RESEARCH ARTICLE

Loss-of-function variants in Kᵥ11.1 cardiac channels as a biomarker for SUDEP

Ming S. Soh[1] (ID), Richard D. Bagnall[2,3], Mark F. Bennett[4,5,6] (ID), Lauren E. Bleakley[1],
Erlina S. Mohamed Syazwan[1], A. Marie Phillips[1,7], Mathew D. F. Chiam[1], Chaseley E. McKenzie[1],
Michael Hildebrand[6,8], Douglas Crompton[6,9], Melanie Bahlo[4,5], Christopher Semsarian[2,3],
Ingrid E. Scheffer[1,6,8,10], Samuel F. Berkovic[6] (ID) & Christopher A. Reid[1,6]*

[1]The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, VIC, Australia
[2]Agnes Ginges Centre for Molecular Cardiology at Centenary Institute, The University of Sydney, Sydney, NSW, Australia
[3]Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia
[4]Population Health and Immunity Division, The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia
[5]Department of Medical Biology, University of Melbourne, Melbourne, VIC, Australia
[6]Epilepsy Research Centre, Department of Medicine, University of Melbourne, Austin Health, Heidelberg, VIC, Australia
[7]School of Biosciences, University of Melbourne, Melbourne, VIC, Australia
[8]Murdoch Children's Research Institute, The Royal Children's Hospital, Parkville, VIC, Australia
[9]Neurology Department, Northern Health, Epping, VIC, Australia
[10]Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Parkville, VIC, Australia

Correspondence

Christopher A. Reid, The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, VIC, Australia. Tel: +61 3 9035 6372; Fax: +61 3 9035 3107; E-mail: christopher.reid@florey.edu.au

Abstract

Objective: To compare the frequency and impact on the channel function of *KCNH2* variants in SUDEP patients with epilepsy controls comprising patients older than 50 years, a group with low SUDEP risk, and establish loss-of-function *KCNH2* variants as predictive biomarkers of SUDEP risk. **Methods**: We searched for *KCNH2* variants with a minor allele frequency of <5%. Functional analysis in *Xenopus laevis* oocytes was performed for all *KCNH2* variants identified. **Results**: *KCNH2* variants were found in 11.1% (10/90) of SUDEP individuals compared to 6.0% (20/332) of epilepsy controls ($p = 0.11$). Loss-of-function *KCNH2* variants, defined as causing >20% reduction in maximal amplitude, were observed in 8.9% (8/90) SUDEP patients compared to 3.3% (11/332) epilepsy controls suggesting about threefold enrichment (nominal $p = 0.04$). *KCNH2* variants that did not change channel function occurred at a similar frequency in SUDEP (2.2%; 2/90) and epilepsy control (2.7%; 9/332) cohorts ($p > 0.99$). Rare *KCNH2* variants (<1% allele frequency) associated with greater loss of function and an ~11-fold enrichment in the SUDEP cohort (nominal $p = 0.03$). In silico tools were unable to predict the impact of a variant on function highlighting the need for electrophysiological analysis. **Interpretation**: These data show that loss-of-function *KCNH2* variants are enriched in SUDEP patients when compared to an epilepsy population older than 50 years, suggesting that cardiac mechanisms contribute to SUDEP risk. We propose that genetic screening in combination with functional analysis can identify loss-of-function *KCNH2* variants that could act as biomarkers of an individual's SUDEP risk.

Introduction

People with epilepsy have a two to threefold increased risk of premature mortality, with Sudden Unexpected Death in Epilepsy (SUDEP) the most common cause of death.[1] SUDEP occurs without warning, most frequently in young adults. Frequent tonic-clonic seizures are the biggest risk factor for SUDEP.[2–4] Other risk factors are the markers of seizure severity including epilepsy duration and poly-therapy.[3–5] However, SUDEP also occurs in patients with mild, well-controlled epilepsy, suggesting that other risk factors exist.[6]

The pathophysiological mechanism(s) responsible for SUDEP are unclear. A systematic retrospective analysis of 10 SUDEP deaths in the Incidence and Mechanisms of Cardiorespiratory Arrests in Epilepsy Monitoring Units (MORTEMUS) revealed that seizure-mediated terminal apnea always preceded terminal asystole.[7] The patient cohort in this study was small and involved individuals who were undergoing long-term video-EEG monitoring, implying refractory epilepsy. These cases may, therefore, not be representative of all individuals with SUDEP. Aside from seizure severity, the presence of abnormal cardiac rhythms has been implicated in SUDEP risk.[8,9] Both human and animal studies show that seizure-mediated changes in cardiac electrophysiology occur, including seizure-driven cortical autonomic dysfunction and longer-term altered cardiac ion channel expression.[10,11] Patients with epilepsy also have an increased risk of sudden cardiac death.[12–14] Furthermore, genetic studies have found variants in genes associated with cardiac arrhythmia syndromes in SUDEP cases, including genes that cause long QT syndrome (LQTS).[9,15–19]

LQTS results from delayed myocardial repolarization that manifests as a prolonged QT interval on the electrocardiography increasing the risk of "torsades de pointes" that can trigger sudden cardiac death.[20] Here we focus on *KCNH2,* which was among the top 30 genes identified in a gene-based rare variant collapsing analysis of SUDEP patients and matched ancestry controls.[16] *KCNH2* encodes the pore-forming α subunit of the voltage-gated potassium channel $K_v11.1$. In vitro screening assays have established that the loss of $K_v11.1$ function leads to familial LQTS type 2 (LQTS2).[21–23] These same assays routinely assess the modulation of $K_v11.1$ by drug candidates, which block a strong predictor of cardiac toxicity.[24] *KCNH2* variants have been identified in SUDEP patients, including both rare pathogenic and common variants although statistically significant enrichment has not been demonstrated.[16–18] We have proposed that when combined, seizures and a risk variant in an arrhythmogenic gene could interact to significantly increase SUDEP risk.[25] This hypothesis predicts that SUDEP individuals will have an enrichment of *KCNH2* variants that impact channel function compared to epilepsy patients at low risk of SUDEP. To test this hypothesis, we measured the $K_v11.1$-generated current of *KCNH2* variants found in SUDEP patients. The results were compared to *KCNH2* variants identified in a control sample comprising epilepsy patients who were over 50 years of age. Death rate is reported to be six times higher between the ages of 16 and 24 in epilepsy patients, with SUDEP accounting for ~40% of deaths under the age of 45.[26,27] Our epilepsy control cohort is, therefore, considered to have "escaped" SUDEP. An enrichment of loss-of-function *KCNH2* variants in SUDEP compared to epilepsy controls argue that cardiac mechanisms may contribute to risk.

Methods

Identification of SUDEP and control variants

Our combined cohorts of 90 unrelated SUDEP patients were recruited from the epilepsy genetics research program in Melbourne, Australia, during life, or from coronial cases investigated at the Departments of Forensic Medicine in New South Wales, Victoria, Queensland, and South Australia, as previously described.[16,18] All exons of *KCNH2* were Sanger-sequenced in 29 SUDEP cases, and the remaining 61 SUDEP cases underwent exome sequencing as previously described.[16] We looked for variants in the KCNH2 protein-coding regions and essential splice site dinucleotides with an allele frequency <5% in the gnomAD reference population database.[28] Variants identified by exome sequencing were Sanger-verified. Eighty-eight out of 90 of the SUDEP events occurred without witnesses. Hence there is limited information on the circumstances surrounding death, but they fulfilled the operational definition of SUDEP (definite or probable SUDEP) and most had post-mortem anti-seizure medication levels performed (Table S1).

KCNH2 variants were identified in our epilepsy control cohort of 332 well-characterized Australian individuals, drawn from a broad spectrum of epilepsy patients, who were over the age of 50 years (born before 1970) and underwent whole-exome sequencing as part of our contribution to the Epi25 Consortium[29] (Table S2). Diagnoses were non-lesional focal epilepsies ($n = 137$), lesional focal epilepsies ($n = 84$), genetic generalized epilepsy ($n = 76$), GEFS+ or febrile seizures ($n = 22$), and developmental and epileptic encephalopathies ($n = 13$). Given that SUDEP is far more likely to occur between the ages of 20 and 40 years of age,[26] with a death rate reported to be six times higher between the ages of 16 and 24 in epilepsy patients and accounting for ~40% of deaths under the age of 45,[27] these patients were considered to have "escaped" SUDEP, providing a suitable comparison population. Given the lack of available clinical details on the SUDEP cases, and IRB constraints, we were unable to match cases and controls for epilepsy duration, seizure burden, frequency of generalized tonic–clonic seizures, and AED use. Variant calling was performed as previously described.[29] Variants in the 332 individuals with epilepsy over the age of 50 were annotated using ANNOVAR[29] and then filtered to obtain a list of single-nucleotide variants in *KCNH2* with an allele frequency <5% in gnomAD.[28]

Ancestry predictions for the exome-sequenced SUDEP and epilepsy controls inferred 95% of samples in each cohort to be European. Exome sequencing achieved mean coverage greater than $20\times$ across 78% f the *KCNH2* coding region in the SUDEP cohort and 94% in the epilepsy controls.

Standard protocol approvals, registrations, and patient consents

The study was approved by the Human Research Ethics Committees of Austin Health and Royal Prince Alfred Hospital. Signed consent was provided by patients themselves, their parents, next-of-kin, or legal guardian in the case of children or patients with intellectual disability. Some of the SUDEP cases were analyzed in a de-identified manner. Some samples from the control cohort had been collected over a 20-year period in some centers, so the consent forms reflected standards at the time of collection. Samples were only accepted if the consent did not exclude data sharing.

KCNH2 site-directed mutagenesis and in vitro cRNA preparation

cDNA encoding a full-length transcript (NM_000238.4 Ensembl database) of human *KCNH2* was subcloned into the *Xenopus* oocyte expression vector pGEMHE-MCS.

Site-directed mutagenesis to create human *KCNH2* variants was completed by GenScript Biotech (Piscataway, NJ, USA). All clones were verified by Sanger sequencing. In vitro synthesis of cRNA was performed using linearized cDNA template and the mMessage mMachine® T7 transcription kit (Ambion, Thermo Fisher Scientific, Waltham, MA). RNA integrity was assessed both spectrophotometrically (NanoDrop) and by gel electrophoresis. All cRNAs were stored at $-80°C$.

Oocyte extraction

Adult female *Xenopus laevis* frogs were housed at the Florey Institute of Neuroscience and Mental Health. Animal procedures and oocyte preparation followed standard procedures in accordance with the conditions approved by the Florey's Ethics Committee. Briefly, frogs were anesthetized with 1.3 mg/mL of tricaine methanesulfonate and oocytes were surgically removed via a small incision to the abdomen. Oocytes were defolliculated with 1.5 mg/mL of collagenase for 2 h and rinsed with OR-2 solution (in mmol/L: 82.5 NaCl, 2 KCl, 1 MgCl$_2$.6H$_2$O, 5 HEPES, pH 7.4). Healthy mature oocytes stage V or VI were isolated.

Channel expression

The NM_000238.4 *KCNH2* transcript was set as our control sequence and designated as the wild-type (WT) channel. cRNAs of 0.5 ng (in 50 nL) coding for *KCNH2* were manually injected into the oocytes. Injected oocytes were maintained in ND96 storage solution (in mmol/L: 96 NaCl, 2 KCl, 1 MgCl$_2$·6H$_2$O, 1.8 CaCl$_2$·2H$_2$O, 5 HEPES, 50 mg/L of gentamicin, pH 7.4) at 17°C for 2 days to allow translation and trafficking of channels prior to recording.

Two-electrode voltage clamp electrophysiology

Standard two-electrode voltage clamp hardware was used (TEC-05X or TEC10X, NPI, Tamm, Germany). Oocytes were impaled with microelectrodes with an input resistance of between 0.2 and 2.0 MΩ, containing 3 mol/L KCl. During experiments, oocytes were continually perfused with high K$^+$ solution (in mmol/L: 100 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, pH 7.4) and clamped at -90 mV. Incremental 10 mV voltage steps from -90 to $+50$ mV were applied for 0.5 s, followed by a test pulse at -100 mV for 3.2 s. Data were sampled at 5000 Hz and low-pass-filtered at 1 kHz. Voltage clamp control and data acquisition were obtained using pCLAMP v8.10 software (Molecular Devices, USA). All recordings were performed at 18–20°C. Currents from WT channels were always measured contemporaneously using the same batch of oocytes.

Electrophysiology analysis

Raw currents were baseline-adjusted and leak-subtracted, and peak current amplitudes were analyzed offline using AxoGraph v1.7.4 (AxoGraph Scientific, Sydney, AU). Half-maximal activation voltage and slope values were obtained from current–voltage curves that were fitted with the Boltzmann equation after normalizing the peak test pulse current after each voltage step to the maximum peak test pulse current. Wild-type normalized current–voltage curves were obtained by normalizing peak test pulse current after each voltage step to the average maximum wild-type peak test pulse current and fitted with the Boltzmann equation.

Statistical analysis

Functional analysis

An *F*-test was used to check for variance equality. Standard one-way ANOVA with Dunnett's post hoc correction was used for statistical comparison to wild-type values if variances were approximately equal ($p > 0.05$). Welch's ANOVA with Dunnett's post hoc correction was used instead, for unequal variances ($p < 0.05$). Statistical analysis for association used the Fisher's Exact Test. Significance was set at $p < 0.05$. Graphs and statistical tests were generated and performed using Prism v8.1.0 (GraphPad, CA, USA). All data points are shown as mean $\pm$ SEM.

Results

KCNH2 variants in SUDEP patients and a control epilepsy population

KCNH2 variants previously described in our SUDEP cohorts are listed in Table 1. Ten out of 90 individuals suffering SUDEP carried a missense or truncation *KCNH2* variant with <5% minor allele frequency.[16,18] These variants have a range of pathogenicity classifications according to the ClinVar database.[30] R744X is reported as pathogenic; Y54H, G924A, R176W, and R1047L are variants of uncertain clinical significance; while G749A is not reported in the ClinVar database. Clinical metadata available for the SUDEP cohort are presented in Table S2.

In our epilepsy control population of 332 living patients with epilepsy over 50 years of age with whole-exome sequencing data, variants in *KCNH2* that satisfied the filtering criteria were found in 20 out of 332 subjects (Table 1). These included the common R1047L variant also found in SUDEP cases (Table 1). Variants A913V, K897R, S871C, T436M, R397H, P347S, and D259N were classified as variants of uncertain significance in ClinVar,

while A193V and S140F are not reported in ClinVar. Clinical metadata available for the control cohort are presented in Table S2.

KCNH2 variants were found in 11.1% of SUDEP cases (10/90) compared to 6.0% of epilepsy controls (20/332; $p = 0.11$).

In silico predictions of $K_V11.1$ channel dysfunction and SUDEP risk

In silico prediction tools provide a method of estimating the detrimental impact of a given variant on protein function. We determined the Combined Annotation Dependent Depletion (CADD) score for each *KCNH2* variant (Table 1). Only the *KCNH2* R744X variant had a CADD score above 30 indicating, as in ClinVar, that it was pathogenic.

Functional characterization of *KCNH2* variants

Manual two-electrode voltage clamp was used to record currents from wild-type (WT; NM_000238.4 *KCNH2* transcript) and mutated $K_V11.1$ channels expressed in *Xenopus* oocytes (Fig. 1). Channels were activated by a series of depolarizing voltage excursions from a holding potential of −90 mV. Maximal tail currents were used to measure channel activity. In our SUDEP cohort, functional analysis revealed a significant reduction in current amplitude (> 20%) for R1047L, G924A, G749A, and R744X mutated channels relative to wild-type channels (Fig. 1A–E and H). R176W and Y54H variants had no effect (Fig. 1F–H). In the epilepsy control group, the R1047L (also in the SUDEP cohort) and the S140F variant reduced current amplitude (Fig. 2). The other nine epilepsy control variants were without effect on $K_V11.1$ channel current amplitude (Fig. 2). Other biophysical parameters measured, including the half-maximal activation voltage and Boltzmann slope, are reported in Figure 3. In the SUDEP group, only G924A and G749A showed altered biophysical properties (Fig. 3A and C), whereas R744X could not be measured. No variants in the epilepsy control group showed any differences in other biophysical parameters (Fig. 3B and D). Changes in biophysical properties for SUDEP *KCNH2* variants may contribute to overall channel dysfunction and consequently increase the risk of sudden death.

Enrichment of loss-of-function *KCNH2* variants in SUDEP cases

Our functional data allow the classification of each variant as either loss-of-function, defined as a statistically

Table 1. *KCNH2* variants found in SUDEP and epilepsy control populations.

Cohort	KCNH2 variant (number of cases)	Combined Annotation-Dependent Depletion (CADD) score	gnomAD allele count	gnomAD allele frequency	Functional analysis
SUDEP (n = 90)	R1047L (5)	25.9	3117	0.0180	Loss-of-function
	G924A (1)	22.8	8	0.0000505	Loss-of-function
	G749A (1)	26.5	0	0	Loss-of-function
	R744X (1)	39.0	0	0	Loss-of-function
	R176W (1)	23.4	44	0.000406	No change
	Y54H (1)	26.6	0	0	No change
Epilepsy control (n = 332)	R1047L (10)	25.9	3117	0.0180	Loss-of-function
	A913V (1)	20.3	73	0.000482	No change
	G903R (1)	22.4	19	0.000120	No change
	K897R (1)	13.2	3	0.0000126	No change
	S871C (1)	28.0	0	0	No change
	T436M (1)	13.9	9	0.0000318	No change
	R397H (1)	27.0	4	0.0000159	No change
	P347S (1)	19.9	281	0.000998	No change
	D259N (1)	22.5	1	0.0000323	No change
	A193V (1)	19.9	2	0.0000286	No change
	S140F (1)	23.7	0	0	Loss-of-function

significant reduction in a current amplitude of >20% ($p < 0.05$), or no change in function (Table 1). Based on these criteria, 8 out of 90 (8.9%) SUDEP cases carried a *KCNH2* loss-of-function variant, while 2 out of 90 (2.2%) carried a variant in which function was not changed. In contrast, our epilepsy patient control population had 11 out of 332 (3.3%) patients with a loss-of-function variant, and 9 out of 332 (2.7%) carried a variant that did not alter function. The SUDEP cohort has approximately threefold enrichment for loss-of-function *KCNH2* variants (OR = 2.7, 95% confidence interval (1.1, 7.4), Fisher's exact test nominal $p = 0.04$) compared with the epilepsy control population (Fig. 4A). There was no enrichment of *KCNH2* variants that did not change channel function in the SUDEP cohort compared to the epilepsy control cohort (OR = 0.8, 95% confidence interval 0.17 to 3.8, Fisher's exact $p > 0.99$).

In the SUDEP cohort, rare *KCNH2* variants with a minor allele frequency of <1% associate with greater loss of function implicating increased risk (Fig. 1). Consistent with this, rare *KCNH2* variants are enriched with a greater odds ratio (OR = 11.4, 95% confidence interval 1.2–111.1, Fisher's exact $p = 0.03$) (Fig. 4B). R1047L is the most common variant that we tested. It is observed in approximately 3.5% of the general population[28] and was found in 5 out of 90 (5.6%) SUDEP cases and 10 out of

332 (3.0%) epilepsy control patients. The reduction in current amplitude for the R1047L variant shows that loss-of-function is not confined to rare *KCNH2* variants.

Discussion

Previous genetic screening efforts in SUDEP have identified rare variants in genes that are associated with severe developmental and epileptic encephalopathies.[3] This is to be expected, as these variants cause severe epilepsies which carry a high SUDEP risk.[2–4] Whether other genetic risk factors contribute to SUDEP risk is less clear. Screening efforts in SUDEP cases have identified variants in cardiac genes that cause arrhythmia syndromes.[9,15–19] We have proposed that when combined, seizures and a risk variant in an arrhythmogenic gene could interact to significantly increase SUDEP risk.[25] Here we focused on *KCNH2*, in which loss-of-function variants are an established cause of LQTS leading to sudden death. We show an approximate threefold enrichment in loss-of-function *KCNH2* variants in our SUDEP cohort relative to an older epilepsy control cohort that has "escaped" SUDEP. These data support the premise that *KCNH2* loss-of-function variants act as genetic biomarkers of SUDEP risk and motivates the need to examine this hypothesis in additional, independent SUDEP cohorts.
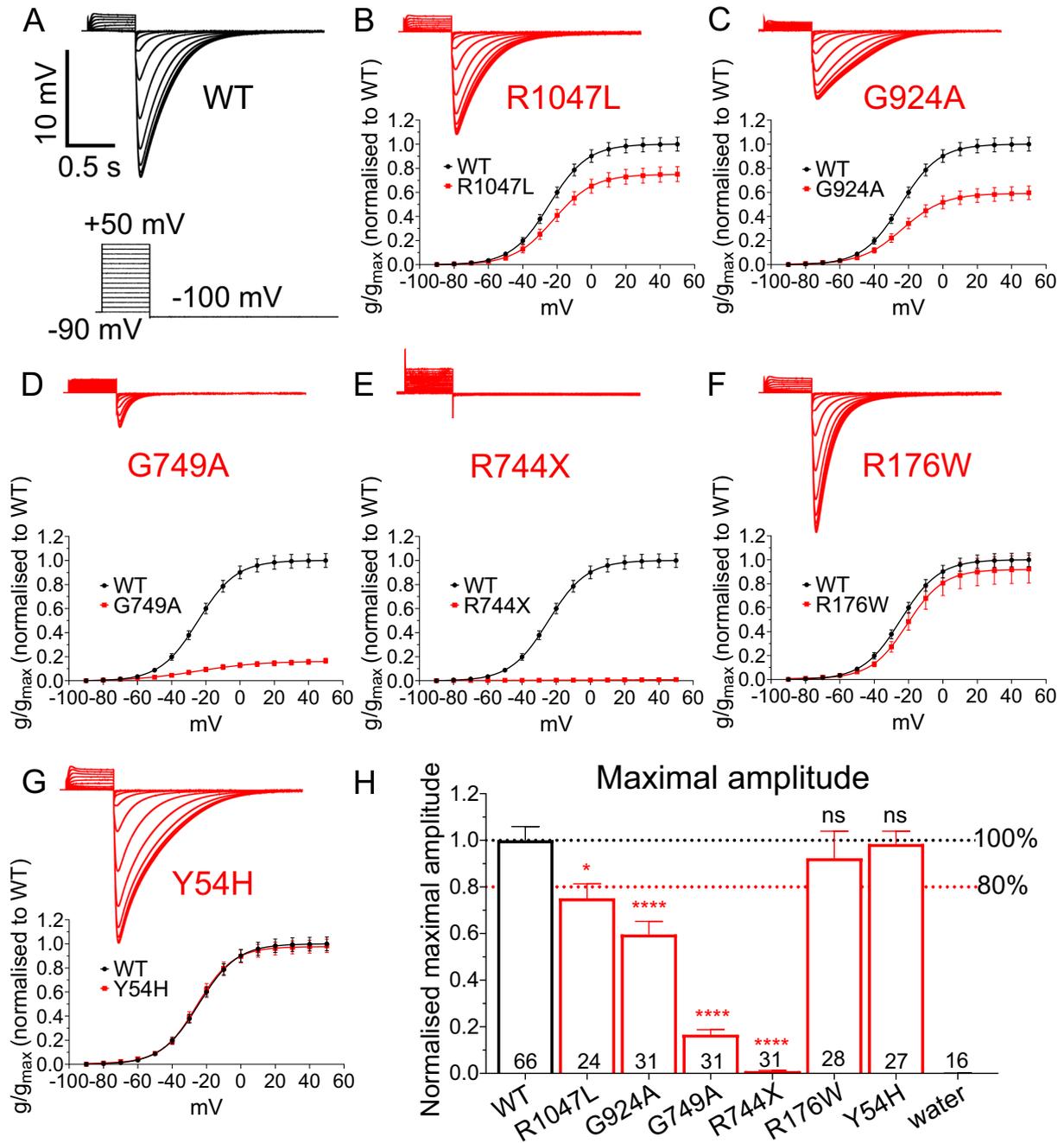
Figure 1. Functional analysis of *KCNH2* variants from SUDEP patients. (A) Sample recording traces of $K_v11.1$ wild-type (WT) channels. *Insert:* cartoon of the voltage protocol applied. (B–G) Sample recording traces of $K_v11.1$ variant channels (*top*) and average normalized conductance–voltage relationships (*below*) comparing $K_v11.1$ WT and variant channels for (B) R1047L, (C) G924A, (D) G749A, (E) R744X, (F) R176W, and (G) Y54H variants. (H) Average maximal amplitude for each variant. Number in each bar represents the number of independent oocytes recorded for each variant. Black and red dashed lines indicate 100% and 80%, respectively, of maximal current amplitude of $K_v11.1$WT channel. *$p < 0.05$, ****$p < 0.0001$.

A systematic retrospective analysis of 10 SUDEP deaths in the Incidence and Mechanisms of Cardiorespiratory Arrests in Epilepsy Monitoring Units (MORTEMUS) revealed that seizure-mediated terminal apnea always preceded terminal asystole.[7] This is strong evidence implying respiratory factors as a cause of death. However, the
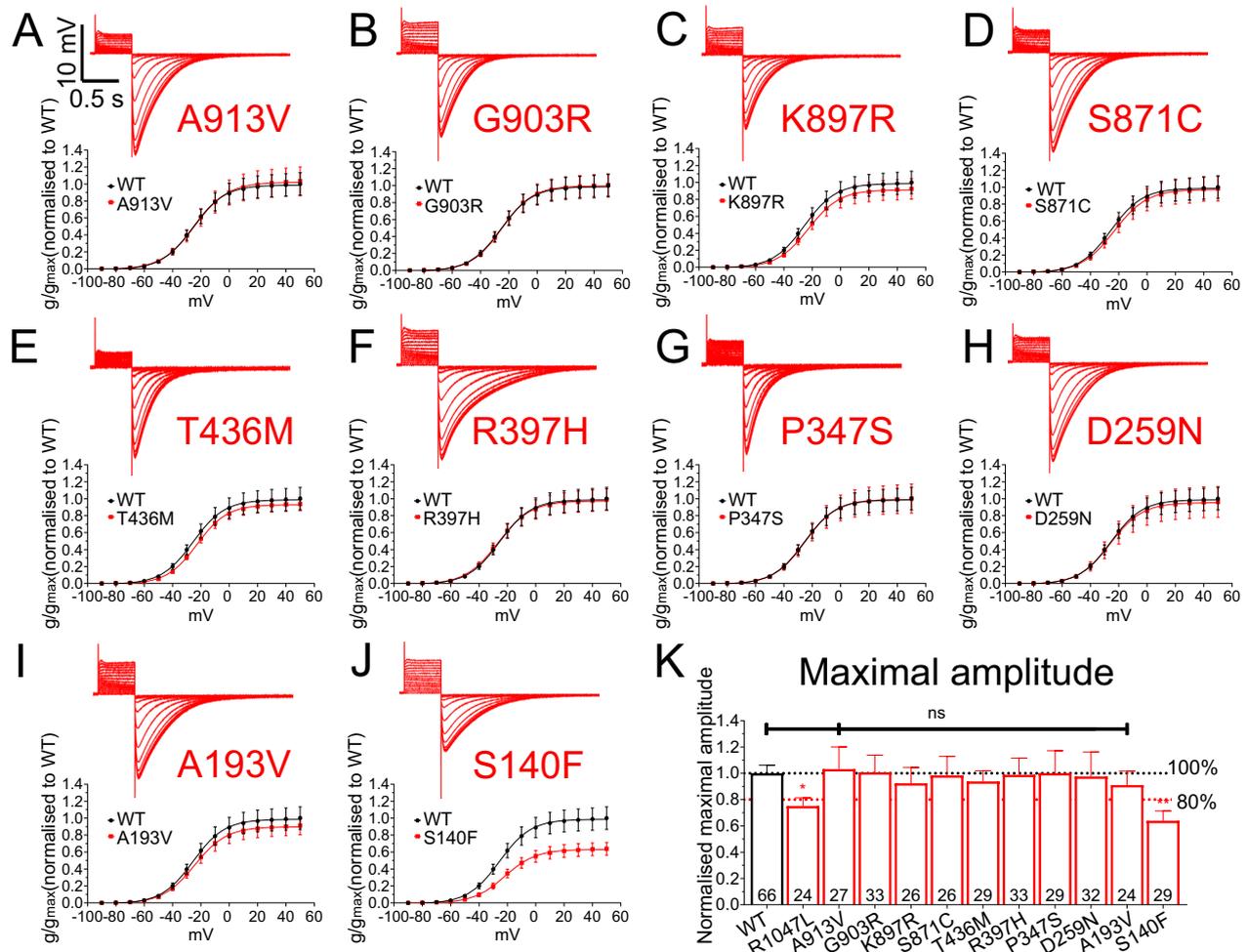
Figure 2. Functional analysis of *KCNH2* variants from epilepsy control cohort. Sample recording traces of K$_v$11.1 variant channels (*top*) and average normalized conductance–voltage relationships (*below*) comparing K$_v$11.1 WT and variant channels for (A) A913V (B) G903R, (C) K897R, (D) S871C, (E) T436M, (F) R397H, (G) P347S, (H) D259N (I), A193V, and (J) S140F variants. (K) Average maximal amplitude for each variant in the epilepsy control cohort. Number in each bar represents the number of independent oocytes recorded for each variant. Black and red dashed lines indicate 100% and 80%, respectively, of maximal current amplitude of the K$_v$11.1 WT channel. *$p < 0.05$, **$p < 0.01$.

patient cohort in this study was small and involved individuals who were undergoing long-term video-EEG monitoring, implying refractory epilepsy. These cases may, therefore, not be representative of all individuals with SUDEP and imply that the pathophysiological mechanism (s) responsible for SUDEP are likely to be multifactorial. Our data suggest that cardiac factors are important, at least in a subset of SUDEP cases.

Characterization of *KCNH2* variants based on in silico predictions of protein dysfunction using the CADD method was uninformative. Only the truncation variant, R744X, had a CADD score greater than 30 and is therefore predicted to be deleterious. CADD scores for the G924A and G749A variants were relatively low yet functional analysis in *Xenopus* oocytes revealed significant

reductions in current amplitude. As noted below, functional analysis methods are not without limitation. However, our findings highlight that functional analysis should remain the gold standard by which to judge potential pathogenicity.

In this study, we have categorized *KCNH2* variants into either loss-of-function or no change in function. However, individual variants do vary in the degree of functional impairment shown in vitro and thus are unlikely to contribute equally to the risk of sudden death. Other factors are also likely to contribute to SUDEP. In the case of rare pathogenic variants, such as R744X, G749A, and G924A there is a large loss-of-function observed in the expression assay and we infer a stronger likelihood that cardiac arrhythmia contributed to death. Increased

A Half-maximal activation voltage- SUDEP variants

B Half-maximal activation voltage- control variants

C Slope- SUDEP variants

D Slope- control variants
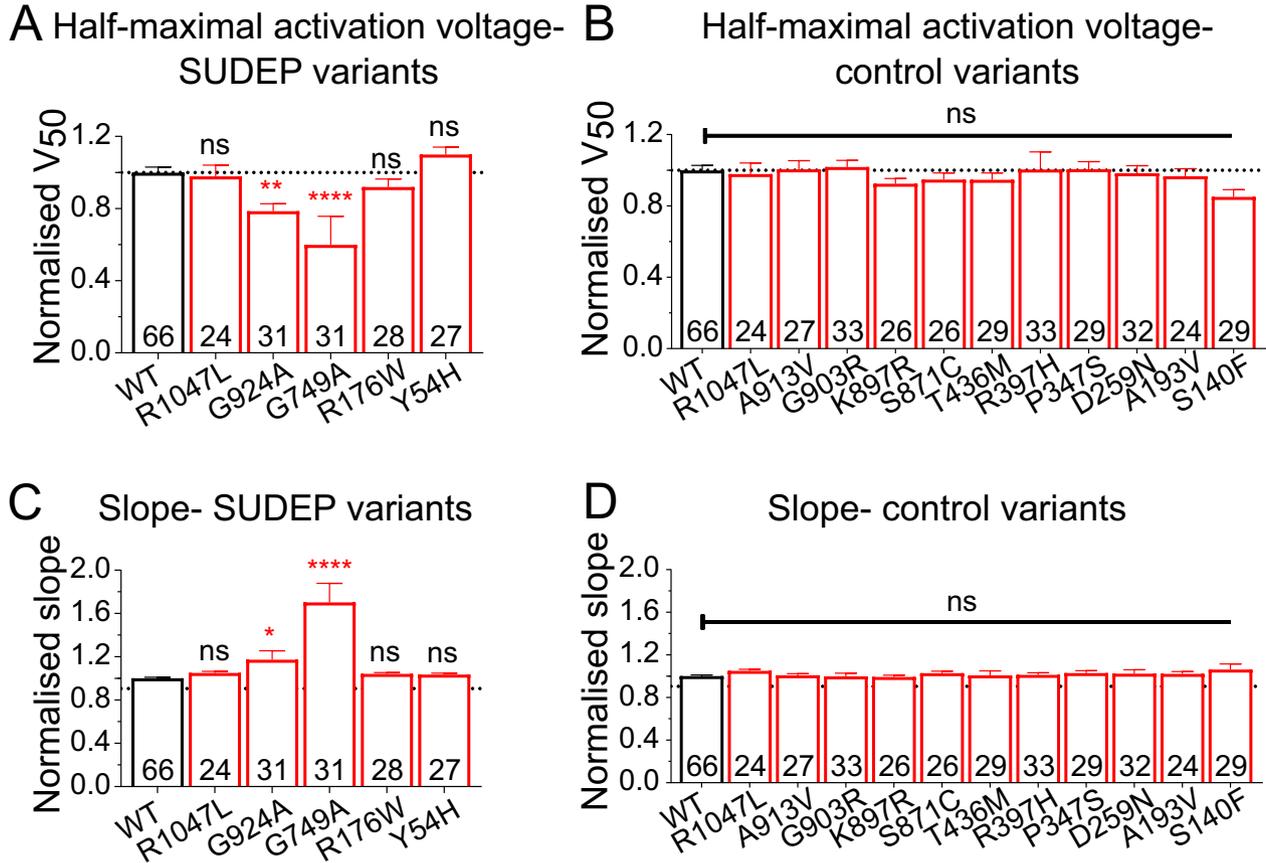
Figure 3. Biophysical properties of *KCNH2* variants from SUDEP cases and epilepsy control population. (A) Average half-maximal voltage of activation for each variant in SUDEP cohort. (B) Average half-maximal voltage of activation for each variant in the epilepsy control cohort. (C) Average slope from the Boltzmann fit for each variant in the SUDEP cohort. (D) Average slope from the Boltzmann fit for each variant in the epilepsy control cohort. Number in each bar represents the number of independent oocytes recorded for each variant. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

A < 5% allele frequency

B < 1% allele frequency
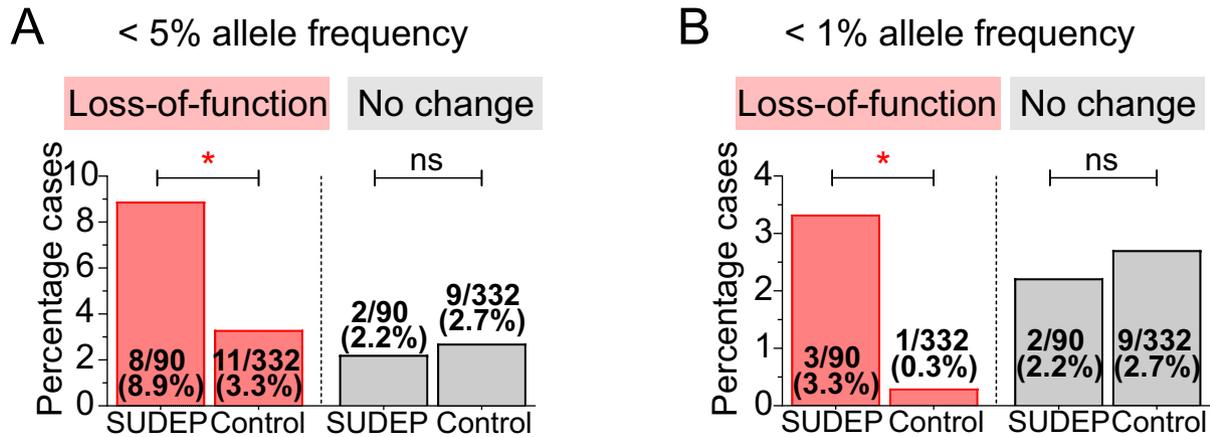
Figure 4. Enrichment of *KCNH2* variants in SUDEP and epilepsy control cohorts. (A) *KCNH2* variants with less than 5% allele frequency are enriched approximately three times in SUDEP compared to the epilepsy control cohort. (B) Enrichment of rare *KCNH2* variants with less than 1% allele frequency is further increased to about 10 times in the SUDEP cohort. *$p < 0.05$.

enrichment of rare *KCNH2* variants (OR = 11.4) in the SUDEP cohort is consistent with a correlation between the extent of loss of function and increased risk. More common variants with lesser in vitro functional impairment may contribute less to individual risk. For example, R1047L has an allele frequency of 1.8% in the gnomAD database of population controls and has a smaller impact on channel function. The R1047L variant is likely to increase individual risk less when compared to variants that cause a large impact on channel function. However, at the population level, the R1047L variant impact is likely to be significant given its common nature, increasing risk to a small degree in many people. Additional studies looking at the functional impact of *KCNH2* variants in a greater number of SUDEP patients will be required to fully understand attributable risk. It is also important to note that heterologous expression systems cannot report on more complex cell functions. More sophisticated model systems such as cardiac myocytes derived from stem cells will help further define the relationship between *KCNH2* variants and arrhythmia risk.

There is surprisingly little evidence that directly links acute seizures to genetically caused cardiac arrhythmia and sudden death. The Kcnq1 T311I mouse model of LQTS provides some evidence with over half of the recorded abnormalities in cardiac rhythm associated with epileptiform discharges.[31] This has implications for more common variants, such as R1047L, that are unlikely to be pathogenic alone but may increase the risk of death in the context of seizures. Furthermore, both human and animal studies show that seizure-mediated changes in cardiac electrophysiology occur.[10] This includes seizure-driven dysfunction that can alter acute cardiac rhythm.[11,32] Studies have also observed altered cardiac ion channel expression with ongoing seizure activity.[33] Either acute and/or chronic changes in cardiac function due to seizures may increase susceptibility to cardiac arrhythmias in people with a "loss-of-function" *KCNH2* variant to significantly increase SUDEP risk.

We acknowledge the small sample size of the SUDEP cohort and the retrospective nature of our study. Adequate seizure and medication metadata are also frequently missing. As such we cannot match our control and SUPDEP cohort precisely. We propose a framework on which to design prospective studies. Whole-genome and exome sequencing (that include *KCNH2*) are becoming routine, providing an opportunity to identify variants. These could be functionally characterized or matched to the growing datasets of characterized *KCNH2* variants that are now available.[34] This would allow the stratification of epilepsy patients and enable prospective cohorts to be captured that could be matched for seizure load, number, and types of antiepileptics and other variables. It would also be informative to identify a cohort with *KCNH2* variants of similar functional deficits but without epilepsy to directly assess the impact of the combination of seizures plus arrhythmic predisposition on the risk of death.

The ability to identify patients at risk of SUDEP has important clinical implications. In patients with epilepsy carrying loss-of-function *KCNH2* variants, prolonged electroencephalogram with cardiac monitoring might be informative to explore ictal and interictal changes in cardiac rhythm, while prolonged cardiac monitoring with cardiac loop recorders could be considered for the continuous interrogation of cardiac rhythm. This would allow the detection of additional biomarkers of risk, including arrhythmogenic markers such as prolonged QT intervals, especially during seizures. Patients identified to be at risk of cardiac arrhythmia could start prophylactic treatment with β-blockers which are used effectively in LQTS. Drugs known to impact QT intervals should also be avoided in such epilepsy patients.

KCNH2 variants will only ever be one biomarker that will be part of a risk assessment. Seizure severity and frequency remain significant predictors of SUDEP risk.[2,3] Further investigation into other potential genetic biomarkers, including arrhythmogenic genes such as *KCNQ1* and *SCN5A* is required, as are studies into how acute seizures or long-term seizure-related changes in cardiac function interact with genetic causes of arrhythmia. An ultimate goal is to develop a SUDEP risk matrix integrating the various clinical, genetic, and environmental factors, and to prevent SUDEP by targeting all modifiable risk factors.

In conclusion, our data provide evidence that both rare and more common *KCNH2* variants that cause loss-of-function may act as biomarkers of SUDEP in epilepsy patients. These data need to be replicated in larger independent study cohorts. Our data motivate more focused clinical studies investigating the impact of loss-of-function *KCNH2* variants on cardiac rhythm. Our study also motivates the development of more complex in vitro models, as well as animal models, that will allow the interaction between seizures and genetic cardiac abnormalities to be investigated. These models will also provide an opportunity to test novel therapeutic strategies for the prevention of SUDEP.

Acknowledgments

Conflict of Interest

SFB declares unrestricted educational grants from UCB Pharma, SCIgen, and Eisai and consultancy fees from Praxis Precision Medicines. IES has served on scientific advisory boards for UCB, Eisai, GlaxoSmithKline, BioMarin, Nutricia, Rogcon, and Xenon Pharmaceuticals; has received speaker honoraria from GlaxoSmithKline, UCB, BioMarin, Biocodex, and Eisai; has received funding for travel from UCB, Biocodex, GlaxoSmithKline, BioMarin, and Eisai; has served as an investigator for Zogenix, Zynerba, Ultragenyx, GW Pharma, UCB, Eisai, Anavex Life Sciences, Ovid Therapeutics, Epygenix, Encoded Therapeutics, and Marinus; and has consulted for Zynerba Pharmaceuticals, Atheneum Partners, Ovid Therapeutics, Epilepsy Consortium, and UCB. IES may accrue future revenue on pending patent WO61/010176 (filed: 2008): Therapeutic Compound; has a patent for SCN1A testing held by Bionomics Inc and licensed to various diagnostic companies; has a patent molecular diagnostic/theranostic target for benign familial infantile epilepsy (BFIE) [PRRT2] 2011904493 & 2012900190 and PCT/AU2012/001321 (TECH ID:2012-009) with royalties paid. The remaining authors have no conflicts of interest.

Author Contributions

MSS, RDB, MFB, LEB, AMP, MH, DC, MB, CS, IES, SFB, and CAR developed the concept and designed the study. MSS and ESMS performed the experiments and analyzed the data. CAR drafted a significant portion of the manuscript. MSS prepared the figures and drafted the methods section. MSS, AMP, MDFC, and CEM performed the molecular biology. All authors contributed to revising and editing the manuscript and approved the submitted version.

References

1. Thurman DJ, Logroscino G, Beghi E, et al. The burden of premature mortality of epilepsy in high-income countries: a systematic review from the Mortality Task Force of the International League Against Epilepsy. Epilepsia 2017;58:17–26.

2. DeGiorgio CM, Markovic D, Mazumder R, et al. Ranking the leading risk factors for sudden unexpected death in epilepsy. Front Neurol 2017;8:1–6.

3. Devinsky O, Hesdorffer DC, Thurman DJ, et al. Sudden unexpected death in epilepsy: epidemiology, mechanisms, and prevention. Lancet Neurol 2016;15:1075–1088.

4. Hesdorffer DC, Tomson T, Benn E, et al. Combined analysis of risk factors for SUDEP. Epilepsia 2011;52:1150–1159.

5. Nilsson L, Farahmand BY, Persson P-G, et al. Risk factors for sudden unexpected death in epilepsy: a case control study. Lancet 1999;353:888–893.

6. Verducci C, Hussain F, Donner E, et al. SUDEP in the North American SUDEP Registry: the full spectrum of epilepsies. Neurology 2019;93:e227–e236.

7. Ryvlin P, Nashef L, Lhatoo SD, et al. Incidence and mechanisms of cardiorespiratory arrests in epilepsy monitoring units (MORTEMUS): a retrospective study. Lancet Neurol 2013;12:966–977.

8. Tomson T, Nashef L, Ryvlin P. Sudden unexpected death in epilepsy: current knowledge and future directions. Lancet Neurol 2008;7:1021–1031.

9. Bagnall RD, Crompton DE, Semsarian C. Genetic basis of sudden unexpected death in epilepsy. Front Neurol 2017;8:348.

10. Ravindran K, Powell KL, Todaro M, et al. The pathophysiology of cardiac dysfunction in epilepsy. Epilepsy Res 2016;127:19–29.

11. Nei M. Cardiac effects of seizures. Epilepsy Curr 2009;9:91–95.

12. Bardai A, Lamberts RJ, Blom MT, et al. Epilepsy is a risk factor for sudden cardiac arrest in the general population. PLoS One 2012;7:e42749.

13. Lamberts RJ, Blom MT, Wassenaar M, et al. Sudden cardiac arrest in people with epilepsy in the community: circumstances and risk factors. Neurology 2015;85:212–218.

14. Stecker EC, Reinier K, Uy-Evanado A, et al. Relationship between seizure episode and sudden cardiac arrest in patients with epilepsy. Circ Arrhythm Electrophysiol 2013;6:912–916.

15. Aurlien D, Leren TP, Taubøll E, et al. New SCN5A mutation in a SUDEP victim with idiopathic epilepsy. Seizure 2009;18:158–160.

16. Bagnall RD, Crompton DE, Petrovski S, et al. Exome-based analysis of cardiac arrhythmia, respiratory control, and epilepsy genes in sudden unexpected death in epilepsy. Ann Neurol 2016;79:522–534.

17. Partemi S, Cestèle S, Pezzella M, et al. Loss-of-function KCNH2 mutation in a family with long QT syndrome, epilepsy, and sudden death. Epilepsia 2013;54:e112–e116.

18. Tu E, Bagnall RD, Duflou J, et al. Post-mortem review and genetic analysis of sudden unexpected death in epilepsy (SUDEP) cases. Brain Pathol 2011;21:201–208.

19. Tu E, Waterhouse L, Duflou J, et al. Genetic analysis of hyperpolarization-activated cyclic nucleotide-gated cation channels in sudden unexpected death in epilepsy cases. Brain Pathol 2011;21:692–698.

20. Tester DJ, Ackerman MJ. Genetics of long QT syndrome. Methodist Debakey Cardiovasc J 2014;10:29–33.

21. Kozek KA, Glazer AM, Ng C-A, et al. High-throughput discovery of trafficking-deficient variants in the cardiac potassium channel K$_V$11.1. Heart Rhythm 2020;17:2180–2189.

22. Bellin M, Casini S, Davis RP, et al. Isogenic human pluripotent stem cell pairs reveal the role of a *KCNH2* mutation in long-QT syndrome. EMBO J 2013;32:3161–3175.

23. Smith JL, Anderson CL, Burgess DE, et al. Molecular pathogenesis of long QT syndrome type 2. J Arrhythm 2016;32:373–380. https://doi.org/10.1016/j.joa.2015.11.009

24. Witchel H. Drug-induced hERG block and long-QT syndrome. Cardiovasc Ther 2011;29:251–259.

25. Bleakley LE, Soh MS, Bagnall RD, et al. Are variants causing cardiac arrhythmia risk factors in sudden unexpected death in epilepsy? Front Neurol 2020;11:925.

26. Langan Y. Sudden unexpected death in epilepsy (SUDEP): risk factors and case control studies. Seizure 2000;9:179–183.

27. Morrish P, Duncan S, Cock H. Epilepsy deaths: learning from health service delivery and trying to reduce risk. Epilepsy Behav 2020;103:106473.

28. Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature 2020;581:434–443.

29. Epi25 Collaborative; Feng YC, Howrigan DP, Abbott LE, et al. Ultra-rare genetic variation in the epilepsies: a whole-exome sequencing study of 17,606 individuals. Am J Hum Genet 2019;105:267–282.

30. Landrum MJ, Lee JM, Riley GR, et al. ClinVar: public archive of relationships among sequence variation and human phenotype. Nucleic Acids Res 2014;42(D1):D980–D985.

31. Goldman AM, Glasscock E, Yoo J, et al. Arrhythmia in heart and brain: *KCNQ1* mutations link epilepsy and sudden unexplained death. Sci Transl Med 2009;1:2ra6.

32. Seyal M, Pascual F, Lee C-Y, et al. Seizure-related cardiac repolarization abnormalities are associated with ictal hypoxemia. Epilepsia 2011;52:2105–2111.

33. Li MCH, O'Brien TJ, Todaro M, et al. Acquired cardiac channelopathies in epilepsy: evidence, mechanisms, and clinical significance. Epilepsia 2019;60:1753–1767.

34. Ng C-A, Perry MD, Liang W, et al. High-throughput phenotyping of heteromeric human ether-à-go-go-related gene potassium channel variants can discriminate pathogenic from rare benign variants. Heart Rhythm 2020;17:492–500.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Characteristics of SUDEP patients without *KCNH2* variant (orange-shaded patients carried loss-of-function *KCNH2* variants).

Table S2. Characteristics of control epilepsy patients with *KCNH2* variant (orange-shaded patients carried loss-of-function *KCNH2* variants).

Table S3. Types of epilepsies in the control cohort.