A mutation in the CACNA1C gene leads to early repolarization syndrome with incomplete penetrance: A Chinese family study

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Abstract

Background

Early repolarization syndrome (ERS) may be a near-Mendelian or an oligogenic disease; however, no direct evidence has been provided to support this theory.

Methods and results

We described a large Chinese family with nocturnal sudden cardiac death induced by ERS in most of the young male adults. One missense mutation (p.Q1916R) was found in the major subunit of the L-type calcium channel gene CACNA1C by the direct sequencing of candidate genes. A concomitant gain-of-function variant in the sodium channel gene SCN5A (p.R1193Q) was found to rescue the phenotype of the female CACNA1C-Q1916R mutation carriers, which led to the incomplete penetrance. The functional studies, via the exogenous expression approach, revealed that the CACNA1C-Q1916R mutation led to a decreasing L-type calcium current and the protein expression defect. The decreased calcium current produced by the mutant channel was improved by isoproterenol but exacerbated by testosterone. The effects of CACNA1C-Q1916R mutation and testosterone on cellular electrophysiology were further confirmed by the human ventricular action potential simulation.

Conclusions

Our results demonstrated that the loss-of-function CACNA1C-Q1916R mutation contributed to ERS-related sudden cardiac death, and the phenotypic incomplete penetrance was modified by the SCN5A-R1193Q variant and sex. These findings suggest that phenotypes of ERS are modified by multiple genetic factors, which supports the theory that ERS may be an oligogenic disease.
Introduction

An early repolarization (ER) pattern on electrocardiography (ECG) is characterized by ST-segment elevation and a terminal QRS slur or notch with J-point elevation ≥1 mm in 2 or more contiguous inferior and/or lateral leads [1]. The ER has been reported to be associated with a risk of arrhythmic death [2–5]. Accordingly, this clinic entity of idiopathic ventricular fibrillation and sudden cardiac death (SCD) with ER pattern is called ER syndrome (ERS) [1, 6]. Although the underlying arrhythmic substrates of ERS are not completely understood, the gene mutations are now considered a major pathogenic factor for developing ERS.

In several recent studies, ERS has been described as a kind of heritable disease. However, only a few ERS-related genes have been identified, including the cardiac inward rectifier potassium channel gene KCNJ8; the cardiac L-type calcium channel (LTCC) subunit genes CACNA1C (encoding the α subunit of LTCC, Cav1.2α1C), CACNB2b and CACNA2D1; the sodium channel α-subunit gene SCN5A; and the ATP-sensitive potassium channels subunit gene ABCC9 [7–12]. Previously, most of the ERS-causing gene mutations were detected in the sporadic cases. A typical ERS family with Mendelian inheritance is extremely rare, and the identified mutations hardly account for the clinical traits regarding male predominance and incomplete penetrance. Therefore, the current understanding of the genetic basis of ERS remains limited, and it has recently been proposed that ERS may be a near-Mendelian or oligogenic inheritance disease [13]. Here we studied a large ERS-related SCD family via the genetic analysis and the functional analysis. The results suggested that the incomplete penetrance of ERS could be modified by the interaction of digenic variations and sex.

Methods

Clinical evaluation

This study conformed to the principles outlined in the Declaration of Helsinki and was prospectively reviewed and approved by the Medical Research Ethics Committee of the Second Affiliated Hospital of Nanchang University. Written informed consent was obtained from all participants whose blood samples were collected for genetic tests. All studied subjects underwent clinical assessments and examinations, including 12-lead ECG, 24-hour ambulatory Holter monitoring, treadmill exercise test, ultrasound echocardiography, and laboratory blood tests were performed in the Second Affiliated Hospital of Nanchang University. The diagnosis of ERS was based on the ECG parameters measured by two independent cardiologists. The peak of a terminal QRS notch or onset of a terminal QRS slur served as the J-point [1].

Variant analysis

Genomic DNA was extracted from peripheral lymphocytes using the RelaxGene Blood DNA System (TIANGEN Biotech, Beijing, China). All exons and exon–intron boundaries of candidate genes, including SCN5A, SCN1B, SCN3B, KCNJ8, ABCC9, KCNJ11, ABCC8, KCNQ1, KCNH2, KCND3, KCNE1, KCNE2, CACNA1C, CACNB2b and CACNA2D1 were amplified by polymerase chain reaction and analyzed by direct sequencing. The genomic DNA of 800 chromosomes from 400 healthy ethnically matched individuals were used as controls.

Mutagenesis and cell transfection

The full-length human WT CACNA1C cDNA containing exon 8a (GenBank accession no. AJ224873) cloned in pcDNA3.1 was a kind gift from Dr. Ganxin Yan’s Laboratory (Jefferson...
Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania). The mutagenesis was performed using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA, USA) and the plasmids were transfected into Human Embryonic Kidney (HEK) 293T cells using Lipofectamine® 2000 reagent (Invitrogen™, Carlsbad, CA, USA). Detailed methods are given in the supporting information, S1 File.

**Cellular electrophysiology and drug intervention**

The HEK293T cells were purchased from the Shanghai Cell Bank of the Type Culture Collection Committee of the Chinese Academy of Science (Shanghai, China) and authenticated using short tandem repeat profiling by the Cell Bank. L-type calcium current ($I_{\text{Ca-L}}$) through wild-type (WT) or mutant LTCC in HEK293T cells was measured by the whole-cell patch clamp recording at room temperature with an EPC10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) as the protocol previously described [14]. Furthermore, testosterone (10 μM) and isoproterenol (10 μM) were used to determine the biological effects on the mutated $I_{\text{Ca-L}}$, respectively.

**Membrane fraction isolation and Western blot**

These experiments were performed as previously described [15]. Briefly, 24 hours after transfection, the protein samples (separate membrane and cytosolic samples) were extracted from cells using the temperature-induced phase separation method. A total of 100 μg lysates or subcellular fractions were separated using 6 to 10% sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene fluoride (PVDF) membranes and probed with specific antibodies. The following antibodies were used: CaV1.2α1C (Alomone Labs, Jerusalem, Israel), ATP1A2 (Proteintech) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Proteintech). Signals of blots were collected through a Bio-RAD ChemiDox XRS detection system and analyzed by Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

**Immunofluorescence**

The immunofluorescence staining images were obtained as previously described [15]. The transfected HEK293T cells was probed by CaV1.2α1C antibody (Alomone Labs), and imaged with a Leica TCS SP5 confocal laser scanning microscopy (Leica Microsystems, Heidelberg, Germany). The detailed methods for immunofluorescence staining are given in the supplementary information S1 File.

**Cardiac myocyte modeling**

The action potentials (AP) of endocardium and epicardium simulations were performed based on a human ventricular cell model developed by Tusscher and Panfilov [16]. The model was accessed from https://models.physiomeproject.org/workspace/tentusscher_panfilov_2006 (Jul 25, 2016). In this cell model, a stimulus frequency of 1 Hz, an amplitude of -52 pA/pF, and a duration of 1 millisecond (ms) was chosen to generate an AP of cardiac myocyte. The effects of mutant calcium channel were simulated by reducing the maximal conductance of $I_{\text{Ca-L}}$ ($G_{\text{Ca}}$) from 0.9 mS/μF to 0.63 mS/μF (by 30%) in a heterozygous pattern. The effects of testosterone (10 μM) treatment were modified by adjusting the electrophysiological experiment data. Consequently, testosterone reduced WT $G_{\text{Ca}}$ from 0.9 mS/μF to 0.74 mS/μF (by 26%) and reduced mutant $G_{\text{Ca}}$ from 0.63 mS/μF to 0.554 mS/μF (by 35%).
Statistical analysis

Numerical data are presented as the means ± standard error of the mean (SEM) and were analyzed using the Statistical Package for the Social Sciences version 19.0 (SPSS19.0). Statistical differences were determined with t-tests for single comparison, and one-way analysis of variance ANOVA followed by the Student-Newman-Keuls test was used for multiple comparisons. P values <0.05 were considered significant.

Results

Clinical characteristics

The pedigree of 4 generations of the Chinese family is presented (Fig 1A). Five seemingly healthy males (I-1, II-2, II-4, III-1 and III-5) died during sleep at a mean age of 34 ± 8.4 years old. The proband (III-5) suffered from nocturnal sudden death at age 34, and his autopsy report (provided by the Department of Forensic Medicine the Medical College of Nanchang University) revealed no pathogenic abnormality of the major organs. His previous medical records indicated that he had suffered from occasional dizziness and palpitations, but no chest pain or episodes of syncope had been observed. On his 24-hour ambulatory Holter monitoring, terminal QRS notches with J point ≥ 1.5 mm in leads II and aVF under the condition of bradycardia were documented (Fig 1B).

Of those affected adult males in this family, the proband’s 33-year-old younger brother (III-7), implanted with an implantable cardioverter defibrillator, was the only survivor. As shown in Fig 1C, the ER ECG pattern with terminal QRS notches in leads II, III, aVF, and V3-V5 (maximum J-point elevation 3 mm) was observed. Besides, the Holter tests of the other 2 surviving family members (IV-3, IV-4) manifested bradycardia-dependent J-point elevation with or without ST-segment elevation. In the Holter of the proband’s 7-year-old son (IV-3), concave ST-segment elevation (1 mm) in leads II, III, aVF, and V4-V6 and terminal QRS notches in leads III and aVF (J-point elevation: 1 mm) were observed (Fig 1D). The ST-segment elevation with terminal QRS slurs in leads II, III, aVF, and V4, and terminal QRS notches in lead V3 (maximum J-point elevation 1.5 mm) were also registered on the Holter of the 5-year-old daughter (IV-4) of III-7 (Fig 1E). The ECGs of other individuals (II-3, II-6, III-4 and IV-1) did not show any ER patterns (S1 Fig). The major ECG parameters of affected members are shown in Table 1.

Genetic analysis

A rare heterozygous mutation (c.A5747G, p.Q1916R) in CACNA1C located in the intracellular C terminus of CaV1.2α1C with high evolutionary conservation was identified in the proband (Fig 2A–2C, Table 2). Five other common polymorphisms in CACNA1C with benign clinical significance were also detected (Table 2). The allele frequency of this variant was 0.000149 according to the Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org) [17] and 0.000799 (G) according to 1000 Genomes (http://www.internationalgenome.org/1000-genomes-browsers). Furthermore, this CACNA1C-Q1916R mutation was absent in 800 chromosomes from 400 healthy controls. A concomitant variant (p.R1193Q, rs41261344) with an allele frequency of 0.006215 (data form ExAC) in SCN5A was also found in the proband (Fig 2D, Table 2). This SCN5A-R1193Q variant, located in a highly conserved spot in the linker region between domains II and III (Fig 2E and 2F), has been reported to be “gain-of-function” and associated with long QT syndrome (LQTS) [18].

The result of sequencing screening revealed that all genotyped members with ancestry originating from I-1 and I-2 carried the CACNA1C-Q1916R mutation. Unfortunately, the genetic
Fig 1. Pedigree and Holter (25 mm/s, 10 mm = 1 mV) results of the family. (A) Phenotypic and genotypic traits are represented by specific symbols in the pedigree. (B) Terminal QRS notch in leads II and aVF (arrows) without ST-segment elevation shown in the Holter of the proband (III: 5). (C) J-point elevation from 1 mm to 3 mm in leads II, III, aVF, and V3—V5 (arrows), which behaved as a terminal QRS notch without ST-segment elevation in III: 7. (d) Terminal QRS notch in leads III and aVF and concave ST-segment elevation (1 mm) in leads II, III, aVF, and V4-V6 (arrows) is presented for IV: 3. (E) Terminal QRS slur with ST-segment...
tests were not performed for the deceased II-4 and III-1, and therefore their genetic states were not defined. Given their offspring was mutation carriers, we might infer that II-4 and III-1 also harbored the CACNA1C-Q1916R mutation. Notably, not all CACNA1C-Q1916R carriers (II-3, II-6, III-4, III-5, III-7, IV-1, IV-3, IV-4 and obligate carriers II-4 and III-1) manifested the positive phenotypes (ER pattern in ECG or nocturnal SCD). This phenotypic incomplete penetrance might be modified by SCN5A-R1193Q variant and sex. As shown in Table 3, all male individuals carrying the CACNA1C-Q1916R mutation with (II-4, III-1, III-5 and IV-3) or without (III-7) concomitant SCN5A-R1193Q showed the ERS phenotypes. The female CACNA1C-Q1916R mutation carriers with SCN5A-R1193Q variant (II-3, II-6, III-4 and IV-1) were not affected, while the female member only carrying the CACNA1C-Q1916R mutation (IV-4) showed the ER ECG pattern. Other family members without the two variations had no ERS phenotypes.

Dysfunctional electrophysiology and drug intervention in mutated CaV1.2α1C

To determine the molecular consequences of the CACNA1C-Q1916R mutation, we transfected CACNA1C with the other 2 subunits (CACNB2b and CACNA2D1) forming the LTCC into HEK293T cells and performed whole cell patch-clamp experiments. The Q1916R mutation reduced the amplitude of the current produced by LTCC when compared with the WT sequence (Fig 3A). The current density of the mutant LTCC over the voltage range of -10 mV to 20 mV was also reduced by 60% at 0 mV (-23.3±1.1 pA/pF for WT LTCC versus -9.4±1.6 pA/pF for mutant LTCC, n = 17–28, *P<0.05, **P<0.01; Fig 3B). However, the voltage-dependence of steady state activation (SSA) and steady state inactivation (SSI) were unchanged (Fig 3C and 3D). The results suggested that the CACNA1C-Q1916R mutation showed a "loss-of-function" in LTCC.

To explore the underlying pathogenic role of sex and the possible therapy for the ERS with CACNA1C-Q1916R, HEK293T cells exogenously expressing the LTCC were treated with the principal male sex hormone, testosterone, or the non-selective β-adrenoceptor agonist, isoproterenol. As reported by Scragg JL et al., testosterone can directly interact with Cav1.2α1C protein and blockade the I_Ca,L in a concentration-dependent manner in the HEK293T cells [19].

Table 1. ECG characteristics of affected individuals.

| Individual | sex | Age (yrs) | CACNA1C-Q1916R Carrier | SCN5A-R1193Q Carrier | HR (beats/min) | PR (ms) | QRS (ms) | J-point elevation (mm) | ST-segment elevation | QTc (ms) | Tp-ed (ms) | Tp-Te (ms) |
|------------|-----|-----------|------------------------|----------------------|----------------|---------|---------|-----------------------|---------------------|---------|-----------|-----------|
| II-4       | M   | 32*       | ●                      | NA                   | NA             | NA      | NA      | NA                    | NA                  | NA      | NA        | NA        |
| III-1      | M   | 32*       | ●                      | NA                   | 68 (160)       | 90      | 3       | No                    | 436                 | 60      | 100       |
| III-5      | M   | 34*       | ●                      | 88 (140)            | 80             | 80      | 1       | Yes                   | 413                 | 20      | 80        |
| III-7      | M   | 33        | ●                      | 94 (120)            | 80             | 80      | 1.5     | Yes                   | 388                 | 40      | 60        |
| IV-3       | M   | 7         | ●                      | 80 (160)            | 80             | 80      | 1       | Yes                   | 437                 | 40      | 120       |
| IV-4       | F   | 5         | ●                      | 94 (120)            | 80             | 80      | 1.5     | Yes                   | 388                 | 40      | 60        |

*Age of death
● positive variant carrier. M = Male; F = Female; NA = records not available; HR = heart rate; Tp-ed = Tp-e dispersion; Tp-Te = Tpeak-Tend.

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The inhibitory effects of 10 μM testosterone were clearly independent of the activation voltage range, thus we chose 10 μM as a working concentration [19]. In the WT group, treatment with testosterone significantly reduced the current densities by 51.5% at 0 mV (-43.1±4.0 pA/pF for WT-vehicle group versus -20.9±0.04 pA/pF for the WT-testosterone group, n = 6, *P<0.05; Fig 4A and 4B). In the CACNA1C-Q1916R group, treatment with testosterone reduced the current densities by 69% at 30 mV (-11.4±1.7 pA/pF for the Q1916R-vehicle group versus -3.5±1.2 pA/pF for the Q1916R-testosterone group, n = 8, *P<0.05; Fig 4A and 4B). Treatment with isoproterenol (10 μM) enhanced the current density of WT channels by 68.5% at -20 mV (-39.1±4.8 pA/pF for the WT-isoproterenol group versus -23.2±4.2 pA/pF for the WT-vehicle group, n = 12, *P<0.05) and mildly increased the current density of CACNA1C-Q1916R channels.
(-11.8±3.8 pA/pF for the Q1916R-isoproterenol group versus -9.5±3.5 pA/pF for the Q1916R-vehicle group, n = 10, P > 0.05; Fig 4E and 4F). The voltage-dependent SSA displayed a left-shift in the Q1916R-isoproterenol group compared with that in the Q1916R-vehicle group, and the half-activation voltage (V_{1/2}) shifted from -6.1 mV to -17.0 mV (P < 0.01; Fig 4G). These results suggested an exacerbated role of testosterone but an improved effect of isoproterenol in the mutant LTCC.

Decreased expression of mutated Ca_{1.2α1C} protein

To further study the mechanism of the decrease in Q1916R I_{Ca-L}, protein expression and subcellular localization were detected in the exogenously expressing HEK293T cells. As shown in Fig 5A and 5B, the expression of mutant Ca_{1.2α1C} was reduced by 54% in the entire cellular lysate, 51% in the membrane fraction and 48% in the cytoplasm fraction, respectively, when compared with the expression of WT Ca_{1.2α1C}. In the similar manner, a decrease in the fluorescent intensity but normal localization was observed via immunofluorescence in HEK293T cells (Fig 5C).

Human ventricular AP simulation

To evaluate the effects of CACNA1C-Q1916R mutation on the AP shape and the potential electrophysiological mechanisms of ER pattern formation, a human ventricular cell model was used to simulate APs with the CACNA1C-Q1916R mutation in the heterozygous status. In this model, CACNA1C-Q1916R was represented by a 30% reduction in the conductance of total I_{Ca-L}. The APs of WT cardiac M (midmyocardial) cell and mutant M cell are shown in Fig 6A, in which a deeper notch of ER was observed in the mutant condition. Additionally, the APD90 (AP duration at the level of 90% of repolarization) was reduced by 14.29% in the mutant M

| Gene      | Rs ID       | Exon | Nucleotide change | Amino-acid change | Allele Frequency | ExAC    | Clinical significance |
|-----------|-------------|------|------------------|-------------------|------------------|---------|----------------------|
| CACNA1C   | rs186867242 | 48   | c.5747A>G        | p.Q1916R          | 0.000799         | 0.000149| NA                   |
|           | rs215976    | 17   | c.2436C>T        | p.D812D           | 0.14557          | 0.1621  | Benign               |
|           | rs216008    | 30   | c.3786C>T        | p.F1262F          | 0.25919          | 0.2095  | Benign               |
|           | rs1051375   | 44   | c.5361G>A        | p.T1787T          | 0.502636         | 0.6953  | Benign               |
| rs201777030 | 47       | c.5609C>T        | p.T1870M          | NA                | 0.003012 | Benign               |
| rs56270948 | 47       | c.5649G>A        | p.P1883P          | 0.03594           | 0.02804 | Benign               |
| SCN5A     | rs41261344  | 20   | c.3578G>A        | p.R1193Q          | 0.01238          | 0.006215| Pathogenic           |

ExAC = The Exome Aggregation Consortium; NA = Not available.

[Table 2. The feature of the variants that identified.](https://doi.org/10.1371/journal.pone.0177532.t002)

[Table 3. The correlation between genotype and phenotype.](https://doi.org/10.1371/journal.pone.0177532.t003)

Table 2. The feature of the variants that identified.

| Phenotype penetrance | CACNA1C-Q1916R +/- | SCN5A-R1193Q +/- | CACNA1C-Q1916R +/- | SCN5A-R1193Q +/- | CACNA1C-Q1916R +/- | SCN5A-R1193Q +/- |
|----------------------|---------------------|------------------|---------------------|------------------|---------------------|------------------|
| Male                 | 100% (4/4 cases)    | 100% (1/1 case)  | 0 (0/1 cases)       | II-4, III-1, III-5, IV-3 | II-7                   | III-3 |
| Female               | 0 (0/4 cases)       | 100% (1/1 case)  | 0 (0/4 case)        | II-3, II-6, IV-1   | IV-4                   | II-5, III-2, III-6, III-8 |

+/- heterozygous for variant; −/− wild type.
cell. J waves are associated with the prominent voltage gradients between the endocardium and epicardium in the ER period. Accordingly, we compared the AP shape between the endocardial cell and the epicardial cell in WT or CACNA1C-Q1916R mutant condition, respectively (Fig 6B and 6C). Then, the transmural voltage gradient from the endocardial cell to the epicardial cell within the ER interval (from 12 ms to 55 ms of AP duration) was analyzed in the WT and the mutant conditions (Fig 6D). In the ER period, overall, the value of voltage gradient between endocardial cell and epicardial cell in the CACNA1C-Q1916R mutant condition was...
Fig 4. Effects of testosterone and isoproterenol on the WT and mutant LTCC. (A-B) The representative whole-cell Ca2+ current traces and average current-voltage relationship show a decrease in current density in both the WT (n = 6) and Q1916R (n = 8) LTCC after treatment with testosterone (*P<0.05, Q1916R or WT+tes versus WT-vehicle; #P<0.05, Q1916R+tes versus Q1916R-vehicle). (C-D) The voltage-dependence of SSA and SSI were not significantly altered among the 4 groups. (E-F) The representative whole-cell Ca2+ current traces and average current-voltage relationship show an increase in current density in the WT (n = 12) LTCC but not the Q1916R (n = 10) LTCC after treatment with isoproterenol (*P<0.05, **P<0.01, Q1916R or WT+ISO versus WT-vehicle). (G-H) The voltage-dependence of SSA displayed a left-shift in both the WT and Q1916R LTCC after treatment with isoproterenol, whereas no change was observed in SSI. SSA: steady state activation, SSI: steady state inactivation, tes: testosterone, ISO: isoproterenol.
greater than that in the WT condition (16.1 mv for the CACNA1C-Q1916R mutation versus 14.44 mv for the WT at 18 ms).

We also modeled the effects of testosterone on the APs in these 3 cell types of cardiac tissue, according to the previous data of patch clamp experiment. The repolarization was earlier and the AP duration was shorter in the testosterone treated cell than that in non-treated cell, due to the more pronounced reduction of $I_{\text{Ca-L}}$ (Fig 6E–6G). Testosterone enhanced the transmural voltage gradient (15.47 mv for testosterone treatment versus 9.93 mv for non-treatment at 23 ms) in the mutant cell (Fig 6H). These results highlighted that the ventricular transmural heterogeneity of electrical activity in the ER stage might be responsible for the generation of the ER pattern on ECG, and testosterone might aggravate the voltage gradient between different myocardium layers.

**Discussion**

Here, we presented a rare large Chinese family with ER-associated SCD, in which the disease phenotypes were mainly caused by a CACNA1C-Q1916R mutation and modulated by the SCN5A-R1193Q variant and sex (Fig 7).

From the clinical perspective, ERS and Brugada syndrome (BrS) share some similar clinical traits, such as male predominance, mean age of first arrhythmic onsets, and circumstances of
arrhythmic episodes. In genetics, loss-of-function mutations in CACNA1C, CACNB2, CACNA2D, SCN5A, and SCN10A, and gain-of-function mutations in KCNJ8 have been shown to lead to both ERS and BrS. Hence, the two entities are referred to as J-wave syndrome by some investigators, but the concept is still controversial. The European Society of Cardiology (ESC) Guidelines for management of patients with ventricular arrhythmias and the prevention of SCD do not include the J-wave syndrome [20]. Furthermore, the differential response to sodium-channel blocker suggests that ERS and BrS are caused by the different basic pathophysiological mechanisms [21]. Indeed, the ST-segment elevation in the right precordial leads
Fig 7. A graphical illustration of the balances represented by the relationships of three genetic contributors linked to the ERS phenotype in this study. (A) The disequilibrium represented the male patients with the CACNA1C mutation and SCN5A variation; the protective effect of the SCN5A variation was less than the pathogenic effects of CACNA1C mutation plus male sex, so the indicator turned pointed left toward the ERS phenotype. (B) The disequilibrium represented the female patients with the CACNA1C mutation. The pathogenic effect of the CACNA1C mutation was greater than the protective effect of female sex; therefore, the indicator pointed left toward the ERS phenotype. (C) The equilibrium state represented the female case with the CACNA1C mutation plus the SCN5A variation. The pathogenic effect of the CACNA1C mutation was neutralized by the SCN5A variation plus female sex, so the indicator consequently stopped in the middle pointing to the normal phenotype.

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in BrS can be explained by abnormalities of both repolarization and depolarization in right ventricular outflow [22]. The affected family members were diagnosed based on the scientific statement referring to ERS, and no BrS was found during the familial clinical screening. Therefore, the mechanisms we studied here were specifically responsible for ERS rather than J-wave syndrome.

The ERS in this pedigree was inherited in an incomplete penetrance manner, with a penetrance of only 60%. The ERS caused by an SCN5A mutation G4297C also showed the inherited pattern of incomplete penetrance; however, the contributory factor for this phenomenon was not elucidated[10]. In another study of some small pedigrees with BrS caused by mutations in CACNA1C, male mutation carriers were more inclined to be symptomatic, which suggests that non-segregation of genotypes and phenotypes may be associated with sex [23]. BrS and ERS share a similar genetic complexity, and not all familial cases provide evidence for Mendelian inheritance [10, 24]. There are always multiple genetic and environmental factors that cause or modulate the phenotype; therefore, a more complex genetic inheritance known as the oligogenic model has recently been hypothesized to explain the particular clinical presentation of inherited cardiac disorders [25]. In this family, the evidence-based on the genetic and functional findings indicated that the loss-of-function mutation CACNA1C-Q1916R was the detrimental variation, and that the gain-of-function variant SCN5A-R1193Q modulated the phenotype. The candidate genes of ERS included CACNA1C and SCN5A, which suggests that the interaction of variations in these 2 genes may potentially modify the penetrance of ERS phenotypes.

As previously reported, SCN5A-R1193Q channels showed inactivation gating and generated a persistent, non-inactivating inward sodium current (I_{Na}), which was associated with LQTS [18]. The QT interval on the ECG represents the depolarization and the repolarization phases of the cardiac AP. Decreases in repolarizing outward potassium currents or increases in depolarizing inward I_{Na} or I_{Ca-L} can result in the prolonged QT interval [26]. Type 3 LQTS is caused by the gain-of-function variation in the SCN5A (e.g., R1193Q), which evokes a sustained inward I_{Na} during the repolarization phase, leading to the deceleration of the repolarization and the prolongation of AP. In contrast, a decrease in I_{Na} or I_{Ca-L} or an increase in outward potassium currents may generate a prominent epicardial notch, which results in a greater transmural voltage gradient in the initiation of the repolarization phase. This voltage gradient registering as a J-point elevation on ECG may be clinically associated with lethal ventricular arrhythmias. The loss-of-function variation in SCN5A or CACNA1C (e.g., CACNA1C-Q1916R), which produces inadequate inward hybrid currents, is responsible for the pathophysiology of ERS. Thus, from the mechanistic point of view, I_{Na} and I_{Ca-L} show a synergistic effect on the repolarization as two ingredients of the inward currents. In this study, we speculated that, during the repolarization phase, the inadequate inward current caused by the detrimental CACNA1C-Q1916R mutation might be partly compensated by the persistent inward tail $I_{Na}^\text{tail}$ produced by the SCN5A-R1193Q channel. That may be how SCN5A-R1193Q plays a protective role against the detrimental phenotype induced by the CACNA1C-Q1916R mutation. Unfortunately, additional experiment of an interaction between the rare mutation and the common variant was not feasible based on the in vitro experimental system that we used, the further in vivo study may be needed to confirm this assumption.

In the oligogenic model of ERS, sex is another high-impact contributor to the phenotypic differences. The male sex predominance has been observed in both the patients with ERS and healthy individuals with an ER pattern, and testosterone levels are significantly increased in men exhibiting an ER pattern than those without the ER pattern [27, 28]. The ER pattern was demonstrated to be the result of a transmural voltage gradient between the epicardium and endocardium, caused either by an increased outward ATP-sensitive potassium current...
$I_{K_{ATP}}$ or transient outward current ($I_{to}$), or by a decreased $I_{Na}$ or $I_{Ca-L}$ [29, 30]. Testosterone has been reported to have a directly inhibitory effect on the $I_{Ca-L}$ [19]. Our findings also suggest that male sex facilitates the formation of ER pattern under certain circumstances, and it is an independent risk factor of ERS in the family.

The CACNA1C-Q1916R variant was not considered to be "pathogenic", although it has been found in two small Japanese cohorts with inherited arrhythmias associated with sudden death [23, 31]. The ethnic formation of Eastern Asian subjects in the ExAC data set is not explicit, hence we cannot know the accurate incidence of CACNA1C-Q1916R in Chinese Han population based on ExAC. According to the data from the 1000 Genomes Project Phase 3, only 1 allele count is observed in Chinese Han population, and the allele frequency of CACNA1C-Q1916R in the Chinese population (including Chinese Dai in XiShuangbanna, Han Chinese in Beijing and Southern Han Chinese) is 0.0017 (1/601). Furthermore, in our study, the mutation was absent in 800 chromosomes from 400 healthy controls, which together indicates that CACNA1C-Q1916R might be considered a rare variant in the Chinese population, with a frequency lower than 0.001. We also found that the allele frequency of CACNA1C-Q1916R in the Japanese population was higher than that in other ethnic populations (Japanese: 3/208, Chinese: 1/601, African: 0, American: 0, European: 0, South Asian: 0) according to the 1000 Genomes data, which suggested that there might be a spatial diversity in the distribution of the CACNA1C-Q1916R mutation. In our study, the CACNA1C-Q1916R mutation was identified in a pedigree and was absent from the 800 control alleles. Additionally, in vitro experiments revealed that the Q1916R mutation impaired the electrophysiological function of the LTCC, which supported the known pathogenic mechanisms of ERS. Based on Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology [32], we consider CACNA1C-Q1916R a pathogenic variation.

The mechanism of ER-related ventricular arrhythmias involves the development of phase 2 re-entry derived from heterogeneous loss of the epicardial AP dome, which is accentuated by the elevated vagal tone due to the elevated $I_{to}$ that is caused by the slow heart rate [29]. This explains why the arrhythmic events occur during sleep in ERS patients. Isoproterenol is widely used to suppress ERS-related VF, and the augmentation effect of $I_{Ca-L}$ by isoproterenol may be one of its pharmacological actions [33, 34]. We observed that isoproterenol could enhance the activity of LTCC in the HEK293T cells, which may be associated with the evocation of cAMP/protein kinase A pathways by the activation of the endogenous β2 adrenoreceptors [35, 36].

In summary, we investigated an extremely rare large ERS family with a high incidence of nocturnal SCD, in which we found a pathogenic mutation in CACNA1C (p.Q1916R) with loss-of-function. The penetrance was also incomplete, which was modified by a gain-of-functional SCN5A-R1193Q variant and sex. The result strengthens the evidence that males are typically at higher risk of SCD, and the inheritance of ERS may be an oligogenic pattern rather than a monogenic model. The targeted mutation screening will effectively guide disease prevention and offspring fertility in the family. Isoproterenol, in theory, may be a putative pharmacologic option to improve the function of CACNA1C-Q1916R mutant channel. However, further translational studies are needed to explore the therapeutic feasibility to human. One limitation of our study is that the sodium channel blocker challenging test used for excluding BrS was refused by the family members. Another limitation is the lack of the induced pluripotent stem cell-derived myocytes from affected individuals and further in vivo study, which is preferable for interpreting the cumulative effects of complex genetic variants.
Supporting information

S1 Fig. Normal ECG of the family members. The ECG of II-3, II-6, III-4, IV-1 showed normal manifestation without ER pattern.
(TIF)

S1 File. Details of methods.
(DOCX)

S1 Data. The original data relating to Figs 3–5.
(XLSX)

Author Contributions

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Investigation: JX ZX WZ.
Methodology: XL YS.
Project administration: KH.
Resources: HB XX JH.
Supervision: KH.
Validation: QC RW.
Visualization: YS JX.
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References

1. Patton KK, Ellinor PT, Ezekowitz M, Kowey P, Lubitz SA, Perez M, et al. Electrocardiographic Early Repolarization: A Scientific Statement From the American Heart Association. Circulation. 2016; 133 (15):1520–9. Epub 2016/04/14. https://doi.org/10.1161/CIR.0000000000000388 PMID: 27067089

2. Aizawa Y, Chinushi M, Hasegawa K, Naiki N, Horie M, Kaneko Y, et al. Electrical storm in idiopathic ventricular fibrillation is associated with early repolarization. Journal of the American College of Cardiology. 2013; 62(11):1015–9. Epub 2013/06/12. https://doi.org/10.1016/j.jacc.2013.05.030 PMID: 23747791

3. Derval N, Simpson CS, Birnie DH, Healey JS, Chauhan V, Champagne J, et al. Prevalence and characteristics of early repolarization in the CASPER registry: cardiac arrest survivors with preserved ejection fraction registry. Journal of the American College of Cardiology. 2011; 58(7):722–8. Epub 2011/08/06. https://doi.org/10.1016/j.jacc.2011.04.022 PMID: 21816308

4. Rosso R, Adler A, Halkin A, Viskin S. Risk of sudden death among young individuals with J waves and early repolarization: putting the evidence into perspective. Heart rhythm: the official journal of the Heart Rhythm Society. 2011; 8(6):923–9. Epub 2011/02/08.

5. Haissaguerre M, Derval N, Sacher F, Jesel L, Deisenhofer I, de Roy L, et al. Sudden cardiac arrest associated with early repolarization. The New England journal of medicine. 2008; 358(19):2016–23. Epub 2008/05/09. https://doi.org/10.1056/NEJMoa071968 PMID: 18463377

6. Priori SG, Wilde AA, Horie M, Cho Y, Behr ER, Berul C, et al. HRS/EHRA/APHRS expert consensus statement on the diagnosis and management of patients with inherited primary arrhythmia syndromes:
document endorsed by HRS, EHRA, and APHRS in May 2013 and by ACCF, AHA, PACES, and AEPC in June 2013. Heart rhythm: the official journal of the Heart Rhythm Society. 2013; 10(12):1932–63. Epub 2013/09/10.

7. Burashnikov E, Pfeiffer R, Barajas-Martinez H, Deplon E, Hu D, Desai M, et al. Mutations in the cardiac L-type calcium channel associated with inherited J-wave syndromes and sudden cardiac death. Heart rhythm: the official journal of the Heart Rhythm Society. 2010; 7(12):1872–82. Epub 2010/09/08. PubMed Central PMCID: PMCPMC2999985.

8. Medeiros-Domingo A, Tan BH, Crotti L, Tester DJ, Eckhardt L, Cuoretti A, et al. Gain-of-function mutation S422L in the KCNJ8-encoded cardiac K(ATP) channel Kir6.1 as a pathogenic substrate for J-wave syndromes. Heart rhythm: the official journal of the Heart Rhythm Society. 2010; 7(10):1466–71. Epub 2010/06/19. PubMed Central PMCID: PMCPMC3049900.

9. Watanabe H, Nogami A, Ohkubo K, Kawata H, Hayashi Y, Ishikawa T, et al. Electrocardiographic characteristics and SCN5A mutations in idiopathic ventricular fibrillation associated with early repolarization. Circulation Arrhythmia and electrophysiology. 2011; 4(6):874–81. Epub 2011/10/27. https://doi.org/10.1161/CIRCEP.111.963983 PMID: 22028457

10. Li N, Wang R, Hou C, Zhang Y, Teng S, Pu J. A heterozygous missense SCN5A mutation associated with early repolarization syndrome. International journal of molecular medicine. 2013; 32(3):661–7. Epub 2013/06/27. https://doi.org/10.3892/ijmm.2013.1422 PMID: 23799537

11. Guo Q, Ren L, Chen X, Hou C, Chu J, Pu J, et al. A novel mutation in the SCN5A gene contributes to arrhythmogenic characteristics of early repolarization syndrome. International journal of molecular medicine. 2016; 37(3):727–33. Epub 2016/01/29. PubMed Central PMCID: PMCPMC4771109. https://doi.org/10.3892/ijmm.2016.2488 PMID: 26820605

12. Hu D, Barajas-Martinez H, Terzic A, Park S, Pfeiffer R, Burashnikov E, et al. ABCC9 is a novel Brugada and early repolarization syndrome susceptibility gene. International journal of cardiology. 2014; 171(3):431–42. Epub 2014/01/21. PubMed Central PMCID: PMCPMC3947869. https://doi.org/10.1016/j.ijcard.2013.12.084 PMID: 24439875

13. Reinhard W, Kaess BM, Debiec R, Nelson CP, Stark K, Tobin MD, et al. Heritability of early repolarization: a population-based study. Circulation Cardiovascular genetics. 2011; 4(2):134–8. Epub 2011/02/02. https://doi.org/10.1161/CIRCGENETICS.110.958298 PMID: 21282333

14. Hu ST, Liu GS, Shen YF, Wang YL, Tang Y, Yang YJ. Defective Ca(2+) handling proteins regulation during heart failure. Physiological research. 2011; 60(1):27–37. Epub 2010/10/16. PMID: 20945956

15. Xiong Q, Cao Q, Zhou Q, Xie J, Shen Y, Wan R, et al. Arrhythmogenic cardiomyopathy in a patient with a rare loss-of-function KCNQ1 mutation. Journal of the American Heart Association. 2015; 4(1):e001526. Epub 2015/01/27. PubMed Central PMCID: PMCPMC4330077. https://doi.org/10.1161/JAHA.114.001526 PMID: 24516976

16. ten Tusscher KH, Panfilov AV. Alternans and spiral breakup in a human ventricular tissue model. American journal of physiology Heart and circulatory physiology. 2006; 291(3):H1088–100. Epub 2006/03/28. https://doi.org/10.1152/ajpheart.00109.2006 PMID: 16565318

17. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016; 536(7616):285–91. Epub 2016/08/19. PubMed Central PMCID: PMCPMC5018207. https://doi.org/10.1038/nature19057 PMID: 27355333

18. Wang Q, Chen S, Chen Q, Wan X, Shen J, Hoeltge GA, et al. The common SCN5A mutation R1193Q causes LQTS-type electrophysiological alterations of the cardiac sodium channel. Journal of medical genetics. 2004; 41(5):e66. Epub 2004/05/04. PubMed Central PMCID: PMCPMC1618878. https://doi.org/10.1136/jmg.2003.013300 PMID: 15121794

19. Scragg JL, Dallas ML, Peers C. Molecular requirements for L-type Ca2+ channel blockade by testosterone. Cell calcium. 2007; 42(1):11–5. Epub 2006/12/19. https://doi.org/10.1016/j.ceca.2006.11.003 PMID: 17173968

20. Priori SG, Blomstrom-Lundqvist C, Mazzanti A, Blom N, Borggreve M, Camm J, et al. 2015 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: The Task Force for the Management of Patients with Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death of the European Society of Cardiology (ESC). Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC). European heart journal. 2015; 36(41):2793–867. Epub 2015/09/01. https://doi.org/10.1093/eurheartj/ehv316 PMID: 26320108

21. Kawata H, Noda T, Yamada Y, Okamura H, Satomi K, Aiba T, et al. Effect of sodium-channel blockade on early repolarization in inferior/lateral leads in patients with idiopathic ventricular fibrillation and Brugada syndrome. Heart rhythm: the official journal of the Heart Rhythm Society. 2012; 9(1):77–83. Epub 2011/08/23.
22. Shimizu W, Aiba T, Kamakura S. Mechanisms of disease: current understanding and future challenges in Brugada syndrome. Nature clinical practice Cardiovascular medicine. 2005; 2(6):408–14. Epub 2005/08/27. PMID: 16119703

23. Fukuyama M, Ohno S, Wang Q, Kimura H, Makiyama T, Itoh H, et al. L-type calcium channel mutations in Japanese patients with inherited arrhythmias. Circulation journal: official journal of the Japanese Circulation Society. 2013; 77(7):1799–806. Epub 2013/04/12.

24. Probst V, Wilde AA, Barc J, Sacher F, Babuty D, Mabò P, et al. SCN5A mutations and the role of genetic background in the pathophysiology of Brugada syndrome. Circulation Cardiovascular genetics. 2009; 2(6):552–7. Epub 2009/12/25. https://doi.org/10.1161/CIRCGENETICS.109.853374 PMID: 20031634

25. Bezzi CR, Lahrouchi N, Priori SG. Genetics of sudden cardiac death. Circulation research. 2013; 77(7):1799–806. Epub 2013/04/12. https://doi.org/10.1007/CRD.0b013e3182203504 PMID: 21808164

26. Haissaguerre M, Chatel S, Sacher F, Weerasooriy A R, Probst V, Loussouarn G, et al. Ventricular fibrillation with prominent early repolarization associated with a rare variant of KCNJ8/KATP channel. Journal of cardiovascular electrophysiology. 2009; 20(1):93–8. Epub 2009/01/06. https://doi.org/10.1111/j.1540-8167.2008.01326.x PMID: 19120683

27. Sager S J, Hoosien M, Junttila MJ, Tanawuttithat W, Perry AC, Myerburg RJ. Comparison of inferolateral early repolarization and its electrocardiographic phenotypes in pre- and postadolescent populations. The American journal of cardiology. 2013; 112(3):444–8. Epub 2013/05/07. https://doi.org/10.1016/j.amjcard.2013.03.052 PMID: 23642382

28. Giudicessi JR, Ackerman MJ. Potassium-channel mutations and cardiac arrhythmias—diagnosis and therapy. Nature reviews Cardiology. 2012; 9(6):319–32. Epub 2012/02/01. https://doi.org/10.1038/nrcardio.2012.3 PMID: 22290238

29. Koncz I, Gurabi Z, Patocska B, Panama BK, Szel T, Hu D, et al. Mechanisms underlying the development of the electrocardiographic and arrhythmic manifestations of early repolarization syndrome. Journal of molecular and cellular cardiology. 2014; 68:20–8. Epub 2014/01/01. PubMed Central PMCID: PMCPMC3943882. https://doi.org/10.1016/j.yjmcc.2013.12.012 PMID: 24378566

30. Hata Y, Kinoshita K, Mizumaki K, Yamaguchi Y, Hiroko K, Ichida F, et al. Postmortem genetic analysis of sudden unexplained death syndrome under 50 years of age: A next-generation sequencing study. Heart rhythm: the official journal of the Heart Rhythm Society. 2016; 13(7):1544–51. Epub 2016/03/24.

31. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in medicine: official journal of the American College of Medical Genetics. 2015; 17(5):405–24. Epub 2015/03/06. PubMed Central PMCID: PMCPMC4544753.

32. Macfarlane PW, Antzelevitch C, Haissaguerre M, Huikuri HV, Potse M, Rosso R, et al. The Early Repolarization Pattern: A Consensus Paper. Journal of the American College of Cardiology. 2015; 66(4):470–7. https://doi.org/10.1016/j.jacc.2015.05.033 PMID: 26205599

33. Weiss S, Oz S, Benmocha A, Dascal N. Regulation of cardiac L-type Ca(2)(+) channel CaV1.2 via the beta-adrenergic-cAMP-protein kinase A pathway: old dogmas, advances, and new uncertainties. Circulation research. 2013; 113(5):617–31. Epub 2013/09/21. https://doi.org/10.1161/CIRCRESAHA.113.301781 PMID: 23948586

34. Gao T, Yatani A, Dell’Acqua ML, Sako H, Green SA, Dascal N, et al. CAMP-dependent regulation of cardiac L-type Ca2+ channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron. 1997; 19(1):185–96. Epub 1997/07/01. PMID: 9247274

35. Binkowski BF, Butler BL, Stecha PF, Eggers CT, Otto P, Zimmerman K, et al. A luminous biosensor with increased dynamic range for intracellular cAMP. ACS chemical biology. 2011; 6(11):1193–7. Epub 2011/09/22. https://doi.org/10.1021/cb200248h PMID: 21932825