Novel CALM3 mutations in pediatric long QT syndrome patients support a CALM3-specific calmodulinopathy

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Introduction
The long QT syndrome (LQTS) is an inherited cardiac arrhythmia characterized by a prolonged heart rate–corrected QT interval (QTc) on the surface electrocardiogram (ECG). It is associated with syncope and sudden cardiac death from torsades de pointes, and is estimated to affect 1 per 2000 individuals.1 Fifteen genes have been implicated in familial LQTS, most of which encode cardiac ion channels or channel-interacting proteins.2 To date, mutations in the KCNQ1, KCNH2, and SCN5A genes (causing LQTS 1, 2, and 3) have been found in about 70% of cases, while mutations in other genes (LQTS 4–15) are found in only 5%, indicating that for ~25% of cases the genetic cause remains unknown.1

LQTS 14 and 15 are caused by defects in the calmodulin-encoding genes CALM1 and CALM2,3,4 respectively. More recently, Reed and colleagues5 described the first case of an infant with LQTS resulting from a CALM3 mutation. The similarities in clinical characteristics observed in LQTS patients with calmodulin gene mutations (very early onset, bradycardia detectable during fetal life, conduction abnormalities, and extremely long QTc) has led to the description of a “calmodulinopathy” phenotype.6

In this study we describe 2 gene-elusive early-onset LQTS subjects, showing sinus bradycardia in utero and profound QT prolongation after birth (QTc intervals >600 ms). Based on this extreme LQTS phenotype and the negative family history, we expected the causal variants in these subjects to be de novo and to negatively impact protein amino acid sequence and function. We carried out a sequencing study of these subjects and identified 2 novel variants in CALM3 potentially responsible for the LQTS.

Case report
The 2 subjects were diagnosed at the Hospital for Sick Children in Toronto with a high probability of LQTS based on Schwartz scores ≥3.5 points.7 Informed consent was obtained for each subject and family members enrolled according to the procedures approved by the Hospital for Sick Children Ethics Review Board. Both subjects are of European descent.

Subject 1 is female and was diagnosed with fetal bradycardia at 30 weeks of gestation. Fetal ECG identified a sinus bradycardia with a heart rate of 109 beats per minute (bpm) at 32 weeks. Fetal echocardiogram was normal and anti-Ro and anti-La antibodies were absent. At 36 weeks, 2:1 atrioventricular block in conduction with a ventriculat rate of 50–55 bpm was noted. An echocardiogram at 37 weeks was suggestive of endocardial fibroelastosis, and a coronary artery–to–left atrial fistula was also identified. The rhythm had reverted to sinus bradycardia with 1:1 conduction, with a slow heart rate of 85 bpm.

Following delivery by caesarean section, the newborn showed a saturation of 94% and an ECG demonstrated sinus rhythm with a very long QT interval (QTc = 623 ms) and 2:1 atrioventricular block (Figure 1). At 1 day of age, she reverted to sinus bradycardia with 1:1 conduction (heart rate = 92 bpm) and a very long QT interval (QTc = 645 ms) (Figure 1). The coronary artery–to–left atrial fistula was
KEY TEACHING POINTS

- The human genome contains 3 distinct CALM genes (CALM1, CALM2, CALM3) located on different chromosomes that encode identical ubiquitously expressed calcium-binding protein calmodulin (CaM).
- Multiple de novo mutations in the 3 calmodulin genes have been described in infants with severe early-onset long QT syndrome (LQTS), all of which are located within the C-terminal Ca$^{2+}$-binding motifs of the protein.
- An LQTS-specific “calmodulinopathy” phenotype associated to calmodulin mutations has been described with a very early onset of the disease (average age of onset: 3.7 years), with fetal or neonatal presentation of the disease described in 50% of cases.
- CaM-encoding genes should be considered for genetic diagnostic testing of patients with LQTS, especially in young patients with severe symptoms.

surgically ligated, an epicardial DDD pacemaker was implanted, and she was treated with propranolol 2.4 mg/kg/day.

She has remained well, without symptoms or arrhythmia, for 14 years, with ECGs always demonstrating sinus bradycardia and normal corrected QT intervals (QTc = 380 and 399 ms, respectively); both parents were available for genetic testing.

Subject 2 is female, born by caesarean section for a low biophysical profile score with intrauterine growth retardation and fetal bradycardia. An ECG identified sinus bradycardia at 83 bpm with a very long QT interval (QTc = 627 ms) at 3 days of age (Figure 1). A VVI pacemaker was implanted and she was treated with propranolol 2.8 mg/kg/day. She had an episode of cyanosis while she was in her car seat at the age of 1 month, diagnosed as an episode of gastric reflux and inhalation. She has remained well for the last 15 years with only adjustments in her beta-blockade medication and pacing rate. The QTc intervals have remained in the 480–660 ms range (Figure 1). The parents have normal ECGs without bradycardia and normal corrected QT intervals; only the mother was available for genetic testing.

Methods and results

In order to increase our chances to discover novel LQTS mutations, we focused our study on 2 very early-onset LQTS subjects that shared a similar phenotype of sinus bradycardia in utero with profound QT prolongation after birth. The 2 subjects were found to be negative on genetic testing of the 5 most frequently implicated genes in LQTS (KCNQ1, KCNH2, SCN5A, KCNE1, and KCNE2). We thus opted to use a whole exome sequencing approach in order to identify the underlying genetic causes for LQTS in these probands. For this, genomic DNA samples were sequenced on the HiSeq2000 high-throughput sequencing platform (illumina, San Diego, CA)) according to the manufacturer’s protocols. Briefly, the DNA samples were fragmented and linked to indexed adapters, then amplified using illumina’s “Low Throughput” protocol. Exome enrichment was performed using illumina’s “Truseq exome enrichment” kit and exome-enriched fragments were sequenced using a paired-end (2 × 100 bp) protocol at the Beaulieu-Saucier Université de Montréal Pharmacogenomics Centre. For subjects 1 and 2, the percentage of bases with a coverage superior to 20× was 80.7% and 82.7%, respectively.

Overall, our whole exome sequencing identified 59,541 high-quality single nucleotide variants and in/dels for the 2 subjects. Given the prevalence of LQTS, the fact that LQTS can independently be caused by many different genes and that most mutations identified to date are nonsynonymous and familial in nature, we opted to focus our analyses exclusively on novel nonsynonymous variants. To achieve this we filtered out all previously reported variants from public SNP databases (dbSNP138, 1000 Genome, HapMap, and ESP6500), as well as all synonymous variants. Following this filtering strategy, we identified 222 novel variants for the 2 subjects (98 for subject 1 and 109 for subject 2), well within the expected range of variants for this type of screening strategy in unrelated individuals.

While we did not identify any causal variant in the 15 known LQTS genes, we identified 2 novel heterozygous mutations, p.Phe142Leu and p.Asp96His, for subjects 1 and 2, respectively, in the novel LQTS candidate gene CALM3 (Supplemental Figure 1, available online). We used the Sequenom Mass Array platform and Sanger sequencing to independently confirm that the mutations were present in the subjects and absent in the available unaffected parents (Supplemental Figure 1), as well as from 540 European-derived chromosomes, indicating that p.Phe142Leu in subject 1 is de novo. The heredity status of the p.Asp96His mutation could not be determined owing to the unavailability of paternal DNA. The 2 variants, p.Phe142Leu and p.Asp96His, were predicted as “probably damaging” and “deleterious” by the pathogenicity scores in Polyphen2 and PROVEAN, respectively (data not shown).

Discussion

The human genome contains 3 distinct CALM genes (CALM1, CALM2, CALM3) located on different chromosomes that show differences at the level of DNA sequences but encoding an identical ubiquitously expressed calcium-binding protein, calmodulin (CaM). All 3 genes are
expressed in cardiac myocytes, with transcription expression levels highest for \textit{CALM3}.\textsuperscript{3} CaM serves as a calcium sensor for Ca\textsuperscript{2+}-dependent inactivation of L-type voltage-gated Ca\textsuperscript{2+} channels (Cav1.2).\textsuperscript{3,10}

In 2013, \textit{3 de novo} mutations in \textit{CALM1} and \textit{CALM2} were described in infants with severe early-onset LQTS. Later studies have found an additional 8 variants in \textit{CALM1} and \textit{CALM2} associated with different arrhythmia syndromes, including LQTS (5 variants), catecholaminergic polymorphic ventricular tachycardia (2 variants), and idiopathic ventricular fibrillation (1 variant) (Supplementary Table 1, available online).\textsuperscript{4,11,12} More recently, a missense \textit{de novo} mutation in \textit{CALM3}, p.Asp130Gly, identical to a previously reported \textit{CALM1} mutation, was described in a patient with early-onset LQTS.\textsuperscript{5} The fact that the same mutation found in 2 different calmodulin genes causes LQTS is not surprising, since these genes encode an identical protein, and thus the pathophysiological impact of the variant is expected to be the same. All 8 LQTS-associated amino acid changes identified to date in CaM are located within the C-terminal (high-affinity, slowly dissociating) Ca\textsuperscript{2+}-binding motifs of the protein (Figure 2).

An LQTS-specific “calmodulinopathy” phenotype associated to calmodulin mutations has been described with a very early onset of the disease (average age of onset: 3.7 years), with fetal or neonatal presentation of the disease described in 50% of cases. Sinus bradycardia or a 2:1 atrioventricular block are also very frequent, with 6 of the 10 LQTS cases (60%) described with calmodulin-associated variants presenting such conduction abnormalities. In the majority of cases (70%), a ventricular arrhythmia is described, with ventricular fibrillation identified in 50% of cases. In all cases, the QTc interval is extremely long in comparison with other LQTS (average QTc: 591 ms [478–690 ms]) (Supplementary Table 1).

Our 2 patients show several characteristics of an LQTS phenotype associated with calmodulin, with very early onset of the disease, conduction abnormalities, and extremely long QT intervals (650 and 660 ms). Similarly to the LQTS subject described by Reed et al\textsuperscript{5} with a \textit{CALM3} mutation, our 2 subjects have not presented any evidence of ventricular arrhythmias in the last 14–15 years of follow-up. All 3 patients have had an early pacemaker implantation, avoiding...
the development of extreme bradycardia, suggesting that the absence of ventricular arrhythmias either could be linked to an early implantation of the pacemaker or is a characteristic of CALM3 mutations.

Similarly to the recently reported p.Asp130Gly mutation in CALM3, the p.Phe142Leu identified in subject 1 has previously been reported in CALM1 for a young patient suffering from LQTS (Supplemental Table 1). We found this mutation to be de novo, which agrees with the negative LQTS family history. Published functional studies of p.Phe142Leu in CALM1 showed that this variant reduces calcium binding, and in vitro studies demonstrated that this variant also impaired Ca$^{2+}$-dependent inactivation of L-type voltage-gated Ca$^{2+}$ channels, leading to prolongation of ventricular action potentials. For these reasons, we strongly believe that this variant is the causal LQTS mutation in this subject. CaM also interacts with the cardiac sodium channel (Na$_v$1.5) and the voltage-gated potassium channel in this subject. CaM also interacts with the cardiac sodium channel (Nav1.5) and the voltage-gated potassium channel in this subject. CaM also interacts with the cardiac sodium channel (Nav1.5) and the voltage-gated potassium channel in this subject. CaM also interacts with the cardiac sodium channel (Nav1.5) and the voltage-gated potassium channel in this subject.

While we do not have direct functional evidence supporting the impact of this mutation on CaM function, the predicted pathogenicity score of the variant, the fact that this position has previously been implicated in LQTS, and the fact that the phenotype of subject 2 matches that of the proposed LQTS “calmodulinopathy” phenotype lead us to believe that this variant is most likely causal.

Our identification of 2 novel mutations in CALM3 further validates the previously reported role of CALM3 in LQTS and supports the concept of an LQTS “calmodulinopathy” phenotype. It should be noted, however, that the lack of severe clinical manifestations of disease in our patients, and in the published CALM3 patient following initial identification and treatment, highlights the treatability of the LQTS “calmodulinopathy” phenotype and thus the importance of rapid and reliable diagnosis. This and the lack of ventricular arrhythmia in these patients may also suggest the existence of a CALM3 LQTS “calmodulinopathy” sub-phenotype. The increasing number of mutations in CaM further illustrates that the CaM-encoding genes should be considered for genetic diagnostic testing of patients with LQTS, especially in young patients with severe symptoms.

Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at http://doi:10.1016/10.1016/j.hrcr.2016.02.002.

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