Published in final edited form as:

Am J Med Genet A. 2018 December ; 176(12): 2733–2739. doi:10.1002/ajmg.a.40657.

Expanding Clinical Phenotype in *CACNA1C* Related Disorders: From Neonatal Onset Severe Epileptic Encephalopathy to Late-onset Epilepsy

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Abstract

CACNA1C (NM_000719.6) encodes an L-type calcium voltage-gated calcium channel (Ca_v1.2), and pathogenic variants have been associated with two distinct clinical entities: Timothy syndrome and Brugada syndrome. Thus far, *CACNA1C* has not been reported as a gene associated with epileptic encephalopathy (EE) and is less commonly associated with epilepsy. We report three individuals from two families with variants in *CACNA1C*. Patient 1 presented with neonatal onset severe epileptic encephalopathy (NOEE) and was found to have a *de novo* missense variant in *CACNA1C* (c.4087G>A (p.V1363M)) on exome sequencing. In family 2, Patient 2 presented with congenital cardiac anomalies and cardiomyopathy and was found to have a paternally inherited

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DISCLOSURE OF CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines

Web Resources: The URLs for data presented include:

UCSC genome browser: [Di et al., 2009; Hedley et al., 2009; Berger and Bartsch, 2014]

OMIM: <http://omim.org/>

dbVar: <http://www.ncbi.nlm.nih.gov/dbvar/browse/>

ClinGen: <http://www.ncbi.nlm.nih.gov/projects/dbvar/clingen/>

ExAC: <http://exac.broadinstitute.org/>

gnomAD: <http://gnomad.broadinstitute.org>

ClinVar: <https://www.ncbi.nlm.nih.gov/clinvar/>

Uniprot: <http://www.uniprot.org>

splice site variant, c.3717+1_3717+2insA, on a cardiomyopathy panel. Her father, Patient 3, presented with learning difficulties, late-onset epilepsy, and congenital cardiac anomalies. Family 2 highlights variable expressivity seen within a family. This case series expands the clinical and molecular phenotype of *CACNA1C*-related disorders and highlights the need to include *CACNA1C* on epilepsy gene panels.

Keywords

CACNA1C; Neonatal onset epileptic encephalopathy (NOEE); Epileptic Encephalopathy (EE); Electroencephalogram (EEG); Exome sequencing (ES)

INTRODUCTION

CACNA1C encodes the alpha-1 subunit (also known as Ca_v1.2) of the L-type voltage-dependent calcium channel, which consists of 24 transmembrane segments that form the pore for ion transport into the cell. It undergoes extensive alternative splicing and has at least 36 different transcripts that are expressed in heart, brain, lung, and smooth muscle [Napolitano and Antzelevitch, 2011; Hedley et al., 2009]. Pathogenic variants in *CACNA1C* are known to be associated with vastly different clinical phenotypes. The first, Timothy syndrome, is characterized by multisystem abnormalities including cardiac, limb anomalies, facial dysmorphisms, and neurologic features including autism, seizures, intellectual disability, and hypotonia [Lo-A-Njoe et al., 2005; Napolitano et al., 1993; Reichenbach et al., 1992; Splawski et al., 2004; Gillis et al., 2012; Napolitano and Antzelevitch, 2011]. The second, Brugada syndrome, is characterized by ST elevation in the right precordial leads with symptoms of ventricular arrhythmia, syncope, and sudden death [Brugada et al., 1993; Napolitano et al., 1993; Lo-A-Njoe et al., 2005; Marks et al., 1995b, 1995a; Reichenbach et al., 1992; Splawski et al., 2004; Hedley et al., 2009]. Importantly, *CACNA1C*'s clinical validity and association with Brugada syndrome was not definitely established when an evidence-based approach was utilized [Hosseini et al., 2018]. Thus far, *CACNA1C* has not been reported as a gene associated with EE, a severe form of epilepsy associated with developmental delay, cognitive dysfunction, and uncontrolled seizures, nor has it been associated with NOEE, which is characterized by neonatal onset intractable epilepsy with a high seizure burden, severe cognitive and psychomotor delays, failure of neurodevelopment, poor prognosis, and high mortality rate at a young age [Beal et al., 2012; Shellhaas et al., 2017]. We describe the clinical and molecular details of 3 individuals from 2 unrelated families with heterozygous variants in *CACNA1C*. This includes an infant with a *de novo* missense variant who presented with NOEE, and a family with variable expressivity, which included tetralogy of Fallot (TOF), cardiomyopathy, and developmental delay in the proband and a father with a ventricular septal defect, late-onset epilepsy and learning disabilities attributed to a splice site variant in *CACNA1C*.

METHODS

Trio exome sequencing (ES) was done in Patient 1 and her nonaffected parents (Family 1), and a cardiomyopathy panel (combined cardiac sequencing and deletion/duplication panel

including 120 genes) was performed on Patient 2 (Family 2). Her father, Patient 3, had targeted testing of the two variants of uncertain significance (VUS) found on panel testing. Informed consent was obtained from all affected individuals or their guardians per the specifications of Seattle Children's Hospital and University of Washington, including consent to publish photographs.

For the *in silico* analysis of p.V1363M, seen in Patient 1, topology of the protein encoded by *CACNA1C* (Ca_v1.2) was predicted and plotted using Protter [Omasits et al., 2014]. Subsequently, we identified the orthologues of Ca_v1.2 using reciprocal blast approach in Chordata species in the Uniprot Proteome database. These orthologues were aligned using Clustal Omega [Sievers et al., 2011] and manually inspected to remove sequences with significant gaps. The resulting sequence alignment was visualized using Jalview [Waterhouse et al., 2009]. Finally, we predicted the 3D structure of Ca_v1.2 using rosettaCM [Song et al., 2013] and Ca_v1.1 as a template (PDB ID: 5GJV_A). p.V1363M and selected pathogenic variants from ClinVar [Landrum et al., 2016] associated with Timothy syndrome were mapped onto the predicted structure and visualized in Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

RESULTS

Clinical Report for Patient 1 (Family 1)

She was born at 38 weeks of gestation to a nonconsanguineous 33-year-old mother and a 30-year-old father. Maternal and paternal ancestry was English and Ukrainian respectively. She had a healthy twin brother and an older brother. Apgar scores were 8 and 9 at one and five minutes, respectively. Growth parameters at birth included a weight of 2.4 kg (z-score -1.93), length of 40.6 cm (z-score -3.92), and an occipitofrontal circumference (OFC) of 31.8 cm (z-score -1.84). Following birth, she was hospitalized for two days for evaluation of hypoglycemia (30mg/dL) and multiple congenital anomalies. She was found to have profound axial and appendicular hypotonia, laryngomalacia with stridor, bilateral clubbed feet, bilateral 3–4 finger and 2–3 toe syndactyly, and camptodactyly. Dysmorphic facial features included micrognathia, downturned corners of the mouth, frontal bossing, very scant hair, and bilaterally inverted nipples (Figure 1A). Ophthalmological evaluation demonstrated mild optic nerve hypoplasia. In addition, she had severe gastroesophageal reflux, constipation, and anal stenosis which required sequential dilation. A rectal biopsy ruled out Hirschsprung disease. At 8 months, she required a gastrostomy tube due to severe dysphagia. At 18 months, she lacked progression of developmental milestones and had profound hypotonia and inability to visually track or fixate. In addition, she had severe disturbances of her sleep-wake cycle. Her seizures started within a few hours of birth with numerous subtle tonic seizures as well as prolonged tonic and tonic-myoclonic seizures per day. She was initially treated with zonisamide, with no response. At 3 months, she was diagnosed with epileptic tonic spasms and did not respond to multiple anti-epileptic drugs (AED) including vigabatrin, perampanel, levetiracetam, lacosamide, rufinamide, pyridoxine, folic acid, and pyridoxal 5'-phosphate. She had a small seizure reduction with topiramate and valproic acid. At 7 months, clobazam was started, which significantly reduced her seizure burden from numerous tonic seizures per day to less than 10 per day and improved

sleep duration of 8 to 10 hours at night. By 18 months, she still had multiple daily tonic seizures and focal seizures with eye blinking and facial twitching. Her neurologic workup included brain magnetic resonance imaging (MRI) at 7 days and 12 months of age and a proton magnetic resonance spectroscopy at 12 months, all of which were normal. Her first EEG at 5 days of life demonstrated very distinctive features with diffuse excessive fast activities admixed with other frequency activities and subtle tonic seizures. These diffuse fast activities and lack of normal organization have persisted beyond the neonatal period. At 4 months, her EEG demonstrated intermittent diffuse fast activity alternated with diffuse slowing with high voltage occipital sharp wave discharges. Her polysomnography at 5-months of age demonstrated both central and obstructive sleep apnea in the severe range with normal sleep architecture. An echocardiogram (ECHO) and a 24-hour Holter monitor was normal at 13 months. She had an extensive biochemical workup which was normal.

Diagnostic testing for Patient 1

Her genetic workup included a normal karyotype, chromosomal microarray (CMA), and mitochondrial DNA (mtDNA) analysis. Trio ES revealed a heterozygous *de novo* variant in *CACNA1C* (NM_000719.6: c.4087G>A, p.V1363M). The amino acid position is highly conserved, is absent from control databases such as gnomAD, ExAC, and 1000 Genomes [1000 Genomes Project Consortium et al., 2015], and has not been previously reported as a pathogenic variant. Therefore, it was interpreted as a likely pathogenic. Additional findings on ES included a heterozygous, *de novo*, terminal deletion of *MSH2*. This deletion involves exons 11–16 ([NM_000251.2] arr[GRCh37] 2p21(47,695,322–47,710,345)x1) of the gene and has been previously reported as pathogenic of hereditary non-polyposis colorectal cancer (HNPCC) (Lynch syndrome) in several families [Lagerstedt-Robinson et al., 2016; Martínez-Bouzas et al., 2007; McPhillips et al., 2005; Romero et al., 2013].

In silico analysis of the *CACNA1C* p.V1363M variant from Patient 1 and 3D structural prediction

In silico analysis predicts this variant is likely pathogenic, including an elevated GERP score of 4.6, SIFT score of 0 (Damaging), DANN score of 0.9989, and MutationTaster predicts that it is disease causing [Quang et al., 2015; Cooper et al., 2005; Schwarz et al., 2014; Kumar et al., 2009]. Moreover, codon position 1363 is highly conserved across multiple Chordata species as well as 100 vertebrates in PhyloP, PhastCons, and Multiz Alignment (Fig. 1D). Taken together, this indicates the functional importance and likelihood of pathogenicity for p.V1363M. Ca_v1.2 consists of four homologous transmembrane domains, and each domain contains six transmembrane helices (TMH). V1363M localizes in the fifth TMH in domain IV (Figure 1C,D (purple circle)). The predicted three-dimensional structure of Ca_v1.2 shows that (Figure 1E,F) the sixth TMH in each of the four domains form the channel to allow passage of calcium ions, and they appear to be critical for the proper function of Ca_v1.2. Three pathogenic variants, p.G402S, p.G406R, and p.A1473G in the 6th TMH have been associated with Timothy syndrome. p.G402S and p.G406A were shown to cause reduced channel inactivation, and thus calcium overload in cells [Splawski et al., 2004, 2005]. The fifth TMH in each domain is packed directly against the sixth TMH, and position 1363 is facing towards the sixth TMH. A change from valine to methionine, a larger

residue, likely disrupts the protein structure in the sixth TMH, affecting the gating or calcium transmission of $Ca_v1.2$.

Clinical report for Patient 2 and 3 (Family 2)

In family 2, patient 2 was born at 39 weeks of gestation to a nonconsanguineous 19-year-old mother and a 24-year-old father (Patient 3) of Irish and German descent. Her birth weight was 3.04kg (z score -0.42) and length was 53cm (z score 1.38). Following delivery, she was found to have an asymptomatic murmur. This worsened following discharge and an ECHO demonstrated TOF which was surgically repaired at 2 months of age. Subsequently, at 4 months of life, she developed acute severe biventricular failure requiring inotropic support and mechanical ventilation. ECHO demonstrated left ventricular noncompaction (LVNC). She has mild dysmorphic features including a flat nasal bridge, low-set ears with cupping of the superior helices, deep-set eyes with shallow orbits, thin vermilion border of the upper lip, and a round face (Figure 1B) and mild bilateral 2-3-4 toe syndactyly. At 8 months, she had mild developmental delays. By 12 months, she spoke 10 words and first walked. Her neurologic exam showed mild diffuse hypotonia. She had an extensive biochemical workup which was normal. Her father, who also carried the splice site variant in *CACNA1C*, had a congenital ventricular septal defect and aortic valve stenosis which was repaired at age 2.5 months. In addition, he had learning disabilities requiring individualized education program at school and attention-deficit hyperactivity disorder (ADHD). By 18 years, he developed seizures. The clinical semiology consists of feeling numbness on the left side which evolves into generalized tonic-clonic seizures. He was treated with levetiracetam and has 1 to 2 seizures per month. His craniofacial features were similar to his daughters and shared many commonalities to described individuals with Timothy syndrome [Napolitano et al., 1993], including low-set ears, thin vermilion border of the upper lip, premaxillary underdevelopment, and a round face.

Diagnostic Testing for Patient 2 and 3

Patient 2's CMA was normal. A 120-gene cardiomyopathy panel with sequencing and duplication/deletion analysis revealed three variants in three genes. The first was a heterozygous *CACNA1C* splice site variant (NM_000719.6: c.3717+1_3717+2insA) that was paternally inherited. This variant has not been previously reported in the literature, is absent from large control population databases, and is predicted to destroy the canonical splice donor site in intron 28, which is invariably a GT at the 5' end of the intron for normal splicing to occur. Given the phenotypes of Patient 2 and 3 was consistent with Timothy Syndrome, the *CACNA1C* splice site variant was thought to be likely pathogenic; however, in the absence of functional mRNA studies this remains VUS. The second variant detected on panel testing was a heterozygous splice site variant in *ALPK3* (NM_020778.4: c.5105+2dupT). This variant destroys the canonical splice donor site in intron 11 and is predicted to cause abnormal splicing. No additional variants in *ALPK3* were detected, including deletion and duplication analysis of the gene. Given inheritance is autosomal recessive, this variant was likely not causing her phenotype, but may be contributing. The third variant was a heterozygous missense variant in *TPM1* (NM_001018005.1: c.389T>C, p.I130T). *In silico* analysis predicts p.I130T is damaging, and the amino acid position is highly conserved across species. It is not present in large control databases and has not been

previously reported in the literature. *TPM1* is associated with autosomal dominant familial hypertrophic cardiomyopathy, dilated cardiomyopathy, and LVNC. It has not been associated with congenital heart defects. Thus, it was considered a VUS. Targeted testing of *TPM1* p.I130T in her father (Patient 3) was negative. Her mother did not undergo targeted testing; therefore, it is uncertain if the variant is *de novo* or inherited in Patient 2. It is possible that the *TPM1* variant was contributing to her cardiomyopathy. In summary, given Patient 2 and 3's phenotype is most consistent with Timothy syndrome, the splice site variant in *CACNA1C* was thought to be causative; however, it is classified as a variant of uncertain significance (VUS) in the absence of functional mRNA studies.

DISCUSSION

CACNA1C gene encodes multiple isoforms of the pore-forming alpha-1C subunit of the long lasting (L-type) voltage gated calcium channel (Ca_v1.2, NM_000719.6) [Takimoto et al., 1997]. Ca_v1.2 is evolutionally conserved protein that is expressed in the heart, brain, lung and smooth muscles, and is critical for Ca²⁺ signaling, cellular and neuronal excitability, muscle contraction, and regulation of gene expression [Dai et al., 2009; Hedley et al., 2009]. The variable clinical spectrum summarized in Table 1 [Splawski et al., 2005; Sepp et al., 2017; Corona-Rivera et al., 2015; Etheridge et al., 2011; Splawski et al., 2004; Gillis et al., 2012; Wemhöner et al., 2015; Boczek et al., 2015; Fukuyama et al., 2014; Liu et al., 2017; Antzelevitch et al., 2007] may be accounted for by the numerous isoforms generated by alternatively spliced exons, variable expression of different transcripts in tissue, and the variant's location in the protein and structural domain of the Ca_v1.2 channel. For example, in some individuals it is predominantly confined to the heart as seen in Brugada syndrome, while in others there is a predominant neurologic phenotype as seen in Patient 1. In the brain, Ca_v1.2 is predominantly expressed in the hippocampus, thalamus, cerebral cortex, suprachiasmatic nucleus, and cerebellum [Berger and Bartsch, 2014; Obermair et al., 2004; Nahm et al., 2005]. As the thalamus and hippocampus are important structures for rhythmicity on EEG [Steriade et al., 1993] and the suprachiasmatic nucleus plays a critical role in circadian rhythms, this could be the biologic underpinnings of disturbed circadian rhythm and seizures observed in some individuals with *CACNA1C*-related disorders. Additional neuropsychiatric impairment such as developmental delay and autism are reported in patients with Timothy Syndrome [Splawski et al., 2005, 2004]. In contrast, seizures are rarely reported in *CACNA1C*-related disorders with only 3 previously reported individuals prior to our case series [Splawski et al., 2005; Boczek et al., 2015; Gillis et al., 2012]. We expand upon this with an infant presenting with NOEE and an adult with late seizure onset. It is possible that Patient 3's seizures are unrelated to the splice site variant in *CACNA1C*; however, given the shared clinical characteristics to individuals with Timothy syndrome and that of his daughter (Patient 2) we hypothesize it may be related. Splicing variants in *CACNA1C* have been associated with Brugada syndrome [Fukuyama et al., 2014, 2013] and schizophrenia [Purcell et al., 2014], but not been previously reported in patients with Timothy syndrome and epilepsy. As summarized in Table 1, the majority are missense variants; however, given the many different isoforms and importance of splicing on tissue specific expression, we propose this splice site variant and haploinsufficiency can also

account for the phenotype in Timothy syndrome. Future mRNA studies are needed to bolster this proposal.

CONCLUSION

This case series expands the molecular and clinical phenotype for *CACNA1C*-related disorders to include NOEE with unique clinical-electrical characterization. Patient 1 had many hallmark features of Timothy Syndrome including cutaneous syndactyly, dysmorphic features, and sleep disturbances; however, she lacked cardiac abnormalities. Her presentation differs from previously reported individuals in regard to her severe refractory epilepsy of neonatal onset, lack of neurodevelopment, and recognizable EEG patterns of diffuse fast activity. With this information, we propose the consideration of including the *CACNA1C* in early-onset epilepsy gene panels. In addition, we report an inherited splice site variant with variable expressivity in the family including congenital heart defects, cardiomyopathy, learning difficulties, epilepsy, and craniofacial features consistent with Timothy syndrome. The clinical phenotype of *CACNA1C*-related calcium channelopathies is broad and complex. Better understanding of the clinical-electrophysiological features and functional consequences of *CACNA1C* variants is needed for the development of targeted treatments and novel therapeutics.

ACKNOWLEDGEMENT

We thank the families for participating and sharing their story. We thank Dr. David Baker's lab at University of Washington for providing the protein structure prediction data.

STUDY FUNDING

Research reported in this publication was supported in part by the National Institute of General Medical Sciences of National Institutes of Health Postdoctoral Training Program in Medical Genetics (5T32GM007454 to J.N.D), and the National Institute of Neurological Disorders and Stroke (NINDS) (K08NS092898 to G.M.M) and Jordan's Guardian Angels (to G.M.M.)

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Figure 1.

Photographs of Patient 1 and 2 and topological diagram of CACNA1C. (A) Patient 1 at 13 months of life demonstrating facial dysmorphisms including micrognathia, downturned corners of mouth, flared nares, frontal bossing, and scant hair. (B) Patient 2 at 9months demonstrating low-set posteriorly rotated ears, prominent eyes with shallow orbits, flat nasal bridge, thin vermillion upper lip, and cupping of superior helices. (C) Topology diagram of CACNA1C. Select pathogenic variants from ClinVar associated with Timothy syndrome (A1473G, I1166T, G406R, G402S) are marked as yellow dots. V1363M, seen in Patient 1, is shown as a magenta dot. (D) Sequence conservation (top) and location of predicted transmembrane helices (bottom). The position of disease-causing variants is indicated by yellow (previously reported), magenta (V1363M seen in Patient 1), and green (splice-site variant, c.3717+1_3717+2insA seen in Family 2) pins. (E-F) Side and bottom view (the cytoplasmic side) of the predicted 3D structure of human CACNA1C. The transmembrane helices forming the calcium channel are colored in pale cyan; other transmembrane helices are colored in light pink; the cytoplasmic and periplasmic portion of the protein is colored in pale green. The calcium ions are shown as black spheres. Atoms of the residues that are associated with severe diseases are shown as spheres. V1363M (colored in magenta) is in direct contact with a variant associated with severe neurologic disease, A1473G (colored in yellow).

Table 1.

Summary of the clinical and molecular information for our patients and previously reported individuals from the literature grouped by (A) Timothy syndrome phenotype and (B) Brugada syndrome phenotype. GRCh37/hg19 assembly and NM_000719.6 was used as the reference sequence for all variants with the exception of NM_001167625.1 for (c.1216G>A, p.G406R)

	Nucleotide (c.)	Amino Acid (p.)	Exon	Inheritance	Clinical Phenotype
A. Timothy Syndrome					
Patient 1	4087G>A	V1363M	33	<i>De Novo</i>	Early onset intractable epilepsy , GDD, hypotonia, dysmorphic features, laryngomalacia, syndactyly, camptodactyly, anal stenosis
Patient 2	3717+1_3717+2insA	N/A	Intron 28	PI	TOF, severe LVNC and heart failure, syndactyly, dysmorphic features
Patient 3	3717+1_3717+2insA	N/A	Intron 28	Unknown	VSD, generalized adult-onset epilepsy , and ID
Splawski 2005	1216G>A	G406R	8A	<i>De novo</i>	13 individuals with classic Timothy syndrome
Corona-Rivera 2015	1216G>A	G406R	8A	<i>De novo</i>	Fetal hydrops due to congenital AV block, postnatal prolonged QTc, SCA
Sepp 2017	1216G>A	G406R	8A	<i>De novo</i>	No syndactyly with QT prolongation and AV conduction abnormalities
Etheridge 2011	1216G>A	G406R	8A	Mosaic	Father (mosaic) with mild phenotype: syndactyly and prolonged QTc and a severely affected child Unrelated child (mosaic) with prolonged QTc, SCA due to VF, syndactyly
Splawski 2004, 2005 *	1216G>A *	G406R *	8 *	<i>De novo</i> /mosaic	<i>De novo</i> : Severe prolong QT, arrhythmias, sudden cardiac death, and cognitive delays; no syndactyly Mosaic: Two affected sibs with an "unaffected" mother
Splawski 2005	1204G>A	G402S	8	<i>De novo</i>	Atypical Timothy syndrome, isolated QTc prolongation and arrhythmias, no syndactyly
Gillis 2012	4418C>G	A1473G	36	<i>De novo</i>	Stroke, early onset intractable epilepsy , GDD, joint contractures, dysmorphic facial features, syndactyly, cortical blindness, and myopathy
Wenhöner 2015	3497T>C	I1166T	27	<i>De novo</i>	1 individual with HCM, syndactyly, ductus arteriosus, SCA
Boczek 2015	1552C>T	R518C	12	Inherited	3 families with LQTS, HCM, SCA, congenital heart defects
B. Brugada Syndrome					
Antzelevitch 2007	1116C>T	A39V	2	Inherited	SCA, short QT interval
	1468G>A	G490R	10	Inherited	SCA, short QT interval
Fukuyama 2014	1141C>T	P381S	8	PI	LQTS
	1368G>A	M456I	9	Presumed <i>de novo</i>	
	1745C>A	A582D	13	MI	
	2573G>A	R858H	19	MI	
	5347G>T	G1783C	42	<i>De novo</i>	

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	Nucleotide (c.)	Amino Acid (p.)	Exon	Inheritance	Clinical Phenotype	
Wenhöner 2015	82G>A	A28T	2	PI	LQTS	
	2578C>G	R860G	19	<i>De novo</i>	SCA, LQTS	
	3496A>G	I1166V	27	Inherited	LQTS, Syncope	
	4425C>G	I1475M	36	MI	SCA, LQTS	
	4486G>A	E1496K	36	De novo	SCA, LQTS	
Liu 2017	5747A>G	Q1916R	45	Inherited	SCA due to early repolarization syndrome	

Abbreviations: GDD: Global Developmental delay; Het: Heterozygous; LQTS: Long QT Syndrome; Maternally Inherited: MI; Paternally Inherited: PI; SCA: Sudden cardiac arrest
* which leads to an alternatively spliced product.