

A Mutation in *CALM1* Encoding Calmodulin in Familial Idiopathic Ventricular Fibrillation in Childhood and Adolescence

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- Objectives** This study aimed to identify the genetic defect in a family with idiopathic ventricular fibrillation (IVF) manifesting in childhood and adolescence.
- Background** Although sudden cardiac death in the young is rare, it frequently presents as the first clinical manifestation of an underlying inherited arrhythmia syndrome. Gene discovery for IVF is important as it enables the identification of individuals at risk, because except for arrhythmia, IVF does not manifest with identifiable clinical abnormalities.
- Methods** Exome sequencing was carried out on 2 family members who were both successfully resuscitated from a cardiac arrest.
- Results** We characterized a family presenting with a history of ventricular fibrillation (VF) and sudden death without electrocardiographic or echocardiographic abnormalities at rest. Two siblings died suddenly at the ages of 9 and 10 years, and another 2 were resuscitated from out-of-hospital cardiac arrest with documented VF at ages 10 and 16 years, respectively. Exome sequencing identified a missense mutation affecting a highly conserved residue (p.F90L) in the *CALM1* gene encoding calmodulin. This mutation was also carried by 1 of the siblings who died suddenly, from whom DNA was available. The mutation was present in the mother and in another sibling, both asymptomatic but displaying a marginally prolonged QT interval during exercise.
- Conclusions** We identified a mutation in *CALM1* underlying IVF manifesting in childhood and adolescence. The causality of the mutation is supported by previous studies demonstrating that F90 mediates the direct interaction of CaM with target peptides. Our approach highlights the utility of exome sequencing in uncovering the genetic defect even in families with a small number of affected individuals. (J Am Coll Cardiol 2014;63:259–66) © 2014 by the American College of Cardiology Foundation

Sudden unexplained death in childhood refers to the unexpected (witnessed or unwitnessed) death of an apparently healthy child over 1 year of age that remains unexplained after a complete post-mortem examination. Most sudden deaths in children and young adults have a primary cardiac cause and occur in the setting of congenital heart disease

(mostly in children <2 years of age), primary rhythm disease, and cardiomyopathy (1,2). Most of these sudden cardiac death (SCD) syndromes are dominantly inherited

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Abbreviations
and Acronyms

CaM = calmodulin
CPVT = catecholaminergic polymorphic ventricular tachycardia
DNA = deoxyribonucleic acid
ECG = electrocardiogram/electrocardiographic
ICD = implantable cardioverter-defibrillator
IVF = idiopathic ventricular fibrillation
LQTS = long-QT syndrome
OHCA = out-of-hospital cardiac arrest

disorders. They are genetically heterogeneous and may be characterized by incomplete penetrance and variable expressivity (3,4).

Widely recognized forms of primary rhythm disorders associated with SCD in childhood exist, including the long-QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT), both characterized by specific electrocardiographic (ECG) features (5). However, at times, SCD from ventricular fibrillation (VF) may occur in the absence of recognized ECG

features; this is referred to as idiopathic ventricular fibrillation (IVF). IVF is difficult to track genetically because the affected status of an individual only becomes apparent following an arrhythmic event. This limits the number of family members available for genetic studies, hindering gene discovery with consequent major implications for genetic testing and risk stratification.

Using an exome sequencing approach, we here identify a mutation in calmodulin encoded by *CALM1* as the causative genetic defect in a family of Moroccan descent presenting with IVF in childhood and adolescence.

Methods

Clinical data analysis. Clinical data of family members, including medical records, ECGs, 24-h Holter recordings, exercise ECGs, and echocardiographic data, were collected and evaluated. Family members who experienced (aborted) sudden cardiac arrest were considered affected. The QT interval was measured using the tangent method (6) and by averaging 3 consecutive beats in lead II or V5. The QT interval was corrected for heart rate using Bazett's formula ($QT_c = QT/\sqrt{RR}$). QT_c duration was considered prolonged if it was >450 ms (males) and >460 ms (females). LQTS scores based on the Schwartz criteria (based on QT_c interval and clinical and family history) were calculated (7,8). Written informed consent was obtained from all participants or their guardians. Peripheral blood samples for deoxyribonucleic acid (DNA) isolation were collected from 7 individuals across 2 generations.

Candidate gene analysis. Prior to exome sequencing, extensive candidate gene analysis was carried out for mutations in genes commonly involved in primary arrhythmia syndromes and arrhythmogenic cardiomyopathy, including *DPP6*, *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, *RYR2*, *CASQ2*, *KCNJ2*, *TMEM43*, *JUP*, *DSG2*, *TNNT2*, and *PKP2*. These genes were polymerase chain reaction-amplified using primers complementary to flanking intronic sequences, and the purified polymerase chain reaction products were Sanger

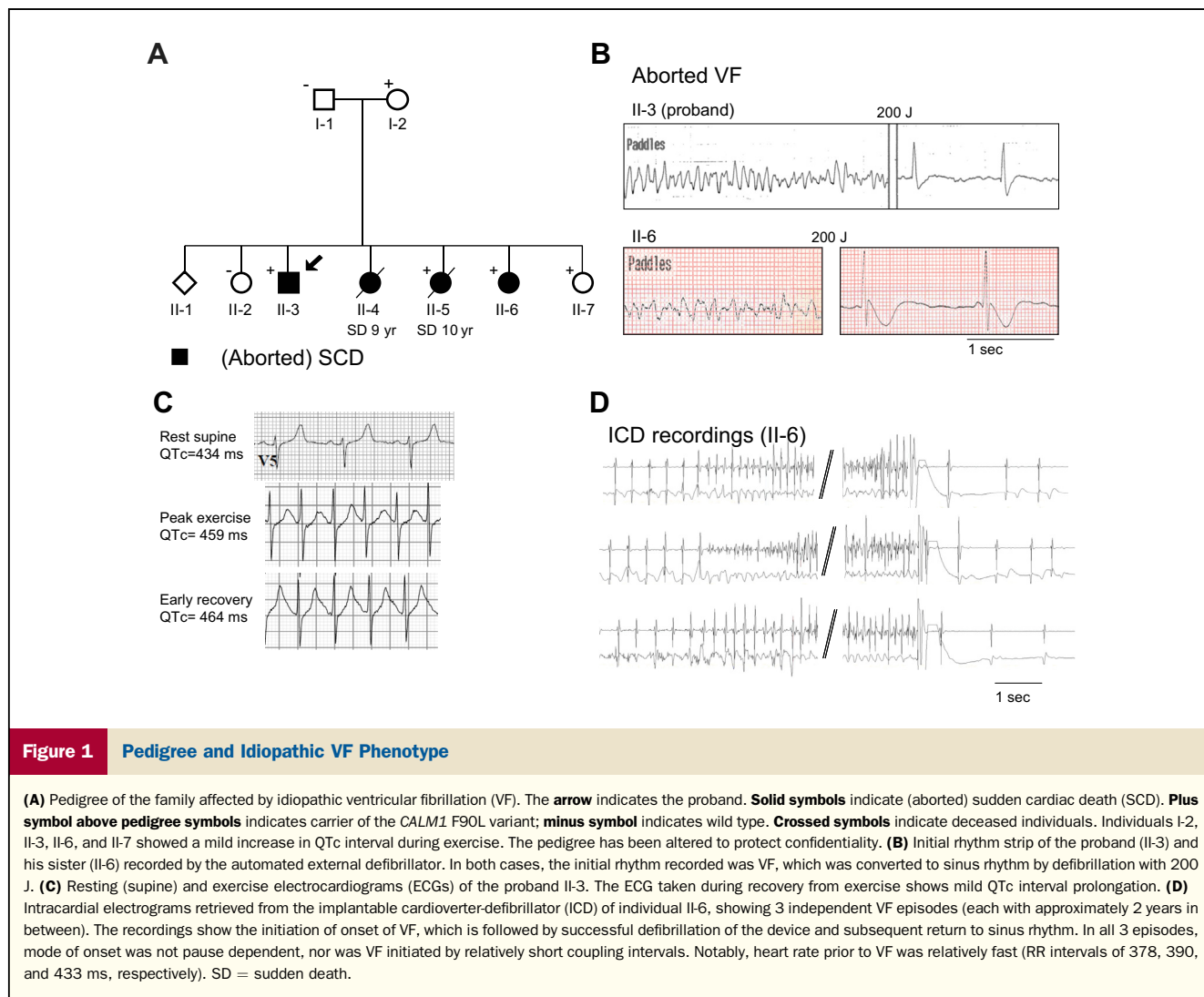
sequenced using BigDye Terminator 3.1 chemistry (Applied Biosystems, Foster City, California).

Exome sequencing. Exome sequencing was carried out on the proband and his sister, who were both successfully resuscitated from a cardiac arrest (subjects II-3 and II-6). Exome sequencing was carried out at the Beijing Genomics Institute (BGI) (Shenzhen, China). In brief, coding exons were captured from 3 μ g of randomly fragmented genomic DNA using the Agilent SureSelect Human All Exon kit following the manufacturer's protocol (Agilent Technologies, Santa Clara, California). Sequencing was carried out on a HiSeq2000 sequencer (Illumina, San Diego, California) for 90-bp paired-end read sequencing to allow a minimum coverage of 75 fold.

Data analysis. Sequence reads were aligned to the human reference genome (UCSC NCBI37.1/hg19) using SOAP-aligner (9) (version 2.21), and functional annotation of high-quality variants was performed using SOAPsnp software for the single nucleotide variation. For insertion/deletion detection, sequence reads were aligned by BWA (10) and annotated by GATK (11) for break-point identification. The Knime4Bio tool (12) was used to manage and filter variants. Synonymous variations that were not located at splice sites were excluded from further analysis. Heterozygote and homozygote coding and splice site variations of each sequenced patient were compared with publicly available variant databases, namely: 1) dbSNP132; 2) Phase 1 version 3 of the 1000 Genomes project (data release October 2012); 3) NHLBI GO Exome Sequencing Project (13); 4) GoNL (14); 5) the 69 genomes from Complete Genomics; and 6) an in-house exome database from the BGI. Genetic variants found in any of these databases were excluded from further analyses. The 2 exomes were subsequently compared and genetic variations found in both patients were retained. **Validation and segregation.** Variants found in both exomes were validated and tested for segregation in the family by Sanger sequencing on an ABI PRISM 3730 DNA Sequencer (Applied Biosystems, Foster City, California). A total of 500 control individuals of Moroccan descent were tested for the presence of the *CALM1* p.F90L variant by Sanger sequencing.

Results

Clinical data and pedigree. The proband, individual II-3 in the pedigree (Fig. 1A), experienced out-of-hospital cardiac arrest at age 16 years while romping with a classmate at school. The initial recorded rhythm was VF that converted to sinus rhythm after 2 defibrillatory shocks (Fig. 1B). He underwent comprehensive clinical evaluation, and baseline testing was unremarkable. Transthoracic echocardiography, cardiac magnetic resonance imaging, and cardiac catheterization did not reveal any structural or functional abnormalities. His 12-lead ECG showed a sinus rhythm of 74 beats/min, normal conduction intervals, and a QT_c interval of 411 ms. Holter monitoring showed 4 ventricular



extrasystoles in a 24-h period. Flecainide provocation testing did not uncover a Brugada ECG pattern. Invasive electrophysiological testing showed normal sinus node and atrioventricular node parameters. Polymorphic ventricular tachycardia that deteriorated into VF was induced with 3 extrastimuli at the right ventricular outflow tract. On exercise testing, the proband reached a maximal heart rate of 176 beats/min, and mild prolongation of the QTc interval was revealed, which was maximal during early recovery (464 ms) (Fig. 1C). The patient received an implantable cardioverter-defibrillator (ICD). During 12 years of follow-up, the patient did not report any syncopal episodes nor did the ICD record any episode of (non-)sustained ventricular tachycardia. Genetic testing was negative for mutations in genes commonly involved in primary arrhythmia syndromes and arrhythmogenic cardiomyopathy.

Just 7 months following the index event of the proband, his younger sister (II-5) died suddenly at age 10 years. ECGs taken at 3 and 6 months before her death showed no abnormalities (QTc = 443 ms, HR = 88 beats/min)

(Online Fig. 1). The family history included a sister (II-4) who died suddenly at the age of 9 years. Another sister (II-6) collapsed while playing at the playground at the age of 10 years and was successfully resuscitated from VF (Fig. 1B). Her ECG revealed a normal QTc interval (QTc = 421 ms, HR = 70 beats/min). She was placed on beta-blocker therapy, and an ICD was implanted. During a follow-up of 8 years, she experienced 3 episodes of VF, all of which were terminated by ICD shocks (Fig. 1D). The youngest sister (II-7) has thus far been asymptomatic and with an unremarkable cardiac evaluation. She was implanted with an ICD at age 7 years, which has not discharged during a follow-up period of 3 years. Both parents are asymptomatic and show no ECG abnormalities at rest. However, upon exercise testing, the mother (I-2) demonstrated prolonged QTc intervals at high heart rates (QTc = 476 ms, HR = 121 beats/min).

None of the individuals displayed significant arrhythmias during 24-h Holter monitoring or exercise testing. All family members that were tested displayed a maximal heart

rate response to exercise. No prominent U waves were observed at rest or during exercise testing. Based on the Schwartz criteria (7,8), all individuals had a low probability of having LQTS. Maximal Schwartz scores of 0.5 were calculated based on the family history of unexplained sudden cardiac death below age 30 years.

In summary, the phenotype of this family is characterized by mild QTc prolongation in the recovery phase after exercise and idiopathic ventricular fibrillation before the age of 17 years (Table 1, Online Fig. 2).

Exome sequencing and variant filtering. The presentation in this family could be compatible with 2 forms of inheritance: either an autosomal recessive pattern or an autosomal dominant pattern with reduced penetrance. In an attempt to uncover the underlying genetic cause of sudden cardiac death in this family, whole-exome sequencing was performed on 2 successfully resuscitated young patients (II-3 and II-6). On average, this yielded more than 85 million reads per sample, 91% of which could be mapped. The mean coverage of the target region was ~80-fold, with over 90% of target regions covered by ≥10 reads (Online Table 1). Over 27,000 small insertion-deletions and single nucleotide (including synonymous, missense, nonsense and splice site) variations were identified in each of the individuals (Table 2).

IVF is a highly malignant disorder, and underlying disease-causing variants are under such strong negative selection that they are unlikely to be detected in public single nucleotide polymorphism (SNP) or exome databases. We thus next filtered out genetic variants found in public, exome and genome databases (dbSNP132, Phase 1 version 3

of the 1000 Genomes project, exome variant server, GoNL, the 69 genomes from Complete Genomics, and an in-house exome database from BGI). This identified 397 and 367 novel variations in the 2 successfully resuscitated young patients: II-3 and II-6, respectively (Table 2). We next looked for the variants that the 2 individuals shared, and we kept the 66 heterozygous variants in 66 different genes that they shared. No variants were shared homozygously among the 2 individuals, which might have been compatible with an autosomal recessive model of inheritance.

Validation and segregation of candidate variants. Of the 66 variants shared between the 2 resuscitated patients (II-3 and II-6), 11 variants were excluded as they occurred in pseudogenes or were mapped to regions that are duplicated in the genome and, therefore, also likely involve pseudogenes. The remaining 55 variants were validated by Sanger sequencing (primers are available upon request). Of these, only 9 variants were also carried by the sibling who died suddenly (II-5) as well as by the mother (I-2). We next investigated the 9 genes harboring these variants for a potential role in cardiac function by conducting a literature search (assessing expression data, insight into function and involvement in disease) and by noting the presence among the 4,054 genes comprising the Cardiovascular Gene Ontology Annotation Initiative gene list (15) (Table 3). This allowed us to exclude 6 genes that have been previously related to a noncardiac disease/phenotype (*AIRE* [16,17], *CLAPIN1* [18], *C10orf112/AD7* [19–21], *SEMA5A* [22–24], *TTC3* [25] and *ZNF764* [26]). Of note, the nucleotides affected by these 6 variants also displayed low genomic

Table 1 Clinical Characteristics of the Family

	I-1	I-2	II-1	II-2	II-3	II-4	II-5	II-6	II-7
Sex	M	F		F	M	F	F	F	F
SCD (age, yrs)						Y (9)	Y (10)		
VF (age first event, yrs)					Y (16)			Y (10)	
Schwartz score	0.5	0.5	NA	0.5	0.5	NA	0.5	0.5	0.5
Age at ECG, yrs	41	45	NA	23	27	NA	9	18	9
Resting QT (ms)	400	360	NA	334	371	NA	372	380	320
Resting heart rate (beats/min)	67	78	NA	83	82	NA	88	77	104
Resting QTc (ms)	423	410	NA	392	434	NA	449	430	421
Age at exercise ECG	NA	57	NA	23	28	NA	NA	19	10
Peak exercise									
QT (ms)	NA	265	NA	225	268	NA	NA	268	245
HR (beats/min)	NA	151	NA	191	176	NA	NA	169	182
QTc (ms)	NA	420	NA	401	459	NA	NA	450	428
Early recovery*									
QT (ms)	NA	335	NA	263	284	NA	NA	291	282
HR (beats/min)	NA	121	NA	149	160	NA	NA	159	162
QTc (ms)	NA	476	NA	414	464	NA	NA	474	463
Late recovery†									
QT (ms)	NA	365	NA	308	320	NA	NA	316	311
HR (beats/min)	NA	92	NA	108	118	NA	NA	129	131
QTc (ms)	NA	452	NA	413	447	NA	NA	464	460
ICD (implanted at age, yrs)					Y (16)			Y (10)	Y (7)

Schwartz scores ≤1 indicate low probability for long-QT syndrome. *Early recovery indicates 1 min of recovery; †late recovery indicates 4 min of recovery. ECG = electrocardiogram; HR = heart rate; ICD = implantable cardioverter-defibrillator; QTc = corrected QT interval; SCD = sudden cardiac death; VF = ventricular fibrillation.

Table 2 Summary of Variant Filtering

	II-3	II-6
Coding single-nucleotide variants (exons, exon boundaries, splicing)	27,958	28,001
Homozygous	9,171	9,090
Heterozygous	18,787	18,911
Synonymous variants filtered out	17,285	17,290
Novel variants	397	367
Homozygous	5	6
Heterozygous	392	361
Shared variants by the 2 patients		66
Homozygous		0
Heterozygous		66
Not in duplicated regions or in pseudogenes		55
Carried by II-5 and I-2		9
Variants in genes not involved in other phenotypes		3
Carried by II-7 and not by II-2		1

evolutionary rate profiling scores (27) (Table 3), indicating low evolutionary conservation.

Of the remaining 3 variants (in *CALM1* [28,29], *CLTC* [30], and *ZFYVE9* [31]; in fact the only 3 genes of the original 66 shared variants that are listed in the Cardiovascular Gene Ontology Annotation Initiative gene list), only 1 variant, in the *CALM1* gene, was also inherited by the youngest sister (II-7), who displayed borderline QTc interval prolongation during exercise, and was not inherited by individual II-2. Notably, the genomic position affected by the *CALM1* variant displays high evolutionary conservation, supporting its importance (32). In addition, the amino acid residue affected by the mutation (phenylalanine at position 90 (F90)) is highly conserved throughout evolution (from yeast to mammals) (Figs. 2A and 2B). Finally, we excluded the presence of the *CALM1* variant in 500 control individuals of Moroccan descent.

Discussion

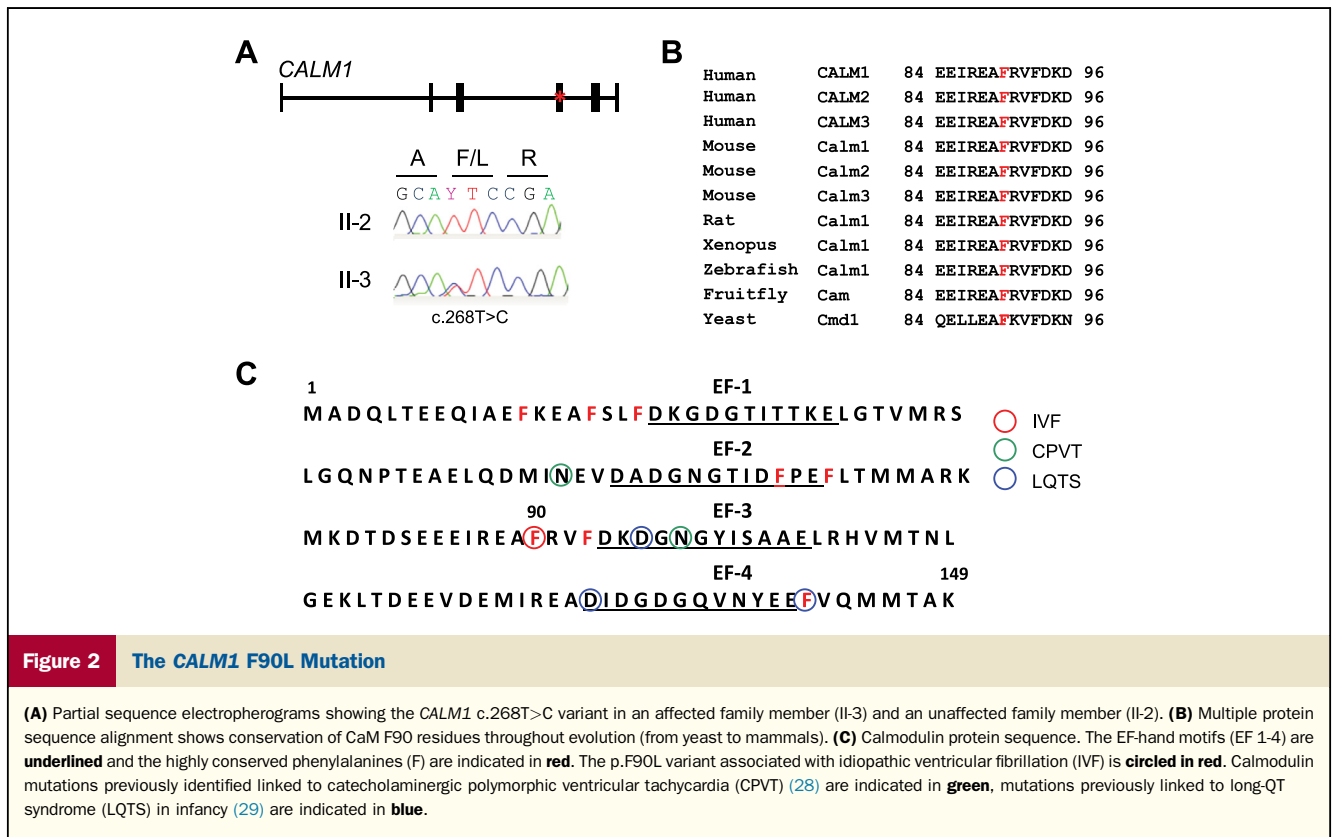
IVF, a rare rhythm disorder, is characterized by VF in the absence of ECG abnormalities or structural heart disease is characterized by VF in the absence of ECG abnormalities or structural heart disease. Due to this elusive clinical presentation, the disorder is difficult to track genetically. To date, only 1 haplotype encompassing the *DPP6* gene showed evidence for strong genetic linkage to the disease (33). Using whole-exome sequencing, we have, for the first time, linked a mutation in *CALM1* to IVF presenting in childhood and adolescence.

CALM1 encodes calmodulin (CaM), a ubiquitously expressed, evolutionarily conserved protein that is an essential regulator of calcium-dependent processes in virtually every cell type (34). Two other genes (*CALM2* and *CALM3*), each located on different chromosomes, encode CaM. Although the coding regions differ to some extent, as do the regulatory flanking noncoding regions, the protein product of these 3 genes is identical.

Table 3 The 9 Variants Shared by Individuals I-2, II-3, II-5, and II-6

Chr	Position (Hg19)	Ref Allele	Alt Allele	Gene	Amino Acid Change/Splice Site	Expression	CV GO List	Physiological Role	Pathophysiological Involvement	GERP Score	Ref. #
chr1	52704299	T	G	ZFYVE9	L404V	Muscle, brain, pancreas, heart, kidney, placenta, and lung	Yes	TGFβ signaling	Unknown	1.44	28
chr5	9154609	C	T	SEMA5A	R491H	Brain and heart	No	Neuronal development	Autism (OMIM 609297)	-2.13	19-21
chr10	19678186	G	A	C10orf112	A897T	Brain, placenta, and heart	No	Unknown	Alzheimer (OMIM 606187)	2.34	16-18
chr14	90870295	T	C	CALM1	F90L*	Ubiquitously expressed	Yes	Regulator of calcium-dependent processes	Catecholaminergic polymorphic ventricular tachycardia (OMIM 614916); LQTS (ref 26)	5.22	25-26
chr16	30567369	C	A	ZNF764	D125Y	Unknown	No	Hormonal regulation	Hormonal dysregulation	1.06	23
chr16	57474740	G	A	CIAPIN1	A34V	Ubiquitously expressed	No	Apoptosis processes	Hematopoiesis defect (OMIM 608943)	-9.96	15
chr17	57754310	C	T	CLTC	splice site	Ubiquitously expressed	Yes	Endocytosis	Unknown	-3.01	27
chr21	38568081	A	G	TTC3	S1775G	Spleen, thymus, prostate, and ovary	No	Neuronal development	Down syndrome (OMIM 602255)	-0.84	22
chr21	45713720	G	A	AIRE	V11M	Lymph nodes, spleen, leukocytes, cartilage, bone, and skin (not in heart)	No	Immunity	APECED syndrome (OMIM 240300)	1.86	13-14

*Only this variant was also carried by II-7 who displayed borderline QTc interval prolongation during exercise and not by individual II-2. Alt = alternative; Chr = chromosome; CV GO list = Cardiovascular Gene Ontology Annotation Initiative gene list; GERP score = genomic evolutionary rate profiling score, ranging from -12.3 to 6.17 (low > high conservation) (27); LQTS = long-QT syndrome; Ref = reference.



Mutations in *CALM1* and *CALM2* have recently been linked to 2 other primary rhythm disorders (28,29). In 1 study, Nyegaard et al. (28) linked mutations (N53I and N97S) in *CALM1* with early onset CPVT. In another study, Crotti et al. (29) identified 3 de novo mutations in *CALM1* (D130G and F142L) and *CALM2* (D96V) in 4 infants presenting with severe ventricular arrhythmias and markedly prolonged QTc intervals (>600 ms) in the first year of life (29). A number of features distinguish the clinical presentation in the family we studied from patients of these 2 studies. First, the *CALM1* mutation carriers in our study displayed no bidirectional or polymorphic ventricular tachycardia during repeated exercise tests. Furthermore, their QTc intervals were normal at baseline and only became mildly prolonged with exercise. Second, in the family we studied, events to date occurred between 9 and 16 years of age. Finally, the patients studied by Crotti et al. (29) exhibited neurodevelopmental delay, whereas the patients in our study did not present with such disabilities. When considered in the light of these 2 previous studies, our findings point to the occurrence of a broad spectrum of cardiac rhythm disease that could arise from mutations in genes encoding CaM.

CaM is a cytoplasmic molecule that can bind 4 calcium ions with high affinity at its EF-hand motifs and subsequently interact with target proteins. In the heart, CaM binds and regulates a number of ion channels and their regulators (35). Disruption of these interactions may lead to

disturbances in several processes, including excitability, excitation-contraction coupling, and refractoriness, which may cause arrhythmia. Indeed, mutations affecting CaM-binding sites (termed “IQ domains”) in ion channels have long been associated with inherited arrhythmia disorders. For instance, mutations in the IQ domain of *Kv7.1* (encoded by *KCNQ1*), *Ca_v1.2* (*CACNA1C*), and *Na_v1.5* (*SCN5A*) have been shown to affect CaM binding and thereby affect the function of these channels to cause disease (36–39). Dysregulated binding of CaM to ion channels can have different consequences on ion channel function. These include altered calcium-sensitive gating, channel assembly, and cell surface expression (36,40,41).

CALM mutations linked thus far to CPVT or LQTS in infancy predominantly affect residues within the EF-hand domains of the protein and have been shown to affect the binding of calcium in studies conducted in vitro (28,29). In addition, abnormal interaction of CaM with the ryanodine receptor was described for the *CALM1*-N97S linked to CPVT. The mutation causing IVF in the family we describe is located between 2 EF domains, namely, EF-2 and -3 (Fig. 2C). This inter-EF hand linker contains 3 highly-conserved aromatic phenylalanine residues and is important for positioning the EF hands for high-affinity cooperative calcium binding (42,43). The mutation in our IVF family causes the substitution of 1 of these phenylalanines at position 90 for leucine. Of note, all 8 phenylalanines residues in CaM are highly

conserved throughout evolution (from yeast to mammals), and structural studies have uncovered a crucial role of each of these phenylalanines residues, including F90, in mediating direct interaction of CaM with target peptides (44,45). Although this requires further studies, one could speculate that mutations in CaM cause disease through different mechanisms that could occur through effects on calcium binding and/or binding of target proteins.

All 3 CaM genes are expressed in the human fetal, infant, and adult left ventricle (29). However, differences in the relative expression of the CaM product arising from the 3 respective genes may occur across the different compartments in the heart. Interestingly, the *CALM1* messenger ribonucleic acid transcript was found to be more abundant in Purkinje fibers as compared to the working atrial and ventricular myocardium in the nondiseased human heart (46). This raises the intriguing possibility that the *CALM1* F90L defect is more pronounced in the Purkinje system.

Of note, the mother (I-2) displayed mild exercise-induced QTc prolongation but has remained asymptomatic. This points to a role for other, possibly genetic, factors in modulation of the arrhythmia risk (47). The mild exercise-induced QTc prolongation is also found among the 2 siblings who were resuscitated. Although none of the family members met with the diagnostic criteria for LQTS (7,8), because ECG recordings are not available in a large number of mutation carriers in this family, it is difficult to rule out LQTS with certainty. Nevertheless, the occurrence of VF in 4 siblings from this family highlights the importance of the discovery of this variant in pre-symptomatic genetic diagnosis to guide therapy.

Study limitations. In this study we did not carry out functional studies on the *CALM1* F90L mutant. Such studies will be necessary to provide insight into the mechanism by which this mutation leads to the particular clinical presentation in this family.

Conclusions

Our results suggest that the *CALM1* F90L mutation, identified by exome sequencing, is causal to VF and SCD in childhood and adolescence. This study highlights the fact that exome sequencing is a powerful and efficient way to drastically reduce the number of candidate genes, even in a family with a small number of definitely affected individuals.

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Key Words: calmodulin ■ exome sequencing ■ genetics ■ idiopathic ventricular fibrillation ■ ventricular arrhythmia.

APPENDIX

For a supplemental table and figures, please see the online version of this article.