A novel trafficking-defective *HCN4* mutation is associated with early-onset atrial fibrillation

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BACKGROUND Atrial fibrillation (AF) is the most common arrhythmia, and a recent genome-wide association study identified the hyperpolarization-activated cyclic nucleotide-gated channel 4 *(HCN4)* as a novel AF susceptibility locus. *HCN4* encodes for the cardiac pacemaker channel, and *HCN4* mutations are associated with familial sinus bradycardia and AF.

OBJECTIVE The purpose of this study was to determine whether novel variants in the coding region of *HCN4* contribute to the susceptibility for AF.

METHODS We sequenced the coding region of *HCN4* for novel variants from 527 cases with early-onset AF from the Massachusetts General Hospital AF Study and 443 referents from the Framingham Heart Study. We used site-directed mutagenesis, cellular electrophysiology, immunocytochemistry, and confocal microscopy to functionally characterize novel variants.

RESULTS We found the frequency of novel coding *HCN4* variants was 2-fold greater for individuals with AF (7 variants) compared to the referents (3 variants). We determined that of the 7 novel *HCN4* variants in our AF cases, 1 (p.Pro257Ser, located in the amino-terminus adjacent to the first transmembrane spanning domain) did not traffic to cell membrane, whereas the remaining 6 were not functionally different from wild type. In addition, the 3 novel variants in our referents did not alter function compared to wild-type. Coexpression studies showed that the p.Pro257Ser mutant channel failed to colocalize with the wild-type HCN4 channel on the cell membrane.

CONCLUSION Our findings are consistent with *HCN4* haploinsufficiency as the likely mechanism for early-onset AF in the p.Pro257Ser carrier.

KEYWORDS *HCN4*; Mutation; Atrial fibrillation; Electrophysiology

ABBREVIATIONS **AF** = atrial fibrillation; **CHO** = Chinese hamster ovary; **CNBD** = cyclic nucleotide-binding domain; **FHS** = Framingham Heart Study; **GFP** = green fluorescent protein; **HCN4** = hyperpolarization-activated cyclic nucleotide-gated channel 4; **I** = current; **k** = slope; **MGH** = Massachusetts General Hospital; **SA** = sinoatrial; **V** = voltage; $\mathbf{V_{1/2}}$ = midpoint of activation

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Introduction

In recent years, compelling evidence has emerged from epidemiologic studies suggesting that atrial fibrillation (AF) is a heritable disease.[1] The advent of genome-wide association studies has considerably enhanced our ability to identify common genetic variants underlying complex traits such as AF. In a recent meta-analysis of genome-wide association studies, we identified 6 novel genetic loci for AF.[2] At 1 of the loci on chromosome 15q24, the risk variants are intronic to the hyperpolarization-activated cyclic

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nucleotide-gated 4 *(HCN4)* gene. *HCN4* encodes for the cardiac HCN4 channel and represents a compelling candidate gene for AF.

The HCN4 channel is abundantly expressed in the sinoatrial (SA) node and cardiac conduction tissue, and it underlies the funny current (I_f). I_f is an inward current that contributes to spontaneous pacemaking during the diastolic depolarization phase of the SA node action potential.[3] Several studies have reported an association between loss-of-function mutations in the HCN4 channel and familial sinus node dysfunction and sinus bradycardia.[4–8] The association between sinus node dysfunction and AF is well recognized.[9,10] Interestingly, dominant negative mutations in the HCN4 channel previously have been associated with familial bradycardia and AF.[5,7,11]

Therefore, we sought to determine if genetic variation in the coding region of the *HCN4* gene is associated with AF. We identified 7 novel HCN4 variants in our cohort with early-onset AF and 3 novel HCN4 variants in our referent population. The variants in the referents had no functional effect. One of the novel AF variants (p.Pro257Ser) had defective trafficking to the cell membrane. Coexpression of the p.Pro257Ser mutant did not alter the activity of the wild-type HCN4 channel, consistent with haploinsufficiency as the likely mechanism of disease.

Methods

Study samples

Cases included unrelated individuals with early-onset AF from the Massachusetts General Hospital (MGH) AF Study. Early-onset AF was defined as a first occurrence of AF at age <66 years and no history of myocardial infarction, heart failure, or structural heart disease as assessed by echocardiography. Referent individuals were drawn from the Framingham Heart Study (FHS) and had no history or ECG evidence of AF on serial examinations. Baseline characteristics of both study populations are listed in Table 1. Written informed consent for genetic research was obtained from each individual, and the Institutional Review Boards at MGH and FHS approved the study.

Table 1 Baseline characteristics of early-onset AF cases and referents

Study	Massachusetts General Hospital AF cases	Framingham Heart Study referents
Affection status	Early-onset AF	No AF
No.	527	443
Female	103 (19.5)	227 (51.2)
Age (years)	54.2 ± 10.5	66.0 ± 9.2
Age at onset of AF (years)	47.2 ± 10.9	NA
Family history of AF	211 (40.9)	176 (39.7)
Hypertension	163 (30.9)	259 (58.6)
Congestive heart failure	16 (3.2)	14 (3.2)
Diabetes	26 (5.0)	57 (13.0)
Body mass index	28.2 ± 5.3	28.3 ± 5.1

Values are given as no. (%) or mean ± SD.
AF = atrial fibrillation; NA = not applicable.

Screening and mutagenesis

The coding region of *HCN4*, which consists of 8 exons and 3612 base pairs, was screened using a combination of high-resolution melting and Sanger sequencing. Oligonucleotide primers were designed using the genomic sequence from the University of California, Santa Cruz Genome Browser (hg19 assembly).

High-resolution melting was performed using LightScanner technology (Idaho Technology, BioFire Defense, Salt Lake City, UT) following the manufacturer's recommendations. All variants identified by high-resolution melting were confirmed using standard Sanger sequencing. Exons 2 to 6 of the *HCN4* gene were screened using high-resolution melting, and the remaining exons (1, 7, and 8) were sequenced directly using Sanger sequencing.

Nonsynonymous variants that had not been reported previously in publically available databases (dbSNP, 1000 Genomes Project, exome variant server) and hence were deemed to be novel were prioritized for further analysis. Common nonsynonymous and synonymous variants with a mean allele frequency >1% are reported in the Online Supplementary Data. Each novel rare variant was introduced into the human *HCN4* clone (Open Biosystems, Pittsburgh, PA) using the QuikChange mutagenesis kit (Stratagene Inc, La Jolla, CA) with appropriate mutagenic primers. For immunocytochemistry and confocal microscopy studies of coexpressed wild-type and p.Pro257Ser mutant channel, the constructs were tagged with C-terminal epitopes for myc and V5 using pcDNA4/myc-His and pcDNA4/V5-His (Invitrogen, Carlsbad, CA). All constructs were followed by confirmatory sequencing of the entire clone.

Cell culture and electrophysiology

Chinese hamster ovary (CHO) cells were transfected with 2 μg wild-type or variant *HCN4* with 0.6 μg green fluorescent protein (GFP) using FuGENE (Promega, Valencia, California) according to the manufacturer's instructions. For coexpression experiments, 1 μg HCN4 and 1 μg p.Pro257Ser with 0.6 μg GFP were used. GFP-positive cells were used for whole-cell patch-clamp experiments 1 to 2 days after transfection. The extracellular solution used contained 135 mM KCl, 5 mM NaCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM HEPES, pH 7.4 with KOH. The intracellular solution contained 130 mM potassium aspartate, 10 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA, and 5 mM HEPES, pH 7.4 with KOH. Signals were amplified using an Axon 200B amplifier, (Sunnyvale, CA) digitized with a Digidata 1322A A/D converter, and analyzed with Clampex 9.2, Clampfit 9.2 (Molecular Devices Inc, Sunnyvale, CA), and SigmaPlot 9.0 (Systat Software Inc, Point Richmond, CA). Normalized current–voltage (I–V) relationships were determined from tail currents elicited at –35 mV for 2 seconds following 4- to 20-second test pulses ranging from