Sick sinus syndrome with HCN4 mutations shows early onset and frequent association with atrial fibrillation and left ventricular noncompaction

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BACKGROUND Familial sick sinus syndrome (SSS) is often attributable to mutations in genes encoding the cardiac Na channel SCN5A and pacemaker channel HCN4. We previously found that SSS with SCN5A mutations shows early onset of manifestations and male predominance. Despite recent reports on the complications of atrial fibrillation (AF) and left ventricular noncompaction (LVNC) in patients with SSS caused by SCN5A mutations, their overall clinical spectrum remains unknown.

OBJECTIVE The purpose of this study was to investigate the clinical and demographic features of SSS patients carrying HCN4 mutations.

METHODS We genetically screened 38 unrelated SSS families and functionally analyzed the mutant SCN5A and HCN4 channels by patch clamping. We also evaluated the clinical features of familial SSS by a meta-analysis of 48 SSS probands with mutations in HCN4 (n = 16) and SCN5A (n = 32), including previously reported cases, and 538 sporadic SSS cases.

RESULTS We identified two HCN4 and three SCN5A loss-of-function mutations in our familial SSS cohort. Meta-analysis of HCN4 mutation carriers showed a significantly younger age at diagnosis (39.1 ±21.7 years) than in sporadic SSS (74.3 ± 0.4 years; P < .001), but a significantly older age than in SCN5A mutation carriers (20.0 ± 17.6 years; P = .003). Moreover, HCN4 mutation carriers were more frequently associated with AF (43.8%) and LVNC (50%) and with older age at pacemaker implantation (43.5 ± 22.1 years) than were SCN5A mutation carriers (17.8 ± 16.5 years; P < .001).

CONCLUSION SSS with HCN4 mutations may form a distinct SSS subgroup characterized by early clinical manifestation after adolescence and frequent association with AF and LVNC.

KEYWORDS Mutation; HCN4; Pacemaker; Sick sinus syndrome; Left ventricular noncompaction; SCN5A; Atrial fibrillation

Introduction

Sick sinus syndrome (SSS) is a clinically common and heterogeneous arrhythmia comprising sinus bradycardia, sinus arrest, and bradycardia–tachycardia syndrome. SSS is...
often associated with primary structural heart disorders, and its occurrence increases with age.\textsuperscript{1} SSS can also occur in a familial form as a genetic disorder.\textsuperscript{2} The most prevalent genes responsible for familial SSS are those encoding the cardiac sodium channel \(\alpha\) subunit (SCN5A) and hyperpolarization-activated cyclic nucleotide-gated channel \((HCN4)\).\textsuperscript{3,4} Mutations in SCN5A result in conduction delay or exit block without disturbing impulse generation in the sinoatrial node (SAN). However, HCN4 mutations reduce the pacemaker current and suppress diastolic depolarization and automaticity in the SAN.\textsuperscript{5} We previously showed a male predominance and early onset of manifestations in familial SSS caused by SCN5A mutations.\textsuperscript{6} However, the clinical features of SSS caused by HCN4 mutations have not been statistically evaluated, mainly because of the scarcity of individuals with this condition.

Mutations in HCN4 are thought to predominantly underlie pure SAN disorders because HCN4 expression is mostly limited to the cardiac conduction system, especially the SAN area.\textsuperscript{6} However, several recent genetic studies have revealed associations between HCN4 mutations and atrial fibrillation (AF) and left ventricular noncompaction (LVNC) of SSS.\textsuperscript{7–9} These studies suggest that HCN4 mutations constitute a subgroup of familial SSS associated with AF and LVNC, although their detailed clinical and demographic properties remain to be determined.

In this study, we investigated the clinical spectrum of familial SSS probands carrying HCN4 mutations, those with SCN5A mutations, and those with sporadic SSS, consisting of our cohort and those from previous publications. We found that the familial SSS subgroup with HCN4 mutations had an intermediate age of onset between the subgroup with SCN5A mutations and sporadic SSS cases. Permanent pacemaker (PPM) implantation was usually required after adolescence in the HCN4 subgroup, whereas it was frequently required during the first decade of life in the SCN5A subgroup. Complications of AF and LVNC were also observed more frequently in the HCN4 subgroup than in the SCN5A subgroup. Finally, HCN4 mutations appeared to confer more complex cardiac disorders comprising electrical and structural abnormalities, which were distinct from those with SCN5A mutations.

### Methods

#### Genetic analysis

Thirty-eight unrelated familial SSS probands and their family members who participated in the study provided written informed consent in accordance with the Declaration of Helsinki and local ethics committees. Genetic screening was performed on genomic DNA extracted from peripheral blood cells using standard methods.\textsuperscript{5} All probands underwent genetic testing for mutations in 3 major bradyarrhythmia genes (SCN5A, LMNA, HCN4) by polymerase chain reaction and direct sequencing (see Online Supplemental Table S1). For the probands with positive HCN4 mutations, we extensively sequenced for other mutations in 31 and 8 genes responsible for AF and LVNC, respectively, by target exon sequencings. Mutations were validated by public variation databases, dbSNP, the 1000 Human Genome Project Database (1000 Genomes), NHLBI GO Exome Sequencing Project (ESP6500), Exome Aggregation Consortium (ExAC), and Japanese-registered Human Genetic Variation Database (HGVD), and \textit{in silico} SNP prediction tools. The study was approved by a review committee of each institution. Further detailed information is given in the Online Supplemental Materials.

#### Plasmid construction, and electrophysiologic and confocal microscope imaging

To investigate the electrophysiologic properties of HCN4 and SCN5A mutations identified in the genetic screening, we used the transient transfection system with tsA-201 cells as previously described.\textsuperscript{5,10,11} The modified protocol was used for HCN4 current recordings.\textsuperscript{11} In some experiments, HCN4 currents were recorded in the presence of cyclic adenosine monophosphate (cAMP). Membrane trafficking of the mutant HCN4 channels was determined by a confocal microscope in tsA-201 cells transfected with EGFP-tagged HCN4 plasmids of wild-type (WT) or R393H. Further details are available in the Online Supplemental Materials.

#### Study cohorts for evaluating the clinical characteristics of familial SSS

We performed a meta-analysis of familial SSS by obtaining clinical and demographic data from the following study cohorts: (1) 5 genotype-positive probands of familial SSS identified in the present study (HCN4: \(n = 2\); SCN5A: \(n = 3\)); and (2) previously published families with SSS caused by HCN4 mutations (\(n = 14\)),\textsuperscript{8–10,12–16} in addition to individuals with familial SSS caused by SCN5A mutations (\(n = 29\)) and sporadic SSS cases (\(n = 538\)) that we previously reported.\textsuperscript{5,10,11} Accordingly, there were 3 cohorts in our study, including families with SSS caused by HCN4 mutations (\(n = 16\)), families with SSS caused by SCN5A mutations (\(n = 32\)), and sporadic SSS cases (\(n = 538\)) (see Online Supplemental Table S2). Detailed information on the 29 patients with SCN5A mutations and 538 sporadic cases is provided in our previous study.\textsuperscript{5} Clinical variables were extracted for sex, age, causative \textit{HCN4} mutation, consciousness of bradycardia since childhood, and presence of AF and LVNC.

#### Statistical analysis

Data are given as mean ± SE and were analyzed by \(\chi^2\) test, Student \(t\) test, and 1-way analysis of variance with Bonferroni post hoc test. \(P < .05\) was considered significant.

#### Results

Candidate gene screening for mutations in SCN5A, LMNA, and HCN4 identified two \textit{HCN4} mutations for families 1 and 2, and three SCN5A mutations for families 3, 4, and 5. No mutation was detected in LMNA. The profiles of identified \textit{HCN4} and SCN5A mutations are given in Online Supplemental Table S3. None of these mutations are registered...
in the human genetic variations databases, including dbSNP, 1000 Genomes, ESP6500, ExAC, or the ethnic-specific Japanese database HGVD, and these variations inferred by in silico variation prediction tools were deleterious. HCN4-R393H, SCN5A-N1354K, and SCN5A-N1372R were novel mutations (see Online Supplemental Table S3). Target exon sequencing in the probands of families 1 and 2 excluded mutations in other AF and LVNC genes than HCN4.

**Case presentations**

**Family 1**

A 3-year-old girl (II:3, Figure 1A) was referred to the hospital because of bradyarrhythmia, which was identified at a physical checkup at her kindergarten school. She was asymptomatic, but her ECG recordings showed junctional rhythm with retrograde P waves (heart rate [HR] 68 bpm) (Figure 1A and Online Supplemental Figure S1A). Her
brother (II:2) was asymptomatic, but his ECG showed intermittent sinus arrest with supraventricular escape beats. His father (I:1) had dilated cardiomyopathy and AF but did not show apparent bradycardia (see Online Supplemental Figure S1B). Genetic screening identified a p.R393H mutation (c.G1178A) in exon 2 of HCN4 in the proband as well as in her brother and father (Figure 1E). The arginine-393 residue was predicted to be one of the positively charged residues on the voltage sensor domain S4 (Figure 1E). This residue shows perfect evolutionary conservation among HCN channels and their paralog Shaker Kv channels (see Online Supplemental Figure S2).

**Family 2**

A 13-year-old girl (II:2; Figure 1A) was admitted to the hospital for evaluation of syncope during exercise. ECG showed sinus bradycardia (37 bpm) (Figure 1C and Online Supplemental Figure S1C). Echocardiography and magnetic resonance imaging revealed LVNC with normal ventricular wall systolic function (Figure 1D and Online Supplemental Figure S1D) and normal left ventricular ejection fraction (65%) on left ventriculography. Twenty-four-hour Holter ECG recordings showed severe sinus bradycardia (minimum HR 26 bpm). Electrophysiologic study revealed a prolonged sinus node recovery time of 1943 ms (corrected sinus node recovery time 601 ms) and prolonged sinoatrial conduction time of 357 ms. Four years later, she underwent PPM implantation. A missense mutation p.G482R (c.G1444A) in exon 4 of HCN4 was identified. The patient had no family history of bradycardia or sudden death, and her mother and brother were negative for the mutation and cardiac disorders including bradycardia and LVNC. Paternal inheritance was suspected, but the father declined the genetic test (Figure 1A). The G482 residue consists of the consensus triplet GYG, which is located at the pore of the HCN channel and controls ion selectivity (Figure 1E and Online Supplemental Figure S2). The mutation HCN4 G482R was previously reported in multiple European families with SSS associated with LVNC (families 5 and 6 in Online Supplemental Table S2).8,9

**Functional characterization of HCN4 mutations**

To evaluate the functional outcome of the HCN4 mutation R393H, pacemaker currents were recorded from tsA-201 cells transfected with WT-HCN4, R393H-HCN4, or both constructs. Using the voltage clamp protocol, cells transfected with 1.6 µg of WT-HCN4 showed a robust pacemaker current (Figure 3A). In contrast, cells transfected with 1.6 µg of R393H mutant plasmids showed markedly reduced pacemaker currents (Figure 3B). The current density of mutant HCN4 channels measured at −60 mV, a physiologic membrane voltage during the diastolic depolarization phase of SAN, was significantly lower in the R393H (−1.5 ± 0.3 pA/pF, n = 8; P < .001) than in WT (−6.5 ± 1.3 pA/pF, n = 8) (Figure 3C). When cotransfected with WT equal amount (0.8 µg) of WT and R393H, heteromeric channel (WT/R393H) showed moderately reduced current density intermediate between those of WT and R393H (Figure 3B). The current density of the measured at −60 mV was significantly smaller in WT/R393H (−2.8 ± 0.3 pA/pF, n = 10; P < .05) (Figure 3C and Online Supplemental Table S4). Activation properties between WT and WT/R393H showed comparable half maximal voltage (V1/2) and time constants (Figures 3D and 3E, and Online Supplemental Table S4). However, WT/R393H showed significantly smaller slope factor k than WT, suggesting that the mutation of an important positive residue at the voltage sensor domain S4 altered the voltage dependence of the HCN4 channel. We found that the membrane-permeable cAMP deliberative dibutyryl-cAMP increased the current levels of both WT and R393H to a similar extent (Figure 3G), suggesting that the loss of function of R393H is not attributable to the membrane trafficking defects but to the activation gating dysfunction, which may be associated with reduced single-channel conductance.

G482R-HCN4 was previously shown to severely abolish pacemaker currents with a hyperpolarization shift in voltage dependence of activation.8,9 Two HCN4 mutations, R393H and G482R, identified in this study had the typical loss-of-function properties of HCN4 channels that would be expected to be responsible for SSS.

**Functional characterization of SCN5A mutations**

To evaluate the biophysical properties of N1354K and P1372R, sodium currents were recorded from tsA-201 cells transiently transfected with WT or mutant SCN5A plasmids (see Online Supplemental Table S5 and Online Supplemental Figure S4). N1354K and P1372R demonstrated the loss-of-function properties of sodium channels, and the other mutation S910L was previously reported to be nonfunctional.18 Overall, three SCN5A mutations identified in this study have the typical loss-of-function properties of sodium channels, consistent with other SCN5A mutations responsible for SSS.5
Meta-analysis of clinical and demographic parameters

We performed a meta-analysis of two familial SSS subgroups with HCN4 mutations (n = 16) and SCN5A mutations (n = 32), as well as sporadic SSS cases (n = 538). Adding three probands with SCN5A mutations who were newly identified in the present study did not change the male predominance of the SCN5A cohort, as we previously reported (78.1%, n = 32 vs 79.3%, n = 29 males). A sex difference was not found in the HCN4 subgroup (Table 1).

The age at diagnosis in the HCN4 subgroup showed a broader distribution (39.1 ± 21.7 years, range 3–74 years; Figure 4) than in the other subgroups. The age at diagnosis in the HCN4 subgroup was significantly younger than that of sporadic SSS cases (74.3 ± 0.4 years; P < .001) but was significantly older than that of the SCN5A subgroup (20.0 ± 17.6 years; P = .003). The age at PPM implantation was also older in the HCN4 subgroup than in the SCN5A subgroup (43.5 ± 22.1 years vs 17.8 ± 16.5 years; P < .001) (Table 1), although the prevalence of PPM implantation was comparable between the 2 subgroups (56.3% vs 65.6%; P = .29). Cardiac arrest was the initial event for 2 probands in the HCN4 subgroup (families 5 and 8 in Online Supplemental Table S2).
whereas no cardiac arrest was found in the SCN5A subgroup. AF was more frequently observed in the HCN4 subgroup (43.8%) than in the SCN5A subgroup (9.4%; \( P < 0.001 \)). However, the initial AF event tended to occur later in life in the HCN4 subgroup (55.0 ± 18.8 years) than in the SCN5A subgroup (11.3 ± 12.5 years; Table 1). LVNC was found in 8 of 16 probands (50%) in the HCN4 subgroup. LVNC was perfectly matched with the HCN4 genotype in each family, indicating a perfect penetrance of LVNC (see Online Supplemental Table S2), whereas there was no case associated with LVNC in the SCN5A subgroup. In contrast, atrial flutter (12.5%), AV block (43.8%), and intraventricular conduction block (37.5%) were significantly more frequently associated with the SCN5A subgroup than in the HCN4 subgroup (Table 1).

Figure 3  Biophysical evaluation of the novel HCN4 mutation R393H. A: Representative whole-cell patch clamp recordings from tsA-201 cells expressing WT, WT/R393H, or R393H of HCN4 channels. Currents were elicited by voltage steps of increasing amplitude (from −60 mV to −140 mV in 20-mV decrements) and decreasing duration (from 11.6 to 2 seconds with 1.2-second decrements) from a holding potential of 0 mV, followed by 5-second voltage steps to −140 mV. B: Current–voltage relationship of HCN4 channels. Shaded bar indicates voltage of a slower diastolic depolarization phase in the human sinoatrial node. C: Current densities of HCN4 channels measured at −60 mV were significantly smaller in the heteromeric channel WT/R393H (\( P < 0.05 \)) as well as in the homomeric channel R393H (\( P < 0.01 \)) than WT. D: Voltage-dependent activation of WT/R393H showed significantly steeper voltage sensitivity than WT. E: Time constants of activation. F: Current levels of WT and WT/R393H (elicited by −60 mV) were increased by 1 μM cAMP. G: Confocal images of EGFP-tagged WT and R393H channels showed predominant expression in the cell membrane. cAMP = cyclic adenosine monophosphate; WT = wild type.
Table 1  Comparisons of clinical profiles of familial SSS probands with HCN4 and SCN5A mutations

<table>
<thead>
<tr>
<th></th>
<th>HCN4 mutations (n = 17)</th>
<th>SCN5A mutations (n = 32)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probands</td>
<td>16</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>7/9</td>
<td>25/7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.1 ± 21.7*</td>
<td>20.0 ± 17.6</td>
<td>.003</td>
</tr>
<tr>
<td>Initial symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syncope</td>
<td>11 (68.8%)</td>
<td>21 (65.6%)</td>
<td></td>
</tr>
<tr>
<td>Cardiac arrest</td>
<td>2 (12.5%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>2 (12.5%)</td>
<td>11 (34.4%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (6.3%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PMI</td>
<td>9 (56.3%)</td>
<td>21 (65.6%)</td>
<td>.29</td>
</tr>
<tr>
<td>Age at PMI (years)</td>
<td>43.5 ± 22.1†</td>
<td>17.8 ± 16.5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>LVNC</td>
<td>8 (50%)</td>
<td>0</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>AF</td>
<td>7 (43.8%)</td>
<td>3 (9.4%)</td>
<td>.013</td>
</tr>
<tr>
<td>Age at AF onset (years)</td>
<td>55.0 ± 18.8‡</td>
<td>11.3 ± 12.5</td>
<td>.009</td>
</tr>
<tr>
<td>AFL</td>
<td>0</td>
<td>4 (12.5%)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>AV block</td>
<td>1 (6.3%)</td>
<td>14 (43.8%)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Intraventricular conduction block</td>
<td>0</td>
<td>12 (37.5%)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Values are given as n, mean ± SE, or n (%), unless otherwise indicated.

Age was calculated in only 14 probands (*), 8 PMI (†), and 6 AF (‡) because the age information was not available for the probands with the HCN4 mutations G480R, S672R, and P883R.

AF = atrial fibrillation; AFL = atrial flutter; LVNC = left ventricular noncompaction; PMI = pacemaker implantation.

Discussion

In this study, we identified two HCN4 and three SCN5A mutations in a familial SSS cohort by candidate gene approaches. Epidemiologic meta-analysis based on our cohort, and 8 publications showed that a moderately older phenotype manifested in SSS and that a higher prevalence of AF and LVNC was associated with familial SSS caused by HCN4 mutations than in familial SSS caused by SCN5A mutations. The other clinical characteristics of SSS resulting from SCN5A mutations, male predominance and higher prevalence of atrial flutter and intraventricular conduction block, were not found in the HCN4 subgroup.

HR gradually decreases with age, and the prevalence of SSS progressively increases after the sixth decade of life in the general population. This finding is consistent with the mean age of 74.3 ± 0.4 years in a Japanese sporadic SSS cohort. The mean age of familial SSS (35.5 ± 5.4 years) is much younger than that of sporadic cases, whereas that in the subgroup carrying SCN5A mutations is even younger (20.0 ± 17.6 years). In our study, because the age at PPM implantation was as young as that of the SSS diagnosis in probands with SCN5A mutations, familial SSS with SCN5A mutations was characterized by severe SSS in childhood. In contrast, the diagnosis of familial SSS probands with HCN4 mutations was made mostly after adolescence, except in 2 cases (families 1 and 7 in Online Supplemental Table S2). Several HCN4 mutation carriers did not always show abnormally slow HR or symptoms, even though the mutant HCN4 channels showed biophysically deleterious properties when expressed in cultured cells. Based on these observations, we conclude that familial SSS probands with HCN4 mutations rarely manifest clinically during childhood and generally show a more benign clinical course than those with SCN5A mutations.

The intermediate suppression of HR in SSS probands with HCN4 mutations may be consistent with the observation that the mean HR in conditional Hcn4 knockout mice was not significantly different from that in control mice. This incomplete suppression of HR may be explained by the fact that automaticity is jointly regulated by 2 distinct clock mechanisms: the membrane clock and the calcium clock in the SAN. Even though the membrane clock is suppressed by HCN4 mutations, automaticity of the SAN could be compensated for by the...
744 calcium clock. Sinus node dysfunction of the HCN4 mutation
745 carrier (family 1, I:1) may be compensated by the increased
746 sympathetic activities due to heart failure. In a clinical setting,
747 some HCN4 mutation carriers may exhibit only LVNC or AF
748 with minor sinus node dysfunction. Therefore, these carriers
749 might be overlooked during a regular checkup unless they show
750 symptoms.

751 We carried out a meta-analysis of 48 familial SSS
752 probands. We confirmed previous findings that the association
753 of LVNC or AF is more common in patients with
754 familial SSS caused by HCN4 mutations.7–9 These findings
755 also highlighted 2 cardiac ion channel genes, SCN5A and
756 HCN4, as underlying the phenotypic overlap of primary
757 arrhythmias and structural cardiac abnormalities, as the
758 association of dilated cardiomyopathy was previously dem-
759 onstrated in SCN5A mutation carriers.21,22

760 Even though there is a frequent association of SSS with
761 LVNC and AF, the underlying mechanisms are not known.7–
762 9,22 HCN4 in the adult heart is predominantly expressed in the
763 restricted region of the cardiac conduction system, especially
764 in the SAN, and plays an essential role in the slow diastolic
765 depolarization responsible for automatic.6 However, during
766 the early embryonic stage, HCN4 is specifically expressed at
767 the first heart field, and HCN4-positive cells in this area give
768 rise to primitive heart tubes. These heart tubes eventually form
769 most myocytes of the left ventricle and parts of the atrium in
770 the adult heart.23,24 Mutations in HCN4 may disrupt delin-
771 eation of progenitor cells in the primitive heart, which in turn
772 disturbs the structural and electrical properties of the left
773 ventricle and both atria. This could lead to a pathogenic
774 substrate for LVNC and AF.

775 Conclusion

776 HCN4 mutations form a clinically distinct familial SSS subset.
777 This subset is distinguishable from familial SSS with SCN5A
778 mutations with a postadolescent diagnosis and frequent
779 association with AF and LVNC without sex differences.

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785 Appendix

789 Supplementary data

792 Supplementary data associated with this article can be found in
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