HCN4 Mutations in Multiple Families With Bradycardia and Left Ventricular Noncompaction Cardiomyopathy

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ABSTRACT

BACKGROUND Familial forms of primary sinus bradycardia have sometimes been attributed to mutations in HCN4, SCN5A, and ANK2. In these studies, no structural cardiac alterations were reported in mutation carriers. However, a cluster of reports in the literature describe patients presenting with sinus bradycardia in association with left ventricular noncompaction cardiomyopathy (LVNC), pointing to a shared genetic cause.

OBJECTIVES This study sought to identify the genetic defect underlying the combined clinical presentation of bradycardia and LVNC, hypothesizing that these 2 clinical abnormalities have a common genetic cause.

METHODS Exome sequencing was carried out in 2 cousins from the index family that were affected by the combined bradycardia–LVNC phenotype; shared variants thus identified were subsequently overlaid with the chromosomal regions shared among 5 affected family members that were identified using single nucleotide polymorphism array analysis.

RESULTS The combined linkage analysis and exome sequencing in the index family identified 11 novel variants shared among the 2 affected cousins. One of these, p.Gly482Arg in HCN4, segregated with the combined bradycardia and LVNC phenotype in the entire family. Subsequent screening of HCN4 in 3 additional families with the same clinical combination of bradycardia and LVNC identified HCN4 mutations in each. In electrophysiological studies, all found HCN4 mutations showed a more negative voltage dependence of activation, consistent with the observed bradycardia.

CONCLUSIONS Although mutations in HCN4 have been previously linked to bradycardia, our study provides the first evidence to our knowledge that mutations in this ion channel gene also may be associated with structural abnormalities of the myocardium. (J Am Coll Cardiol 2014;64:745–56) © 2014 by the American College of Cardiology Foundation.
**ABBREVIATIONS AND ACRONYMS**

- cDNA = complementary deoxyribonucleic acid
- CHO = Chinese hamster ovary
- ECG = electrocardiogram
- GoNL = Genome of the Netherlands
- ICD = implantable cardioverter-defibrillator
- LVNC = left ventricular noncompaction cardiomyopathy
- NHLBI = National Heart, Lung, and Blood Institute
- OHCA = out-of-hospital cardiac arrest
- SAN = sinoatrial node
- SNP = single nucleotide polymorphism
- WT = wild-type

Sinus bradycardia, arbitrarily defined as a heart rate lower than 60 beats/min, can occur at any age and in a variety of cardiac diseases (1). Although familial forms of primary sinus bradycardia are recognized, they have been attributed to mutations in HCN4 (2-4), SCN5A (5,6), and ANK2 (7). In these studies, no structural cardiac alterations were reported in mutation carriers. However, a cluster of reports in the literature describe patients presenting with sinus bradycardia in association with left ventricular noncompaction cardiomyopathy (LVNC) (8-11), raising the possibility that these 2 defects could have a common cause.

LVNC is an increasingly recognized cardiomyopathy characterized by a noncompacted left ventricular myocardial layer with deep intertrabecular recesses (12,13). These clinical manifestations may be highly variable. LVNC may occur in isolation; however, besides sinus bradycardia, it may present together with structural congenital heart defects, neuromuscular disorders, or mitral valve abnormalities (13,14). LVNC may be associated with heart failure, (potentially) lethal arrhythmias, and systemic embolic events. It may be sporadic or familial, and thus far, has mainly been associated with mutations in genes encoding sarcomere proteins (TMP1, MYH7, ACTC1, TNNT2, and MYBPC3) (10,15-17), although mutations in other genes have also been reported: TAZ (18), DTNA (19), LDB3 (20), TNNT3, PLN (21), and LMNA (22). Most recently, mutations were identified in MIB1 (23) and RYR2 (24).

In this study, we characterized a large Dutch family presenting with sinus bradycardia in combination with other cardiac abnormalities, including LVNC. In an effort to identify the causal genetic defect underlying this combined phenotypic manifestation in this family, we integrated exome sequencing with linkage analysis, and identified a novel mutation in HCN4. HCN4 encodes the hyperpolarization-activated cyclic nucleotide-gated channel 4, which conducts the hyperpolarization-activated “funny” current (I_{f}), in the sinoatrial node (SAN) (25,26). In line with the role of this current in SAN pacemaker activity, mutations in HCN4 have been reported as a cause of sinus bradycardia (2-4). However, the association between HCN4 mutations and structural cardiac alterations has never been described before. To confirm our results, we subsequently screened HCN4 in 3 additional families with the same clinical combination of bradycardia and LVNC and have identified HCN4 mutations in all 3.

**METHODS**

**FAMILY A.** In the index family, 7 individuals within 2 generations were affected with bradycardia in combination with LVNC (Fig. 1). Available data on medical history, physical examination, 12-lead electrocardiogram (ECG), echocardiography, Holter monitoring, and exercise testing were collected. Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood according to standard procedures. Written informed consent was obtained from all participating family members.

**ECHOCARDIOGRAPHY.** Two cardiologists blinded to the described phenotype and to the HCN4 mutation status analyzed the echocardiographic images. LVNC was called as present or absent by criteria described previously (27,28). Ejection fractions were calculated using the Teichholz M-mode method (Table 1).

**LINKAGE ANALYSIS.** Genome-wide single nucleotide polymorphism (SNP) genotyping was carried out in 5 individuals (II:1, II:3, II:9, III:2, and III:4) from family A, presenting with bradycardia and LVNC using the Illumina HumanOmni2.5 array (Illumina, San Diego, California). Genotypic data were used for linkage analysis to identify chromosomal regions shared among affected individuals. Patient II:6 was excluded from the linkage analysis because at the time of our study, he had persistent atrial fibrillation, and his sinus rhythm could therefore not be assessed (29). Patient II:7 joined the study at a later stage. Linkage analysis was performed using the interface easyLinkage V5.08 (30) running Merlin 1.1.2 (31), with the assumption of an autosomal dominant inheritance pattern, a disease allele frequency of 0.001, and a disease penetrance of 0.9. Mendelian inheritance was checked using PedCheck 1.1 (32). Gene frequency was assumed to be equal between males and females.

have no relationships relevant to the contents of this paper to disclose. Ms. Milano and Dr. Vermeer contributed equally to this work.

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EXOME SEQUENCING. Exome sequencing was carried out on 2 genetically distant family members (cousins III:2 and III:4) at the Beijing Genomics Institute (BGI, Beijing, China). The coding region of the genome was captured using the Agilent SureSelect Target Enrichment system (Agilent Technologies, Santa Clara, California), followed by sequencing on the Illumina HiSeq 2000 platform. The SOAPsnp (for single nucleotide variants) and the GATK (for copy number variants) genome analysis algorithms were used for genotype calling (33,34). Variants were compared using publicly available variant databases—namely: 1) dbSNP132; 2) Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, Washington; 3) Phase 1v3 of the 1,000 Genomes project (data release 10/2012); 4) Genome of the Netherlands (GoNL) (35); and 5) the 69 genomes from Complete Genomics (Mountain View, California)—in addition to 15 in-house exome datasets, using KNIME open-source software version 2.1 with the Knime4Bio plugin (36). Genetic variants found in any of these databases were excluded from further analysis. The 2 exomes were subsequently compared, and genetic variations found in both patients were retained. Variants that remained after filtering were validated by Sanger sequencing and were tested for segregation with the combined (bradycardia-LVNC) phenotype in the family (e.g., testing for presence in all affected and absence in all unaffected family members).

SEQUENCING OF HCN4 IN ADDITIONAL FAMILIES. The HCN4 gene (Ensembl Transcript ID ENSG00000138622) was screened for coding region mutations in 3 additional families by polymerase chain reaction amplification of the coding regions following Sanger sequencing. Primer sequences of all markers were obtained from the UniSTS database. Genomic DNA was amplified in the presence of FAM, and fragments were separated on an ABI3500 genetic analyzer (Applied}

HAPLOTYPE ANALYSIS IN FAMILIES B AND D. We carried out haplotype analysis to check whether the HCN4 c.1441T>C mutation in families B and D had a common origin. Ten microsatellite markers flanking HCN4 were selected (D15S967, D15S981, D15S1050, D15S980, D15S204, D15S211, D15S1041, D15S206, D15S115, and D15S154) and typed in individuals from these families. Primer sequences of all markers were obtained from the UniSTS database. Genomic DNA was amplified in the presence of FAM, and fragments were separated on an ABI3500 genetic analyzer (Applied
Biosystems, Foster City, California). Results were processed with GeneMapper software 4.1 (Applied Biosystems).

**COMPLEMENTARY DNA CONSTRUCTS, MUTAGENESIS, AND HETEROLOGOUS EXPRESSION.** The construct (pcDNA3.1) containing wild-type (WT) hHCN4 complementary DNA (cDNA) was described before (39). The c.1241C>G (p.Ala414Gly) point mutation was introduced into the WT hHCN4 cDNA by site-directed mutagenesis (QuikChange kit, Stratagene, Agilent Technologies). The hHCN4 cDNAs containing the mutants c.1441T>C (p.Tyr481His) and c.1444G>C (p.Gly482Arg) were synthesized at Life Technologies (Carlsbad, California) and subsequently cloned into pcDNA3.1.

Chinese hamster ovary (CHO) (Sigma-Aldrich, St. Louis, Missouri) cells were cultured in Ham’s F-12 medium with 2 mmol/l glutamine, 10% fetal bovine serum (Lonza, Basel, Switzerland), 1% P/S (5,000 U/ml penicillin and 5,000 U/ml streptomycin sulfate, Lonza) in 5% CO2 at 37°C. CHO cells were transiently transfected with 2 μg of WT-HCN4 cDNA or, to recapitulate the heterozygous state, 1 μg of WT and 1 μg of mutant HCN4 construct, using lipofectamine (Gibco BRL, Life Technologies). Successfully transfected cells were visualized by coexpressed green fluorescent protein (GFP) (0.5 μg eGFP plasmid) (pcDNA3-eGFP, Life Technologies).

**WHOLE-CELL-PATCH ELECTROPHYSIOLOGICAL RECORDINGS AND ANALYSIS.** HCN4 currents were recorded 2 days after transfection at 37 ± 0.2°C using the amphotericin-perforated patch-clamp technique (Axopatch 200B amplifier, Molecular Devices, Sunnyvale, California). Signals were low-pass filtered (cutoff frequency 5 kHz) and digitized at 5 kHz. Series resistance was compensated by 70% to 80%, and potentials were corrected for the estimated liquid junction potential. Voltage control, data acquisition, and analysis were accomplished using custom software. Superfusion solution contained (in mmol/l): NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1.0, glucose 5.5, HEPES 5.0 (pH 7.4) (NaOH). Pipettes (borosilicate glass; resistance 2 to 2.5 MΩ) were filled with solution containing (in mmol/l): K-gluc 125, KCl 20, NaCl 10, amphotericin-B 0.88, HEPES 10 (pH 7.2) (KOH). Cell membrane capacitance (7.6 ± 0.6 pF [average ± SEM, n = 35]) was estimated by dividing the decay time constant of the capacitive transient in response to 5 mV hyperpolarizing voltage clamp steps from –20 mV by the series resistance.

HCN4 currents were evoked by hyperpolarizing steps (range –20 to –160 mV, increments of 10 mV) from a holding potential of 0 mV. To prevent membrane instability and cell death at very negative potentials, the duration of the voltage steps was progressively reduced from 12 s (at –20 mV) to 1.5 s (at –160 mV). The voltage step durations were sufficient to reach steady-state activation at all voltages, because activation kinetics of HCN4 channels become faster at more negative potentials (40). Tail currents (at

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**TABLE 1** Clinical Data of the Families Analyzed in This Study

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Age (yrs)</th>
<th>Min HR</th>
<th>Max HR</th>
<th>Average HR</th>
<th>Site of Noncompaction*</th>
<th>EF (%)</th>
<th>Medical History†</th>
<th>IVSd</th>
<th>LVIDd</th>
<th>LVPWd</th>
<th>LAEDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-II:1</td>
<td>57</td>
<td>31</td>
<td>107</td>
<td>62</td>
<td>1, 2</td>
<td>60</td>
<td>OHCA (57 yrs), ICD with pacing capacity</td>
<td>15</td>
<td>46</td>
<td>12</td>
<td>21.6</td>
</tr>
<tr>
<td>A-II:2</td>
<td>55</td>
<td>30</td>
<td>103</td>
<td>44</td>
<td>1, 2</td>
<td>64</td>
<td>ICD advised</td>
<td>11</td>
<td>51</td>
<td>8</td>
<td>31.2</td>
</tr>
<tr>
<td>A-II:4</td>
<td>54</td>
<td>38</td>
<td>103</td>
<td>63</td>
<td>0</td>
<td>62</td>
<td></td>
<td>11</td>
<td>60</td>
<td>10</td>
<td>24.4</td>
</tr>
<tr>
<td>A-II:6</td>
<td>54</td>
<td>48</td>
<td>175</td>
<td>85</td>
<td>1</td>
<td>38</td>
<td>MV reconstruction, maze</td>
<td>10</td>
<td>68</td>
<td>12</td>
<td>71.0</td>
</tr>
<tr>
<td>A-II:7</td>
<td>49</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1, 2</td>
<td>70</td>
<td>MV reconstruction, maze, OHCA</td>
<td>9</td>
<td>53</td>
<td>10</td>
<td>30.7</td>
</tr>
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<td>A-II:9</td>
<td>47</td>
<td>33</td>
<td>102</td>
<td>46</td>
<td>1, 2</td>
<td>61</td>
<td>Pacemaker</td>
<td>11</td>
<td>46</td>
<td>6</td>
<td>19.1</td>
</tr>
<tr>
<td>A-II:10</td>
<td>42</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
<td>51</td>
<td></td>
<td>9</td>
<td>46</td>
<td>8</td>
<td>30.7</td>
</tr>
<tr>
<td>A-II:11</td>
<td>45</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
<td>50</td>
<td>OHCA (39 yrs), ICD with pacing capacity</td>
<td>8</td>
<td>60</td>
<td>7</td>
<td>29.3</td>
</tr>
<tr>
<td>A-III:2</td>
<td>20</td>
<td>15</td>
<td>126</td>
<td>46</td>
<td>1, 2</td>
<td>50</td>
<td>Pacemaker</td>
<td>11</td>
<td>47</td>
<td>8</td>
<td>17.8</td>
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<tr>
<td>A-III:4</td>
<td>16</td>
<td>26</td>
<td>110</td>
<td>41</td>
<td>1, 2</td>
<td>73</td>
<td>Pacemaker</td>
<td>11</td>
<td>56</td>
<td>12</td>
<td>25.2</td>
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<tr>
<td>B-1:5</td>
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<td>28</td>
<td>94</td>
<td>50</td>
<td>1, 2</td>
<td>40</td>
<td>AF, myocardial valve, pacemaker</td>
<td>8</td>
<td>54</td>
<td>9</td>
<td>55.0</td>
</tr>
<tr>
<td>C-1:4</td>
<td>74</td>
<td>32</td>
<td>169</td>
<td>66</td>
<td>1, 2</td>
<td>30</td>
<td>AF, ICD with pacing capacity</td>
<td>9</td>
<td>49</td>
<td>13</td>
<td>26.9</td>
</tr>
<tr>
<td>C-1:1</td>
<td>44</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
<td>70</td>
<td>LV hypertrophy (IVS – 13 mm)</td>
<td>13</td>
<td>NA</td>
<td>13</td>
<td>23.3</td>
</tr>
<tr>
<td>C-1:2</td>
<td>42</td>
<td>30</td>
<td>111</td>
<td>55</td>
<td>1, 2</td>
<td>70</td>
<td>LV hypertrophy (IVS – 13 mm)</td>
<td>13</td>
<td>55</td>
<td>11</td>
<td>35.1</td>
</tr>
<tr>
<td>D-II:4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Pacemaker</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>D-II:3</td>
<td>36</td>
<td>27</td>
<td>92</td>
<td>44</td>
<td>1, 2</td>
<td>41</td>
<td>Pacemaker</td>
<td>11</td>
<td>75</td>
<td>11</td>
<td>22.1</td>
</tr>
</tbody>
</table>

*Numbering for the site of noncompaction: none = 0, left ventricular apex = 1, right ventricular involvement = 2. †The number in parentheses indicates the patient’s age in years at the time of OHCA. ‡Holter report shows extreme bradycardia.

AF = atrial fibrillation; EF = ejection fraction; HR = heart rate; ICD = implantable cardioverter defibrillator; IVS = interventricular septum; IVSd = interventricular septum in diastole; LV = left ventricular; LAEDV = left atrial end diastolic volume indexed for body surface area; LVIDd = left ventricular internal diastolic dimension; LVPWd = left ventricular posterior wall in diastole; MV = mitral valve; NA = not assessed; OHCA = out-of-hospital cardiac arrest.
0 mV), plotted against test voltage, provided the activation-voltage relationship; the latter was normalized by maximum amplitude and fitted with the Boltzmann function: \( I/I_{\text{max}} = A/(1.0 + \exp[(V-V_{1/2})/k]) \) to determine the half-maximum activation voltage \( V_{1/2} \) and slope factor \( k \).

**STATISTICAL ANALYSIS.** Data are mean ± SEM. Groups were compared using 1-way analysis of variance followed by Tukey’s test or 2-way repeated measures analysis of variance followed by pairwise comparison using the Student-Newman-Keuls test. A p value of <0.05 defined statistical significance.

**RESULTS**

**FAMILY A.** The pedigree of the index family is shown in Figure 1. The proband (II:1) visited our cardiogenetics outpatient clinic because of an out-of-hospital cardiac arrest (OHCA) as a result of ventricular fibrillation at the age of 57 and a positive family history for cardiomyopathy. A previously made Holter recording showed episodes of bradycardia for 7% of the 24 h, with a minimum heart rate of 31 beats/min. Further evaluation initially did not reveal any abnormalities, and an implantable cardioverter-defibrillator (ICD) (with pacing capacity) was implanted for secondary prevention of sudden cardiac death. LVNC of the myocardium was detected after evaluation of her echocardiographs.

Three siblings (II:3, II:7, and II:9) and 2 nephews (III:2 and III:4) of the proband presented with the same combination of bradycardia and LVNC. Anamnestically, the sinus bradycardia of III:2 was first detected in utero during Doppler echocardiographic evaluation. A pacemaker was implanted in II:7, II:9, and III:2, because of bradycardia-related symptoms. Clinical data of family A are presented in Table 1.

Two individuals (II:6 and II:7) had mitral valve reconstruction because of a mitral valve prolapse. Post-operative atrial fibrillation occurred in II:6, and he was unsuccessfully treated by a surgical maze procedure; consequently, we could not determine the occurrence of bradycardia in this individual (29). His echocardiogram showed LVNC. In 2013, II:7 collapsed during breakfast; he regained consciousness after 1 to 2 min and was admitted to the hospital. The ECG record showed atrial fibrillation. Later, a sinus rhythm with a total atrioventricular block, without an adequate escape rhythm, was observed. Given his LVNC, worsening systolic function with signs of progressive conduction disorders, and the expectation of frequent pacing, his pacemaker was replaced by a biventricular ICD. Cardiac evaluation of II:4 and II:10 did not reveal any abnormalities.

**TABLE 2** Overview of Known Human HCN4 Mutations

<table>
<thead>
<tr>
<th>Residue Change</th>
<th>HCN4 Domain (Ref. #)</th>
<th>Patients’ Clinical Characteristics</th>
<th>HCN4 Domain Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Ala195Val</td>
<td>Cytoplasmic</td>
<td>Sinus bradycardia in sudden infant death syndrome (51)</td>
<td></td>
</tr>
<tr>
<td>p.Gly482Arg</td>
<td>Pore</td>
<td>Family A</td>
<td>This study</td>
</tr>
<tr>
<td>p.Tyr481His</td>
<td>Pore</td>
<td>Families B and D</td>
<td>This study</td>
</tr>
<tr>
<td>p.Ala414Gly</td>
<td>54-55 linker</td>
<td>Family C</td>
<td>This study</td>
</tr>
<tr>
<td>p.Ile404fs</td>
<td>Extracellular 55-pore</td>
<td>ST-segment elevation and complete right bundle branch block (52)</td>
<td></td>
</tr>
<tr>
<td>p.Gly480Arg</td>
<td>Pore</td>
<td>Sinus bradycardia</td>
<td>(4)</td>
</tr>
<tr>
<td>p.Ala485Val</td>
<td>Pore</td>
<td>Sinus bradycardia</td>
<td>(3)</td>
</tr>
<tr>
<td>p.Lys530Asn</td>
<td>C-linker</td>
<td>Tachycardia-bradycardia syndrome and AF (53)</td>
<td></td>
</tr>
<tr>
<td>p.Asp533Asn</td>
<td>C-linker</td>
<td>Sinus bradycardia with torsade de pointes ventricular tachycardia (54)</td>
<td></td>
</tr>
<tr>
<td>p.Pro544fs</td>
<td>C-linker</td>
<td>Sinus bradycardia</td>
<td>(6)</td>
</tr>
<tr>
<td>p.Ser672Arg</td>
<td>cAMP binding domain</td>
<td>Sinus bradycardia</td>
<td>(55)</td>
</tr>
<tr>
<td>p.Thr644fs</td>
<td>cAMP binding domain</td>
<td>Sinus bradycardia with premature beats linked to adrenergic stress</td>
<td>(56)</td>
</tr>
<tr>
<td>p.Val759Ile</td>
<td>Cytoplasmic</td>
<td>Sinus bradycardia in sudden infant death syndrome (51)</td>
<td></td>
</tr>
</tbody>
</table>

AF = atrial fibrillation; cAMP = cyclic adenosine monophosphate.

The youngest sister of the proband (II:11) had an OHCA at 39 years old as a result of ventricular fibrillation. During her hospital admission, she was repeatedly defibrillated because of sustained polymorphic ventricular tachycardias, initiated by short-coupled ventricular extrasystole. After an electrophysiology study, in which a right bundle branch block morphology with extreme axis focus was ablated, no further arrhythmias were observed. Echocardiographic investigation did not reveal signs of cardiomyopathy. The parents of the proband, who were distantly related (seventh-degree relatives), were deceased, and no clinical information was available.
Representative ECG traces are presented in Figure 2 (III:4). Representative echocardiographic findings of an affected family member (III:4) are presented in Figure 3 and Online Videos 1, 2, and 3.

**GENOME-WIDE LINKAGE ANALYSIS.** We set out to identify the genetic defect underlying the combined phenotype of bradycardia and LVNC. Although the parents are distantly related, the pedigree of family A is suggestive of an autosomal dominant pattern of inheritance. Linkage analysis in 5 relatives with combined bradycardia and LVNC was performed. All loci with suggestive linkages were checked for sharing in all 5 individuals by haplotype analysis; we thus uncovered 30 loci that were shared among all affected individuals (Online Fig. 1). In total, these shared regions span ~492 Mb and contain >2,000 coding genes.

**EXOME SEQUENCING.** Exome sequencing was carried out on cousins III:2 and III:4 in family A. Coverage and quality scores of the exome sequencing experiment are shown in Online Table 1. The number of exonic variants identified per sequenced individual is shown in Online Table 1. We identified 72 novel shared variants not previously reported in public variant databases, including the ethnically matched GoNL study (35). We overlaid these variants with the 30 linkage regions, thus narrowing the list down to 11 novel shared variants (Online Table 3). Of these, only 1 variant in HCN4 (c.1444G>C, p.Gly482Arg) cosegregated with the combined phenotype in the remaining affected family members and was absent in all unaffected family members. Subject II-6, who had LVNC, but in whom the presence of sinus bradycardia could not be evaluated, carried the mutation.

**ADDITIONAL FAMILIES: PEDIGREES.** To further explore the possible link between mutations in HCN4 and the combined phenotype of bradycardia and LVNC, we screened HCN4 in 3 additional families with this combined clinical presentation. The pedigrees of families B, C, and D are shown in Figure 4.

**FAMILY B.** The proband of family B (individual II:5) was referred to the cardiologist after she collapsed in the shower. The cardiovascular examination...
revealed bradycardia, LVNC, and a myxoid degeneration of the mitral valve. Moreover, polymorphic ventricular extrasystoles occurred during exercise. A few months later, she presented with atrial fibrillation at age 53 years. A loop recorder showed more than 800 episodes of bradycardia in 64 days (heart rate <30 beats/min). Because of the syncpe and the frequent episodes of bradycardia, a pacemaker was implanted. Her son also presented with the combined phenotype. Both were carriers of a mutation in HCN4 (c.1441T>C, p.Tyr481His).

**FAMILY C.** The proband of family C (individual II:4) is a 74-year-old man who presented with atrial fibrillation; echocardiographic evaluation revealed LVNC. His 2 sons, presented with the combined phenotype of LVNC and bradycardia. On Holter monitoring, 1 displayed severe sinus bradycardia involving 12 episodes of standstill with a maximum duration of 2.88 s, which most of the time occurred during episodes of bradycardrythmia. All 3 carried a mutation in HCN4 (c.1241C>G, p.Ala414Gly).

**FAMILY D.** The proband of family D (individual III:2) has been previously described in a case report (10). A 36-year-old man was referred because of progressive fatigue. Cardiovascular examination revealed a severe sinus bradycardia (40 beats/min), LVNC, and moderate aortic valve regurgitation. His mother (D:II:4) has a pacemaker because of bradyarrhythmias. Echocardiographic reports of the mother were not available. The LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes.

**ADDITIONAL GENETIC ANALYSIS.** To ensure that the LVNC phenotype in families A through D was not due to mutations in any of the known LVNC genes, the probands from these families were screened for mutations in these genes. No novel or rare (minor allele frequency <1%) mutations in the known LVNC genes were found.

![FIGURE 5 Haplotype Analysis of 10 Microsatellite Markers at the HCN4 Locus in Families B and D, With the p.Tyr481His Mutation in HCN4](image)

The haplotype containing the HCN4 mutation is colored red. Markers D15S206 and D15S115 flanking the recombination event telomeric of the mutation are 200 kb apart.

**ELECTROPHYSIOLOGY OF MUTANT HCN4 CHANNELS.** WT and mutant HCN4 channels were transiently expressed in CHO cells, and the biophysical properties were examined using the voltage clamp protocol as depicted in Figure 7A, top panel. Tail currents (Fig. 7A, bottom, inset) were used to analyze the voltage dependence of activation (Fig. 7B, left panel). Half-maximal activation voltages (V_{1/2}) in all mutant HCN4 channels were significantly more negative as compared with WT channels, whereas no differences in slope factor (k) were observed (Fig. 7B, right panels). The current density at ~160 mV, the potential at which all groups in all channels are activated (Fig. 7B, left panel), did not differ significantly, although there is a tendency to smaller densities (Fig. 7C, left panel) (~184 ± 35 pA/pF [WT, n = 11], ~120 ± 30 pA/pF [WT+p.Ala414Gly, n = 9], ~100 ± 18 pA/pF [WT+p.Tyr481His, n = 8], and ~97 ± 21 pA/pF...

FIGURE 6 Overview of the Protein Changes in HCN4

(A) Schematic diagram of the HCN4 mutation locations with respect to the channel domains. (B) Structural model of HCN4 channel obtained from a ribbon SwissProt model of amino acids 264–520. Positions of the mutated amino acids are indicated by hydrogen bonds. Arrows show the locations of the novel HCN4 mutations identified in this study. (C) p.Ala414Gly, p.Tyr481His, and p.Gly482Arg are evolutionary conserved amino acid residues.

For the first time, we provide strong evidence linking mutations in HCN4 to the combined clinical presentation of bradycardia and LVNC. Using a strategy entailing linkage analysis and exome sequencing, we first identified the causal HCN4 mutation in a large pedigree with individuals affected by the combined phenotype. Follow-up screening of HCN4 in 3 other families with the same combined clinical presentation uncovered 2 additional HCN4 mutations in the 3 families. Although mutations in HCN4 have been previously linked to bradycardia (Table 2), our study provides the first evidence to our knowledge that mutations in this ion channel gene also may be associated with structural abnormalities of the myocardium.

HCN4 encodes the fourth isoform of the hyperpolarization-activated cyclic nucleotide-gated channel 4, which conducts the hyperpolarization-activated “funny” current, among others in the SAN (25,26). In line with the role of this current in SAN pacemaker activity, the HCN4 locus has been identified as a modulator of heart rate in a recent genome-wide association study (41), and mutations in HCN4 have been reported as a cause of sinus bradycardia (2–4). None of these studies, however, describe structural cardiac alterations, although it is unclear whether patients involved were actively screened for this.

The HCN4 channel is composed of 6 transmembrane domains and a pore-forming loop—the P domain—located between transmembrane domains S5 and S6, acting as the ion conducting pore and selectivity filter (40). Two of the identified mutations, p.Tyr481His and p.Gly482Arg, affect highly conserved residues within this channel domain (Fig. 6). Our heterologous expression studies of WT HCN4 with mutant HCN4 carrying, respectively, the p.Tyr481His and the p.Gly482Arg mutations (reflecting the heterozygous situation in mutation carriers), uncovered a large negative shift of the voltage dependence of activation compared with the expression of only WT channels. This finding is in agreement with previous observations for 2 other pore-region HCN4 mutations, namely p.Gly480Arg (4) and p.Ala485Val (3), both located close to p.Tyr481His and p.Gly482Arg, indicating the importance of the pore region of the channel for the voltage dependence of activation. The other mutation we identified, p.Ala414Gly, which is also evolutionarily highly conserved, is the first mutation described in the S4–S5 linker. The cytoplasmic S4–S5 linker of HCN channels is thought to play a role in voltage-activated gating (42). Consistent with this hypothesis, p.Ala414Gly resulted in a negative shift of the voltage dependence of activation. The shifts of the activation voltage dependence toward more negative...
potentials observed for the 3 mutations result in a significantly lower HCN4 current density in the potential range of the diastolic depolarization (i.e., the action potential phase responsible for the spontaneous activity of SAN cells). The very small inward current that drives the diastolic depolarization is the net result of a complex interaction of a large number of inward and outward currents, including the inwardly directed HCN4-mediated current underlying If (40). The reduced If observed for the 3 mutations is
thus compatible with the clinically observed bradycardia.

Although the involvement of HCN4 mutations in bradycardia is in line with the established role of HCN4 in cardiac pacing, the mechanism underlying the LVNC observed in the described families is unclear. We formed 2 possible hypotheses that will require further testing (Central Illustration):

1. LVNC may be congenital and the direct result of the HCN4 mutations in these families. In humans, the trabecular myocardium compacts around 8 weeks of gestation, following ventricular septation (43). In mice, at this developmental stage, Hcn4 is expressed within the SAN (44) and in humans also is detectable in the trabecular (subendocardial) layer, although at a low level in comparison with that of the SAN (45). Furthermore, a recent study demonstrated that Hcn4 is expressed in an early progenitor pool of cells that ultimately gives rise to the left ventricle (both compact and trabecular) (46). The presence of HCN4 in precursors of the ventricular wall myocardium would allow for a signaling function of HCN4 in the process of normal compaction of the human fetal ventricles.

2. A second hypothesis may be that the observed LVNC is an acquired feature in response to sinus bradycardia, as an adaptive remodeling to enable increased stroke volume and improved cardiac oxygen uptake. This hypothesis is supported by the fact that mild trabeculations are a physiological response to exercise (47). Possible future studies could entail longitudinal studies in patients taking the I$_1$-blocker ivabradine—a common angina pectoris medication for which bradycardia is a common side effect—to check the possible development of LVNC.

The possibility that LVNC may be an incidental finding seems unlikely, given the very low frequency in the population (0.05%) (48) and the consistent observation of this feature in all the HCN4 mutation carriers across all families, whereas it was absent in the noncarriers. This study will further aid the diagnostic challenges associated with LVNC. Other cardiac complications are present in the families, including myxoid mitral valve, atrial fibrillation, left ventricular hypertrophy, and sudden cardiac arrest. The latter occurred in 2 individuals from family A, one with and the other without the familial HCN4 mutation. The occurrence of OHCA in the individual who tested negative for the familial HCN4 mutation could point to an additional genetic defect in the family. Screening of a large panel of genes associated with primary electrical disease or cardiomyopathy in this individual, however, did not identify any putative mutation (data not shown).

Cardiac ion channel mutations thus far have been mainly identified in association with arrhythmic disorders of the heart, attributed to primary arrhythmia syndromes without myocardial abnormalities. However, in line with the findings presented in this study, evidence is now accumulating that primary channelopathies also can be associated with the development of myocardial structural abnormalities (49,50).

**STUDY LIMITATIONS.** In this study, although the genetic evidence for the involvement of the HCN4 mutations in the combined bradycardia–LVNC phenotype is very strong, and the electrophysiological data provide strong evidence for causality of the HCN4 mutations in the pathogenesis of the observed bradycardia, the mechanism whereby HCN4 mutations lead to LVNC remains unknown.

**CONCLUSIONS**

For the first time, we link mutations in HCN4 to a combined phenotype of bradycardia and LVNC. Our findings implicate mutations in this ion channel gene in cardiac structural abnormalities in addition to sinus bradycardia.
REFERENCES


Mutations in Patients With Bradycardia & LVNC

HCN4

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APPENDIX For supplemental tables, videos, and a figure, please see the online version of this article.


KEY WORDS exome sequencing, genetics, HCN4, ion channel, left ventricular noncompaction cardiomyopathy, sinus bradycardia