Original Article

Novel Calmodulin Mutations Associated With Congenital Arrhythmia Susceptibility

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Background—Genetic predisposition to life-threatening cardiac arrhythmias such as congenital long-QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) represent treatable causes of sudden cardiac death in young adults and children. Recently, mutations in calmodulin (*CALM1*, *CALM2*) have been associated with severe forms of LQTS and CPVT, with life-threatening arrhythmias occurring very early in life. Additional mutation-positive cases are needed to discern genotype–phenotype correlations associated with calmodulin mutations.

Methods and Results—We used conventional and next-generation sequencing approaches, including exome analysis, in genotype-negative LQTS probands. We identified 5 novel de novo missense mutations in *CALM2* in 3 subjects with LQTS (p.N98S, p.N98I, p.D134H) and 2 subjects with clinical features of both LQTS and CPVT (p.D132E, p.Q136P). Age of onset of major symptoms (syncope or cardiac arrest) ranged from 1 to 9 years. Three of 5 probands had cardiac arrest and 1 of these subjects did not survive. The clinical severity among subjects in this series was generally less than that originally reported for *CALM1* and *CALM2* associated with recurrent cardiac arrest during infancy. Four of 5 probands responded to β-blocker therapy, whereas 1 subject with mutation p.Q136P died suddenly during exertion despite this treatment. Mutations affect conserved residues located within Ca^{2+}-binding loops III (p.N98S, p.N98I) or IV (p.D132E, p.D134H, p.Q136P) and caused reduced Ca^{2+}-binding affinity.

Conclusions—*CALM2* mutations can be associated with LQTS and with overlapping features of LQTS and CPVT. **(*Circ Cardiovasc Genet*. 2014;7:466-474.)**

Key Words: calmodulin ■ long QT syndrome

Congenital long-QT syndrome (LQTS) is a recognizable and treatable genetic predisposition to sudden cardiac death in children and young adults.[1] Considerable genetic heterogeneity underlies this syndrome, although a large fraction of successfully genotyped LQTS subjects belong to 3 major subtypes (LQT1, LQT2, and LQT3) associated with mutations in 3 genes encoding plasma membrane ion channels (*KCNQ1*, *KCNH2*, and *SCN5A*, respectively). Distinct genotype-specific patterns of T-wave morphology,[2,3] triggers for cardiac events,[4] clinical outcomes,[5] and response to the epinephrine provocation test[6,7] have been observed. Importantly, genotype can also influence the response to specific drug therapy.[8–11] Genetic testing for known arrhythmia susceptibility genes has become standard of care for some disorders including LQTS, but despite the rapid progress in understanding the genetic basis, the cause remains unknown in many cases.[12] Additional studies are needed to reveal the

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missing heritable factors and to elucidate genotype–phenotype correlations.

Recently, mutations in 2 of 3 genes encoding identical peptide sequences for the essential Ca^{2+}-signaling protein calmodulin were associated with life-threatening arrhythmia predisposition, including malignant forms of LQTS, catecholaminergic polymorphic ventricular tachycardia (CPVT), and idiopathic ventricular tachycardia.[13–15] Nyegaard et al[13] identified 2 distinct missense *CALM1* (p.N54I, p.N98S) mutations in association with CPVT. Crotti et al[14] used exome sequencing and targeted resequencing to discover novel *CALM1* (p.D130G, p.F142L) and *CALM2* (p.D96V) missense mutations in subjects with infantile or perinatal presentations of severe LQTS associated with recurrent cardiac arrest. Most recently, Marsman et al[15] identified a novel *CALM1* mutation (F90L) segregating with idiopathic ventricular tachycardia and sudden death in a Moroccan family. Although this limited number of calmodulin mutations suggests preliminary genotype–phenotype correlations, additional mutations are needed to establish the spectrum of clinical features and severity of arrhythmia phenotypes associated with calmodulin mutations.

Here we report the discovery of 5 novel de novo missense *CALM2* mutations associated with congenital arrhythmia susceptibility in probands of varying ancestry. The mutations alter conserved residues that directly coordinate Ca^{2+} ions in the carboxyl-terminal domain of calmodulin and cause significant reductions in Ca^{2+}-binding affinity. Clinical and electrophysiological findings in these subjects suggested that *CALM2* mutations can be associated with less severe forms of LQTS compared with our previous report[14] as well as with overlapping clinical features of LQTS and CPVT.

Methods

Study Subjects

The QT interval was corrected for heart rate using Bazett formula ($QTc=QT/\sqrt{RR}$), and the diagnosis of LQTS was made by the Schwartz criteria.[1] All individuals who participated in the study gave written informed consent before genetic and clinical investigations in accordance with the standards of the Declaration of Helsinki and the local ethics committees at each participating institution. We studied 2 Japanese cohorts, 1 consisting of 12 unrelated LQTS subjects who were without a genetic diagnosis after sequencing genes previously associated with life-threatening arrhythmias (*KCNQ1*, *KCNH2*, *SCN5A*, *SCN1B*, *SCN2B*, *SCN3B*, *SCN4B*, *KCNE1*, *KCNE2*, *KCNJ2*, and *CAV3*)[13,14,16] and another cohort consisting of 190 unrelated patients with LQTS in whom whole exome sequencing was performed. Exome sequencing was performed on a parent/child trio in which the proband was a child who suffered cardiac arrest at age of 17 months. A German mutation-negative LQTS proband and a Moroccan girl with sudden cardiac death were also screened for mutations in *CALM1*, *CALM2*, and *CALM3*.

Candidate Gene and Exome Sequencing

Targeted exon capture was performed for 240 candidate arrhythmia susceptibility genes (Table I in the Data Supplement) using the SureSelect Target Enrichment System according to the manufacturer's suggestions (Agilent Technologies, Inc., Santa Clara, CA). The captured DNA was sequenced on the Genome Analyzer IIx platform (Illumina Inc., San Diego, CA) with paired-end reads of 101 bp for insert libraries consisting of 150 to 200 bp fragments. On average for targeted capture sequencing, 1.1 Gbp of short-read sequence data were generated and 98.9% were mapped to the reference human genome. Whole exome capture was performed using Agilent SureSelect Human All Exon V4 reagent, and captured DNA was sequenced on Illumina HiSeq2000 (performed at RIKEN) or HiSeq2500 (performed at Vanderbilt University) platforms. For data obtained on the HiSeq2000, an average of 6.4 Gbp of short-read sequence data were generated, with 98.6% mapped successfully to the reference human genome and 66-fold average coverage for all captured exons. For data obtained on the HiSeq2500, an average of 5.8 Gbp was generated per subject with 99.8% mapped and 50-fold average coverage.

Sequence Data Analysis

Sequence reads were mapped to the human reference genome (GRCh37) using the Burrows-Wheeler Aligner (version 0.6.1).[17] Possible duplicate reads were removed using SAMtools[18] and custom software, leaving an average of 0.8 and 5.5 Gb for targeted capture and exome sequencing, respectively. More than 93% of targeted regions were covered by ≤10 reads. After filtering by pair mapping distance, mapping uniqueness, and orientation between paired reads, the mapping result files were converted into the pileup format using SAMtools.[18] Variant calling was conducted in part on the basis of published methods.[19–21] We further used the following quality control filters: (1) alignments near putative indels were refined using GATK,[22] and (2) a strand bias filter excluded variants whose alternative allele was preferentially found on 1 of the 2 available read orientations at the site. Variants found in dbSNP Build 137, 1000 Genomes,[23] or Exome Variant Server (EVS)[24] databases were excluded from further analyses. Synonymous and intronic (other than canonical splice sites) variants were also excluded. Three other exome databases (RIKEN database of 731 noncardiac disease Japanese exomes, Human Genetic Variation Browser database including exome data obtained from 1208 Japanese subjects [http://www.genome.med.kyoto-u.ac.jp/SnpDB], and the Institute of Human Genetics Helmholtz Zentrum München database of >3000 exomes of European ancestry) were also queried for candidate mutations.

Additional Mutation Detection

Targeted PCR–Sanger sequencing was performed as described previously[14] on DNA from a German woman with clinical features of LQTS and CPVT, as well as in a Moroccan girl with sudden cardiac death and a presumptive diagnosis of CPVT to search for variants in the coding exons of *CALM1*, *CALM2*, and *CALM3*. Variants discovered by exome sequencing were also confirmed by Sanger sequencing using an automated capillary electrophoresis DNA sequencing platform (Applied Biosystems, Foster City, CA), then further annotated based on evolutionary amino acid conservation (Mutation Taster),[25] and predicted impact on protein function (Polyphen2, SIFT).[26,27] Mutation position in calmodulin was based on RefSeq NP_001734 counting the predicted translational start codon (Met) as position 1.

Expression of Recombinant Calmodulins and Measurement of Ca^{2+} Affinity

Biochemical studies of recombinant calmodulin proteins were performed as previously described.[14] Briefly, recombinant wild-type and mutant calmodulins were expressed in *Escherichia coli* and purified by standard chromatographic approaches. Macroscopic affinity constants for Ca^{2+} binding in the amino-terminal and carboxy-terminal domains were determined by measuring changes in intrinsic fluorescence as reported by Shea et al.[28,29] The data were analyzed by plotting the normalized fluorescence signal versus free Ca^{2+} concentration and fitting to a 2-site Adair function for each domain.[30,31]

Results

Case Presentations

Case 1

A 6-year-old Japanese girl was admitted to the hospital for evaluation of syncope and a markedly prolonged QT interval. She had a history of fetal bradycardia but had an uneventful birth. She had her first episode of syncope at the age of 19 months. An ECG at that time showed marked QT prolongation (QTc=579 ms) with atypical notched, late-peaking T waves (Figure 1A). Atrial pacing at 100 bpm prolonged QTc from 596 to 619 ms, whereas mexiletine shortened QTc from 596 to 550 ms (Figure 1B). Subsequently, she experienced multiple episodes of cardiac arrest during exertion when she failed to take mexiletine, prompting placement of an implantable cardioverter defibrillator (ICD) at the age of 14 years. Medical therapy with mexiletine and a β-adrenergic receptor blocker atenolol was generally effective in preventing ventricular arrhythmias, although there was an episode of appropriate ICD discharge that occurred during exertion. The patient had no history of seizures or developmental delay. Genetic testing for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *SCN1B*, *SCN2B*, *SCN3B*, *SCN4B*, *KCNE1*, *KCNE2*, *KCNJ2*, and *CAV3* was negative. There was no family history of LQTS or sudden death, and both parents had normal QTc intervals (father 369 ms, mother 394 ms) as did her 2 brothers (368, 388 ms).

Case 2

A 5-year-old Japanese boy had an episode of syncope with seizure while running. Two months later, he had a similar episode and was evaluated in an emergency room. An ECG exhibited QTc prolongation (478 ms; Figure 2A), whereas echocardiogram, electroencephalogram, and brain MRI were normal. He showed no developmental delay. There was no family history of arrhythmias or sudden death, and both parents (father 364 ms, mother 396 ms) and his brother (340 ms) had normal QTc intervals. Epinephrine infusion test did not induce ectopic beats but caused marked QTc prolongation (baseline heart rate/QT/QTc, 56 bpm/484 ms/466 ms; peak heart rate/QT/QTc, 94 bpm/446 ms/558 ms; steady-state heart rate/QT/QTc, 73 bpm/484 ms/535 ms; Figure 2B). This subject did not tolerate exercise testing because of dizziness. Genetic testing for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, *KCNJ2*, and *AKAP9* was negative. Treatment with propranolol alone or in combination with mexiletine shortened the QTc interval to 471 to 473 ms (Figure 2C), but he continued to experience syncope and dizziness while running. The drugs were replaced with metoprolol at the age of 11 years. Subsequently, he had no further episodes of syncope, and there was normalization of the QTc (449 ms) with elimination of the notch in the descending limb of the T wave (Figure 2C).

Case 3

A 29-year-old German woman, who was previously diagnosed with perinatal bradycardia and neonatal LQTS, had been treated with pindolol. Her family history was negative for cardiac arrest and sudden death. On β-blocker therapy, she remained asymptomatic until the age of 9 years when she suffered syncope while swimming after an interruption of therapy. At that time, there was evidence of exercise-induced polymorphic ventricular ectopy. She became asymptomatic for several years after resumption of treatment with various β-blockers (pindolol, propranolol, atenolol). Her resting ECG exhibited QT prolongation (465–578 ms) with persistent biphasic T waves in leads III, aVF, aVL, V3, and negative T waves in V4 to V6 (Figure 3A). The patient never suffered seizures, and she had normal physical and mental development. At the age of 22 years, exercise-induced polymorphic ventricular ectopy and one 3-beat run of polymorphic ventricular tachycardia was documented (Figure 3B; Figure I in the Data Supplement). Echocardiographic evaluation was normal, but MRI revealed features consistent with noncompaction of the left ventricle myocardium. Both parents had normal QTc intervals (father 407 ms, mother 377 ms) with no signs of polymorphic ventricular arrhythmias. Directed screening of genes involved with LQTS and CPVT (*KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, *KCNJ2*, *ANK2*, *CAV3*, *KCNE3*, *SNTA1*, *RYR2*, *CASQ2*) was negative for mutations.

Case 4

A Moroccan girl from a family with no history of cardiac arrhythmia was hospitalized at the age of 8 years after an episode of syncope associated with prolonged period of unconsciousness. At that time, she had a prolonged QTc interval (500 ms) with ventricular bigeminy. A Holter recording demonstrated prolonged QTc interval (ECG images were not available). Echocardiographic evaluation was normal. Both parents and 4 female siblings were asymptomatic. The subject was

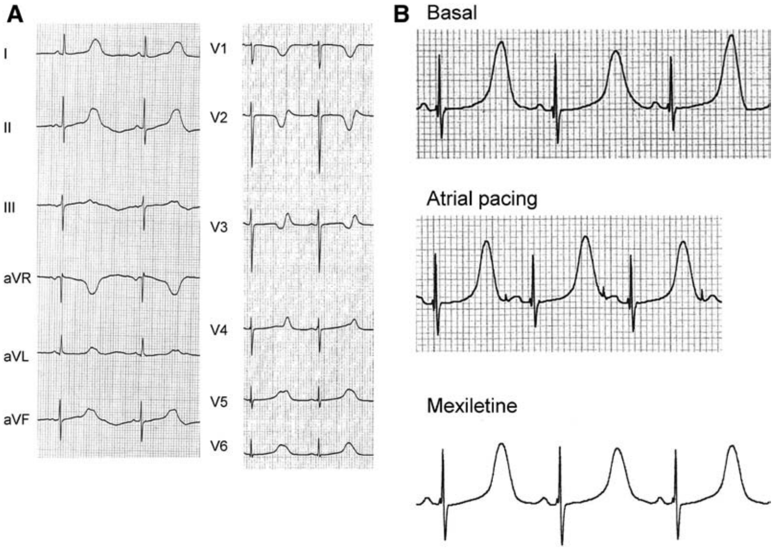

Figure 1. Electrocardiographic abnormalities in case 1. **A**, Standard 12-lead ECG recorded at age 6 y showing marked QTc prolongation (579 ms) with atypical T-wave morphology (late-peaking with notch on the descending limb). **B**, Atrial pacing at 100 bpm prolonged QTc from 596 to 610 ms. By contrast, mexiletine treatment shortened QT interval from 596 to 550 ms.

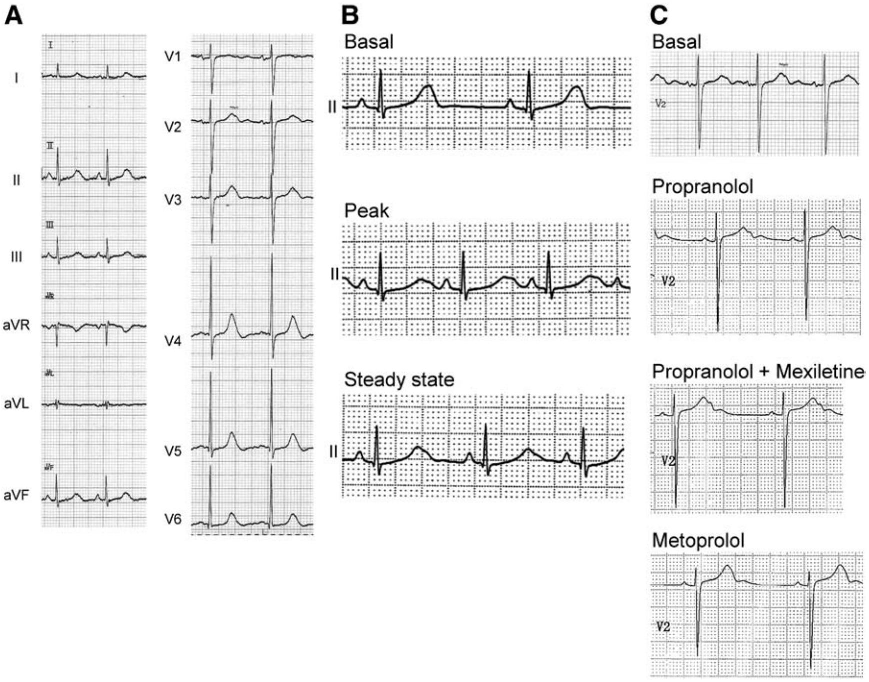

Figure 2. Electrocardiographic abnormalities in case 2. **A**, Standard 12-lead ECG recorded at age 5 y showing QTc prolongation (478 ms). **B**, Epinephrine challenge test prolonged QTc at peak (466 to 558 ms) and at steady state (535 ms). **C**, Propranolol or propranolol with mexieltine caused QTc shortening from 517 to 471 and 473 ms, respectively. Metoprolol normalized QTc to 449 ms.

treated with nadolol (40 mg per day) and she remained asymptomatic with QTc intervals ranging from 420 to 450 ms without ventricular ectopy. Unfortunately, she died suddenly at the age of 11 years while dancing at a wedding in Morocco. The initial diagnosis was LQTS, and later a diagnosis of CPVT was considered because of clinical circumstances and ventricular ectopy. No exercise stress test was performed. No neurological dysfunction was reported and a head computed tomographic scan was normal. Genetic testing was negative for *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *RYR2*, *CASQ2*, and *TRDN*.

Case 5

A previously healthy white boy from England suffered cardiac arrest secondary to ventricular fibrillation at the age of 17 months, and he was promptly resuscitated. ECG showed bradycardia and a prolonged QTc interval (555 ms; Figure 4). There was no family history of cardiac arrhythmia, and both parents were healthy with normal QTc interval duration. There were no siblings. An ICD was placed soon after the cardiac arrest, and no discharges were documented over the ensuing 13 months. The subject was also treated with β-blockers. Genetic testing for mutations *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2* was negative.

A summary of clinical features observed in the 5 cases is presented in Table.

Discovery of Novel *CALM2* Mutations

To identify mutations in candidate arrhythmia susceptibility genes, a custom targeted exon capture probe panel

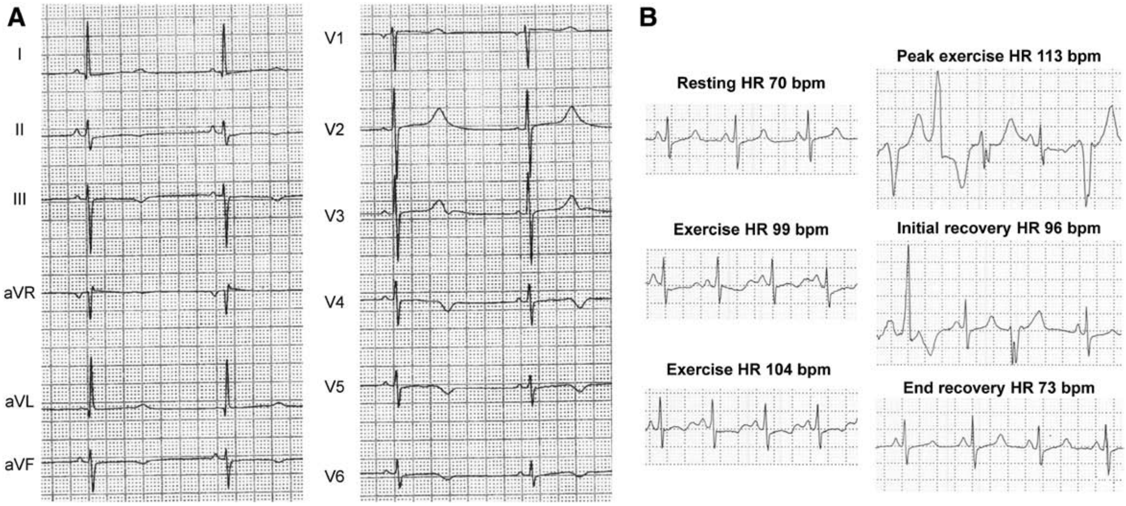

Figure 3. Electrocardiographic features of case 3. **A**, Standard 12-lead ECG recorded at age 27 y showing QTc prolongation (567 ms). **B**, Polymorphic ventricular ectopy recorded (lead III) during exercise (step test) at age 28 y. During exercise, a progressive increase of heart rate was observed with no arrhythmias until 110 bpm was reached. A 3-beat episode of polymorphic ventricular tachycardia was recorded at 113 bpm. A representative 12-lead ECG during exercise is provided as Figure I in the Data Supplement.

interrogating 240 genes (Table I in the Data Supplement) was used to screen 12 unrelated mutation-negative Japanese LQTS probands using a next-generation sequencing platform resulting in an average 187-fold coverage of targeted regions (additional details of the method will be reported elsewhere). A heterozygous nonsynonymous single-nucleotide variant (c.400G>C; Figure 5A) in exon 5 of *CALM2* was identified in a 6-year-old girl (described above as case 1). The nucleotide change predicts the substitution of a conserved aspartic acid residue with histidine (p.D134H) within the fourth EF-hand Ca^{2+}-binding motif in the C-terminal domain of the encoded calmodulin protein. The location of this variant within the protein was 4 residues away from a mutation (p.D130G) previously associated with a very severe form of infantile LQTS (Figure 5B and 5C).[14] This variant was not found in her parents nor her 2 brothers and is absent in dbSNP, 1000 Genomes, EVS, RIKEN, and Human Genetic Variation Browser exome databases consistent with a novel de novo mutation.

Motivated by this finding, we searched for other calmodulin gene (*CALM1*, *CALM2*, *CALM3*) mutations in exome sequence data (coverage was 35X, 92X, and 59X for the 3 calmodulin genes, respectively) obtained from 190 unrelated mutation-negative Japanese LQTS probands. A second heterozygous nonsynonymous variant (c.293A>G; Figure 5A) was found in *CALM2* exon 5 in a 5-year-old boy (described above as case 2) diagnosed with LQTS. This variant is predicted to replace a conserved asparagine at position 98 with serine (p.N98S) within the third EF-hand Ca^{2+}-binding motif in calmodulin (Figure 5B and 5C). Interestingly, de novo p.N98S mutation in a different calmodulin gene (*CALM1*) was previously associated with CPVT in an Iraqi female child without QT prolongation.[13] This variant was not found in her parents or brother and was absent in exome data of the other 189 LQTS probands as well as in databases of genetic variation (dbSNP, 1000 Genomes, EVS, RIKEN, and Human Genetic Variation Browser exome databases) consistent with a de novo missense mutation. The probability that 2 private nonsynonymous mutations occurred in *CALM2* among 190 Japanese samples by chance was estimated as 0.0258 (see the Data Supplement).

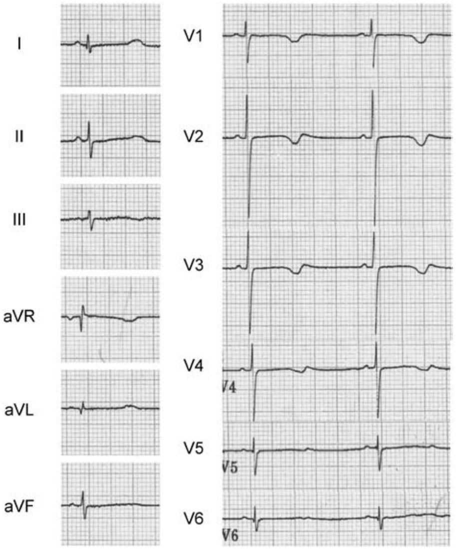

Figure 4. Electrocardiographic features of case 5. Standard 12-lead ECG recorded at age 2 y showing QTc prolongation (555 ms) and bradycardia (heart rate [HR]=55 bpm) during β-blocker treatment. Because of bradycardia, only 1 beat was recorded in the limb leads.

Exome sequencing was also performed on a 17-month-old English boy who suffered cardiac arrest in the context of LQTS (case 5) and his healthy parents (coverage averaged 30X for the 3 calmodulin genes). After excluding all variants shared with at least 1 parent, synonymous variants and common variants, the proband was found to have de novo nonsynonymous variants in 4 protein-coding genes (*CALM2*, *OBSCN*, *DLG1*, *GOLGA3*). However, only the variant identified in *CALM2* (c.A293>T; Figure 5A) predicting substitution of asparagine at position 98 in calmodulin with isoleucine (p.N98I; Figure 5B and 5C) was predicted to be deleterious by SIFT and probably damaging by PolyPhen-2. This variant occurs at the same position as the *CALM2* mutation discovered in case 2 (LQTS) and a previously reported *CALM1* mutation (N98S) found in a child with CPVT.[13] *CALM2*-p.N98I was absent in the previously mentioned databases.

By candidate gene screening of *CALM1*, *CALM2*, and *CALM3*, we identified 2 other heterozygous missense *CALM2* variants. One variant was discovered in a 29-year-old German woman who was diagnosed initially with neonatal LQTS and later exhibited exercise-induced polymorphic ventricular ectopy (case 3). The variant (c.396T>G; Figure 5A) predicted the replacement of a conserved aspartic acid residue at position 132 with glutamate (p.D132E) within the fourth EF-hand Ca^{2+}-binding motif in calmodulin (Figure 5B and 5C). The location of the variant is 2 amino acids N-terminal of p.D134H (case 1) and 2 residues C-terminal of the previously identified p.D130G.[14] This variant was predicted to be damaging by SIFT and Mutation Taster, whereas it was predicted to be benign by Polyphen2. The mutation was not found in the aforementioned databases of genetic variants (dbSNP, 1000 Genomes, EVS) and was also absent in the Helmholtz exome database in which mean coverage of *CALM2* was greater than 95-fold.[14] The variant was not found in her parents and, therefore, D132E was considered a likely novel de novo missense *CALM2* mutation.

The second *CALM2* variant discovered by targeted sequencing was found in an 8-year-old Moroccan girl (case 4) with presumptive diagnoses of LQTS and CPVT who died suddenly during exertion despite ongoing treatment with β-blockers. The variant (c.A407>C; Figure 5A) predicted the replacement of glutamine at position 136 with proline (p.Q136P) in the fourth EF-hand Ca^{2+}-binding motif (Figure 5B and 5C). The mutation was not found in the aforementioned databases of genetic variants (dbSNP, 1000 Genomes, EVS) and was absent in the parents and 4 siblings consistent with a de novo mutation.

CALM2 Mutations Confer Impaired Ca^{2+} Affinity

We previously demonstrated that calmodulin mutations associated with early-onset LQTS confer reduced affinity for Ca^{2+}.[14] Similarly, Nyegaard et al[13] examined Ca^{2+} affinity for *CALM1* p.N98S, which they observed in a de novo case of CPVT and found a slight depression in C-domain Ca^{2+}-binding affinity. To determine the biochemical consequences

Table. Summary of Clinical Characteristics and the *CALM2* Mutations of the Probands

Subject	Sex	Age at Diagnosis (Current Age)	CA	QTc, ms	Treatment	Mutation
Case 1	F	1 y (16 y)	Yes	579	MEX, BB, ICD	*CALM2*-p.D134H
Case 2	M	5 y (12 y)	No	478	MEX, BB	*CALM2*-p.N98S
Case 3	F	Perinatal (29 y)	No	578	BB	*CALM2*-p.D132E
Case 4	F	8 y (died at age 11 y)	SCD	500	BB	*CALM2*-p.Q136P
Case 5	M	17 mo (30 mo)	Yes	555	BB, ICD	*CALM2*-p.N98I

BB indicates β-blocker; Abbreviations: CA, cardiac arrest; F, female; ICD, implantable cardioverter defibrillator; M, male; MEX, mexiletine; PVC, premature ventricular contractions; PVT, polymorphic ventricular tachycardia; and SCD, sudden cardiac death.

of the 4 novel *CALM2* mutations we identified, recombinant calmodulin proteins were generated and purified, and in vitro Ca^{2+} binding affinities were measured. None of the 4 mutations significantly affected Ca^{2+} affinity in the N-domain, but substantial effects on affinity in the C-domain were observed (Figure 6). Dissociation constants for Ca^{2+} (K_d) of 2.1±0.1, 15±1, 48±10, 27±5, and 19±2 µmol/L were determined for wild-type, N98I, D132E, D134H, and Q136P, respectively, corresponding to a 7- to 23-fold reduction in Ca^{2+}-binding affinity to the C-domain. These data demonstrate a significant functional impairment caused by the novel calmodulin variants consistent with disease-causing mutations that will likely disrupt the ability to transduce intracellular Ca^{2+} signals leading to cardiac arrhythmia susceptibility.

Discussion

The identification of new arrhythmia susceptibility genes and mutations will facilitate the prevention of sudden cardiac death through the rapid identification of at-risk populations and may illuminate new molecular targets for therapy. Here we expand the spectrum of mutations in calmodulin, a recently demonstrated cause of life-threatening heart rhythm disorders.

Calmodulin functions as a Ca^{2+} sensor in a wide range of intracellular Ca^{2+}-signaling pathways. The protein sequence is completely conserved among all vertebrates, and in humans, 3 unique genes (*CALM1*, *CALM2*, *CALM3*) encode for identical calmodulin protein.[32] In the recent reports of human calmodulin gene mutations, there was only 1 *CALM2* allele identified compared with 5 *CALM1* mutations.[13–15] The previously identified calmodulin mutations associated with LQTS phenotypes along with those we report here affect conserved residues within the 2 EF-hand motifs of the C-domain and cause substantially impaired Ca^{2+} affinity. The mutations with the greatest impact on Ca^{2+} affinity involve substitutions of conserved aspartic acid residues (D130G, D132E, D134H) known to be directly involved in coordinating Ca^{2+} ions in Ca^{2+}-binding

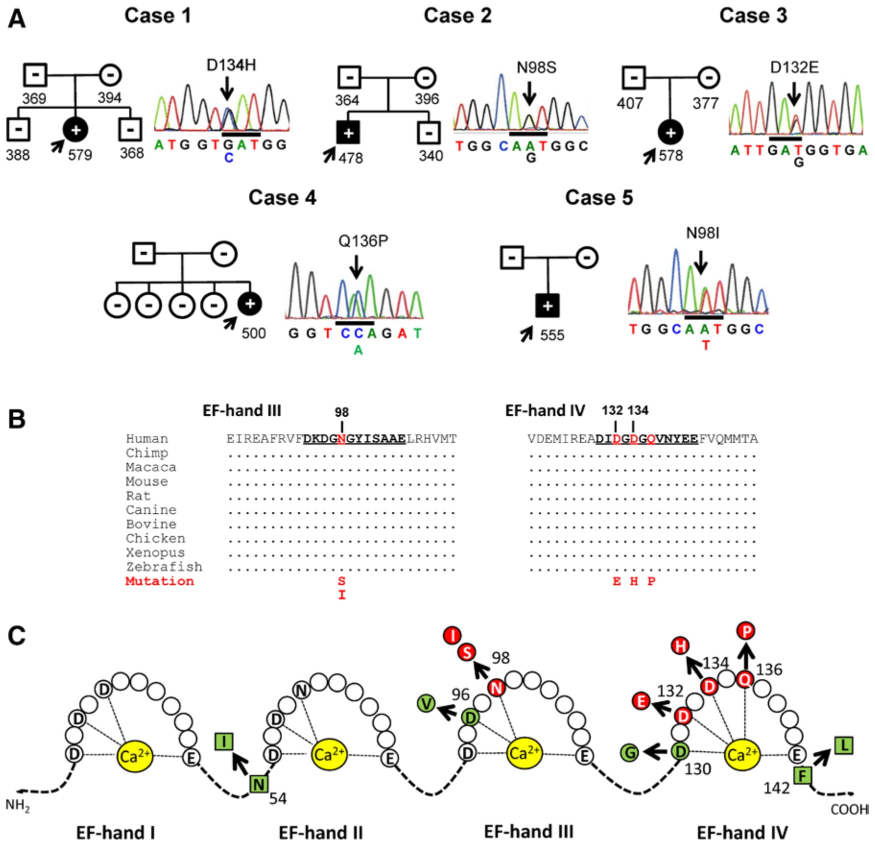

Figure 5. Novel de novo *CALM2* mutations. **A**, Pedigrees and Sanger sequence electropherograms of each proband (marked by arrow). QTc values for each individual are shown underneath the corresponding pedigree symbol. **B**, Amino acid sequence alignments for calmodulins from different species with location of the 5 missense mutations. Amino acid sequence of EF-hands III and IV are underlined. **C**, Schematic model of Ca^{2+} binding loops in the N-terminal (I and II) and C-terminal (III and IV) domains of calmodulin showing the locations of mutations. Red circles represent the *CALM2* mutations (p.N98S, p.N98I, p.D132E, p.D134H, p.Q136P) identified in our present study; green symbols represent previously reported mutations.

loop IV.[33,34] Notably, even substitution with the highly similar glutamic acid side chain in D132E has an influence on Ca^{2+} affinity. Similar effects of this subtle Asp to Glu mutation on the Ca^{2+} affinity of calmodulin have been reported.[35] Although a functional effect was not predicted in silico by PolyPhen2, it is well established that each residue in the Ca^{2+}- binding loops of calmodulin and other EF-hand proteins contribute to the biochemical functions of the protein.[36]

The cellular mechanisms responsible for arrhythmia susceptibility in the setting of calmodulin mutations are likely to be complex given the multitude of molecular interactions possible for this critically important signaling molecule. As previously speculated for LQTS,[14] dysfunctional calmodulin may disrupt Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels leading to increased depolarizing current during the plateau phase of the cardiac action potential. Impaired regulation of voltage-gated sodium channels may also be evoked by certain LQTS-associated calmodulin mutations. For calmodulin mutations associated with CPVT, aberrant regulation of the sarcoplasmic reticulum ryanodine receptor/Ca^{2+} release channel (*RYR2*) is a plausible mechanism based on previous studies.[37] Interestingly, the CPVT mutations do not impair Ca^{2+} affinity to the same extent as those associated with LQTS.[13] The molecular and cellular pathophysiology of arrhythmia susceptibility in the setting of calmodulin mutations is currently under intense investigation.[38–40]

Our findings further expand the phenotypic spectrum of cardiac arrhythmias associated with calmodulin mutations. Three of the probands (cases 1, 2, 5) had a later onset of LQTS compared with what was described in the study by Crotti et al,[14] in which calmodulin mutation-positive subjects had highly malignant ventricular arrhythmias beginning very early in life. Furthermore, none of the *CALM2* mutation-positive subjects we report here had significant neurological findings, other than syncope-associated seizures (case 1), in contrast to the original report in which most subjects had seizures or developmental delays. The previously observed neurological impairments were speculated to be the result of brain injury secondary to hypoxia in the setting of recurrent cardiac arrest. The minimal or absent neurological symptoms in the probands we describe here may reflect fewer episodes of cardiac arrest or more rapid resuscitation. These new observations further imply that neurological symptoms may not be an intrinsic manifestation of calmodulin mutations.

Genotype–phenotype correlations among the calmodulin mutation-positive subjects we described may provide clues to the pathophysiological mechanisms. In particular, *CALM2*-p.D132E was identified in an adult with a history of neonatal LQTS who later developed exercise-induced polymorphic ventricular arrhythmia consistent with CPVT. Similarly, *CALM2*-p.Q136P was identified in a child with LQTS and ventricular ectopy somewhat suggestive of CPVT. We speculate that the combined clinical features of LQTS and CPVT reflect the impact of p.D132E and possibly p.Q136P on 2 principal molecular targets. Abnormal calmodulin regulation of L-type Ca^{2+} channels would account for impaired myocardial repolarization similar to Timothy syndrome,[41] whereas dysregulation of *RYR2* would lead to altered regulation of intracellular Ca^{2+} homeostasis as expected in CPVT.[42,43]

Our study also revealed that an identical amino acid substitution in 2 distinct calmodulin genes can present with different clinical phenotypes. Whereas *CALM1*-p.N98S was originally found in an Iraqi female with CPVT,[13] we identified *CALM2*-p.N98S in a Japanese male with an unambiguous LQTS phenotype (case 2). The physiological basis for this genotype–phenotype disparity is unknown, but may involve differences in the corresponding proteomes of different probands because of sex or ethnicity, or differences in regional or cell type–specific expression of *CALM1* and *CALM2*.

Except during periods of medication noncompliance, all *CALM2* mutation-positive probands described in this report were responsive to β-blockers administered alone or in combination with mexiletine. However, because of recurrent cardiac arrests during treatment lapses, cases 1 and 5 had implantation of an ICD. In our prior report of calmodulin mutations in severe LQTS, probands with de novo *CALM1* or *CALM2* mutations experienced arrhythmia recurrence on pharmacological therapy and were eventually treated with ICD implantation to reduce the risk for sudden cardiac death.[14] Similarly, symptomatic mutation-positive subjects with idiopathic ventricular tachycardia reported by Marsman et al[15] had ICD implantation. By contrast, the Swedish family segregating CPVT with mutation *CALM1*-N54I described by Nyegaard et al[13] exhibited variable responses to β-blockers alone, and only 1 of 10 living mutation-positive subjects received an ICD. None of the cases we report here underwent left cardiac sympathetic denervation.

In conclusion, we report discovery of 5 novel de novo *CALM2* mutations in association with LQTS and exertion-induced arrhythmias. The encoded mutant calmodulin proteins have impaired C-domain Ca^{2+}-binding affinity that will presumably cause dysfunction in Ca^{2+} signaling with resulting

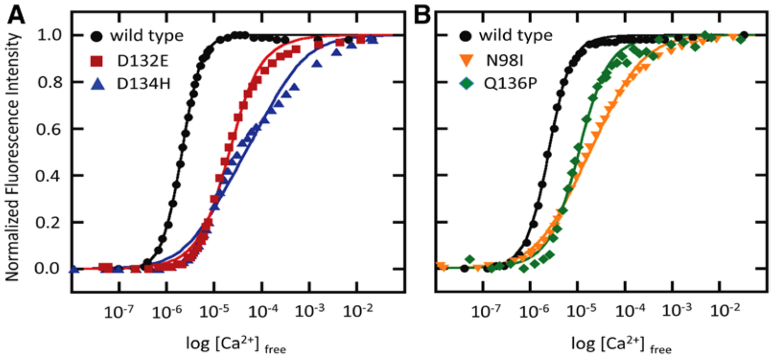

Figure 6. Impaired Ca^{2+} binding by mutant calmodulin C-domains. **A**, Representative Ca^{2+} titrations monitored by intrinsic fluorescence for recombinant wild-type (WT; black circles) and mutant calmodulins (D132E, blue triangles; D134H, red squares). K_d values derived by fitting data from 3 replicates to a 2-site model were (mean±SEM) 2.1±0.1, 48±10, and 27±5 μmol/L for WT, D132E, and D134H, respectively. **B**, Representative Ca^{2+} titrations for WT (black circles) and mutant (N98I, inverted orange triangles; Q136P, green diamonds) calmodulins. K_d values derived by fitting data from 3 replicates to a 2-site model were (mean±SEM) 2.3±0.1, 19±2, and 15±1 μmol/L for WT, N98I, and Q136P, respectively.

adverse effects on plasma membrane ion channels, intracellular membrane ion channels, or possibly both. Therapy with β-blockers was successful in preventing life-threatening exertion-triggered arrhythmias. Our study provides new evidence of congenital arrhythmia susceptibility caused by calmodulin mutations, provides further information regarding genotype–phenotype correlation, and expands the allelic diversity within *CALM2*. Calmodulin gene mutations should be sought in pediatric cases of LQTS and CPVT for whom other genetic candidates have been excluded. Because of the predominance of de novo mutations, calmodulin genes could be considered especially when both parents are unaffected.

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Disclosures

None.

Appendix

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CLINICAL PERSPECTIVE

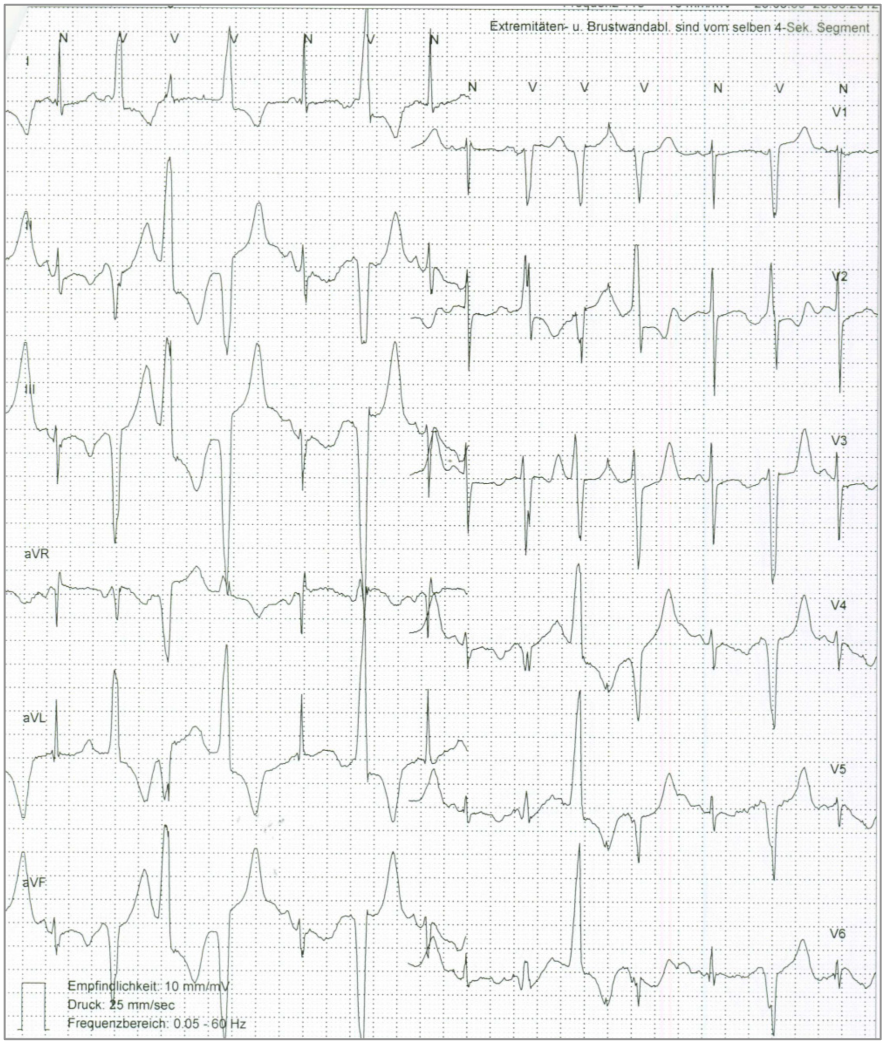
Calmodulin is an essential Ca^{2+}-binding protein that transduces intracellular Ca^{2+} signals to influence activity of ion channels, kinases, and other target proteins necessary for cardiac function. The essential nature of calmodulin is emphasized by its conservation and redundancy among vertebrates and in the recent discovery of calmodulin mutations in life-threatening genetic arrhythmia syndromes. In humans, 3 unique genes (*CALM1*, *CALM2*, *CALM3*) encode for identical calmodulin proteins, and mutations have been described in 2 of these genes (*CALM1*, *CALM2*) associated with either congenital long-QT syndrome or catecholaminergic polymorphic ventricular tachycardia. Previously, only 1 mutation was identified in *CALM2* and here we contribute additional genotype–phenotype correlation with discovery of 5 novel mutations in this gene. All mutations were de novo, altered highly conserved amino acid residues within Ca^{2+}-binding domains located in the carboxyl-terminal half of calmodulin and caused significant impairment of ion binding. Phenotypes of the mutation-positive subjects shared prolonged QTc intervals and a tendency for cardiac events (eg, syncope, cardiac arrest) to occur during physical exertion. Two of the 5 novel *CALM2* mutation-positive subjects also exhibited features consistent with catecholaminergic polymorphic ventricular tachycardia. Importantly, all subjects with *CALM2* mutations received β-adrenergic receptor blockers, and this therapy was successful in preventing life-threatening exertion-triggered arrhythmias. Calmodulin gene mutations should be sought in pediatric cases of long-QT syndrome and catecholaminergic polymorphic ventricular tachycardia for whom other genetic candidates have been excluded.

SUPPLEMENTAL MATERIAL

Estimation of false discovery rate

To estimate the false discovery rate, we first estimated the rate of private non-synonymous mutations in all exons of all other genes, disregarding variants archived in public and in-house databases. On the basis of this background mutation rate, we estimated the expected number of mutations found within a length equal to that of the *CALM2* exons in 190 samples. By assuming a random Poisson distribution for the expected number of mutations, we calculated the expected value of observing two private non-synonymous mutations occurring in *CALM2* among 190 samples by chance. As we observed one event of the two private non-synonymous mutations occurring in *CALM2* among 190 samples, the false discovery rate, estimated from the number of false positives, was 0.0258.

Supplemental Fig. S1 – Twelve lead ECG tracing from Case 3 illustrating exercise-induced polymorphic ventricular ectopy and arrhythmia (HR = 126 bpm).



Supplemental Table S1 - Candidate arrhythmia susceptibility genes interrogated by custom DNA capture and next-generation sequencing.

Gene	Protein
ABCC8	SUR1 (Co-assembles with Kir 6.2 to form K_{ATP})
ABCC9	SUR2A (Co-assembles with Kir 6.2)
ACADVL	very long-chain acyl CoA dehydrogenase
ACE	angiotensinogen-1 converting enzyme
ACOT7	acyl-CoA thioesterase 7
ACTC1	actin, alpha, cardiac muscle 1
ACTN2	actinin, alpha 2
ADRA1A	adrenergic, alpha-1A, receptor
ADRA1B	adrenergic, alpha-1B, receptor
ADRA2A	adrenergic, alpha-2A, receptor
ADRA2B	adrenergic, alpha-2B, receptor
ADRA2C	adrenergic, alpha-2C, receptor
ADRB1	adrenergic, beta-1, receptor
ADRB2	adrenergic, beta-2, receptor, surface
ADRB3	adrenergic, beta-3, receptor
ADRBK1	adrenergic, beta, receptor kinase 1
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
AGTR1	angiotensin II receptor, type 1
AKAP6	A-kinase protein anchor 6
AKAP7	A-kinase protein anchor 7
AKAP9	A-kinase protein anchor 9 (yotiao)
AMPD1	adenosine monophosphate deaminase 1
ANK1	ankyrin 1
ANK2	ankyrin 2
ANKRD1	cardiac ankyrin repeat domain 1
ANKRD46	ankyrin repeat domain 46
ARHGAP24	Rho-GTPase-activating protein 24
ASF1A	ASF1 anti-silencing function 1 homolog A
ASPH	cardiac junctin/junctate/aspartate beta-hydroxylase (alternative splice variants)
ASTN2	astrotactin 2
ATP1A1	ATPase, Na^+/K^+ transporting, alpha 1 polypeptide
ATP1A2	ATPase, Na^+/K^+ transporting, alpha 2 polypeptide
ATP1A3	ATPase, Na^+/K^+ transporting, alpha 3 polypeptide
ATP1B1	ATPase, Na^+/K^+ transporting, beta 1 polypeptide
ATP2A2	SERCA2; ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2
ATP2A3	SERCA3; ATPase, Ca^{2+} transporting, ubiquitous
ATP2B1	PMCA1; ATPase, Ca^{2+} transporting, plasma membrane 1
ATP2B2	PMCA2; ATPase, Ca^{2+} transporting, plasma membrane 2
ATP2B4	PMCA4; ATPase, Ca^{2+} transporting, plasma membrane 4
BMP2	bone morphogenetic protein 2
C18orf20	chromosome 18 open reading frame 20

C6orf204	chromosome 6 open reading frame 204
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit ($Ca_V1.2$)
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit; $Ca_V1.3$
CACNA1G	calcium channel, voltage-dependent, T type, alpha 1G subunit, $Ca_V3.1$
CACNA1H	calcium channel, voltage-dependent, T type, alpha 1H subunit; $Ca_V3.2$
CACNB1	calcium channel, voltage-dependent, beta 1 subunit
CACNB2	calcium channel, voltage-dependent, beta 2 subunit
CACNB3	calcium channel, voltage-dependent, beta 3 subunit
CACNB4	calcium channel, voltage-dependent, beta 4 subunit
CALM1	calmodulin 1 (phosphorylase kinase, delta)
CALM2	calmodulin 2 (phosphorylase kinase, delta)
CALM3	calmodulin 3 (phosphorylase kinase, delta)
CALR	calreticulin
CAMK2B	calcium/calmodulin-dependent protein kinase II beta
CAND1	cullin-associated and neddylation-dissociated 1
CAND2	cullin-associated and neddylation-dissociated 2 (putative)
CANX	calnexin
CASQ1	calsequestrin 1 (fast-twitch, skeletal muscle)
CASQ2	calsequestrin 2 (cardiac muscle)
CAV1	caveolin 1, caveolae protein, 22kDa
CAV3	caveolin 3
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CH25H	cholesterol 25-hydroxylase
CHRM2	cholinergic receptor, muscarinic 2
CHRM3	cholinergic receptor, muscarinic 3
CHRM4	cholinergic receptor, muscarinic 4
CLEC16A	C-type lectin domain family 16, member A
CMYA5	cardiomyopathy-associated protein 5
CNOT1	CCR-NOT transcription complex, subunit 1
CNOT3	CCR4-NOT transcription complex, subunit 3
CRYAB	crystallin, alpha B
CSRP3	cysteine and glycine-rich protein 3 (cardiac LIM protein)
DBC1	deleted in bladder cancer 1
DES	desmin
DKK1	dickkopf homolog 1
DPP6	dipeptidyl-peptidase 6
DSC2	desmocollin-2
DSG2	desmoglein-2
DSP	desmoplakin
DTNA	dystrobrevin
EPAS1	endothelial PAS domain protein 1
EPB41	erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)
ET1	endothelin
EYA4	eyes absent homolog 4 (Drosophila)
FAS	Fas (TNF receptor superfamily, member 6)

FHL2	four and a half LIM domains 2
FKBP1A	FK506 binding protein 1A, 12kDa
FKBP1B	FK506 binding protein 1B, 12.6 kDa (calstabin-2)
FKTN	fukutin
GATA4	GATA binding protein 4
GINS3	GINS complex subunit 3
GJA1	gap junction protein, alpha 1, 43kDa
GJA4	gap junction protein, alpha 4, 37kDa
GJA5	gap junction protein, alpha 5, 40kDa
GJC1	gap junction protein, gamma 1, 45kDa
GOT2	glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)
GPD1L	glyerol-3-phosphate dehydrogenase 1-like protein
GPR133	G protein-coupled receptor 133
GSK3B	glycogen synthase kinase 3 beta
HCN1	hyperpolarization activated cyclic nucleotide-gated potassium channel 1
HCN2	hyperpolarization activated cyclic nucleotide-gated potassium channel 2
HCN3	hyperpolarization activated cyclic nucleotide-gated potassium channel 3
HCN4	hyperpolarization activated cyclic nucleotide-gated potassium channel 4
HIP1	huntingtin interacting protein 1
HTR4	5-hydroxytryptamine (serotonin) receptor 4
ITPR1	inositol 1,4,5-triphosphate receptor, type 1
ITPR2	inositol 1,4,5-triphosphate receptor, type2
ITPR3	inositol 1,4,5-triphosphate receptor, type 3
JPH2	junctophilin 2
JUP	junction plakoglobin
KCNA5	potassium voltage-gated channel, shaker-related subfamily, member 5
KCNAB1	potassium voltage-gated channel, shaker-related subfamily, beta member 1
KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2
KCNB1	potassium voltage-gated channel, Shab-related subfamily, member 1; K_V2.1
KCNB2	potassium voltage-gated channel, Shab-related subfamily, member 2; K_V2.2
KCND3	potassium voltage-gated channel, Shal-related subfamily, member 3; K_V4.3
KCNE1	potassium voltage-gated channel, Isk-related family, member 1; minK peptide
KCNE2	potassium voltage-gated channel, Isk-related family, member 2; MiRP1
KCNE3	potassium voltage-gated channel, Isk-related family, member 3; MiRP2
KCNE4	potassium voltage-gated channel, Isk-related family, member 4; MiRP3
KCNH2	potassium voltage-gated channel, subfamily H (eag-related), member 2; hERG; K_V11.1
KCNIP1	K_V channel interacting protein 1
KCNIP2	K_V channel interacting protein 2
KCNIP3	K_V channel interacting protein 3
KCNIP4	K_V channel interacting protein 4
KCNJ11	potassium inwardly-rectifying channel, subfamily J, member 11
KCNJ12	potassium inwardly-rectifying channel, subfamily J, member 12
KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2
KCNJ3	potassium inwardly-rectifying channel, subfamily J, member 3
KCNJ4	potassium inwardly-rectifying channel, subfamily J, member 4

KCNJ5	potassium inwardly-rectifying channel, subfamily J, member 5
KCNJ8	potassium inwardly-rectifying channel, subfamily J, member 8
KCNN2	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2
KCNN3	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3
KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member 1; K_V7.1
KCNV1	potassium channel, subfamily V, member 1; K_V8.1
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAMP2	lysosome-associated membrane glycoprotein 2
LDB3	LIM domain binding 3
LIG3	ligase III, DNA, ATP-dependent
LITAF	DNA-binding protein
LMNA	lamin A/C
MAML2	mastermind-like 2
MEIS1	Meis homeobox 1
MTMR2	myotubularin related protein 2
MTTG	transfer RNA Glycine
MTTI	transfer RNA Isoleucine
MYBPC3	myosin binding protein C, cardiac
MYH16	myosin, heavy chain 16 pseudogene (MYH5)
MYH6	myosin, heavy chain 6, cardiac muscle, alpha
MYH7	myosin, heavy chain 7, cardiac muscle, beta
MYL2	myosin, light chain 2, regulatory, cardiac, slow
MYL3	myosin, light chain 3, alkali; ventricular, skeletal, slow
MYLK2	myosin light chain kinase 2
MYOZ2	myozenin 2
MYPN	myopalladin
NDRG4	NDRG family member 4
NEURL	neuralized homolog (Drosophila)
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NKX2-5	NK2 transcription factor related, locus 5 (Drosophila)
NOS1AP	nitric oxide synthase 1 (neuronal) adaptor protein
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)
NPHP4	nephronophthisis 4
NPPA	natriuretic peptide precursor A
NPPB	natriuretic peptide precursor B
ORAI1	ORAI calcium release-activated calcium modulator 1
PITX2	paired-like homeodomain 2
PKP2	plakophilin-2
PLEKHG5	pleckstrin homology domain containing, family G (with RhoGef domain) member 5
PLN	phospholamban
PMVK	phosphomevalonate kinase
POU1F1	POU domain, class 1, transcription factor 1
PPP2R3A	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha
PPP3CC	protein phosphatase 3, catalytic subunit, gamma isozyme

PPP3R1	protein phosphatase 3 (formerly 2B), regulatory subunit B, alpha isoform
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit
PRKCE	protein kinase C, epsilon
PSEN1	presenilin 1
PSEN2	presenilin 2
PTPN11	protein tyrosine phosphatase, non-receptor type 11
PTRF	polymerase I and transcript release factor
RAF1	V-RAF-1 murine leukemia viral oncogene homolog 1
RBM20	RNA binding motif protein 20
RETN	resistin
RFFL	ring finger and FYVE-like domain containing 1
RNF19A	ring finger protein 19A
RNF207	ring finger protein 207
RYR1	ryanodine receptor 1 (skeletal)
RYR2	ryanodine receptor 2 (cardiac)
S100A1	S100 calcium binding protein A1
SCN10A	Na_V1.8,sodium channel, voltage-gated, type X, alpha subunit
SCN1B	sodium channel, voltage-gated, type 1, beta
SCN2B	sodium channel, voltage-gated, type II, beta
SCN3B	sodium channel, voltage-gated, type III, beta
SCN4A	Na_V1.4 sodium channel, voltage-gated, type IV, alpha subunit
SCN4B	sodium channel, voltage-gated, type IV, beta
SCN5A	Na_V1.5, sodium channel, voltage-gated, type V, alpha subunit
SCN7A	sodium channel, voltage-gated, type VII, alpha
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein
SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)
SLC22A5	OCTN2 carnitine trasnporter
SLC25A4	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4
SLC30A1	solute carrier family 30 (zinc transporter), member 1
SLC35F1	solute carrier family 35, member F1
SLC38A7	solute carrier family 38, member 7
SLC6A2	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2
SLC8A1	solute carrier family 8 (sodium/calcium exchanger), member 1
SLN	sarcolipin
SNN	stannin
SNTA1	syntrophin, alpha 1
SOS1	son of sevenless homolog 1
SOX5	SRY (sex determining region Y)-box 5
STIM1	stromal interaction molecule 1
SYNE2	spectrin repeat containing, nuclear envelope 2
TAZ	tafazzin
TBX20	T-box 20
TBX5	T-box 5
TCAP	titin-cap (telethonin)
TGFB3	transforming growth factor, beta 3

TMEM38A	transmembran protein 38A/ trimeric intracellular cation channel type A
TMEM38B	transmembrane protein 38B/trimeric intracellular cation channel type B
TMEM43	transmembrane protein 43
TMOD1	tropomodulin 1
TMPO	thymopoietin
TNFRSF17	tumor necrosis factor receptor superfamily, member 17
TNNC1	troponin C type 1 (slow)
TNNI1	troponin I type 1 (skeletal, slow)
TNNI3	troponin I type 3 (cardiac)
TNNT2	troponin T type 2 (cardiac)
TPM1	tropomyosin 1 (alpha)
TRDN	triadin
TRPM4	transient receptor potential cation channel, subfamily M, member 4
TTN	titin
TTR	transthyretin
USP25	ubiquitin specific peptidase 25
VCL	vinculin
WNT11	wingless-type MMTV integration site family, member 11
ZC3H7A	zinc finger CCCH-type containing 7A
ZFHX3	zinc finger homeobox 3

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