Calmodulin (CaM) is an essential Ca\(^{2+}\) sensing, signal-transducing protein. Calcium-induced activation of CaM regulates many calcium-dependent processes and modulates the function of cardiac L-type calcium channel (Ca\(_V\)1.2). CaM also modulates Na\(_V\)1.5 and the ryanodine receptor, RyR2. All these interactions may play a role in disease pathogenesis. Here, we determine the spectrum and prevalence of pathogenic CaM variants in a cohort of genetically elusive LQTS, and functionally characterize the novel variants.

**Methods and Results**—Thirty-eight genetically elusive LQTS cases underwent whole-exome sequencing to identify CaM variants. Nonsynonymous CaM variants were over-represented significantly in this heretofore LQTS cohort (13.2%) compared with exome aggregation consortium (0.04%; \(P<0.0001\)). When the clinical sequelae of these 5 CaM-positive cases were compared with the 33 CaM-negative cases, CaM-positive cases had a more severe phenotype with an average age of onset of 10 months, an average corrected QT interval of 676 ms, and a high prevalence of cardiac arrest. Functional characterization of 1 novel variant, E141G-CaM, revealed an 11-fold reduction in Ca\(^{2+}\)-binding affinity and a functionally dominant loss of inactivation in Ca\(_V\)1.2, mild accentuation in Na\(_V\)1.5 late current, but no effect on intracellular RyR2-mediated calcium release.

**Conclusions**—Overall, 13% of our genetically elusive LQTS cohort harbored nonsynonymous variants in CaM. Genetic testing of \(CALM1-3\) should be pursued for individuals with LQTS, especially those with early childhood cardiac arrest, extreme QT prolongation, and a negative family history. (Circ Cardiovasc Genet. 2016;9:136-146. DOI: 10.1161/CIRCGENETICS.115.001323.)

**Key Words:** calmodulin ■ long QT syndrome ■ L-type calcium channels ■ ryanodine receptor ■ sodium channels

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**Clinical Perspective on p 146**

identical 149 amino acid CaM protein,\(^5,6\) which are expressed differentially in the human heart.\(^7\)

Variants in all 3 of the calmodulin genes (\(CALM1, CALM2,\) and \(CALM3\)) have been described recently in LQTS,\(^2-6\) LQTS is a disorder of ventricular myocardial repolarization characterized by the prolongation of the heart-rate corrected QT interval (QTc) on a resting ECG, manifesting clinically as syncope, seizures, or sudden death in the setting of a structurally normal heart. In 2013, whole-exome sequencing (WES) was
utilized on 2 parent–child trios of severe cases of LQTS presenting during infancy yielding de novo variants in CALM1 and CALM2. Follow-up cohort analysis identified 2 additional LQTS patients with variants in CALM1. Since then, additional CALM2 variants have also been identified in cases of LQTS and LQTS/CPVT overlap phenotypes. Finally, a variant was identified in CALM3-encoded CaM in a severe case of LQTS.

All the identified LQTS-associated variants in CaM characterized, to date, have reduced affinity for Ca2+ and attenuated Ca, 1.2 inactivation through the loss of calcium-dependent inactivation (CDI).7,8,10,11 Here, we describe the spectrum, prevalence, and functional consequence of novel CaM variants identified within our cohort of 38 unrelated patients with genetically elusive LQTS.

Methods

Study Subjects

The study population consisted of 38 unrelated patients with clinically diagnosed LQTS with a Schwartz score of ≥3.5 (Table 1) that were referred to the Windland Smith Rice Sudden Death Genomics Laboratory at Mayo Clinic, Rochester, MN for genetic testing. All 38 patients were genotype negative for all known LQTS-susceptibility genes: AKAP9, ANKB, CACNA1C, CAV3, KCNE1, KCNE2, KCNH2, KCNJ2, KCNJ5, KCNQ1, SCN4B, SCN5A, SNTA1, and TRDN. Thus, the 38 cases tested here represent those patients that would fall within the estimated 20% remnant of patients with a clinically certain diagnosis of LQTS, yet remain without an identified genetic cause after genetic testing of all currently known LQTS susceptibility genes. This study was approved by the Mayo Foundation Institutional Review Board and informed consent was obtained for all patients.

WES With Targeted Calmodulin Gene Analysis

All 38 patients with LQTS underwent WES and subsequent calmodulin (CaM) gene (CALM1, CALM2, and CALM3)–specific analysis. Briefly, paired-end libraries were prepared using the manufacturer’s protocol (Illumina, San Diego, CA and Agilent, Santa Clara, CA) using the Bravo liquid handler from Agilent. Whole-exome capture was carried out using the protocol for Agilent’s SureSelect Human All Exon v4+UTRs kit. Exome libraries were loaded onto TruSeq Rapid run pair-end flow cells at concentrations of 9 pmol/L to generate cluster densities of 600 000 to 800 000 per mm2 using Illumina’s standard protocol using the Illumina cBot and TruSeq Rapid Paired end cluster kit version 1. The flow cells were sequenced as 100×2 paired-end reads on an Illumina HiSeq 2500 using TruSeq Rapid SBS kit. The media was replaced with fresh OPTI-MEM after 4 to 6 hours. Transfected TSA201 cells were cultured in OPTI-MEM and incubated for 48 hours and cells exhibiting green, red, and yellow fluorescence were selected for electrophysiologically experiments. Standard whole-cell patch-clamp technique was used to measure Ca, 1.2 WT currents coexpressed with WT- or E141G-CaM.

All CaM variants identified through WES were Sanger sequence verified. Primer sequences and conditions are available on request.

Functional Analysis

The novel CaM variant (p.E141G) was characterized functionally using Ca2+-binding assays and patch-clamp electrophysiological recording to assess its pathogenicity.

Generation of Recombinant CaM and Measurement of Ca2+ Binding to CaM

Wild-type (WT) and mutant CaM proteins were prepared, and the Ca2+ affinities for WT- and E141G-CaM were determined using previously described methods.7

CaM Mammalian Expression Vectors and Site-Directed Mutagenesis

The EGFP-HA-CaM (enhanced green fluorescent protein [EGFP]) vector, kindly provided by Dr Emanuel Strehler, Mayo Clinic, Rochester, MN, was used for the heterologous expression electrophysiology studies of Ca, 1.2 and Na, 1.5 in TSA201 cells. The p.E141G missense variant was engineered into EGFP-HA-CaM vector using primers containing the missense variant (available on request) in combination with the Quickchange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The integrity of all constructs was verified by DNA sequencing.

Heterologous Expression and Electrophysiological Analysis of Ca, 1.2 Coexpressed With WT- or E141G-CaM

The constructs utilized to recapitulate Ca, 1.2 have been described previously.13 TSA201 cells were cultured in Dulbecco Modification of Eagle’s Medium supplemented with 10% fetal bovine serum, 1.0% -glutamine, and 1.2% penicillin/streptomycin solution in a 5% CO2 incubator at 37°C. Heterologous expression of Ca, 1.2 was accomplished by cotransfecting 1-μg CACNA1C ([pYFP][Nα1C,77]) in pcDNA,13 1-μg CACNB2b (in pIRE2-dsRED2),13 and 1-μg CACNA2D1 (in pcDNA3.1) vectors with either 0.5 μg a green fluorescent protein empty vector (GFP-EV; kindly provided by Dr Gianrico Farrugia, Mayo Clinic, Rochester, MN), EGFP-HA-CaM-WT or EGFP-HA-CaM-E141G vectors using 9-μL lipofectamine 2000 (Invitrogen, Carlsbad, CA). The media was replaced with fresh OPTI-MEM after 4 to 6 hours. Transfected TSA201 cells were cultured in OPTI-MEM and incubated for 48 hours and cells exhibiting green, red, and yellow fluorescence were selected for electrophysiologically experiments. Standard whole-cell patch-clamp technique was used to measure Ca, 1.2 WT currents coexpressed with WT- or E141G-CaM at room temperature (22–24°C) as described in the Methods section.

Table 1. Demographics of Our Genotype Negative LQTS Cohort

| No. of Probands | 38 |
|-----------------|----|
| Age at Diagnosis, y±SD | 20±18 |
| Range | 0–65 |
| <5 y of age (%) | 9 (24) |
| ≥ 5 y of age (%) | 29 (76) |
| Males (%) | 16 (42) |
| QTc, ms ± SEM | 535±13 |
| Syncope (%) | 15 (39) |
| Cardiac arrest (%) | 13 (34) |
| Positive family history (%) | 14 (37) |

LQTS indicates long QT syndrome; and QTc, corrected QT interval.
Animal Use and Experiments in Ventricular Myocytes

The use of animals in this study was approved by the Animal Care and Use Committees of Vanderbilt University, Nashville, TN, and performed in accordance with National Institutes of Health guidelines. Single ventricular myocytes from 10- to 16-week-old C57BL/6 mice were isolated by enzymatic digestion using collagenase as previously described. Inactivation of Ca,1.2 current was studied in freshly isolated murine ventricular myocytes using whole-cell patch-clamp technique. CaM (total concentration 6 μmol/L) was used. Tau values were determined as the time it took for 50% of the CaM current to be inactivated. Events were conducted at room temperature. For mixing studies, a 1:1 mixture of WT CaM and 25% mutant CaM (total CaM concentration 6 μmol/L) was used. Tau values represent monoexponential fit of the last 350 ms of the current during depolarizing step. In addition, Ca"² release spark measurements to examine RyR2 activity were completed as previously described.

Heterologous Expression and Electrophysiological Analysis of Na,1.5 Coexpressed With WT- or E141G-CaM

To recapitulate the SCN5A-encoded Na,1.5 sodium channel, 1 μg of the human cardiac voltage-dependent Na+ channel α subunit (HS58/ Q1077del, Genbank accession no. AY148488) in the pcDNA3 vector (Invitrogen) was cotransfected with either 0.5-μg GFP-EV, EGFP-HA-CaM-WT, EGFP-HA-CaM-E141G, or 0.25-μg EGFP-HA-CaM-E141G with the use of 4-μL lipofectamine in TSA201 cells. Transfected TSA201 cells were cultured in OPTI-MEM (Invitrogen) with 10% FBS. After 24 hours, cells exhibiting green fluorescence were selected for electrophysiological experiments. Standard whole-cell patch-clamp technique was used to measure Na,1.5 WT currents coexpressed with WT-CaM, E141G-CaM, or WT-CaM+E141G-CaM (to mimic the heterozygous state of the patient) at room temperature as previously described. Late I_{Na} was measured at the end of 700-ms long depolarization.

Analytical Techniques

Data were presented as mean values±S.E.M or ±95% confidence intervals. Comparisons were made using Wilcoxon signed rank test and Student t-test where appropriate and P value <0.05 was considered significant. Chi-square test with Yates correction was utilized to derive the P value to test for the enrichment of variants in the 38 cases versus ExAC. Categorical comparisons were made using a Fisher exact test and P value <0.05 were considered significant.

Results

Genetic Analysis

Our genetically elusive LQTS cohort consisted of 38 individuals (42% males, average age at diagnosis was 20±18 years, 24% were <5 years of age). The average QTc was 535±13 ms, 39% experienced syncope, 34% cardiac arrest, and 37% had a positive family history of cardiac arrhythmias or sudden unexplained death (Table 1).

WES revealed 4 CaM missense variants, p.D130G (c.389A>G, CALM2), p.D130V (c.389A>T, CALM2), p.E141G (c.422A>G, CALM1), and 2 cases with p.F142L (c.426C>G, CALM1), in a total of 5 of 38 cases (13.2%; Table 2, Figure 1). Both p.D130V and p.E141G represent novel CaM variants. The p.D130G and p.F142L CaM variants have been characterized functionally and described previously as LQTS-susceptibility variants (Table 2). Our previously discovered CALM3-variant–positive child was not included in this cohort of unrelated cases as the child's variant had been discovered by WES and genomic triangulation (proband/parent trio) akin to the previous CALM1 and CALM2 discoveries.

The p.D130G variant was identified in a female born at term that was noted to have bradycardia (Figure 1). An ECG, recorded 12 hours after birth, revealed a QTc of 740 ms and 2:1 atrioventricular (AV) block. She was treated with β-blockers, phenytoin, spironolactone, potassium, and a single chamber pacemaker in the first week of life. At the age of 6 years, a single-chamber implantable cardioverter-defibrillator was implanted and β-blocker therapy continued. At the age of 11 and 14 years, she experienced appropriate implantable cardioverter-defibrillator discharges for ventricular fibrillation (VF). The family history was negative. Parental DNA was unavailable.

The p.D130V variant was identified in a male born with 2:1 AV block in utero (Figure 1). QT prolongation (QTc=800 ms) was documented at birth, macroscopic T-wave alternans was observed, and β-blocker therapy was initiated. Echocardiograms at the first month of life showed restrictive cardiomyopathy with borderline systolic function. In his second month of life, he developed torsades de pointes, and subsequently had a left T2–T4 sympathetic denervation and minimally invasive epicardial implantable cardioverter-defibrillator was implanted. His β-blocker was combined with sodium channel blockers. At the age of 6 years, he experienced an appropriate VF-terminating shock after a medication dose change by his parents. His echocardiograms demonstrated dilated cardiomyopathy with hypertrophy and continued QT prolongation. At the age of 7 years, he had an additional VF-terminating shock after missing a single β-blocker dose. The family history was negative. The p.D130V was absent in both parents, thus confirming a de novo occurrence in the child.

The p.E141G variant was identified in a male with his first unwatched syncopal event at 3 years of age from which he was found unconscious and spontaneously recovered (Figure 1). He experienced his first sudden cardiac arrest at 4 years of age in which cardiopulmonary resuscitation was initiated, and after 30 minutes, consciousness was regained. After his sudden cardiac arrest, an ECG revealed a QTc of 610 ms, and he was treated subsequently with β-blockers and sodium channel blockers. His QT prolongation has persisted; however, the index case is now 11-years of age and has not experienced an episode since the initiation of treatment. An echocardiogram revealed mild LV dilation; however, the cardiac valves and structure were normal. In addition, it was noted that he has speech and motor skill delay. He has a negative family history. The p.E141G was not identified in either parent confirming a de novo occurrence in the child.
thereafter, she had sudden cardiac arrest which caused anoxic brain injury and seizure-like syncopal episodes. After the initiation of this genetic investigation, the patient died suddenly. She was brought to the emergency room for altered mental status. Echocardiogram revealed severely diminished left ventricular systolic dysfunction. Soon after arrival, she deteriorated to VF and was unable to be resuscitated. Her autopsy revealed cardiomegaly with dilation and hypertrophy. She had no family history of arrhythmias, and her mother was negative for the p.F142L variant. Her father’s sample was unavailable.

Table 2. CaM Variants in LQTS and LQTS/CPVT Overlap Phenotypes

| Study          | Variants | Gene  | Disease | Sex | Race     | QTc, ms | Age at diagnosis | Events                                      | Increased Ca\(^{2+}\)-Dissociation; C Domain | Ca\(_{1.2}\) | Na\(_{1.5}\) | RyR2 |
|----------------|----------|-------|---------|-----|----------|---------|------------------|---------------------------------------------|---------------------------------------------|-----------|-----------|------|
| Crotti et al \(^7\) | p.D96V   | CALM2 | LQTS    | F   | Hispanic | 690     | Prenatal         | Cardiac arrest                              | 14-fold                                     | Loss of CDI | No effect | No effect |
| Makita et al \(^8\) | p.N98I   | CALM2 | LQTS    | M   | White–England | 555   | 17 mo           | Cardiac arrest                              | 7-fold                                       | NA         | NA         | NA      |
| Makita et al\(^8/\) Nyegaard et al \(^\text{a}\) | p.N98S   | CALM2 | LQTS/CPVT | M   | Japanese | 478     | 5 y              | Syncope during exertion                      | 1.7-fold decreased affinity                 | 1.6-fold decrease in CDI                     | NA         | Greater open probability |
| Crotti et al \(^7\) | p.D130G  | CALM1 | LQTS    | F   | White–Italy | 630     | 6 mo            | Cardiac arrest                              | 54-fold                                     | Loss of CDI | 7.5-fold increase in fetal Na\(_{1.5}\) late current | Lower binding affinity |
| Crotti et al \(^7\) | p.D130G  | CALM1 | LQTS    | M   | Grecian   | 610     | 1 mo            | Cardiac arrest                              | 54-fold                                     | Loss of CDI | 7.5-fold increase in fetal Na\(_{1.5}\) late current | Lower binding affinity |
| Reed et al \(^9\) | p.D130G  | CALM3 | LQTS    | M   | White     | 690     | Birth            | None                                         | 54-fold                                     | Loss of CDI | 7.5-fold increase in fetal Na\(_{1.5}\) late current | Lower binding affinity |
| Boczek et al\(^*\) | p.D130G* | CALM2* | LQTS*   | F   | Indian*   | 740*    | Birth*          | Cardiac arrest*                             | 54-fold*                                     | Loss of CDI* | 7.5-fold increase in fetal Na\(_{1.5}\) late current* | Lower binding affinity* |
| Boczek et al\(^*\) | p.D130V* | CALM2* | LQTS*   | M   | White*    | 800*    | Birth*          | Cardiac arrest*                             | NA                          | NA*         | NA*        | NA*    |
| Makita et al \(^8\) | p.D132E  | CALM2 | LQTS/CPVT | F   | White–Germany | 578   | <9 y            | Exercise-induced syncope                     | 23-fold                                     | NA         | NA         | NA      |
| Makita et al \(^8\) | p.D134H  | CALM2 | LQTS    | F   | Japanese | 579     | 6 y             | Cardiac arrest                              | 13-fold                                     | NA         | NA         | NA      |
| Makita et al \(^8\) | p.Q136P  | CALM2 | LQTS/CPVT | F   | Moroccan | 500     | 8 y             | Syncope–SCD age 11 y                        | 9-fold                                      | NA         | NA         | NA      |
| Boczek et al\(^*\) | p.E141G* | CALM1* | LQTS*   | M   | Indian*   | 610*    | 4 y*            | Cardiac arrest*                             | 11-fold decreased affinity*                | 7.1-fold increase in Ca\(_{1.2}\) persistent current* | 1.7-fold increase in Na\(_{1.5}\) Late current* | No effect* |
| Crotti et al \(^7\) | p.F142L  | CALM1 | LQTS    | F   | White–Italy | 600     | Prenatal?       | Cardiac arrest                              | 5-fold                                     | Loss of CDI | No effect | Lower binding affinity |
| Boczek et al\(^*\) | p.F142L* | CALM1* | LQTS*   | F   | Black*    | 612*    | Birth*          | Cardiac arrest–SCD 2 y*                    | 5-fold*                                     | Loss of CDI* | No effect* | Lower binding affinity* |
| Boczek et al\(^*\) | p.F142L* | CALM1* | LQTS*   | M   | Hispanic* | 620*    | Birth*          | SCD–1 y*                                   | 5-fold*                                     | Loss of CDI* | No effect* | Lower binding affinity* |

CDI indicates calcium-dependent inactivation; CPVT, catecholaminergic polymorphic ventricular tachycardia; LQTS, long QT syndrome; NA, when the test has not been performed; and SCD, sudden cardiac death.

*CaM-positive individuals identified in our cohort of 38 patients with LQTS.\(^{3,7-11,18}\)*
combination therapy was associated with an attenuation of his QTc down to 540 ms. Shortly after recruitment into our study at 1 year and 3 months of age, this patient died suddenly. He was placed for a nap and was found gasping for air, unconscious and blue, and unfortunately was unable to be resuscitated. Interrogation of his pacemaker showed sinus rhythm with 1:1 conduction just before a period of fast VF. He had no previous recorded events on his pacemaker. ECGs were obtained for the parents and siblings, all were normal, and both parents were negative for the p.F142L variant, supporting de novo occurrence.

Over-Representation of CaM Variants in LQTS Versus ExAC

Nonsynonymous CaM variants were over-represented significantly in our LQTS cohort (5/38; 13.2%) compared with ExAC (23/60,706; 0.04%; P<0.0001; Figure 1). The 21 rare nonsynonymous CaM variants identified in ExAC are shown in Figure 1 and Table I in the Data Supplement. Interestingly, all 8 of the pathogenic CaM residues (D96, N98, D130, D132, D134, Q136, E141, and F142)^7–9 that are affected in patients with LQTS reside within 1 of the 4 EF hands, where Ca²⁺ binds to CaM. Specifically, these pathogenic variants localize to either the third (D96 and N98) or fourth (D130, D134, Q136, E141, and F142) EF hand located in the C domain (Figure 1). Moreover, the side chains of 7 of these 8 residues interact directly with Ca²⁺. In contrast, only 4 of the 21 unique variants in ExAC (G24, I28, A103, and A104) reside within an EF hand calcium-binding loop; however, none of them involve a residue whose side chain interacts directly with calcium (Figure 1). In addition, there are 2 extremely rare protein truncating variants (G42* and D51Gfs*5) present in the ExAC browser that were identified in 2 of 60,706 individuals. However, given the severity of CaM-related LQTS, this would suggest that having a nonfunctioning CaM protein from 1 of 6 CaM-generating alleles is not pathogenic.14

Demographic and Clinical Characteristics of Patients Harboring CaM Variants

Compared with our original LQTS referral cohort that comprised 541 patients in 2005 where the average age at diagnosis was 24±16 years, the average QTc was 482±57 ms, and 12% had cardiac arrest19; Table 3 details the differences between LQT1–3–positive and LQT1–3–negative cases in this original cohort,19 the CaM-positive cases herein, and the CaM-negative cases that still remain genetically elusive. The average age of CaM-positive patients was significantly younger (0.8 years) compared with CaM-negative (23 years; P<0.01; Figure 2A; Table 3).19 In addition, the yield of CaM variants was significantly higher in patients <5 years of age (5/9, 56%) compared with patients ≥5 years (23/39, 59%).
with patients ≥5 years of age (0/29, 0%; P<0.001; Figure 2B). The average QTc was significantly longer in CaM-positive patients (676±36 ms) compared with CaM-negative patients (514±9 ms; P<0.0001; Figure 2C; Table 3). In addition, the occurrence of cardiac arrest was significantly higher in CaM-positive patients (5/5; 100%) compared with CaM-negative patients (8/33; 24%; P<0.01; Figure 2D; Table 3).

**Functional Characterization**

Because p.D130G and p.F142L are disruptive functionally (Table 2), we confidently conclude that these variants are responsible for the LQTS phenotype observed in our patients. Although p.D130V is novel, we think that because glycine (G) and valine (V) are replacing a critical negatively charged aspartic acid (D) known to interact with the positively charged calcium ion (Figure 1), p.D130V will functionally and clinically mimic p.D130G. The p.E141G variant was the only variant affecting a novel residue, and we therefore characterized it functionally in this study.

**E141G-CaM Impairs Ca²⁺ Binding by CaM**

In line with previous observations, the p.E141G variant, located in EF hand IV of the C domain (Figure 1), decreased the Ca²⁺ affinity of the C domain (by 11-fold) but did not significantly alter the Ca²⁺ affinity of the N domain (Figure 3A).

**E141G-CaM Disrupts Ca₁.2’s CDI**

The previously published LQTS-associated CaM variants significantly impaired Ca₁.2 CDI, leading to a loss of inactivation. Therefore, we examined the effects of E141G-CaM on Ca₁.2 in a heterologous expression system. Typical Ca₁.2 tracings of voltage-dependent activation from CaV₁.2 with GFP-EV, WT- and E141G-CaM are shown in Figure 3B with holding potential at −90 mV to various depolarization potentials. Current–voltage relationship shows that E141G-CaM did not change CaV₁.2 peak current density (Figure 3C). However, E141G-CaM shifted CaV₁.2 V₁/₂ of steady-state inactivation 1.9 mV from −16.8±0.23 mV (CaV₁.2+WT-CaM; n=11) to −14.9±0.27 mV (CaV₁.2+E141G-CaM; n=9). The E141G-CaM also shifted CaV₁.2 V₁/₂ of activation −3.9 mV

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**Table 3. Demographics of CaM Positive, CaM Negative Compared With a Previously Published Cohort of 541 Cases of LQTS**

|                  | CaM Positive (n=5) | CaM Negative (n=33) | LQT1-3 Positive* (n=272) | LQT1-3 Negative† (n=269) |
|------------------|--------------------|---------------------|--------------------------|--------------------------|
| Male/Female      | 3/2                | 13/20               | 94/178                   | 89/180                   |
| Age at diagnosis, y ± SD | 0.8±2       | 23±18               | 23±16                    | 25±16                    |
| QTc, ms±SEM      | 676±36             | 514±9               | 494±51                   | 470±60                   |
| Cardiac arrest (%)| 100                | 24                  | 13                       | 12                       |

CaM indicates calmodulin; LQTS, long QT syndrome; and QTc, corrected QT interval.

*LQT1-3 positive represents individuals who were positive for putative pathogenic variants in KCNQ1, KCNH2, or SCN5A from a larger cohort of 541 unrelated individuals with LQTS from a previously published study.

†LQT1-3 negative represents individuals who were negative for putative pathogenic variants in KCNQ1, KCNH2, or SCN5A from a larger cohort of 541 unrelated individuals with LQTS from a previously published study.

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Figure 2. Demographic and clinical characteristics of the calmodulin (CaM)-positive patients. A, Bar graph comparing average age of diagnosis for our CaM-negative cases (23±3 years) and CaM-positive cases (0.8±0.8 years; P<0.01). B, Bar graph comparing the percent CaM-positive patients <5 years of age (5/9; 56%) to patients ≥5 years of age (0/29; 0%; P<0.001). C, Bar graph comparing the corrected QT interval (QTc) of CaM-negative patients (514±9 ms) to CaM-positive patients (676±36 ms; P<0.0001). D, Pie charts comparing the number of patients who had experienced cardiac arrest in our CaM-negative (8/33; 24%) versus CaM-positive patients (5/5; 100%; P<0.01). Data in (A) and (C) are shown as mean±SEM. LQTS indicates long QT syndrome.
from 16.4±0.97 mV (CaV1.2+WT-CaM, n=12) to 12.5±0.76 mV (CaV1.2+E141G-CaM; n=12; P<0.05; Figure 3D).

CaV1.2+E141G-CaM exhibited much slower fast and slow decay time across the voltages +10 mV to +50 mV compared with CaV1.2+WT-CaM (n=10 for each group, P<0.05; Figure 3E–3F). Typical CaV1.2 current tracings from CaV1.2+EV, WT-CaM, and E141G-CaM are shown in Figure 4A. E141G-CaM increased CaV1.2's persistent current 7.1-fold from 2.1±0.5% (CaV1.2+WT-CaM; n=9) to 17.0±3.0% (CaV1.2+E141G-CaM; n=10, P<0.05; Figure 4B).

We next tested the effect of E141G-CaM in native murine ventricular myocytes. As in our heterologous expression system, E141G-CaM drastically impaired CaV1.2 current inactivation without affecting peak currents (Figure 4C–4E). Even a 25% fraction of E141G-CaM in the presence of 75% WT-CaM was sufficient to significantly impair the CaV1.2 inactivation, which is consistent with a dominant-negative effect.

**E141G-CaMAccentuates NaV1.5 Late Current**

Next, we determined if E141G-CaM affected the electrophysiological characteristics of NaV1.5. Typical I\textsubscript{Na} tracings of voltage-dependent activation from Na\textsubscript{v}1.5 with GFP-EV, WT- and E141G-CaM are shown in Figure 5A with holding potential at –100 mV to various depolarization potentials. Current–voltage relationship shows that WT- and E141G-CaM did not change Na\textsubscript{v}1.5 peak current density, voltage-dependent inactivation or activation, or decay time (Figure 5B–5D).

Typical Na\textsubscript{v}1.5 late current tracings from Na\textsubscript{v}1.5+EV, WT-, E141G-CaM, and WT-CaM+E141G-CaM are shown in Figure 5E. E141G-CaM increased Na\textsubscript{v}1.5’s late current 1.7-fold from 0.13±0.01% (Na\textsubscript{v}1.5+WT-CaM; n=8) to 0.35±0.06% (Na\textsubscript{v}1.5+E141G-CaM; n=10; P<0.05; Figure 5F). However, when WT-CaM was coexpressed with E141G-CaM, there was no longer an effect on the Na\textsubscript{v}1.5 peak late current (Figure 5E–5F). This result suggests that unlike for Cav1.2, there was no functional dominance of E141G-CaM in the regulation of Na\textsubscript{v}1.5 late currents.

**E141G-CaM Has No Effect on RyR2 Ca\textsuperscript{2+} Release Channels and SR Ca\textsuperscript{2+} Handling**

CaM variants have also been identified in patients with CPVT, leading to activation of RyR2 SR Ca\textsuperscript{2+} release channels. However,
to date, the known LQTS-associated CaM variants have exerted no effect or only slightly reduced RyR2 Ca\textsuperscript{2+} release channel activity.\textsuperscript{20} Therefore, we determined the effect of E141G-CaM on intracellular Ca\textsuperscript{2+} handling by measuring Ca\textsuperscript{2+} sparks (a measure of RyR2 Ca\textsuperscript{2+} release channel activity) and found that E141G-CaM had no effect on Ca\textsuperscript{2+} sparks and SR Ca\textsuperscript{2+} content (Figure 6).

**Discussion**

Our examination of 38 patients with genetically elusive LQTS identified 5 individuals (13.2\%) that harbored variants in either CALM1 or CALM2. Two of the identified variants, p.D130G and p.F142L, had been published previously in other cases of LQTS,\textsuperscript{7} 1 variant represented a novel amino acid change at a previously described position, p.D130V, and the final variant, p.E141G, was novel. Within our cohort, CaM variants were over-represented significantly when compared with the publicly available databases in essentially modified case:control analysis, which provides additional evidence that single amino acid substitutions within CaM, particularly those involving calcium-interacting residues in the C domain, are not well tolerated.

When examining the clinical characteristics of these 5 patients harboring CaM variants, we found that they had an early age at onset with the average age of onset at 10 months, average QTc of 676±36 ms, and all experienced cardiac arrest (Figure 2; Table 3).\textsuperscript{19} Moreover, all CaM variants were shown to occur as de novo when parental DNA was available for testing, thus supporting the malignant nature of LQTS-related CaM variants. These clinical characteristics seemed even more severe than those of the previously published LQT1-3 genotype-positive and LQT1-3 genotype-negative LQTS (Table 3),\textsuperscript{19} and the previously described LQTS\textsuperscript{7} and LQTS/CPVT\textsuperscript{8} patients with CaM variants (n=10), where the average age at diagnosis was 3 years, the average QTc was 591±22 ms, and 60\% experienced cardiac arrest (Table 2). However, if we separate the previously described CaM-positive patients into 2 categories, LQTS/CPVT overlap phenotypes and a pure LQTS phenotype, it becomes more apparent that LQTS/CPVT patients (n=3)\textsuperscript{8} may be less severe, as the average age at diagnosis was 7 years, the average QTc was 519±53 ms, with only 33\% experiencing cardiac arrest. In contrast, the patients with a pure LQTS phenotype (n=7) were similar to our LQTS CaM-positive patients, with an average age at diagnosis of 14 months, an average QTc of 622±20 ms, and with 86\% experiencing cardiac arrest. This provides evidence that CaM-mediated LQTS is severe and it may be important to delineate these phenotypes, as they may present with different disease severities, and the
phenotypic presentation may be regulated by distinct underlying mechanisms. Therefore, continued studies will be necessary to elucidate the key differences between CaM variants and their associated phenotypes. In addition, children with CaM-mediated LQTS who survive will need ongoing surveillance for the potential development of a cardiomyopathy as 2 of the CaM-variant–positive children who have survived past 5 years of age, and one of the deceased patients on autopsy, had developed dilated cardiomyopathy. It is possible that the CaV1.2’s impaired CDI may contribute to diastolic calcium excess that could precipitate cardiomyopathic structural remodeling.

CaM modulates the activity of several different ion channels by sensing and transducing Ca^{2+} signals. When Ca^{2+} levels are raised, CaM binds up to 4 calcium ions, which stabilize an open conformation within each domain that mediates interactions with CaM’s targets. Hence, we first examined the effects of the p.E141G variant on the Ca^{2+} affinity of CaM and found that this mutant resulted in an 11-fold reduction in the Ca^{2+} affinity of the C domain. These results are comparable with the Ca^{2+}-binding impairments caused by the 8 variants previously associated with LQTS or LQTS/CPVT overlap phenotypes (5- to 54-fold increased dissociation of Ca^{2+} in the C domain, Table 2), suggesting that impaired Ca^{2+}-binding properties in the C domain is a common underlying pathomechanism of LQTS-associated CaM variants.

Because there are 3 calmodulin genes expressed in the heart, which each encode for an identical protein, our heterozygous variant only affects 1 of 6 of the calmodulin alleles. Therefore, we examined the E141G-CaM variant at reduced levels (25:75; Figure 5).

The disrupted Ca^{2+} affinities have direct implications on CaV1.2’s CDI. Typically, CaV1.2 is inactivated with increasing Ca^{2+} concentrations via a mechanism mediated by the binding of CaM. The altered Ca^{2+}-binding properties of CaM, such as with E141G-CaM, would thereby be expected to impair CaV1.2’s CDI. Using whole-cell patch-clamp experiments, we found that E141G-CaM reduced CaV1.2 inactivation, which closely parallels what has been noted previously with the other LQTS-associated CaM variants.
mutant:WT), which also maintained a significant effect on inactivation. This suggests that p.E141G has a dominant-negative effect and is capable of causing the disease phenotype.

In addition to CaV1.2, it has been established previously that CaM modulates cardiac sodium channel inactivation.21 Previously, Yin et al.11 found that LQTS-associated CaM variants (p.D96V, p.D130G, and p.F142L) did not affect NaV1.5; however, p.D130G led to a 7.5-fold increase in fetal NaV1.5 late current. They hypothesized that because CaM-mediated LQTS has an early age of onset, the variants may only affect the fetal isoform of the sodium channel. However, because there have been associations of CaM with the adult isoform of NaV1.5, we wanted to determine whether E141G-CaM may have an effect on channel gating function. Our studies showed that the p.E141G variant led to a 1.7-fold increase in NaV1.5 late current when expressed as a homozygote. This makes our E141G-CaM variant the first CaM variant identified to also affect the adult isoform of the cardiac sodium channel. Interestingly, however, when p.E141G was expressed with wild-type CaM—mimicking a heterozygote patient—the NaV1.5 late current was normalized. This differs from the effect of E141G-CaM on CaV1.2, where even a 3-fold excess of WT CaM failed to normalize CaV1.2 inactivation.

It has been established that CPVT-associated CaM variants18 affect the functioning of RYR2-encoded ryanodine receptor (RyR2), leading to greater open probability of the channel.3 Because one previously reported LQTS-associated CaM variant (p.N98S) also activates RyR2 and can cause CPVT as well as LQTS,3 we examined the effect of p.E141G on RyR2 Ca2+ release channel activity. The p.E141G variant had no effect on Ca2+ sparks, a measure of RyR2 Ca2+ release channel activity and did not affect SR Ca2+ content. Hence, our results continue to support the emerging evidence that LQTS-associated CaM variants do not affect RyR2, whereas CPVT-associated CaM variants perturb RyR2 function.

Conclusions
We have identified a novel CaM variant p.E141G, which like other LQTS-associated CaM variants, disrupts Ca2+ binding and leads to increased CaV1.2 window/persistent current. In addition, p.E141G is the first CaM variant identified to also affect the adult NaV1.5 isoform, leading to increased sodium late current. Overall, we found that 13% of genetically elusive patients with LQTS harbored a functionally significant CaM variant. The phenotypic characteristics of the CaM-positive individuals from our cohort, combined with the other published cases of LQTS-associated CaM variants, suggest that patients with LQTS harboring variants in either CALM1-, CALM2-, or CALM3-encoded CaM present early in life with profound QT prolongation and have a high predilection for cardiac arrest and sudden death. The existing gene panels for LQTS genetic testing should now be expanded to include CALM1, CALM2, and CALM3.

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Figure 6. E141G-calmodulin (CaM) has no effect on Ca2+ sparks and sarcoplasmic reticulum (SR) Ca2+ content. A, Representative line-scan images of Ca2+ sparks in permeabilized mouse ventricular myocytes in CaM-free (vehicle), WT-CaM, and E141G-CaM in the presence of autocamtide 2-related inhibitory peptide (1 μmol/L). B, Average Ca2+ spark frequency and C, Ca2+ spark amplitude. Data are mean±SD (n=20). D, Line scan (top) and (bottom) line plot (red arrow) examples of SR Ca2+ content evaluated by 10-mmol/L caffeine-evoked Ca2+ transient in CaM-free (vehicle), WT-CaM, and E141G-CaM in the presence of autocamtide 2-related inhibitory peptide (1 μmol/L). E, Average SR Ca2+ content. Data are mean±SD (n=4).
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Disclosures
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Appendix
From the Windland Smith Rice Sudden Death Genomics Laboratory, Department Molecular Pharmacology and Experimental Therapeutics (N.J.B., D.Y., M.L.C., D.J.T., C.G.L., M.J.A.), Division of Cardiovascular Diseases, Department of Medicine (M.J.A.), Division of Pediatric Cardiology, and Department of Pediatrics (M.J.A.), Mayo Clinic, Rochester, MN; Departments of Medicine (N.G.H., D.O.K., H.S.H., B.C.K.) and Biochemistry and Chemistry, Center for Structural Biology (C.N.J., W.J.C.), Vanderbilt University, Nashville, TN; Department of Pediatric, Division of Cardiology, Children’s Hospital of Philadelphia, PA (M.S.); Department of Pediatric Cardiology, Phoenix Children’s Hospital, AZ (A.L.P.); Division of Pediatric Cardiology, Department of Pediatrics, University of Alabama at Birmingham (Y.R.L.); and Division of Cardiology, Nicklaus Children’s Hospital, Miami, FL (R.K.).

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Recently, pathological variants in 3 genes (CALM1, CALM2, and CALM3) encoding for 100% identical calmodulin protein have been associated with early onset long QT syndrome (LQTS). This report sought to characterize the spectrum and prevalence of calmodulin variants in an unrelated cohort of 38 patients with heretofore genetically elusive LQTS. Overall, 5 of these cases harbored rare pathogenic variants in 1 of the 3 calmodulin encoding genes. Two previously published variants (p.D130G and p.F142L identified twice) and 2 novel (p.D130V and p.E141G) variants were identified. Because p.D130V was allelic with a previously characterized pathogenic variant in calmodulin (p.D130G), we elected to functionally characterize the other novel variant. This variant, p.E141G, disrupted Ca2+ binding and led to altered Ca2+ 1,2 window current, similar to previously characterized calmodulin variants, consistent with LQTS pathogenicity. Closer examination of the phenotypic sequence of these 5 patients with calmodulin-mediated LQTS highlighted that such individuals present early in life, have profound QT prolongation, and have a high predilection for cardiac arrest and sudden death when compared with LQTS patients without pathogenic calmodulin variants. These findings replicated what has been previously described in the literature, and there are now at least 10 described variants in one of the 3 calmodulin genes associated with LQTS. Taken altogether, patients with calmodulin-associated LQTS are more severe phenotypically than typical LQTS and genetic testing of all 3 calmodulin genes is warranted when there is evidence of severe, early onset LQTS.

CLINICAL PERSPECTIVE

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