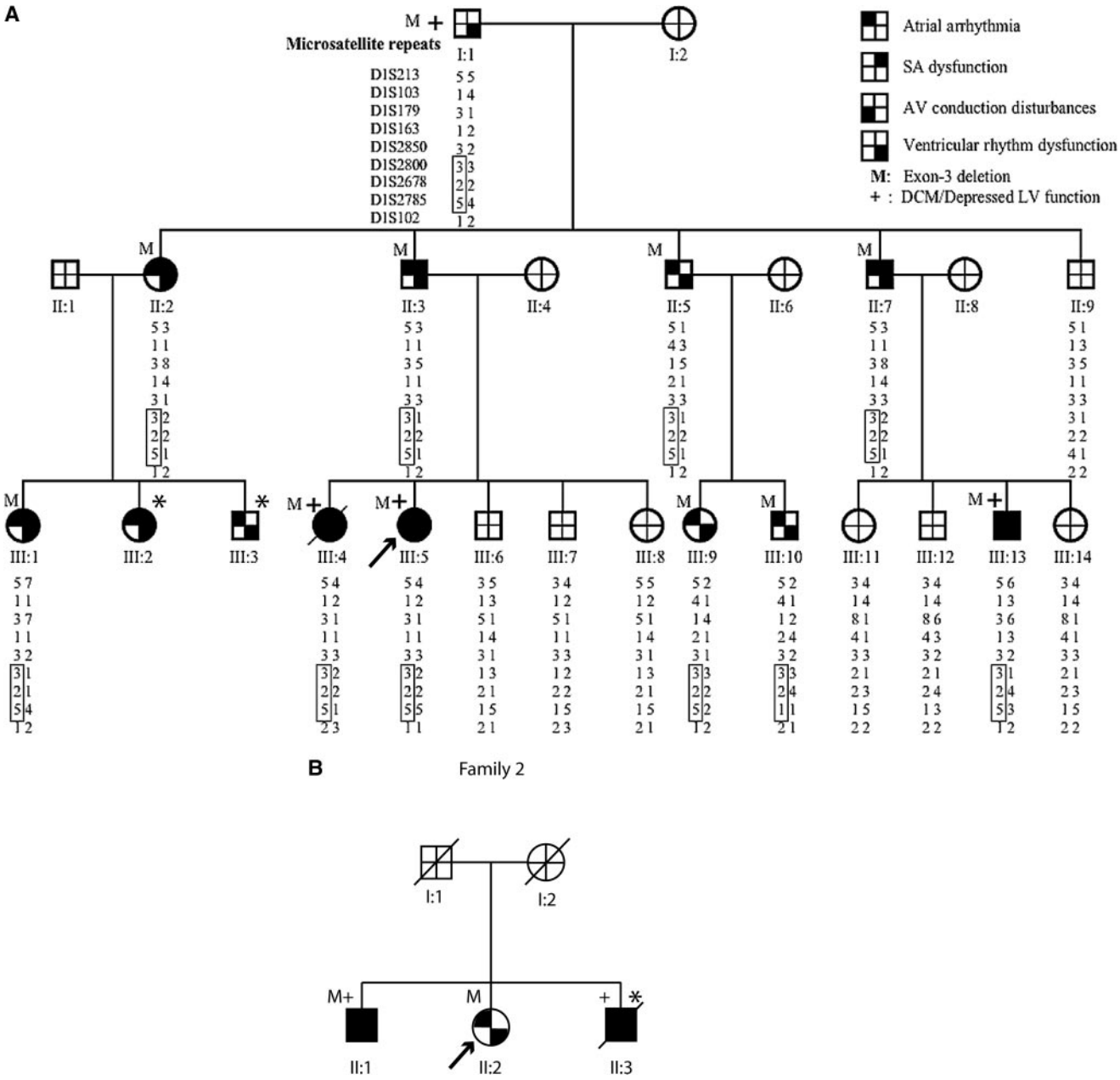

Figure 1. Pedigree structure and clinical features of the 2 families with the *RYR2* deletion mutation. Microsatellite repeats used for haplotype analyses are shown at the top. *Genotype not performed. SA indicates sinoatrial.

course in the proband from family 1 (patient III:5) (Figure 1A) was complicated by progressive AV block, paroxysmal AF (age 20), and later atrial standstill (Figure 2A). During exercise, the patient 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 28 years old, her sister (patient III:4) (Figure 1A) died suddenly, after which the proband had an implantable cardiac defibrillator 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 (patient III:13) (Figure 1A) was also characterized by development of paroxysmal AF and depressed LV function. As part of the initial screening, 9 family members were investigated between 1987 and 1989. In 2 subjects (patients II:9 and III:8) (Figure 1A), no abnormalities were found, whereas 5 subjects (patients II:2, II:5, III:1, III:3, and III:9) (Figure 1A) showed mild abnormalities that ranged from isolated atrial or ventricular premature beats to short bouts of atrial tachycardia stable over the years. In 2 subjects, clinically significant abnormalities were found: a 45-year-old male (patient II:3) (Figure 1A) with chronotropic incompetence, ventricular arrhythmias, and mental stress–related paroxysmal AF and his 14-year-old daughter (patient III:4) (Figure 1A), who developed a similar clinical phenotype that included atrial standstill. At the age of 26 years, 6 months after

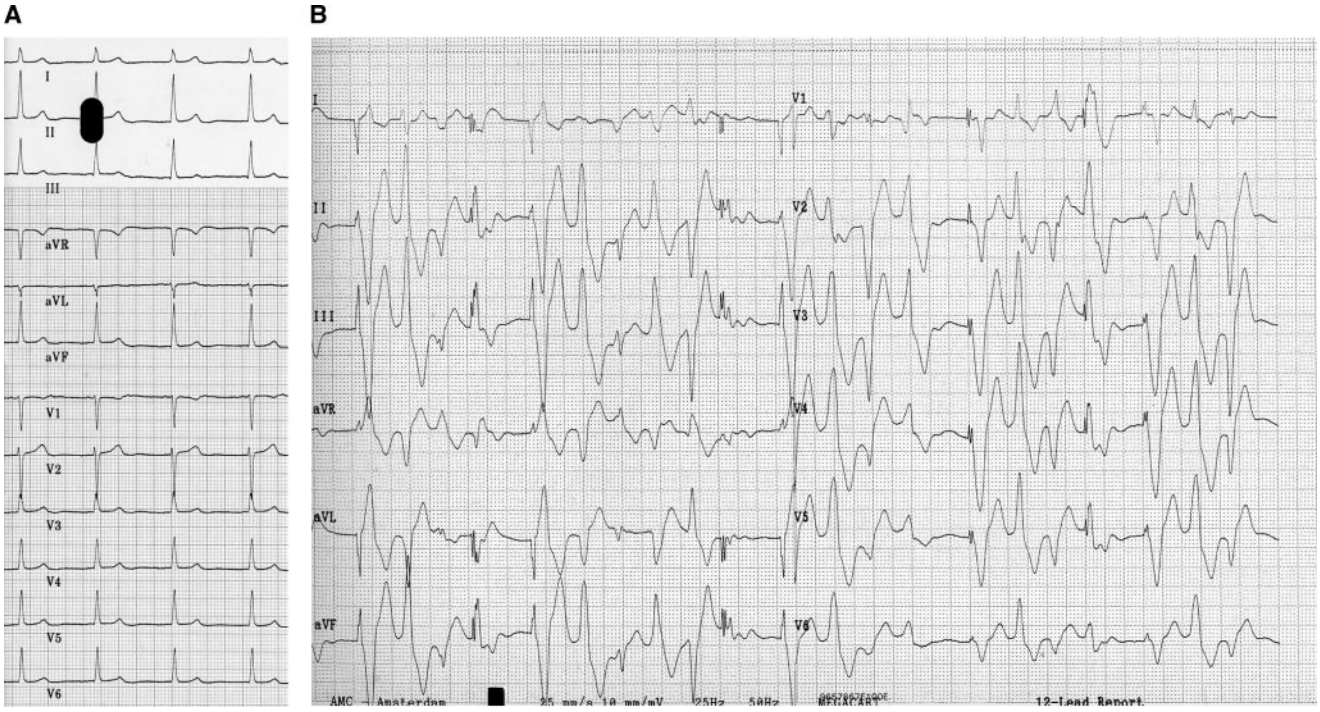

Figure 2. A, Twelve-lead ECG of patient III:5 (standard calibrations) prior to pacemaker implant. P-waves are absent, and AV junctional escape rhythm (CL, 880 ms) is accelerated. Repolarization is normal. B, Twelve-lead ECG of patient III:5 (standard calibrations) during exercise. Pacemaker rhythm is interrupted by polymorphic ventricular tachycardia (maximum 3 beats with frequent fusion of the third ventricular beat with a paced complex).

delivery of her first child, patient III:4 reported progressive dyspnea on exertion. Echocardiography showed DCM (end-diastolic LV dimension, 56 mm) with poor systolic function (ejection fraction, 0.26). 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, her heart weight was 395 g and microscopy showed mild myocyte hypertrophy and interstitial fibrosis, but no other abnormalities were present (although the right ventricle was not investigated at autopsy). In addition to the above initial cohort, other family members have presented in the meantime, either because of symptoms or for screening. These additional family members included several subjects without abnormalities (patients III:7, III:11, III:12, and III:14) (Figure 1A), whereas others were found to have significant abnormalities (patients I-1, II:7, III:2, III-6, and 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 45 years of age (patient II:2) (Figure 1B). Only atrial premature beats were found. Later, after the sudden death of one of her

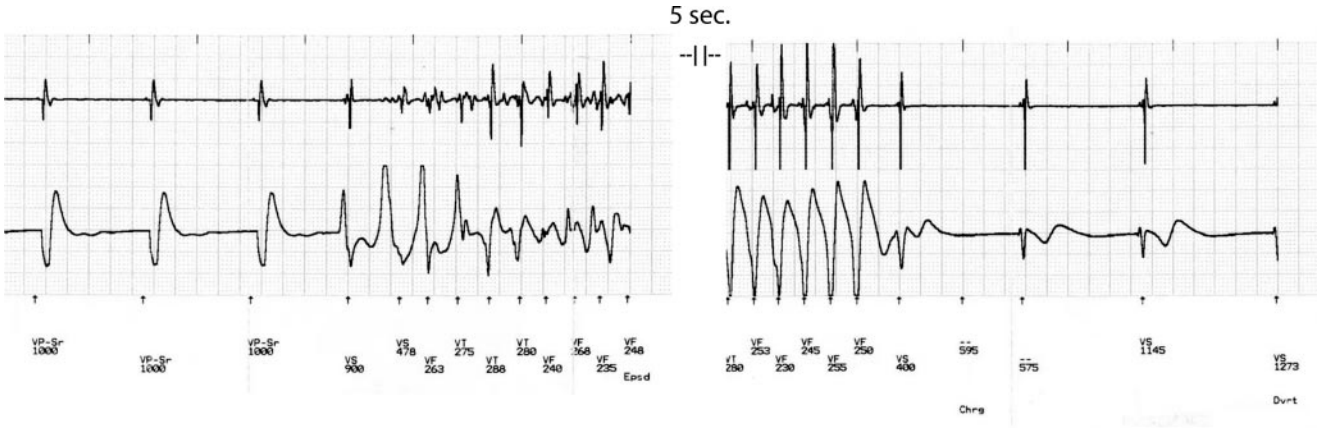

Figure 3. A rapid and presumably polymorphic ventricular tachycardia is initiated during a relatively slow heart rate (60 bpm) by a premature beat with a long coupling interval (left). This initiation sequence is unusual for CPVT. The cycle length of the 36-beat tachycardia is 250 ms (mean). It terminated spontaneously (right).

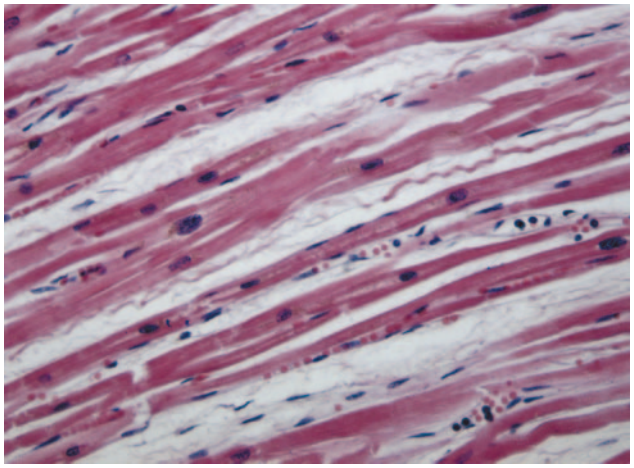

Figure 4. Light microscopy of left ventricular myocardium (see text for description).

brothers (patient II:3, 50 years old) (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 (patient 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.

The proband's oldest brother (patient II:1, 48 years old) (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 LV dimensions increased (LV end-diastolic diameter and end-systolic diameter, 59 mm and 47 mm, respectively) and LVEF decreased. Her younger brother (patient II:3) (Figure 1B) was known to have a non–Q-wave myocardial infarction, AV block, atrial standstill, and depressed LV function; he was given a pacemaker at age 49, but died suddenly at age 50 (Figure 1B).

Histology

Light microscopy of the right ventricular endomyocardial biopsy obtained from patient III:4 (family 1) (Figure 1A) showed no inflammatory changes and no fibrolipomatous changes indicative of arrhythmogenic right ventricular dysplasia features (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, 4.5). In 1 subject (patient III:10) a recombination existed at marker D1S2785. Screening of all the coding exons of *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; Figure I, online-only Data Supplement), which indicated that exon 3 of one of the alleles was deleted. PCR Amplification with the intron 2 and intron 3 primers confirmed this finding and revealed that the region deleted was ≈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.161-236_c.272+781del1126). 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

To delineate the extent of the putative deletion mutation, fine mapping MLPA analyses were done with the probes spread over intron 2, exon 3, and intron 3 (Table I, online-only Data Supplement). MLPA probes int2fr6 and int3fr1 (Figure 6C; Table I, online-only Data Supplement) 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 6, B and C). 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 were performed to see whether this deletion

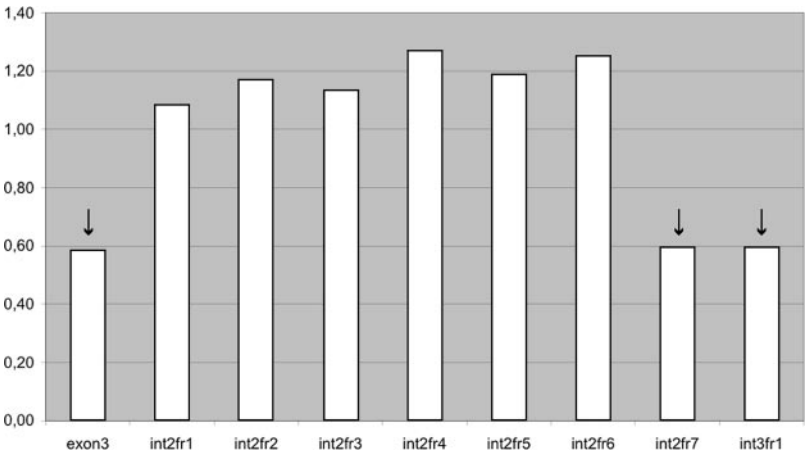

Figure 5. MLPA-based fine mapping analysis of the *RYR2*. The column heights represent the dosage of the respective segments in the genomic DNA with 2 alleles (value of ≈1 corresponds to 2 alleles). The 2-allele dosage for *RYR2* exon 3, intron 2 fragment 7 (immediately upstream from exon 3) and intron 3 fragment 1 (immediately downstream from exon 3) was found in the range of 0.5 (deleted alleles are marked by arrow). Other fragments in intron 2 and also intron 3 (not shown) are near the value of ≈1, which corresponds to 2 alleles.

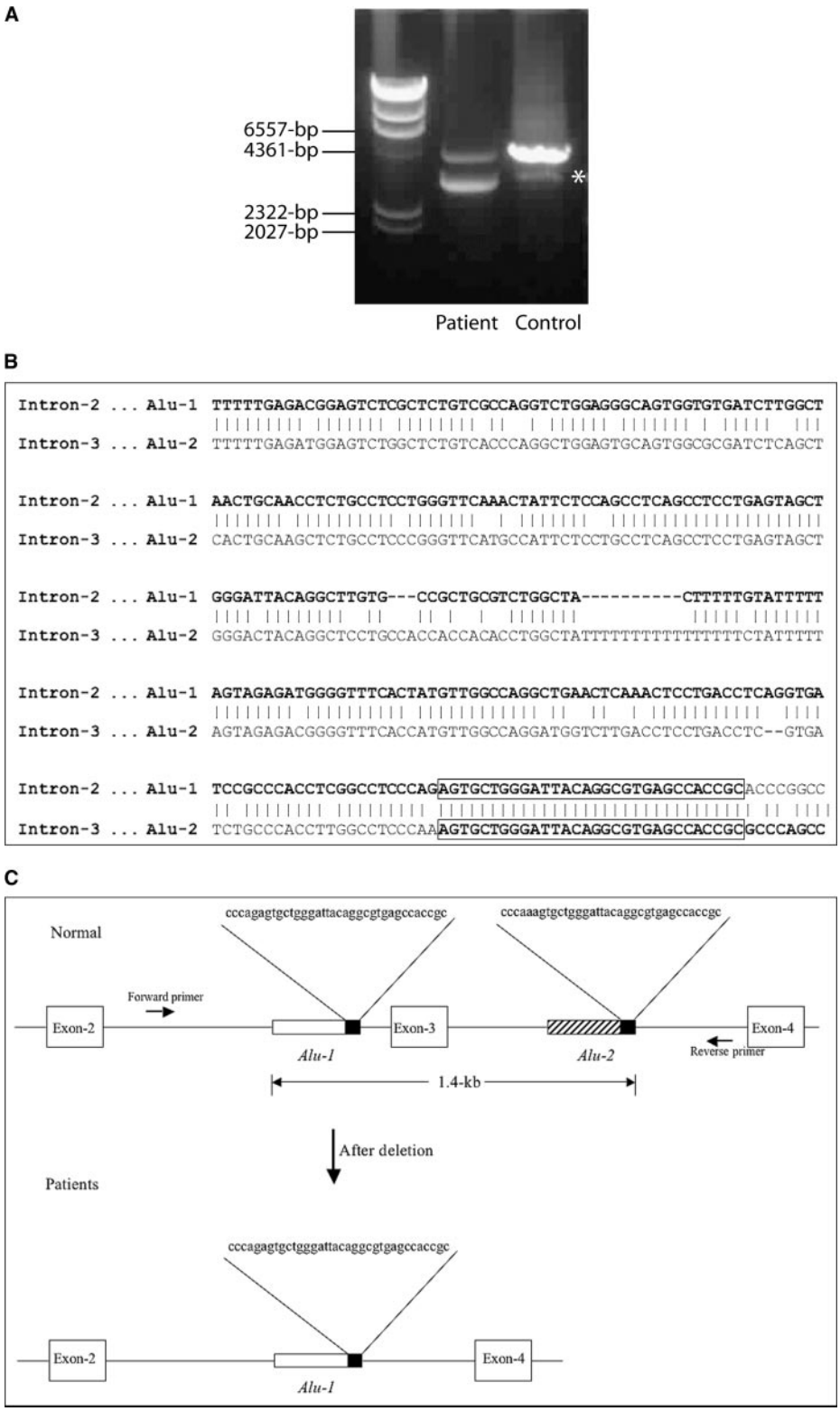

Figure 6. *Alu* repeat–mediated *RYR2* genomic deletion analysis in the patients described. A, PCR amplification with the primers that flank the *Alu* repeats confirmed the deletion and revealed that the region deleted was 1.1 kb. A PCR product of 2.8 kb was obtained with DNA from the probands and the affected family members. PCR product resulted in a 3.9-kb fragment with DNA from a control. Faint band (*) in the control lane shows *Taq* polymerase–mediated slippage in controls (for details see text). B, Comparison of the 2 *Alu* sequences within introns 2 and 3 involved in the rearrangement that led to the deletion of exon 3 of *RYR2*. Sequence identity between the 2 *Alu* repeats is indicated by double dots; the rearranged sequence is shown in bold, the boxed sequence indicates the region where the breakpoint occurred. C, Diagram represents the *Alu-Alu* recombination. *Alu* sequences are located in intron 2, 190 bp upstream from exon 3 and also 536 bp downstream in intron 3. Location of the PCR primers used for amplification and analysis of the breakpoint are shown.

mutation was the result of a founder effect or had occurred in 2 independent events. Both studies showed that the 2 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, whereas MLPA showed no aberration. Sequencing of this faint PCR product (marked by the asterisk 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 artifact that recapitulates the polymerase slippage in vivo in an ancestor of the patients.

Discussion

Here we report 2 families with typical CPVT in conjunction with additional features of progressive AV block, SAN dysfunction, AF, and atrial standstill. In addition, LV function was depressed in several subjects, which included a female with DCM. The causal genetic defect is a large in-frame deletion in the N-terminus region of *RYR2*.

The majority of previous investigators have linked *RYR2* mutations to an electrical phenotype (ie, catecholamine-induced (supra) ventricular arrhythmias in a structurally normal heart). A single group has proposed that *RYR2* is also the gene for arrhythmogenic right ventricular dysplasia features.[8] Our results are significantly different from previously published reports.[2–5] First, our patients have a large genomic deletion, whereas previous reports were all of missense mutations. Second, the clinical presentations of our patients who carry this deletion expand the CPVT phenotypes, with progressive AV block and SAN dysfunction, AF, atrial standstill, and depressed LV function to DCM. We also observed arrhythmias that were apparently unrelated to stress (Figure 3). Several patients are being successfully treated with β-blocker and pacemaker therapy. Later, after the sudden death at 30 years of age of an affected female patient with severe LV dysfunction (patient III:4), prophylactic implantable cardiac defibrillator therapy was installed in patients III:5 and III:13.

Although most patients with missense *RYR2* mutations respond well to β-blocker therapy, some of our patients thus required more aggressive intervention (pacemaker and/or implantable cardiac defibrillator). Actually, bichamber implantable cardiac defibrillator treatment might be the preferred treatment in affected individuals.[5]

At the moment, a wide debate exists about the regulatory mechanism of RYR2 in calcium ion handling.[14–20] It centers around the question whether a mutation in RYR2 alters the affinity for the stabilizing molecule FKBP12.6.[18–20] Despite this debate about the role of FKBP12.6 in RYR2 handling, it is commonly agreed from various functional studies that RYR2 mutations lead to a gain of function that sensitizes the RYR2 to a premature release of calcium from the intracellular stores.[18–20] All of the previous functional studies were performed with various missense mutations in which drastic changes in the conformation of RYR2 were not expected. This conformation is more likely to be distorted when a large chunk of *RYR2* is deleted, as found in the present study. Functional studies with our reported *RYR2* deletion might provide clear insight into the role of the FKBP12.6 in RYR2 handling. Interestingly, despite divergence in clinical phenotypes, CPVT is the common phenotype between all of the described phenotypes. An intriguing question is how this deletion of 35 peptides in RYR2 leads to the pathogenesis of extra features like DCM, SAN, AF, and atrial standstill. Is it only a defect in calcium ion handling or does it involve any other interacting partners in the complex to elicit the divergence in clinical phenotypes?

Alu repeat–mediated genomic deletion that causes different diseases has already been described.[21–22] However, no such deletion has been reported in any ion channel genes, which could well be possible as *Alu* exons are interspersed elements in the whole genome. Our in vitro experiment elucidated that the short 35-nucleotide direct repeats at the end of the upstream and downstream *Alu* elements caused polymerase slippage during chromosomal replication, which deleted the region in between, in this case *RYR2* exon 3. The present finding is novel in that it elucidates and recapitulates 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 *RYR2* elicits a divergence in phenotype that includes DCM, SAN, AF, and atrial standstill combined with CPVT. Furthermore, MLPA testing in *RYR2* is currently not routine practice in a diagnostic setting, but the importance of identification of the causative mutation in individual patients and their relatives pleads for inclusion of MLPA analysis in the routine genetic testing of those individuals in whom a mutation has been excluded by the current exon-scanning methods.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Catecholaminergic polymorphic ventricular tachycardia is a disease characterized by ventricular arrhythmias elicited by adrenergic stress and a high probability for premature sudden cardiac death. Baseline ECGs are typically normal except for marked bradycardia, which is often present. We describe 2 families, with long-term follow-up, who shared these phenotypic characteristics but showed additional abnormalities in sinoatrial node function and atrioventricular nodal function, atrial fibrillation, and atrial standstill. Furthermore, left ventricular dysfunction and dilatation was present in several affected individuals. The disease locus was mapped by linkage analysis to a 4-cM region on chromosome 1q42-q43 that included the Ryanodine receptor 2 gene (*RYR2*). However, conventional polymerase chain reaction–based screening did not reveal a mutation in *RYR2*. Subsequently, multiplex ligation-dependent probe amplification and long-range polymerase chain reaction identified a genomic deletion that involved *RYR2* exon 3. This abnormal sequence segregated in all the affected family members (n=16) in both unlinked families. Further investigation revealed that the genomic deletion occurred in both families as a result of *Alu* repeat–mediated polymerase slippage. This is the first report on a large genomic deletion in *RYR2*, which, in addition to the catecholaminergic polymorphic ventricular tachycardia signature arrhythmias, leads to extended clinical phenotypes (eg, sinoatrial node and atrioventricular node dysfunction, atrial fibrillation, atrial standstill, and dilated cardiomyopathy).

Expanding Spectrum of Human *RYR2*-Related Disease: New Electrocardiographic, Structural, and Genetic Features

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