SCN4B-Encoded Sodium Channel β4 Subunit in Congenital Long-QT Syndrome

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Background—Congenital long-QT syndrome (LQTS) is potentially lethal secondary to malignant ventricular arrhythmias and is caused predominantly by mutations in genes that encode cardiac ion channels. Nearly 25% of patients remain without a genetic diagnosis, and genes that encode cardiac channel regulatory proteins represent attractive candidates. Voltage-gated sodium channels have a pore-forming α-subunit associated with 1 or more auxiliary β-subunits. Four different β-subunits have been described. All are detectable in cardiac tissue, but none have yet been linked to any heritable arrhythmia syndrome.

Methods and Results—We present a case of a 21-month-old Mexican-mestizo female with intermittent 2:1 atrioventricular block and a corrected QT interval of 712 ms. Comprehensive open reading frame/splice mutational analysis of the 9 established LQTS-susceptibility genes proved negative, and complete mutational analysis of the 4 Na\(_{\text{v1.5}}\)-subunits revealed a L179F (C535T) missense mutation in SCN4B that cosegregated properly throughout a 3-generation pedigree and was absent in 800 reference alleles. After this discovery, SCN4B was analyzed in 262 genotype-negative LQTS patients (96% white), but no further mutations were found. L179F was engineered by site-directed mutagenesis and heterologously expressed in HEK293 cells that contained the stably expressed SCN5A-encoded sodium channel α-subunit (hNa\(_{1.5}\)). Compared with the wild-type, L179F-β4 caused an 8-fold (compared with SCN5A alone) and 3-fold (compared with SCN5A + WT-β4) increase in late sodium current consistent with the molecular/electrophysiological phenotype previously shown for LQTS-associated mutations.

Conclusions—We provide the seminal report of SCN4B-encoded Na\(_{\text{v1.5}}\)4 as a novel LQT3-susceptibility gene. (Circulation. 2007;116:134-142.)

Key Words: genetics ■ ion channels ■ long-QT syndrome

Long-QT syndrome (LQTS) represents the prototypic cardiac channelopathy that affects 1 in 3000 individuals and is characterized by QT prolongation, abnormal ventricular repolarization, and increased propensity for sudden cardiac death as a result of its trademark dysrhythmia of torsade de pointes. Since the first discovery in 1995 that pathogenic mutations in cardiac ion channels cause LQTS,1-12 hundreds of mutations distributed among 9 genes comprise 75% of LQTS. Five genes encode critical, pore-forming, ion channel α subunits (KCNQ1, KCNH2, SCN5A, KCN2, and CACNA1C) and 4 encode ion-channel regulatory proteins ( KCNE1, KCNE2, ANKB, and CAV3). However, 25% of patients with LQTS remain genotype-negative after evaluation of all known LQTS-susceptibility genes.3,4

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Because genes that encode cardiac channel interacting or auxiliary proteins can affect channel function, they are attractive candidates for the cause of LQTS. One set of auxiliary proteins belongs to the sodium channel β subunit (Na\(_{\beta}\)) gene family, where these Na\(_{\beta}\) subunits play a critical role in cell adhesion, signal transduction, channel expression at the plasma membrane, and voltage dependence of channel gating.6-9 Presently, 4 different Na\(_{\beta}\) subunits have been described (SCN1B, SCN2B, SCN3B, and SCN4B).10-14 All are detectable in cardiac tissue,15 but none have been linked to any heritable arrhythmia syndrome.
Case Report
The index case was a 21-month-old Mexican-mestizo girl, referred for evaluation of asymptomatic bradycardia with rates <60 bpm. Her 12-lead ECG revealed profound QT prolongation with a heart rate–corrected QT interval (QTc) of 712 ms and intermittent 2:1 atrioventricular (AV) block (Figure 1A). During 1:1 conduction, macroscopic T-wave alternans were observed (Figure 1B). Her past medical history included fetal bradycardia noted at 24 weeks of gestation and small ventricular septal defect that spontaneously closed by 6 months of age. Despite the severe electrocardiographic phenotype, the patient has remained asymptomatic during her first 5 years of life after placement of an epicardial pacemaker. On further inquiry, a family history of premature, unexpected, and unexplained sudden cardiac deaths that involved 2 paternal great aunts was elucidated (sudden cardiac death at 35 years after delivery of twins and sudden cardiac death at 8 years during exercise) (Figure 1C).

On the basis of the ECG findings, the patient’s ECG phenotype was found to be consistent with SCN5A-mediated LQT3, which shows a long isoelectric ST segment with late-onset T wave, and 2:1 AV block, which was shown previously to be associated with defective LQTS-susceptibility genes, in particular the cardiac sodium channel.16–18

Methods
Subjects
The study was performed according to the terms required by the Research Ethics Committee of the National Institute of Cardiology...
TABLE 1. Oligonucleotide Primers, Polymerase Chain Reaction, and Denaturing High-Performance Liquid Chromatography Conditions for Mutational Analysis of Na\textsubscript{4}β Subunits

<table>
<thead>
<tr>
<th>Gene-Exon</th>
<th>Forward Primer (5\textsuperscript{'} to 3\textsuperscript{'} of SCN1B-1)</th>
<th>Reverse Primer (5\textsuperscript{'} to 3\textsuperscript{'} of SCN1B-2)</th>
<th>Size, Base Pairs</th>
<th>MgCl\textsubscript{2}, mmol/L</th>
<th>Thermal Cycling Method†</th>
<th>Gradient 1, %B</th>
<th>Temp °C</th>
<th>Gradient 2, %B</th>
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<td>CGC CCG CCG CCG CAG CAG TGC TGC C</td>
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<td>GAC CAG GGG CTT CAT GCC A</td>
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<td>294</td>
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<td>2</td>
<td>56.5 to 66.5</td>
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Polymerase chain reaction and other reactions were performed in 20-μL volumes with 50 ng of DNA, 16 pmol of each primer, 200 μM of each dNTP, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), and 1.0 U of Amplitaq Gold (Applied Biosystems, Branchburg, NJ). PCR indicates polymerase chain reaction; DHPLC, denaturing high-performance liquid chromatography.

*8% dimethyl sulfoxide was added to the reaction mixture. Polymerase chain reaction amplification was performed with a DNA Engine Tetrad thermal cycler.†Thermal cycling method 1: 94°C for 5 minutes, followed by 5 cycles of 94°C for 20 s, 64°C for 20 s, and 72°C for 30 s; additional 35 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 30 s, and a final extension of 72°C for 10 minutes. Thermal cycling method 2: 94°C for 15 minutes, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 minutes.‡DHPLC was performed with a 5% buffer B/minute gradient. The start and stop % buffer B followed by the temperature at which the gradient was performed is indicated in the table.

“Ignacio Chávez,” Mexico City, and the Mayo Foundation Institutional Review Board; written informed consent was obtained from all participants. Genomic DNA from the index case and all consenting family members was extracted from peripheral blood lymphocytes by use of standard techniques. Control DNA from 200 healthy Mexican-mestizo subjects was obtained from Unidad de Biología Molecular y Medicina Genómica, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City. Mexican-mestizo ethnicity resulted from admixture of racial ancestry that included European (Spanish) and indigenous descent, with smaller contribution from Asian and African groups. Two hundred additional control DNA samples (100 from white donors and 100 from black), obtained from Corell Cell Repositories (Camden, NJ), were also examined.

In addition, 262 genotype-negative patients (180 females; 96% white; age at diagnosis, 25±16 years; mean QTc, 470±60 ms) with a suspected clinical diagnosis of congenital LQTS, who were referred previously for genetic testing to the Mayo Clinic Windland Smith Rice Sudden Death Genomics Laboratory, were included.

Mutational Analysis

Through polymerase chain reaction, denaturing high-performance liquid chromatography, and direct DNA sequencing, we performed comprehensive open reading frame/splice site mutational analysis of all known LQTS-susceptibility genes (\textit{KCQ1}, \textit{KCQ2}, \textit{SCN5A}, \textit{ANK2}, \textit{KCNE1}, \textit{KCNE2}, \textit{KCNJ2}, \textit{CACNA1C}, and \textit{CAV3}) using previously published primers, followed by mutational analysis of the 4 Na\textsubscript{4}β subunits encoded by \textit{SCN1B}, \textit{SCN2B}, \textit{SCN3B}, and \textit{SCN4B}. The flanking primers used in polymerase chain reaction amplification of the β subunits were designed with Oligo software (Molecular Biology Insights, Inc., Cascade, Colo.); primers, polymerase chain reaction, and denaturing high-performance liquid chromatography conditions are shown in Table 1.

Functional Assay

Cloning, mutagenesis, and voltage-clamp techniques were performed as previously described.\textsuperscript{19} Briefly, the WT-β4 was cloned from human heart cDNA with reverse transcriptase–polymerase chain reaction and 2 primers: 5'-AGAGAACAGGACTATGCGCCG-3' and 5'-TTTCATCATCATCAGAAAGG-3'. The WT-β4 was subcloned into pcDNA3 and confirmed by DNA sequencing analysis. L179F-β4 was engineered by site-directed mutagenesis with the following primers: Na\textsubscript{4}-4 L179F sense 5'-CTTGATCAGCAGGATGAAGATGAGGAGCCCGAT-3' and Na\textsubscript{4}-4 L179F antisense 5'-AGAGAACAGGACTATGCGCCG-3'. The WT-β4 or L179F-β4 was subcloned into pIRGFP1 vector, a mammalian expression vector (kindly provided by Dr David Johns from the Johns Hopkins University, Baltimore, Md), which contains IRES and GFP. These WT-β4 and L179F-β4 constructs were expressed heterologously in HEK 293 cells that contain stably expressed SCN5A-encoded sodium channel α subunits (hNa\textsubscript{1.5}, hH4, GeneBank accession #AY148488). Macroscopic sodium current was measured by standard
Results

After a negative mutational analysis of the 9 known LQTS-susceptibility genes, we identified a C to T base mutation at position 535, which yielded a novel L179F-SCN4B missense mutation (leucine [L] to phenylalanine [F] at position 179, when the full-length gene product is considered) by denaturing high-performance liquid chromatography and DNA sequencing (Figure 1D). This mutation was absent in 800 reference alleles, which included 400 ethnicity-matched, Mexico-mestizo alleles. 

L179 localizes to the transmembrane spanning region (Figure 2A), is relatively conserved across species (Figure 2B), and a substitution with phenylalanine is predicted to alter secondary structure (data not shown). L179F cosegregated properly through a 3-generational pedigree with complete penetrance. QTc values and ages are shown in Figure 1C. Although other family members displayed QTc values above normal average, the index case exhibited the only severe ECG phenotype detected on this pedigree, and despite the severe ECG anomalies, the index case has never experienced syncope or torsade de pointes. However, no premortem ECGs were available for either of the sudden-death victims and 1 of the decedents was proven to have been an obligate mutation carrier.

When the wild-type (WT) SCN4B and the L179F mutant SCN4B were transfected transiently into a HEK cell line that stably expressed the most common SCN5A transcript in humans (H558/Q1077del),20 robust sodium current traces were recorded and no significant differences in current amplitude and time course were noted in comparison to SCN5A alone (Figure 3A and Table 2). The steady-state voltage dependence of activation and inactivation of the sodium channel were analyzed with standard protocols and fitted with the use of a Boltzmann function. Voltage dependence of inactivation but not activation was modified by the L179F mutant (Figure 3, B and C). This small but significant 3.42-mV positive shift in inactivation (WT-β4, −82.52±0.74 (n=9), to L179F-β4, −79.10±0.59 (n=9); P<0.05) increases the window current and could be arrhythmogenic. More importantly, this voltage shift in inactivation indicates a consistent effect and an interaction of the α subunit with β4 in the heterologous system.

Compared with SCN5A alone, L179F-β4 caused a dramatic 8-fold increase in late sodium current at −60 mV, which is consistent with an effect on terminal repolarization to prolong the QT interval as previously shown for LQT3-associated mutations in SCN5A. In fact, the increase in late sodium current exceeded that of the classic mutation that causes LTQ3 (ΔKPQ) in the pore-forming, SCN5A-encoded α subunit (Figure 4).21 Table 2 presents a summary of current density and the remaining gating param-
Statistically significant changes in recovery kinetics, particularly a significant increase in the slow Tau of recovery, were noted. Coimmunoprecipitation experiments (Figure 5) showed that both WT-β4 and L179F-β4 were present when SCN5A was specifically immunoprecipitated by a labeled tag.

**Discussion**

We provide the first report to detail SCN4B as a novel LQTS-susceptibility gene on the basis of identification of a novel missense mutation (L179F) that cosegregated properly, was absent in 800 reference alleles, and produced a “gain-of-function” Na1.5 current in a family with no other identifiable LQTS-associated mutations.

L179F cosegregated properly with incomplete penetrance, which is typical for heritable arrhythmia syndromes like LQTS. Genetic testing in LQTS has demonstrated that 40% of carriers of LQTS-related mutations had a QTc within normal limits.4,22 Penetrance, expressivity, and phenotype do not depend solely on the primary LQTS-associated mutation, as these can be influenced by modifier genes.19 Thus far, no modifiers have been identified to account for the extreme QT prolongation and intermittent 2:1 AV block in the index case or the sudden death in the obligate carrier compared with the milder ECG phenotype seen in other genotype-positive living family members. Intermittent functional 2:1 AV block in the setting of LQTS has an incidence of 4% to 5% in pediatric series,23 is usually an isolated disorder, and is associated with a high mortality rate of >50% regardless of treatment.24,25 Symptoms could appear at a very young age; neonatal paroxysmal bradycardia and/or hydrops fetalis are common findings. The phenomenon occurs in the setting of a very long, rate-dependent, effective refractory period.26 QRS complex is usually narrow; however, in spite of this, infrahisian block locations have been documented,26–28 although suprahisian block is not ruled out and the level of the block could depend on the genotype. Until now 3 genes have been associated with 2:1 AV block: SCN5A, KCNH2, and CACNA1.17,27–38 Our data suggest that perturbations in the SCN4B-encoded β4 subunit constitute another pathogenic mechanism for 2:1 AV block in patients with LQTS.

According to the proband’s family history, the 2 sudden deaths occurred during exercise or after delivery; these triggers have been previously associated with the KCNQ1 and KCNH2 genes, respectively; nevertheless, no mutations in these genes were found in our patient. Although exertional syncope more likely suggests LQT1, and the presence of cardiac events during the postpartum period more likely suggests LQT2, 44% of patients referred for LQTS genetic testing because of exertional syncope and 25% with family history of an event that occurred post-partum did not have LQT1 to LQT6.39 Importantly, the genotype-phenotype relationships for the rare subtypes of LQTS, mainly those that involve ion channel interacting proteins, have not been determined.

Ion channels exist as macromolecular complexes with several auxiliary proteins known as channel interacting proteins that localize to or are involved in the plasma membrane, extracellular matrix, intracellular proteins, cytoskeleton anchoring, and signal transduction.9 In principle, perturbations in any component of this complex may affect the proper function of the pore-forming channel subunit itself. Voltage-gated sodium channels have a pore-forming α subunit and 1
or more auxiliary \( \beta \) subunits. Presently, 9 functional \( \alpha \) subunits (Na\(_{\text{1.1}} \) to Na\(_{\text{1.9}} \)) and 4 different Na\(_{\beta}\) subunits have been identified in humans. Numerous mutations in various Na\(_{\alpha}\) subunits have been associated with inherited diseases such as mutations in the SCN5A-encoded Na\(_{1.5}\) subunit that precipitates LQT3 and Brugada syndrome. However, among all the \( \beta \) subunits, only \( \beta 1 \) has been associated with human disease, namely febrile seizures.

Na\(_{\beta}\) subunits are proteins with type I topology characterized by an extracellular N-terminal cleaved region, a transmembrane segment, and a cytoplasmic domain with a C-terminal tail. Na\(_{\beta}\) subunits contain an extracellular Ig-like fold, often found in cell adhesion molecules that target ion channels to the plasma membrane and mediate interactions with signaling molecules. Na\(_{\beta 1}\) and Na\(_{\beta 3}\) are similar in sequence and associate noncovalently with \( \alpha \) subunits. Na\(_{\beta 2}\) and Na\(_{\beta 4}\) are related proteins that are disulfide-linked to \( \alpha \) subunits. Immunocytochemical studies in mouse hearts indicate that the primary cardiac sodium channel in ventricular myocytes is composed of Na\(_{1.5}\) plus \( \beta 2 \) and/or \( \beta 4 \) subunits in intercalated disks, whereas other isoforms, such as Na\(_{1.1}\), Na\(_{1.3}\), and Na\(_{1.6}\), are also expressed in heart plus \( \beta 1 \) or \( \beta 3 \) in the transverse tubules. Knockout mice that lack the sodium channel \( \beta 1 \)-subunit display spontaneous generalized seizures and ataxia, whereas mice that lack the \( \beta 2 \)-subunit have increased susceptibility to seizures.

The \( \beta 4 \) subunit is distinguished by a cytoplasmic tail insert (KKLITIFILKKTREK) from amino acid 184 to 197 when the full gene product is considered, or amino acid 154 to 167 when the mature protein after cleavage is considered, as shown in Figure 2A. This cluster of lysine and hydrophobic residues has been implicated recently with a transient and resurgent sodium conduction contributing to the short refractory period seen in the SCN8A-encoded sodium channel of cerebellar Purkinje cells and may serve as an endogenous open-channel blocker.

Enzymatic removal of this endogenous blocker generated a

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<th>TABLE 2. Summary of Current Density and Gating Parameters</th>
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<tr>
<td>( h_m ) density</td>
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<td>( \tau ) Slow, ms</td>
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<td>Decay, (-20) mV</td>
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<td>( \tau ) Fast, ms</td>
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All data are represented as mean\pmSD. \( h_m \) indicates robust sodium current.

*\( P<0.05 \) vs SCN5A.
†\( P<0.05 \) vs WT-\( \beta 4 \).

Figure 4. Effect of coexpression of L179F-\( \beta 4 \) mutant on late sodium current. A. Representative sodium current traces in response to a step to \(-60\) mV for 700 ms from a holding potential of \(-140\) mV (protocol inset) are shown. Leak-subtracted currents were normalized to peak current and are shown on a scale such that peak current is off-scale to emphasize the small late component. B. Summary data showed that L179F-\( \beta 4 \) mutant increased late sodium current during the window of terminal repolarization as much as the \( \alpha \) subunit sodium channel mutation (\( \Delta KPQ \)) that causes LQT3.
The lysates were obtained from untransfected HEK cells (column 1) and from cells cotransfected with HA-SCN5A and Myc-β4 (column 2). Columns 3 to 6 are immunoprecipitation protein complexes derived from cotransfection of HA-SCN5A and Myc-β4-WT; HA-SCN5A and Myc-β4-L179F; HA-SCN5A and untagged β4-WT; and HA-SCN5A and untagged β4-L179F, respectively. The 38-kDa line recognizes the β4 subunit, and the bands above 38 kDa show nonspecific binding. Column 1 shows no recognition in control; column 2 shows recognition of the expressed Myc label. In columns 3 and 4, the β4 subunit was recognized in the SCN5A precipitate for both WT and L179F (red arrows). In columns 5 and 6, the β4 subunit was not recognized because the complex of unlabeled SCN5A and Myc-β4 were not precipitated.

delay in channel recovery. This resurgent current, induced by the β4 cytoplasmic tail, has been demonstrated also in the cardiac isoform in vitro and a putative S6-binding site within the inner cavity of hNa1.5 has been suggested.47 This mechanism is analogous to that seen in patients with SCN5A-mediated LQT3 and raises the possibility that L179F-β4 represents a primary loss-of-function mutation in SCN4B that secondarily precipitates a gain-of-function on Na1.5. In fact, the accentuation in late sodium current rendered by L179F-β4 is greater than many primary mutations in the SCN5A-encoded α subunit itself. Unlike SCN5A mutations, however, the gain-of-function mechanism in these experiments for L179F-β4 is confined to the window current and would primarily affect and slow terminal repolarization rather than the classic accentuation of late sodium current at more depolarized membrane potentials that would prolong the action potential plateau.

Experiments were performed in heterologous mammalian expression systems, a standard technique for characterization of LQTS mutations. These systems, however, lack the native environment of the cardiac cell and did not include β1, β2, or β3 subunits, or other components of the Na channel macromolecular complex such as caveolin-3. As such, the effects may differ and be more severe in a more complex and native environment. However, direct concordance between the degree of channel dysfunction and the manifest clinical severity is not necessarily present. Nonetheless, the molecular phenotype was consistent with LQT3, and in combination with the clinical data and genetic cosegregation supports the L179F mutation in the sodium channel β4 subunit as disease associated.

In the present report we have provided proof of principle that mutations in SCN4B may contribute to the pathogenic substrate for some cases of LQTS by alteration of the cardiac sodium channel current (Na1.5). Our study provides the first human disease associated with perturbations in the sodium channel β4 subunit. Notably, mutations in CAV3-encoded caveolin-3 were demonstrated recently as a novel LQTS-susceptibility gene (LQT9).48 In coexpression studies that involved heterologous expression of only the α subunit and mutant caveolin-3, marked accentuation of the late sodium current was observed, similar to that seen here with L179F-β4. Thus, SCN4B (LQT10) joins CAV3 (LQT9) as rare LQTS-susceptibility genes that encode cardiac sodium encode cardiac channel interacting proteins that, when disrupted, yield a LQT3-like molecular/electrophysiological phenotype.

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Disclosures
None.

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CLINICAL PERSPECTIVE

Long-QT syndrome (LQTS) is a potentially lethal heritable arrhythmia syndrome that affects an estimated 1 in 3000 persons. Since the sentinel discovery of cardiac channel mutations as its pathogenic basis in 1995, LQTS has been viewed as a “cardiac channelopathy.” To date, 9 LQTS-susceptibility genes have been discovered, and 5 of these genes encode the critical ion channel pore-forming α subunit whereas the other 4 genes encode cardiac channel interacting proteins. Presently, ~20% of LQTS remains genetically elusive, and cardiac channel interacting protein–encoding genes represent the latest targets of investigation. Sodium channel β subunits are crucial regulatory proteins. Four different β subunits (β1 to β4), encoded by SCN1B through SCN4B, respectively, have been described. All are detectable in cardiac tissue but none have been related to any arrhythmogenic disease. In the present study, we discovered that SCN4B is a novel albeit rare LQTS-susceptibility gene (LQT10). Consistent with the Towbin Final Common Pathway Hypothesis, a single missense mutation (L179F) in the 228-amino acid that contains β4 subunit conferred a secondary gain-of-function clinical and biophysical phenotype to the sodium channel macromolecular complex. The mutation converted the α subunit of the otherwise intact Naᵥ1.5 into a channel with accentuated late sodium current that essentially mimicked mutations that cause LQT3.
SCN4B-Encoded Sodium Channel β4 Subunit in Congenital Long-QT Syndrome
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