Mutations in the cardiac L-type calcium channel associated with inherited J-wave syndromes and sudden cardiac death

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BACKGROUND L-type calcium channel (LTCC) mutations have been associated with Brugada syndrome (BrS), short QT (SQT) syndrome, and Timothy syndrome (LQT8). Little is known about the extent to which LTCC mutations contribute to the J-wave syndromes associated with sudden cardiac death.

OBJECTIVE The purpose of this study was to identify mutations in the $\alpha 1$, $\beta 2$, and $\alpha 2\delta$ subunits of LTCC ($Ca_v 1.2$) among 205 probands diagnosed with BrS, idiopathic ventricular fibrillation (IVF), and early repolarization syndrome (ERS). *CACNA1C, CACNB2b,* and *CACNA2D1* genes of 162 probands with BrS and BrS+SQT, 19 with IVF, and 24 with ERS were screened by direct sequencing.

METHODS/RESULTS Overall, 23 distinct mutations were identified. A total of 12.3%, 5.2%, and 16% of BrS/BrS+SQT, IVF, and ERS probands displayed mutations in $\alpha 1$, $\beta 2$, and $\alpha 2\delta$ subunits of LTCC, respectively. When rare polymorphisms were included, the yield increased to 17.9%, 21%, and 29.1% for BrS/BrS+SQT, IVF, and ERS probands, respectively. Functional expression of two *CACNA1C* mutations associated with BrS and BrS+SQT led to loss of function in calcium channel current. BrS probands displaying a normal QTc had additional variations known to prolong the QT interval.

CONCLUSION The study results indicate that mutations in the LTCCs are detected in a high percentage of probands with J-wave syndromes associated with inherited cardiac arrhythmias, suggesting that genetic screening of Ca_v genes may be a valuable diagnostic tool in identifying individuals at risk. These results are the first to identify *CACNA2D1* as a novel BrS susceptibility gene and *CACNA1C, CACNB2,* and *CACNA2D1* as possible novel ERS susceptibility genes.

KEYWORDS Arrhythmia; Calcium; Electrophysiology; Genetics; Ion channels

ABBREVIATIONS **BrS** = Brugada syndrome; **CHO** = Chinese hamster ovary; **ERS** = early repolarization syndrome; **IVF** = idiopathic ventricular fibrillation; **LQTS** = long QT syndrome; **LTCC** = L-type calcium channel; **PCR** = polymerase chain reaction; **SCD** = sudden cardiac death; **SNP** = single nucleotide polymorphism; **SQT** = short QT; **WT** = wild type

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Introduction

Sudden cardiac death (SCD) is often associated with inherited cardiac arrhythmia syndromes.[1] Twenty-five percent of all unexplained sudden deaths may be due to inherited cardiac diseases such as Brugada syndrome (BrS), idiopathic ventricular fibrillation (IVF), and long QT syndrome (LQTS).[2] BrS, early repolarization syndrome (ERS), and some forms of IVF represent a continuous spectrum of phenotypic expression that differ with respect to the magnitude and lead location of abnormal J-wave manifestations, which we and others have proposed be termed *J-wave syndromes.*[3]

The past decade has witnessed a veritable explosion of information linking inherited cardiac arrhythmia syndromes to cardiac ion channel mutations. BrS has been associated with mutations in seven genes classified as BrS1 through BrS7.[4] Mutations in *SCN5A,* which encodes the $Na_v1.5$ protein forming the α subunit of the sodium channel, have been associated with 11% to 28% of BrS probands by different groups.[5] A genotype has not yet been identified in the majority of BrS probands. ERS has thus far been associated with one mutation in *KCNJ8,* a gene encoding the pore-forming subunit of the $I_{K\text{-}ATP}$ channel.[6] Expression studies suggesting a functional effect of this mutation has recently been reported.[7]

Little is known about the contribution of calcium channel gene variations to the etiology of inherited cardiac arrhythmia syndromes. Splawski et al[8,9] first described gain-of-function mutations in *CACNA1C,* a gene encoding $Ca_v1.2$ protein that forms the α subunit of the L-type calcium channel (LTCC), associated with a multiorgan dysfunction causing long QT intervals, arrhythmias, and autism known as Timothy syndrome (LQT8). Our group first described loss-of-function mutations in the α and β subunits of the cardiac LTCC associated with BrS and shorter than normal QT intervals and SCD.[10,11]

The LTCC is composed of four subunits: the main pore-forming $\alpha1$ ($Ca_v1.2$) subunit, which determines the main biophysical and pharmacologic properties of the channel, and three auxiliary subunits, including a cytoplasmic β subunit, encoded by *CACNB,* $\alpha2\delta$ encoded by *CACNA2D,* and a γ subunit, which is present in skeletal, but not cardiac, muscle.[12–14] Although a number of isoforms for the auxiliary subunits have been identified, in this study we focused on $\beta2$ (*CACNB2*), the dominant isoform known to play an essential role in the voltage dependence of LTCC,[15,16] and the extracellular $\alpha2$ and transmembrane $\delta1$ *(CACNA2D1),* which are linked to each other via disulfide bonds.

Few data are available on the extent to which mutations in the various subunits of LTCC contribute to SCD, the extent to which they are associated with ST-segment elevation and QT abbreviation giving rise to the BrS and BrS+SQT phenotypes, and their pathogenicity. The present study sought to identify genetic variations in the $\alpha1$, $\beta2$, and $\alpha2\delta1$ subunits of LTCC among probands diagnosed with BrS, ERS, and IVF and the extent to which they contribute to pathogenesis of these syndromes. We tested the hypothesis that mutations in LTCC genes are relatively common among probands diagnosed with these syndromes. We also examined the hypothesis that LTCC mutation-mediated BrS associated with a normal QTc is attributable to additional genetic variations known to prolong the QT interval.

Material and methods

Diagnosis

The probands and their family members were diagnosed as having BrS, BrS with shorter than normal QT (BrS/SQT), IVF, or ERS based on established criteria.[17–20] Diagnosis was made based on 12-lead ECG, personal history of syncope, seizures, or aborted cardiac death, and family history of SCD or arrhythmic events. BrS patients displayed a coved-type ST-segment elevation in at least one right precordial lead under baseline conditions or after sodium channel block challenge with ajmaline or procainamide. Criteria for BrS with shorter than normal QT included QTc $\leq$360 ms for males and QTc $\leq$370 ms for females. An early repolarization pattern was defined as J-point (QRS–ST junction) elevation $>$0.1 mV manifested as QRS slurring or notching or a distinct J wave. Patients were categorized as having IVF when no clear established phenotype was discernible in individuals experiencing one or more episodes of ventricular fibrillation. Most, but not all, patients underwent a sodium block challenge to rule out BrS and/or an isoproterenol challenge to rule out catecholaminergic polymorphic ventricular tachycardia.

Mutation analysis

A total of 205 BrS, BrS/SQT, ERS, and IVF probands who tested negative for *SCN5A* mutations were included in the study. After obtaining informed consent, blood was collected from the probands and family members. Genomic DNA was extracted from peripheral blood leukocytes using a commercial kit (Puregene, Gentra Systems, Inc., Minneapolis, MN, USA) and amplified by polymerase chain reaction (PCR) on GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). All exons and intron borders of the *CACNA1C, CACNB2,* and *CACNA2D1* genes were amplified and analyzed by direct sequencing. PCR products were purified with a commercial reagent (ExoSAP-IT, USB, Cleveland, OH, USA) and directly sequenced from both directions using an ABI PRISM 3100 Automatic DNA Analyzer (Applied Biosystems). Electropherograms were visually examined for heterozygous peaks and compared with reference sequences for homozygous variations (GenBank accession number NM_000719, NM_201590, NM_000722.2) using CodonCode Aligner Version 2.0.4 (CodonCode Corporation, Dedham, MA, USA). Fifty-five primer pairs were used to screen 55 exons, including splice alternative variants of *CACNA1C*, 20 primer pairs were used for *CACNB2,* and 39 primer pairs were used for *CACNA2D1*. Probands with calcium channel mutations and rare variants were also screened for *KCNH2, KCNQ1, KCNJ8, KCNE1, KCNE2, KCNE3, KCNE4, SCN1B,* and

SCN3B for the purpose of identifying additional mutations and/or polymorphisms. Variations were designated as mutations based on the Human Genome Variation Society's guidelines for nomenclature.[21] To be considered a mutation, a variation must have changed or disrupted the open reading frame (missense, nonsense, insertion/deletion mutation) and been absent in a minimum of 400 reference alleles obtained from more than 200 healthy individuals of similar ethnicity whenever possible. Possible single nucleotide polymorphisms (SNPs) were confirmed in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/projects/SNP). Numbering of residue variations was based on the NCBI database nucleotide reference sequence. The degree to which variations uncovered are conserved among species was determined using VISTA browser (http://pipeline.lbl.gov/cgi-bin/gateway2).

Mutagenesis and functional expression

The human wild-type (WT) *CACNA1C* cDNA [(EYFP)Nα1c,77] in pcDNA vector was a gift from Dr. Nikolai Soldatov. cDNA of *CACNB2b* and *CACNA2D1* genes, both cloned in pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA), were a gift from Dr. Michael Sanguinetti. Site-directed mutagenesis was performed with QuikChange II XL mutagenesis kit (Stratagene Agilent Technologies Co., La Jolla, CA, USA).

Mutated genes were functionally expressed in either Chinese hamster ovary (CHO) cells or human embryonic kidney (TSA201) cells as previously described.[22] CHO cells were transfected with the cDNA encoding WT or p.V2014I *CACNA1C* subunits (3 μg) together with the cDNA encoding *CACNB2b* (12 μg), *CACNA2D1* (5.1 μg),[9] and the CD8 antigen (0.5 μg) using FuGENE 6 (Roche Diagnostics, Pittsburgh, PA, USA). The other two calcium variants were expressed in TSA201 cell line as previously described.[22] cDNA of the three LTCC subunits were transfected in a 1:1:1 molar ratio using FuGENE 6 (Roche Diagnostics). In addition, CD_8 cDNA was cotransfected as a reporter gene for the experiment involving p.D601E *CACNB2b.* Because p.E1829_Q1833dup *CACNA1C* was already tagged with YFP, no extra reporter gene was added. Before experimental use, cells were incubated with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450, Invitrogen Dynal, Carlsbad, CA, USA) for the experiment of p.V2014I *CACNA1C* and p.D601E *CACNB2b.* For protocols involving p.E1829_Q1833dup *CACNA1C*, cells were directly identified by epifluorescence.

CHO cells were perfused with an external solution containing the following (in mmol/L): NMDG 130, KCl 5, $CaCl_2$ 15, $MgCl_2$ 1, and HEPES 10 (pH 7.35 with HCl). Recording pipettes were filled with internal solution containing the following (in mmol/L): $CsCl_2$ 120, $MgCl_2$ 2, MgATP 2, HEPES 10, $CaCl_2$ 5, and EGTA 10 (pH 7.25 with CsOH).[10] Voltage-clamp recordings on TSA201 cells were made with patch pipettes filled with a solution containing the following (in mmol/L): CsCl 110, $CaCl_2$ 0.1, HEPES 10, EGTA 10, MgATP 2, and TEA 10 (pH 7.3 with CsOH). Extracellular solution contained the following (in mmol/L): glucose 10, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and TEA 150 (pH 7.35 with CsOH). Currents were filtered with a four-pole Bessel filter at 5 kHz and digitized at 50 kHz. Series resistance was electronically compensated at 70% to 80%.[23]

Electrophysiology

Calcium currents were recorded in CHO or TSA201 cells using whole-cell, patch-clamp techniques at 21° to 23°C with Axon-200B patch-clamp amplifiers and pCLAMP9 software (Axon Instruments, Chicago, IL, USA). Mean maximum current amplitude and cell capacitance were 0.5 ± 0.1 nA and 12.2 ± 1.5 pF, respectively (n = 16). Capacitance and series resistance were optimized, and ≈80% compensation was usually achieved, leading to uncompensated access resistances of 1.5 ± 0.7 MΩ.

Current–voltage relationships were constructed by applying 500-ms pulses from a holding potential of –70 mV to potentials ranging –50 and +70 mV. Voltage dependence of inactivation was determined using a two-step voltage-clamp protocol with a 500-ms conditioning pulse from –70 mV to potentials between –90 and +50 mV, followed by a test pulse to +20 mV. Inactivation curves were constructed by plotting the current amplitude elicited by the test pulse as a function of the voltage command of the conditioning pulse. Calcium channel conductance (G) was determined from the following relationship:

$$G = I_{tp}/(V_m - V_R),$$

where I_{tp} = peak current amplitude at V_m, and V_R = reversal potential (–67.4 ± 1.1 mV, n = 16). A Boltzmann function was fitted to the conductance–voltage and inactivation curves, yielding the midpoint (V_h) and slope (k) value of the curves.

Statistical analysis

Results are expressed as mean ± SEM. Data were compared using analysis of variance followed by the Newman-Keuls test. $P < .05$ was considered significant.

Results

Clinical characteristics

A total of 205 unrelated probands enrolled at the Masonic Medical Research Laboratory (MMRL) inherited cardiac arrhythmia registry over the past 5 years diagnosed with BrS, BrS/SQT, IVF, or ERS and their families were included in the study. Figure 1 shows representative 12-lead ECGs from BrS, ERS, and BrS/SQT phenotypes. The cohort consisted of 152 probands diagnosed with BrS, 10 with BrS/SQT, 19 with IVF, and 24 with ERS. Demographic characteristics are given in Table 1. Average age ranged between 30 ± 11 and 43 ± 16 years, and all four categories were male dominated (68%–90%).

Identification of mutations

Among all diagnostic groups, 25 probands were identified with one or more mutations in *CACNA1C, CACNB2,* or

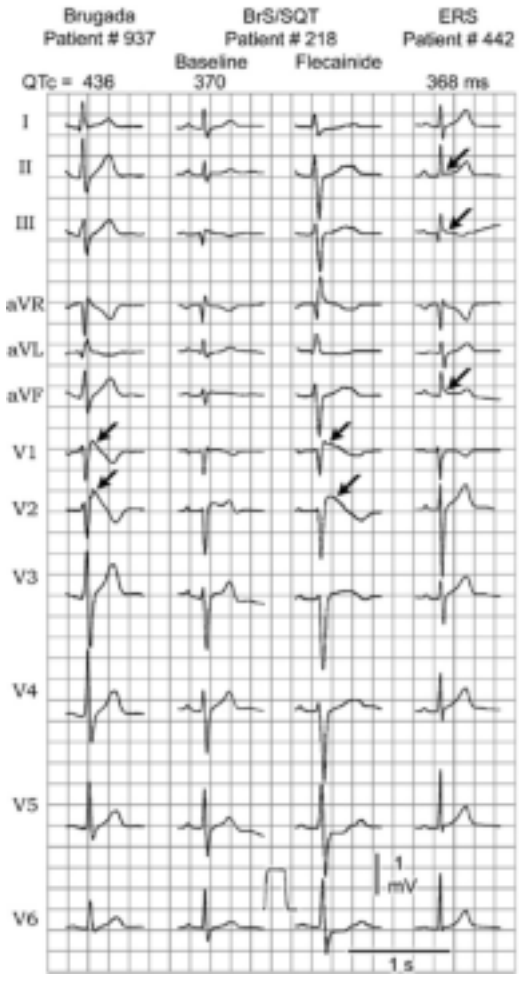

Figure 1 Representative ECGs of Brugada syndrome (BrS), BrS with shorter than normal QT (BrS/SQT), and early repolarization syndrome (ERS). *Arrows* denote type I ST-segment elevation in the BrS patients and early repolarization pattern in the ERS patient.

CACNA2D1 genes encoding the three subunits of the L-type Ca channel: 15 BrS, 5 BrS/SQT, 1 IVF, and 4 ERS. Clinical characteristics and demographics of the probands with mutations are summarized in Table 1. Mean age at time of diagnosis and gender among the probands identified with mutations were similar to those of the entire cohort. A majority of probands in all four diagnostic groups were symptomatic, and there was a high incidence of syncope, ventricular tachycardia/ventricular fibrillation, and family history of SCD in all groups. An early repolarization pattern was observed in one or more of the inferior or lateral leads of 26% of BrS probands. Corrected QT intervals were shorter than normal in the BrS/SQT, ERS, and IVF groups but were in the normal range in the BrS group (Table 1).

Of the 23 mutations uncovered, 21 were missense and 2 were deletion/duplication (Table 3). Four of the mutations (p.A39V, p.G490R, p.T11I, p.S481L) were previously reported by our group.[10,11] Nine mutations were localized in the $\alpha 1$ subunit, 10 in the $\beta 2$ subunit, and 4 in the $\alpha 2\delta$ subunit.

Four of the 9 mutations in *CACNA1C* were identified in BrS probands, 4 in BrS/SQT, and 1 in the ERS group (Table 3). Six of the 10 mutations in *CACNB2* were identified in BrS, 1 each in BrS/SQT and IVF, and 2 in ERS probands (Table 3). The mutation p.S709N was found in two unrelated patients. Two mutations (p.D550Y and p.Q917H) were identified in the same individual. The mutation p.S143F was found in three BrS patients. Two mutations (p.L399F in exon 13 and p.K170N in alternative exon 7b) were genotyped in the same BrS proband (Table 3 and Figure 2H). Three of the 4 mutations identified in the *CACNA2D1* gene were found in BrS patients and 1 in an ERS patient (Table 3). Six rare SNPs were identified in screened probands in two subunits of the calcium channel listed in Table 3. Four of the 6 (p.P817S, p.A1717G, p.T1787M, p.R1973Q) are novel, and two are present in NCBI's dbSNP (p.G37R, rs34534613 in CACNA1C and p.R552G, rs61733968 in CACNB2b). Variation p.G37R has a reported heterozygous frequency of 0.028. The estimated frequencies of other identified rare polymorphisms varied from 0.5% to 1.6%.

Each mutation was tested for degree of conservation among multiple species (Rhesus monkey, dog, horse, mouse, rat, chicken; Table 3). Fourteen (61%) of the 23 mutations were in residues highly conserved among species, 6 (26%) were conserved among large mammals, and 3 (13%) were not conserved. In the case of rare polymorphisms, 2 of the 6 were highly conserved, two was conserved among large mammals, and 2 were not conserved.

Figure 3 shows the predicted topology of the three subunits of LTCC and the location of the mutations. Interestingly, 6 of the 9 mutations in the $Ca_v1.2$ $\alpha 1$ subunit were in either the N-terminus or the C-terminus, with no mutations detected in any of the transmembrane regions. Larger symbols with numbers denote the frequency of appearance the mutation among probands.

Pedigrees of the available families of probands with mutations are shown in Figure 2. Penetrance was complete in five families (A, C, D, G, I). Families B and F showed incomplete penetrance for BrS, which could be explained on the basis of female gender and young age.[24] Family J with a diagnosis of ERS represents a rare case in which both the proband and his wife carried the same mutation (p.S160T in *CACNB2*), resulting in a homozygous appearance of the mutation in one son and heterozygous in the other. Both sons experienced ventricular tachycardia/ventricular fibrillation. Family H, with a diagnosis of BrS, presented with a double mutations in *CACNB2* on the same allele inherited from the mother. The first child (female) died suddenly at age 16 months. The proband, a 9-year-old boy, was diagnosed at age 10 months with a ventricular conduction defect and BrS (procainamide challenge). The same genotype was found in his asymptomatic brother. Such diversity between siblings may be due to protective or deleterious effects of some additional genetic variation, which may be revealed with further genetic testing.

Table 1 Demographic and clinical characteristics of screened probands and probands with mutations

	BrS	BrS/SQT	IVF	ERS
Diagnosis				
No. of probands	152	10	19	24
Age at diagnosis (years)	43 ± 16	41 ± 14	37 ± 11	30 ± 11
Gender (% male)	74%	90%	68%	81%
Clinical characteristics and demographics of probands with mutations				
No. of patients with mutations in all Ca_v subunits	15	5	1	4
Age at diagnosis (years)	34 ± 19	42 ± 15	50	40 ± 4
Range (years)	(1–72)	(25–65)	—	(32-51)
Gender (% male)	80%	100%	0	75%
Symptomatic patients (%)	93%	100%	100%	75%
Patients with syncope (%)	46%	40%	100%	75%
Ventricular tachycardia/ventricular fibrillation (%)	60%	60%	100%	75%
Family history of unexplained sudden death (%)	42%	60%	100%	100%
Type I ST-segment elevation at baseline or with sodium blockers (%)	100%	100%	0	0
Early repolarization pattern (%)	26%	0	0	100%
Average QTc (ms)	432 ± 38	350 ± 15	376	375 ± 13

Age at time of diagnosis and average QTc values are given as mean ± SD.

BrS = Brugada syndrome; BrS/SQT = Brugada syndrome with shorter than normal QT; ERS = early repolarization syndrome; IVF = idiopathic ventricular fibrillation.

Loss-of-function mutations involving LTCC are known to predispose to a phenotype consisting of BrS with an abbreviated QTc. Yet the majority of BrS probands in this study presented with normal QTc intervals. It is noteworthy that a QT-prolonging variation could be identified in 12 (86%) of the 14 BrS cases (Table 3). The most common modulating variation involved the co-presence of a p.D601E polymorphism in *CACNB2b* that augments late I_{Ca} (Figures 5C and 5G). Another common variant modulating the manifestation of the QT interval is a common polymorphism in *KCNH2,* p.K897T. Although this SNP has been reported to exert a modifying effect on QTc, whether it confers risk or a protective effect remains controversial. Some studies have shown that it reduces I_{Kr} and aggravates LQTS,[25,26] whereas others have shown it increases I_{Kr} and confers a protective effect.[27,28] Other additional variations typically associated with LQTS include p.T10M-*KCNE2,* p.R1047L-*KCNH2,* p.D76N-*KCNE1,* and p.G643S-*KCNQ1*.[29–34] In contrast, these QT-prolonging variants are only present in 1 (20%) of the 5 BrS/SQT probands (Table 3).

The total yields of probands with mutations and rare polymorphisms in each of the diagnostic groups is listed in Table 2. A total of 12.3% of BrS and BrS/SQT probands displayed mutations in the α1 (5.5%), β2 (4.9%),

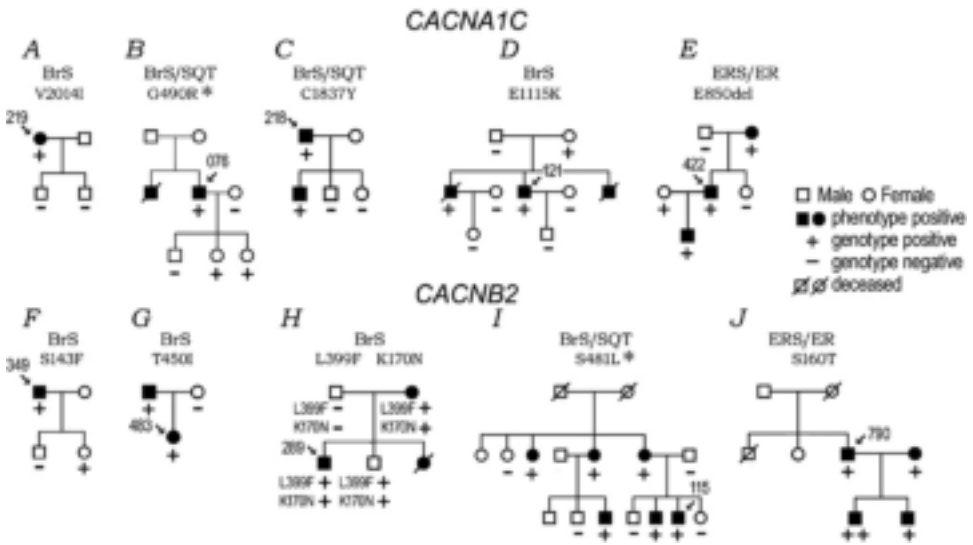

Figure 2 Pedigree of the available families for CACNA1C and CACNB2 mutations. BrS = Brugada syndrome; BrS/SQT = BrS with shorter than normal QT; ER = early repolarization pattern; ERS = early repolarization syndrome; IVF = idiopathic ventricular fibrillation. + = heterozygous for the mutation; ++ = homozygous for the mutation. *Arrows* indicates proband. *Numbers* represent the Masonic Medical Research Laboratory ID number. *Asterisk* denotes previously published mutations.[10,11]

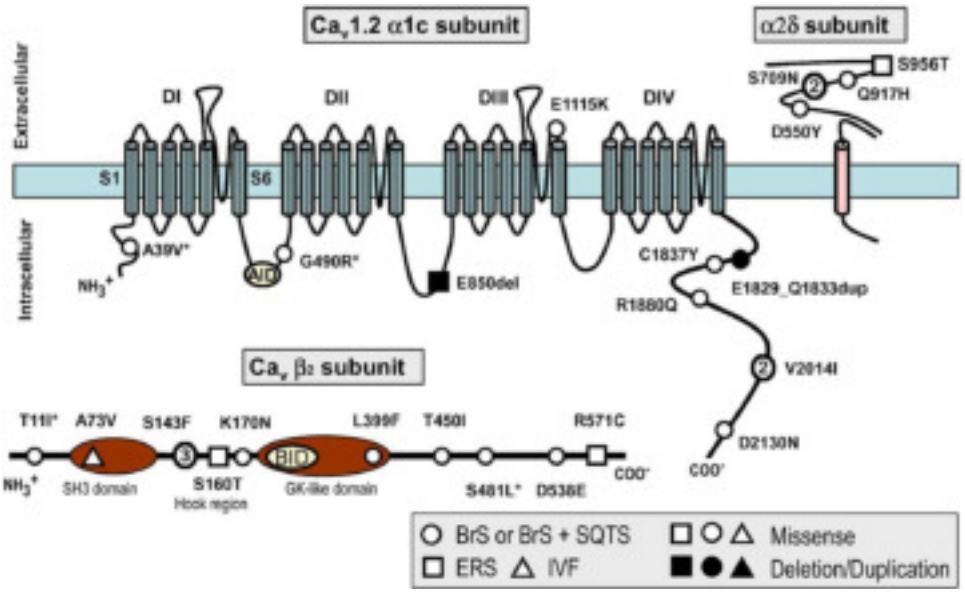

Figure 3 Predicted topology of the Cav1.2 (α1c) subunit with associated β2 and α2δ subunits shows the location of the mutations. AID and BID show the position of interaction of α1c and β2 subunits and the position of the α-subunit interaction domain (AID) and β-subunit interaction domain (BID). GK = guanylate kinase–like domain, SH3 = Src homology domain; Src-. *Larger symbols with numbers* denote multiple probands with the same mutation. *Asterisk* denotes previously published mutations.[10,11]

and α2δ (1.8%) subunits of the LTCC; a total of 5.2% of IVF patients had mutations in the β2 subunit; and 16.0% of ERS patients had mutations in the α1 (4.1 %), β2 (8.3%), and α2δ (4.1%) subunits. The total yield of probands with mutations and rare polymorphisms together was 17.9 % for BrS and BrS/SQT, 21 % for IVF, and 29.1% for the ERS group.

Functional expression studies

Expression studies probing the functional consequences of mutations in LTCC are limited. Previous studies have shown a loss of function of I_{Ca} as the basis for BrS associated with mutations in *CACNA1C* and *CACNB2b*.[10,11] As a further test of the hypothesis that loss-of-function mutations in LTCC underlie BrS as well as ERS and some forms of IVF, we are in the process of performing functional studies of the variants uncovered. We present two cases here.

The first case is a 41-year-old woman of Panamanian descent who presented with palpitations, incomplete right bundle brunch block, and a history of presyncope (MMRL219). A diagnosis of BrS was confirmed following a positive procainamide challenge (Figure 4A). Family history was negative for SCD but positive for stroke and coronary disease. Genetic testing identified a heterozygous substitution of a valine for isoleucine at position 2014 of *CACNA1C* and a polymorphism, p.D601E, in *CACNB2* (Figure 4B and Table 3). The husband and two sons were negative for the p.V2014I mutation (Figure 4A). The same mutation was present in another BrS patient (MMRL793; Table 3) together with a common polymorphism, p.H558R, in *SCN5A*. This proband presented with a BrS type I ECG following sodium block challenge and has a family history of sudden death of undetermined cause at a young age. A valine at position 2014 is highly conserved among species (Figure 4C).

To determine the consequences of this mutation, we evaluated I_{Ca} characteristics in CHO cells transfected with WT or p.V2014I *CACNA1C*. Figure 5A shows that the p.V2014I mutation significantly reduced peak current density at potentials between 0 and +60 mV, with a 61% reduction at +10 mV (–72.3 ± 19.0 pA/pF vs –28.2 ± 10.6 pA/pF, n = 8 in each group, $P < .05$; Figure 5D). The voltage at which the maximum peak current was achieved remained unchanged. As illustrated in Figure 5E, the mutation significantly reduced conductance of the calcium channel at potentials between 0 and +30 mV ($P < .05$) without modifying V_h or k values of the activation curve (–0.5 ± 3.3 mV and 5.9 ± 0.8 mV, n = 8) compared to WT (–1.5 ± 1.4 mV and 6.0 ± 0.9 mV, n = 8, $P > .05$).

Figure 5B shows current traces recorded using a protocol designed to examine the voltage dependence of inactivation. I_{Ca} density recorded during the test pulse to +20 mV was significantly smaller with conditioning pulses to potentials between –90 and –20 mV in cells expressing p.V2014I. The mutation shifted half-inactivation voltage to more negative potentials (–23.0 ± 1.2 mV vs –30.5 ± 4.2 mV, n = 8 in each group, $P < .01$) without modifying k values (7.2 ± 0.4 mV vs 7.9 ± 0.6 mV, $P > .05$; Figure 5F).

Interestingly, this proband also had a p.D601E polymorphism in *CACNB2*. To examine the functional effect of this variant, we expressed it in human embryonic kidney (TSA201) cells. Figures 5C and 5G show the effect of p.D601E in *CACNB2* in significantly increasing late I_{Ca}, which is known to prolong QT. The modulatory effect of

Table 2 Yield of probands with mutations in $\alpha 1$, $\beta 2$, and $\alpha 2\delta$ subunits of L-type calcium channel

Diagnosis	BrS, BrS/SQT			IVF			ERS		
No. of screened probands	**162**			**19**			**24**		
Subunit	$\alpha 1$	$\beta 2$	$\alpha 2\delta$	$\alpha 1$	$\beta 2$	$\alpha 2\delta$	$\alpha 1$	$\beta 2$	$\alpha 2\delta$
No. of probands with mutations for $\alpha 1$	**9**						**1**		
Yield	5.5%						4.1%		
No. of probands with mutations for $\beta 2$		**8**			**1**			**2**	
Yield		4.9%			5.2%			8.3%	
No. of probands with mutations for $\alpha 2\delta$			**3**						**1**
Yield			1.8%						4.1%
Total yield of probands with mutations	20/162			1/19			4/24		
	12.3%			**5.2%**			**16%**		
No. of probands with rare polymorphism for $\alpha 1$	**7**			1			1		
Yield	4.3%			5.2%			4.3%		
No. of probands with rare polymorphism for $\beta 2$		**2**			**2**			**2**	
Yield		1.2%			10.5%			8.3%	
Total yield of probands with mutations and rare polymorphisms	29/162			4/19			7/24		
	17.9%			**21%**			**29.1%**		

Total number (n) and yield (%) of mutations and rare polymorphisms identified for each subunit of calcium channel and diagnostic group.
BrS = Brugada syndrome; BrS/SQT = Brugada syndrome with shorter than normal QT; ERS = early repolarization syndrome; IVF = idiopathic ventricular fibrillation.

this SNP likely accounts for the fact that QTc (449 ms) in this proband is not accompanied by SQT, as is the case with loss-of-function mutations involving LTCC.

The second case is a 33-year-old man who presented with presyncope incomplete right bundle brunch block (MMRL300, Figure 6A). An ajmaline challenge performed was positive, confirming a diagnosis of BrS (data not shown). QTc interval was 346 ms. Genetic analysis showed duplication of five amino acids in exon 43 of *CACNA1C* p.E1829_Q1833dup (Table 3), with no other variations. Family members were not available for genetic screening.

To determine the functional consequences of the mutation, we expressed WT and p.E1829_Q1833dup *CACNA1C* in TSA201 cells. Figure 6C shows I_{Ca} traces recorded during application of 500-ms pulses from –90 mV to potentials ranging between –50 and +50 mV. The p.E1829_Q1833dup mutation reduced peak current density at potentials between –20 and +50 mV, resulting in nearly compete suppression of I_{Ca} (n = 8 in each group, $P < .01$; Figure 6D).

Discussion

This study is the first comprehensive attempt to associate inherited cardiac arrhythmia syndromes with genetic variations in the cardiac LTCC. We identified 23 mutations in three genes encoding the three subunits of the LTCC in 25 unrelated probands and six rare polymorphisms in 17 additional probands diagnosed with BrS, BrS/SQT, IVF, or ERS. A total of 12.3%, 5.2%, and 16% of BrS/BrS+SQT, IVF, and ERS probands displayed mutations in $\alpha 1$, $\beta 2$, and $\alpha 2\delta$ subunits of LTCC, respectively. The total yield of probands with mutations and rare polymorphisms is 17.9% for BrS and BrS/SQT, 21% for IVF, and 29.1% for ERS diagnostic groups (Table 2). The yield of probands with LTCC mutations associated with BrS (12.3%) is second only to *SCN5A* mutations, which have been reported to range between 11% and 28% at different international centers.[5] In the case of ERS, *CACNA1C, CACNB2,* and *CACNA2D1* represent the second, third, and fourth genes proposed to underlie this phenotype, the first one being *KCNJ8*.[6]

Topologically, it is interesting that no mutations were detected in any of the transmembrane regions of $Ca_v1.2$ (Figure 3). Six of the nine mutations were located in the N- or C-terminus of the $\alpha 1$ subunit. Relevant to this finding is the demonstration by Soldatov's group of voltage-gated mobility of the C-and N-cytoplasmic tails of $Ca_v1.2$ and their important regulatory role in voltage- and Ca^{2+}-dependent inactivation.[35,36] In addition, cleavage of the C-terminus of native $Ca_v1.2$ channels has been shown to result in a proteolytic fragment that is able to act as a repressor of $Ca_v1.2$ promoter activity.[37,38] Thus, mutations in the C-terminus could have significant effects on the regulation of expression level and on function of the $Ca_v1.2$ channel. Another mutation of great interest is p.E1115K because it is located in the region of a calcium ion selectivity and permeability site and may cause the appearance of severe SCD in the family (Figure 2D).

The probability of a nonsynonymous mutation causing a genetic disease increases with a higher degree of evolutionary conservation of the mutated site.[39] The majority of our mutated sites were located in highly conserved regions (Table 3), suggesting that many of the variations

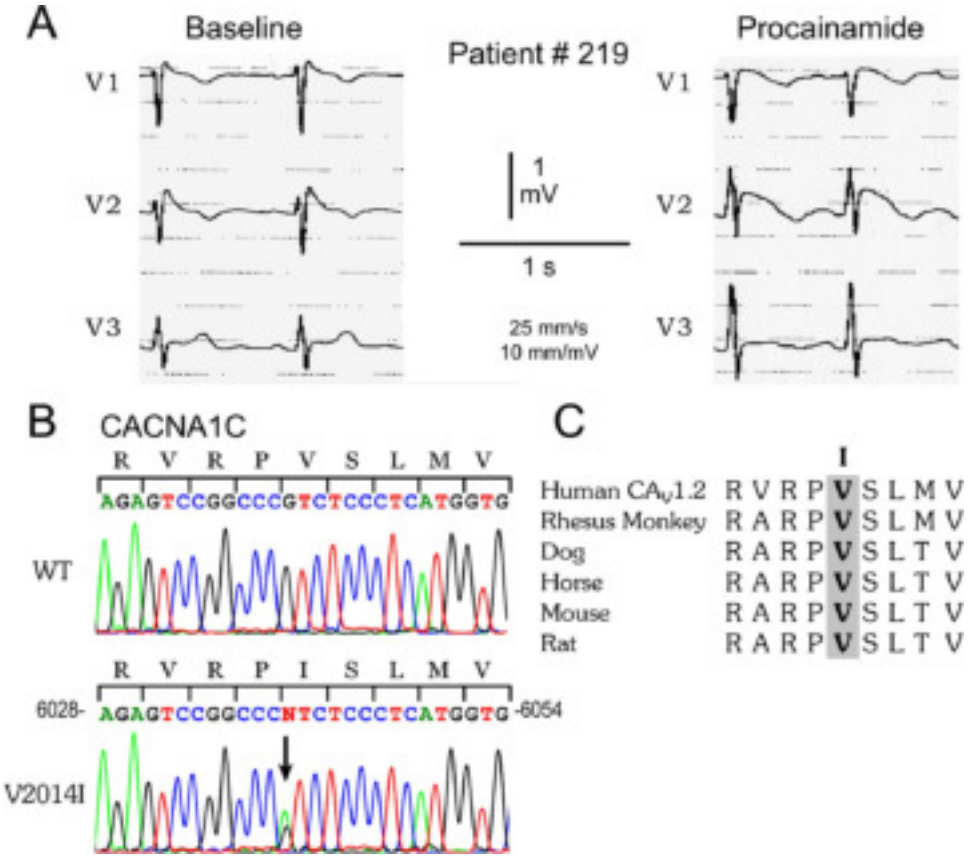

Figure 4 **A:** ECG recorded with leads V_1–V_3 of patient #219 before and after procainamide. **B:** Electropherogram of wild-type (WT) and mutant CACNA1C gene showing heterozygous transition c.6040 G>A predicting replacement of valine by isoleucine at position 2014. **C:** Amino acid sequence alignment showing that valine at position 2014 is highly conserved among multiple species.

uncovered likely are disease-causing. Twelve of the 14 probands with rare polymorphisms had variations in residues that were either highly conserved or conserved among large mammals (Table 3). Moreover, excellent genotype–phenotype correlation was seen among available families, with pathogenic phenotypes co-segregating with a positive genotype (Figure 2). Failure to do so in all but one case could be attributed to female gender and/or young age, both of which are known to diminish expression of the disease phenotype.

In previous studies we demonstrated a loss of function of I_{Ca} for four of these mutations (marked with an asterisk in Figures 2 and Figure 3 and Table 3).[10,11] In the present study, we demonstrated a loss of function of I_{Ca} in BrS and BrS/SQT probands carrying a p.V2014I or p.E1829_Q1833dup mutation in *CACNA1C*. The BrS proband, unlike the BrS/SQT proband, was also found to carry a rare polymorphism, p.D601E, in *CACNB2b,* which when expressed was found to augment late I_{Ca}, thereby explaining the absence of an abbreviated QTc in this proband. QT-prolonging variations (p.D601E-*CACNB2b,* p.K897T-*KNCH2,* p.T10M-*KCNE2,* p.R1047L-*KCNH2,* p.D76N-*KCNE1,* p.G643S-*KCNQ1)* were found in 12 of the 14 BrS probands presenting with a normal QTc (Table 3).[25,26,33]

Our study results suggest that mutations in all three subunits of the LTCCs are detected in a relatively high percentage of probands with inherited cardiac arrhythmia syndromes, including BrS, ERS, and some forms of IVF. These findings suggest that genetic screening of Ca_v genes may prove to be a valuable diagnostic tool for identifying individuals who might be at risk. *CACNA1C, CACNB2,* and *CACNA2D1* should be included in the genotyping of patients who have diseases with a high occurrence of sudden death, particularly in cases where J-wave syndromes are suspected.[3]

Study limitations

The LTCC subunit genes, especially *CACNB,* have multiple isoforms. Our focus on *CACNB2* in this study may have resulted in an underestimation of linkage of LTCC mutations to inherited cardiac arrhythmia disease. Thus far, a total of seven genes have been identified as associated with BrS.[40] Our findings of three BrS probands associated with mutations in highly conserved residues of *CACNA2D1* suggest that it may be a new gene for BrS. In support of this hypothesis, our preliminary functional expression studies indicate that the double mutation in *CACNA2D1* [p.D550Y and p.Q917H (MMRL194)] reduces I_{Ca} to 25% of normal (Barajas et al, unpublished observation).

Mutations in only one gene, *KCNJ8*, have thus far been associated with ERS.[6,7] The present study identifies four probands in whom mutations in highly conserved residues

Table 3 Summary of L-type calcium channel mutations and rare polymorphisms in CACNA1C, CACNB2, and CACA2D1

No.	Amino acid change	Nucleotide change	Mutation type	Conserv.	Exon	Location	Probands MMRL ID no.	Diagnosis	Additional variations
Mutations in CACNA1C									
1	p.E1115K	c.3343 G>A	Missense	HC	26	DIII-S5/S6	121	BrS	KCNH2 p.K897T-SNP
2	p.R1880Q	c.5639 G>A	Missense	NC	44	C-terminus	794	BrS	KCNH2 p.K897T-SNP CACNA1C p.R1973Q-SNP
3	p.V2014I	c.6040 G>A	Missense	HC	46	C-terminus	219 793	BrS BrS	CACNB2 p.D601CACNB2bE-SNP(219) SCN5A p.H558R-SNP(793)
4	p.D2130N	c.6388 G>A	Missense	HC	47	C-terminus	317	BrS	KCNE2 p.T10M CACNA1C p.A1717G-SNP
5	p.A39V*	c.116 C>T	Missense	HC	2	N-terminus	066	BrS/SQT	KCNH2 p.K897T homozygous SNP
6	p.G490R*	c.1468 G>A	Missense	HC	10	DI/DII	076	BrS/SQT	SCN5A-p.H558R-SNP SCN5A-p.S1103Y-SNP
7	p.E1829_Q1833dup	c.5485_5499 dup15	Duplication	NC	43	C-terminus	300	BrS/SQT	
8	p.C1837Y	c.5510 G>A	Missense	NC	42/43 (45)	C-terminus	218	BrS/SQT	SCN5A p.P1090L-SNP
9	p.E850 del	c.2548-550del GAG	Deletion	HC	19	DII/DIII	422	ERS	
Mutations in CACNB2									
1	p.T11I*	c.32 C>T	Missense	CM	2	N-terminus	284	BrS	CACNB2 p.D601E-SNP KCNH2 p.K897T-SNP
2	p.S143F	c.428 C>T	Missense	HC	5	Hook region	015 349 776	BrS BrS BrS	KCNH2 p.K897T-SNP(015) CACNB2 p.D601E-SNP(349) KCNH2 p.R1047L(776) SCN5A p.H558R-SNP(776)
3	p.T450I	c.1349 C>T	Missense	CM	14	C-terminus	483	BrS	
4	p.D538E	c.1614 C>A	Missense	CM	14	C-terminus	249	BrS	KCNE1 p. D76N KCNQ1 p.G643S-SNP KCNH2 p.K897T-SNP
5	p.L399F	c.1195 C>T	Missense	HC	13	GK domain	289	BrS	SCN5A p.H558R-SNP
6	p.K170N	c.510 C>T	Missense	HC	7b	Hook region			
7	p.S481L*	c.1442 C>T	Missense	CM	14	C-terminus	115	BrS/SQT	SCN5A p.H558R-SNP
8	p.A73V	c.218 C>T	Missense	HC	4	SH3 domain	644	IVF	SCN5A p.H558R-SNP
9	p.S160T	c.479 G>C	Missense	HC	6	Hook region	790	ERS	KCNH2 p.K897T Homozygous-SNP CACNB2 p.D601E-SNP
10	p.R571C	c.1711C>T	Missense	HC	14	C-terminus	445	ERS	SCN5A p.H558R-SNP KCNH2 p.K897T-SNP CACNB2 p.D601E-SNP
Mutations in CACNA2D1									
1	p.S709N	c.2126 G>A	Missense	HC	26	Extracellular	387 937	BrS BrS	CACNB2 p.R552G-SNP(937) KCNH2 p.K897T-SNP(937)
2	p.D550Y	c.1648 G>T	Missense	CM	19	Cache domain	194	BrS	KCNH2 p.K897T-SNP CACNB2 p.D601E-SNP
3	p.Q917H	c.2751 A>T	Missense	HC	34	Extracellular			
4	p.S956T	c.2867C>A	Missense	CM	36	Extracellular	954	ERS	

No.	Gene	Amino acid change	Nucleotide change	Mutation type	Conserv.	Exon	Location	Probands (n) Diagnosis
Rare SNP								
1	CACNA1C	p.G37R	c.109 G>A	Missense	HC	2	N-terminus	2 BrS 1 IVF 1 ERS
2	CACNA1C	p.P817S	c.2449 C>T	Missense	NC	17	DII/DIII	2 BrS
3	CACNA1C	p.A1717G	c.5150 C>G	Missense	CM	42	C-terminus	2 BrS
4	CACNA1C	p.T1787M	c.5360 C>T	Missense	NC	42	C-terminus	1 BrS
5	CACNA1C	p.R1973Q	c.5918 G>A	Missense	HC	46	C-terminus	2 BrS
6	CACNB2	p.R552G	c.1654 C>G	Missense	CM	14	C-terminus	2 BrS 2 IVF ERS

Conserv. = degree of conservation for the mutated site among multiple species: CM = conserved among large mammals; HC = highly conserved; NC = not conserved. BrS = Brugada syndrome; BrS/SQT = Brugada syndrome with shorter than normal QT; ERS = early repolarization syndrome; IVF = idiopathic ventricular fibrillation; MMRL ID no. = three-digit Masonic Medical Research Laboratory identification number; SNP = single nucleotide polymorphism.

*Previously published mutations.[10,11]

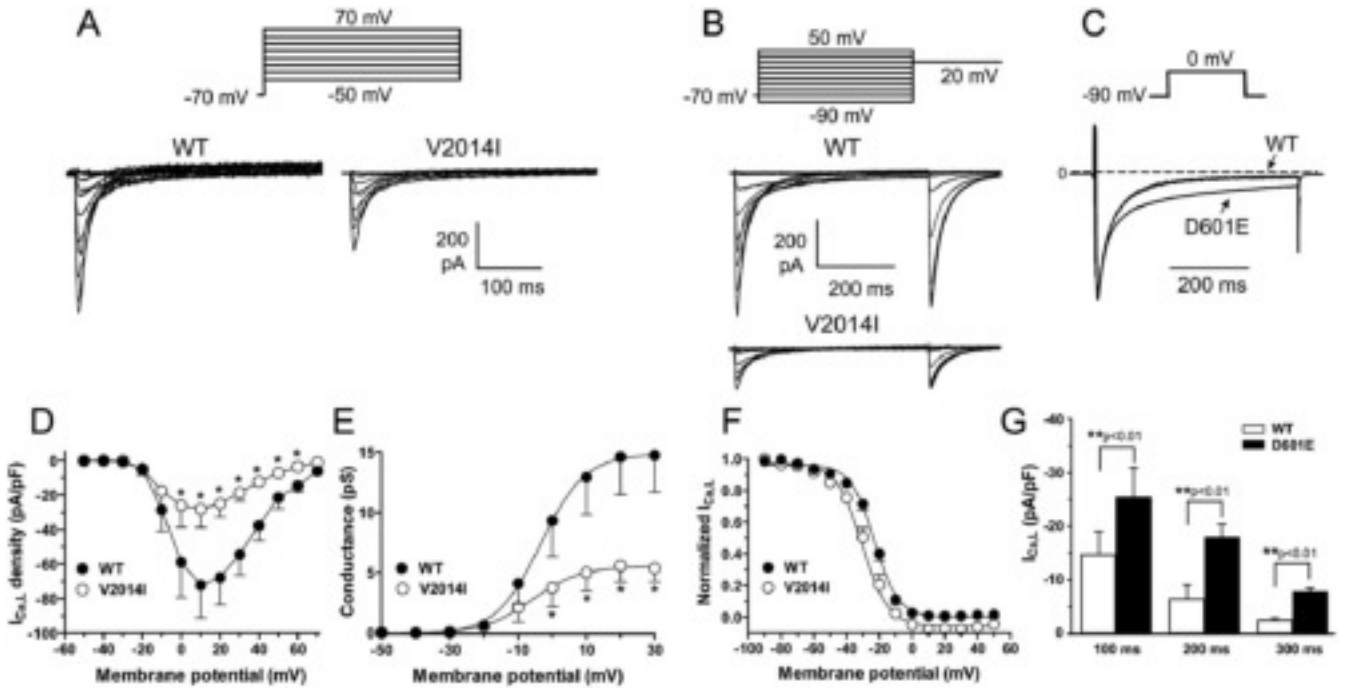

Figure 5 The p.V2014I-CACNA1C mutation causing a loss of function of I_{Ca} together with a p.D601E-CACNB2b single nucleotide polymorphism causing a gain of function of late I_{Ca}, result in Brugada syndrome (BrS) with normal QTc (MMRL219). **A:** Representative calcium current traces recorded in Chinese hamster ovary (CHO) cells transfected with wild-type (WT; **left**) or p.V2014I (**right**) CACNA1C subunits in response to the voltage clamp protocol shown at the **top. B:** I_{Ca} recorded in response to the inactivation protocol shown. **C:** Overlapping calcium traces recorded from human embryonic kidney (TSA201) cells expressing WT and p.D601E-CACNB2b rare polymorphism. **D:** Current–voltage relationship. **E:** Activation curve showing conductance–voltage. **F:** Normalized inactivation curves in WT or p.V2014I CACNA1C. **G:** Bar graph showing I_{Ca} current density recorded with WT versus p.D601E CACNB2b at different times (100, 200, and 300 ms) into the depolarized testing pulse at 0 mV (protocol inset). $^*P < .05$, $^{**}P < .01$ vs WT data. Each datapoint/bar represents mean ± SEM of 6–8 experiments.

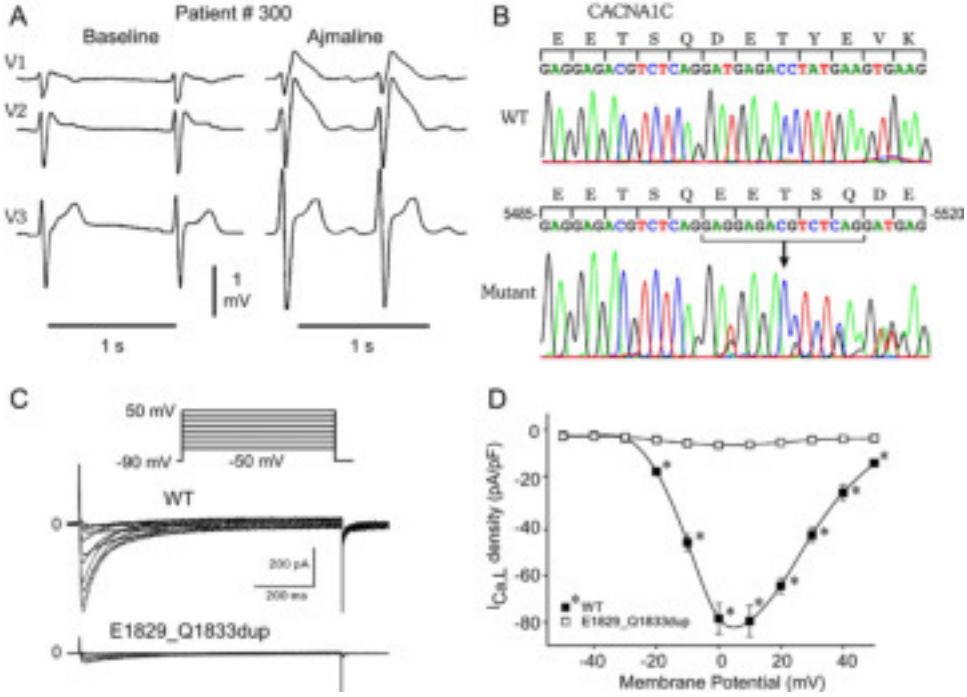

Figure 6 **A:** Duplication of five amino acids in Cav1.2 leads to loss of function of I_{Ca} resulting in Brugada syndrome (BrS) with shorter than normal QT interval (QTc = 346 ms). ECG recorded in leads V_1–V_3 of patient #300 at baseline. **B:** Electropherogram of wild-type (WT) and mutant CACNA1C showing duplication of five amino acids EETSQ. **C:** Representative calcium current traces recorded in human embryonic kidney (TSA201) cells transfected with WT (**left**) and p.E1829_Q1833-dup mutant (**right**) CACNA1C subunits by applying the protocol shown at the **top. D:** Current–voltage relationship (I–V curve) p.E1829_Q1833-dup mutant effect in Cav1.2 channels. Data are given as mean ± SEM of at least eight cells. $^*P < .05$.

of *CACNA1C, CACNB2,* and *CACNA2D1* are associated with ERS, suggesting linkage of these genes with ERS. Although many of the mutations in these genes occur in highly conserved residues and genotype–phenotype correlation among male members of available families is excellent, confirmation of these hypotheses must await the availability of functional expression studies. The requirement for such studies is underscored by the study of Kapa et al[41] suggesting that, in the case of LQTS, mutations of highly conserved residues may not always be disease-causing. It is possible that the same may be true in the case of calcium channel mutations associated with BrS and ERS.[41]

We present functional expressions data for two of the phenotypes evaluated. Although functional studies for the other mutations are in process, the data likely will not be available for many months, and it would be unreasonable to delay reporting of these results until that time.

Although in most cases of IVF we made a diligent effort to exclude the diagnosis of known channelopathies, we recognize that these tests are not always definitive and that patients whom we categorize as IVF may properly belong to another category.

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