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Point Mutation in the HCN4 Cardiac Ion Channel Pore Affecting Synthesis, Trafficking, and Functional Expression Is Associated With Familial Asymptomatic Sinus Bradycardia

Eyal Nof, MD*; David Luria, MD*; Dovrat Brass, MSc*; Dina Marek, MSc;
Hadas Lahat, PhD; Haya Reznik-Wolf, PhD; Elon Pras, MD; Nathan Dascal, PhD;
Michael Eldar, MD; Michael Glikson, MD

Background—The hyperpolarization-activated nucleotide-gated channel-HCN4 plays a major role in the diastolic depolarization of sinus atrial node cells. Mutant HCN4 channels have been found to be associated with inherited sinus bradycardia.

Methods and Results—Sixteen members of a family with sinus bradycardia were evaluated. Evaluation included a clinical questionnaire, 12-lead ECGs, Holter monitoring, echocardiography, and treadmill exercise testing. Eight family members (5 males) were classified as affected. All affected family members were asymptomatic with normal exercise capacity during long-term follow-up. Electrophysiological testing performed on 2 affected family members confirmed significant isolated sinus node dysfunction. Segregation analysis suggested autosomal-dominant inheritance. Direct sequencing of the exons encoding HCN4 revealed a missense mutation, G480R, in the ion channel pore domain in all affected family members. Function analysis, including expression of HCN4 wild-type and G480R in *Xenopus* oocytes and human embryonic kidney 293 cells, revealed that mutant channels were activated at more negative voltages compared with wild-type channels. Synthesis and expression of the wild-type and mutant HCN4 channel on the plasma membrane tested in human embryonic kidney 293 cells using biotinylation and Western blot analysis demonstrated a reduction in synthesis and a trafficking defect in mutant compared with wild-type channels.

Conclusions—We describe an inherited, autosomal-dominant form of sinus node dysfunction caused by a missense mutation in the HCN4 ion channel pore. Despite its critical location, this mutation carries a favorable prognosis without the need for pacemaker implantation during long-term follow-up. (*Circulation*. 2007;116:463-470.)

Key Words: bradycardia ■ genetics ■ ion channels

Familial disease of the sinus node is a rare syndrome, usually associated with other cardiac and extracardiac abnormalities.^{1–8} In most of these reported cases, permanent pacing was required.

Clinical Perspective p 470

Some families with sinus bradycardia (usually without any extracardiac abnormalities) have a more benign course and may even be asymptomatic.^{1,2,9–12} In some of these cases, increased vagal tone rather than intrinsic sinus node dysfunction has been proposed as the responsible mechanism.^{11,12}

Recently, mutations in the gene coding for pacemaker hyperpolarization-activated nonselective cationic HCN4 ion channel were found to be responsible for inherited sinus bradycardia.^{13–15} HCN4 is part of the *f* channels (carrying *I_f*)

participating in the spontaneous diastolic membrane depolarization in the sinoatrial node cells.^{16–19} Mutant channels were found to be activated at voltages more negative than wild-type (WT) channels, decreasing the inward diastolic current depolarization and thus slowing the heart rate.^{13,15} These changes mimic the effect of vagal stimulation. We describe a family with asymptomatic sinus bradycardia with no extracardiac abnormalities who were managed conservatively during long-term follow-up (14±11 years). We hypothesized that a mutation in the HCN4 ion channel is responsible for the clinical manifestations in this family.

Methods

Family pedigree is presented in Figure 1. Sixteen family members were evaluated. All patients gave informed consent for both the

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From the Heart Institute (E.N., D.L., M.E., M.G.) and Laboratory of Human Genetics (D.M., H.L., H.R.-W., E.P.), Chaim Sheba Medical Center, Tel-Hashomer; Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv (D.B., N.D.); and Mina and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Bar Ilan (D.M.), Israel.

*The first 3 authors contributed equally to this work.

Correspondence to Eyal Nof, MD, Heart Institute, Sheba Medical Center, Tel Hashomer 52621 Israel. E-mail ernof@netvision.net.il

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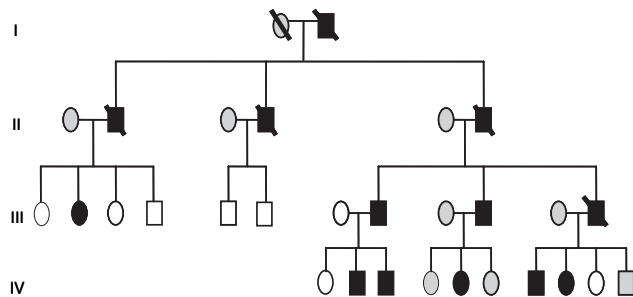


Figure 1. A family tree suggesting autosomal-dominant inheritance. Solid symbols represent affected family members. (Patients II-2, II-4, II-6, and III-12 in the pedigree were considered affected only on family history. Clinical evaluation and blood tests could not be performed because these patients were not alive at the time of the present study). Open symbols indicate family members not carrying the mutant gene; gray symbols, patients whose clinical and genetic status is unknown.

clinical and genetic studies, which were approved by the Institutional Ethics Committee of the Chaim Sheba Medical Center, Tel Hashomer.

Evaluation included a clinical questionnaire, resting ECG, and 24-hour Holter monitoring. Holter monitoring of 12 patients was interpreted by our computer system (Impresario 3.04.0089, DELMAR systems, Irvine, Calif) and confirmed by one of the electrophysiologists in our Heart Institute; Holter monitoring of the other 4 patients was examined by external cardiologists. Two-dimensional echocardiography and treadmill exercise tests were performed in those family members considered to be affected. Two patients underwent electrophysiological testing. Electrode catheters were advanced into the right atrium to record the atrial-His-ventricular intervals. Incremental atrial pacing was done until atrial-His Wenckebach was achieved. In 1 patient, overdrive atrial stimulation was performed to calculate the sinus node recovery time (SNRT) and the corrected SNRT (defined as SNRT minus spontaneous sinus cycle length).

Genetic Analysis

Family members were classified as affected and nonaffected on the basis of results of the average heart rate and minimum heart rate assessed by Holter monitoring. Affected family members were defined as having a minimum heart rate of <40 bpm and an average of <60 bpm. Heparinized blood (20 mL) was drawn from each family member; DNA was extracted with a commercial kit (Gentra System Inc, Minneapolis, Minn); and primers were designed with the Primer3 software.²⁰ The whole coding region and the exon-intron boundaries of HCN4 were amplified and sequenced as previously described¹⁴ with an automated ABI Prism 3100 Genetic Analyzer (PerkinElmer, Waltham, Mass). The presence of the G480R mutation was confirmed with a restriction assay. The segment containing the mutation was amplified with the primers 5'-agttaggttgaggagtg-3' and 5'-ctcttcctcacactggagtg-3' in a 25- μ L reaction containing 50 ng DNA, 13.4 ng each primer, 1.5 mmol/L dNTPs, 1.5 mmol/L MgCl₂, and polymerase chain reaction buffer, with 1.2 U Taq polymerase (Bio-Line, London, UK). After an initial denaturation of 5 minutes at 95°C, 30 cycles were performed (94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds), followed by a final extension of 10 minutes at 72°C. The amplification product was then cut with the *FokI* restriction enzyme (New England Biolabs, Beverly, Mass). A control group of 50 healthy subjects was used to exclude DNA polymorphisms.

Molecular Cloning of HCN4 and In Vitro RNA Synthesis

A human HCN4 cDNA in pCDNA3 vector (hHCN4; accession No. AJ132429) was provided by Dr F. Hofmann (Technical University of München, München, Germany). The HCN4 G480R point mutation

was introduced with the QuickChange Site-Direct Mutagenesis Kit (Stratagene, La Jolla, Calif). The resulting point-mutated clone and the WT were further subcloned into *EcoRI* and *XbaI* restriction enzyme (MBI, Fermentas) sites in pGEM-HJ vector. All mutations and polymerase chain reaction products were verified by nucleotide sequencing at the Tel Aviv University Sequencing Facility.

For *Xenopus* oocyte expression, the RNAs were prepared using the standard procedure, which ensures capping of the 5' end of the RNA and preferential inclusion of noncapped GTP in the rest of the RNA.²¹ Before transcription, the plasmid DNA was linearized with *NheI*. G α_s and β_2 -adrenergic receptors were prepared as described²² and coexpressed with HCN4 in all oocyte experiments.

Oocyte Culture and Electrophysiology

All the experiments were carried out in accordance with the Tel Aviv University Institutional Animal Care and Use Committee (permit 11-05-064). *Xenopus laevis* frogs (Xenopus I, Dexter, Mich) were maintained and operated on, and oocytes were collected, defolliculated, and injected with RNA as described.²³ The oocytes were injected with RNAs of HCN4 WT (2.5 ng/oocyte), HCN4 G480R (2.5 ng/oocyte), or HCN4 WT+G480R (1.25 ng each per oocyte) mRNA, in addition to 50 pg β_2 AR and 500 pg G α_s , and incubated for 4 days at 20°C to 22°C in ND96 solution (96 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 5 mmol/L HEPES, pH 7.5), supplemented with 2.5 mmol/L Na-pyruvate and 50 μ g/mL gentamycin. HCN4 current was measured in high-²⁴-K solution (74 mmol/L NaCl, 24 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 5 mmol/L HEPES, pH 7.5) at room temperature (21°C to 23°C). Whole-cell currents were recorded by a Gene Clamp 500 amplifier (Axon Instruments, Foster City, Calif) using the 2-electrode voltage clamp technique.²² The pipette solution contained 3 mol/L KCl.

Human Embryonic Kidney 293 Cell Culture and Electrophysiology

Human embryonic kidney 293 (HEK293T) cells were cultured in DMEM supplemented with 2 mmol/L glutamine, 10% FCS, 100 U/mL penicillin-G sodium, and 100 μ g/mL streptomycin sulfate in an atmosphere of 95% air, 5% CO₂ at 37°C.

A standard calcium phosphate procedure was used for transfection of HEK293T cells. Amounts of DNA used per 24-well dish were as follows: HCN4 WT and G480R, 0.8 μ g per 24-well dish. In all transfections for electrophysiological studies, the CD8 reporter gene system was used to visualize transfected cells (0.5 μ g DNA per 24-well dish). Dynabeads coated with anti-CD8 antibodies were purchased from Dynal (Carlsbad, Calif). Empty pCDNA3 was used to balance the total amount of cDNA used for transfection to 2.1 μ g per 1.5-cm well.

Patch-clamp experiments were performed 24 hours after transfection. Membrane currents were recorded under voltage-clamp conditions using conventional whole-cell patch-clamp techniques. Whole-cell patch-clamp recordings were performed at room temperature (21°C to 23°C) with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, Calif). Signals were analog filtered with a low-pass Bessel filter (1-kHz corner frequency). Series resistance was compensated by 70% to 80%. Data were digitally stored using an IBM-compatible personal computer with the pCLAMP software package for voltage control, data acquisition, and data evaluation. Patch pipettes were fabricated from glass capillaries (Warner Instrument Corp, Hamden, Conn) using a horizontal puller (P-97, Sutter Instruments Co, Novato, Calif). The DC resistance of the filled pipettes ranged from 2 to 5 mol/L Ω . The external solution contained (in mmol/L) NaCl 54, KCl 90, CaCl₂ 1.8, MgCl₂ 1.2, glucose 11, and HEPES 5.5; pH was adjusted to 7.4 with KOH. The pipette solution contained (in mmol/L) KCl 90, MgCl₂ 1, MgATP 3, EGTA 5, and HEPES 10. Intracellular and extracellular solution osmolality was adjusted to 290 and 310 mosm respectively, with sucrose.

TABLE 1. Baseline Clinical Characteristics of Family Members

Patient (Location in Pedigree)	Sex	Age, y	Holter Recording			Time of Holter Recording, h	Phenotype	Genotype (G480R)	Maximum Heart Rate Achieved During Exercise Testing	Basic ECG Parameters, ms		
			Min	Avg	Max					PR	QRS	QTc
III-8	M	57	27	43	79	24	Affected	Carrier	154	200	100	431
III-10	M	43	26	36	73	24	Affected	Carrier	118	160	100	291
IV-2	M	19	25	37	94	24	Affected	Carrier	163	160	120	378
IV-3	M	10	36	49	97	24	Affected	Carrier	165	160	100	371
IV-5	F	15	30	55	115	24	Affected	Carrier	NA	160	80	440
IV-7	M	31	29	50	123	24	Affected	Carrier	150	120	100	363
IV-8*	F	28	33	41	61	6	Affected	Carrier	NA	120	80	400
III-2	F	49	49	72	128	24	Nonconclusive	Carrier	149	120	80	360
III-6	M	53	36	55	112	24	Nonconclusive	Noncarrier	152	200	120	367
III-1	F	55	67	89	132	24	Nonaffected	Noncarrier	140	160	80	350
III-3	F	43	61	80	160	24	Nonaffected	Noncarrier	152	150	80	320
III-4	M	38	60	81	130	24	Nonaffected	Noncarrier	122	160	80	340
III-5	M	56	49	63	109	24	Nonaffected	Noncarrier	160	160	80	411
III-7	F	55	54	67	121	24	Nonaffected	Noncarrier	NA	160	90	420
IV-1	F	24	56	79	130	24	Nonaffected	Noncarrier	190	120	100	430
IV-9	F	25	57	74	112	24	Nonaffected	Noncarrier	NA	160	110	360

Min indicates minimum; Avg, average, and Max, maximum.

*This patient was not included in the analysis of the average and maximum heart rate in Table 2 because of insufficient number of hours recorded.

Western Blot Analysis and Cell-Surface Biotinylation of HEK293T Cells

HEK293T cells were transfected with a calcium phosphate transfection procedure. Cells were grown in 25-cm² culture dishes for 48 hours after transfection, collected, washed 3 times with ice-cold PBS supplemented with 1 mmol/L MgCl₂ and 0.5 mmol/L CaCl₂ (PB-SCM), and incubated for 15 minutes with 0.5 mg/mL EZ-link Sulfo-NHS-SS-Biotin (Pierce, Rockford, Ill) in cold PBSCM, pH 8.0, with gentle agitation at 4°C. Cells were washed once and incubated for 10 minutes with a quenching buffer (192 mmol/L glycine and 25 mmol/L Tris in PBSCM). Subsequently, cells were rinsed twice in cold PBS, homogenized in lysis buffer (1% Triton X-100, 20 mmol/L HEPES, pH 7.4, and 150 mmol/L NaCl), and centrifuged. Supernatants (100 µg total protein) were incubated with 50 µL of 50% slurry of streptavidin-sepharose (Pierce) overnight at 4°C and then washed 3 times with lysis buffer. Biotinylated proteins were eluted by 5 minutes of boiling in SDS-PAGE sample buffer, separated on 6% gel, and immunoblotted with rabbit polyclonal antibody to HCN4 (Alomone Labs, Jerusalem, Israel) and to calnexin (endoplasmic reticulum protein serving as a protein loading control; Santa Cruz Biotechnology, Santa Cruz, Calif).

Peroxidase-conjugated anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa) were used as secondary antibodies. The bands were visualized with SuperSignal West Pico chemiluminescent solution (Pierce, Rockford, Ill).

Presentation and Analysis of the Experimental Results

Electrophysiology experimental results were analyzed with pCLAMP9 software (Axon Instruments), SigmaPlot 2000, and SigmaStat (both Sigma-Aldrich, St Louis, Mo). Data are presented as mean ± SEM. A 2-tailed unpaired *t* test was used to compare statistically significant differences between the 2 groups (*P* < 0.05). One-way ANOVA, followed by Dunnett or Tukey tests, was used to compare several groups.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

After completion of the clinical questionnaire and 24-hour Holter monitoring, 7 family members (5 males) were initially classified as affected (Table 1). All affected family members had a minimum heart rate of <36 bpm, whereas those not affected had a minimum heart rate of >49 bpm. The average heart rate for all affected family members was <55 bpm compared with >63 bpm for all those not affected (Figure 2). An additional family member known to have bradycardia for many years (patient III-2 in the family pedigree) demonstrated a minimum heart rate of 49 bpm and an average heart rate of 72 bpm on Holter monitoring and therefore could not be classified as affected until genetic testing confirmed that she carried the mutant gene (Table 1).

Another family member (patient III-6), a competitive athlete, demonstrated a low average (55 bpm) and minimum (38 bpm) heart rate on Holter monitoring. Because of the nature of his profession, we did not consider him to be affected, and indeed, genetic testing confirmed that he did not carry the mutant gene.

All affected family members were known to have had bradycardia from a young age (12 ± 4 years). None reported complaints of dizziness or syncope during rest or physical exercise. There were no other rhythm or conduction abnormalities. QTc was within normal limits in all patients (379 ± 47 ms). There were no cases of sudden cardiac death in the family.

Exercise testing demonstrated normal chronotropic and exercise capacity, with all subjects achieving submaximal heart rate (adjusted to age) without any conduction disturbances (Table 1). All family members had a normally structured heart on echocardiography.

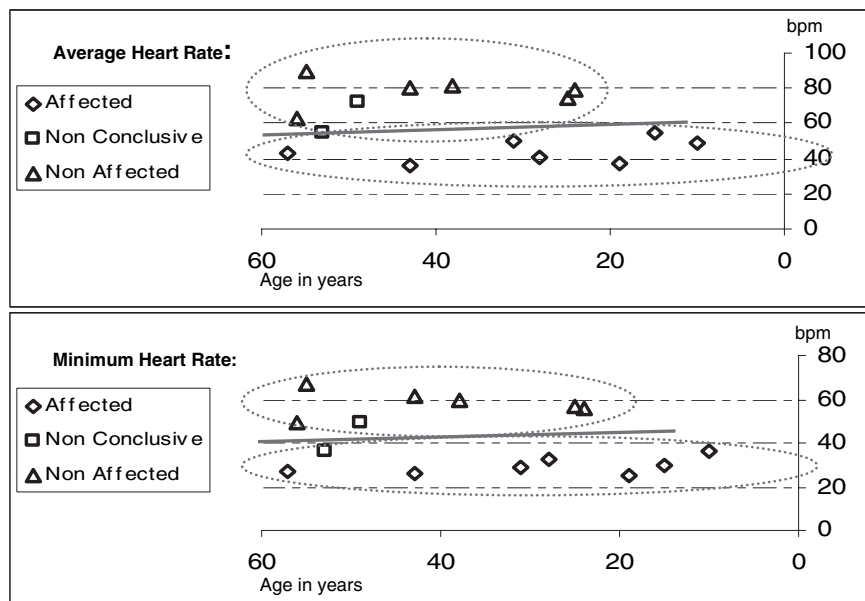


Figure 2. This chart, comparing age with heart rate of family members, demonstrates that there is no congruence of minimum and average heart rate on Holter recording between affected and nonaffected family members. Two family members could not be defined as affected or unaffected until genetic testing was done, as detailed in the text.

Electrophysiological testing performed on 2 affected family members confirmed significant sinus node dysfunction. The average SNRT and corrected SNRT were prolonged (2025 and 800 ms, respectively). Average corrected SNRT completely resolved after atropine (2.5 mg) injection (from 800 to 230 ms). In contrast, the atrioventricular node and His-Purkinje conduction times were within normal ranges.

Sequencing of the HCN4 gene in one of the patients revealed a heterozygous G-to-C change in exon 4 at position 1439. This change resulted in a substitution of glycine to arginine at position 480 of the protein (Figure 3). The HCN4 consists of 6 transmembrane segments containing a pore-forming region between S5 and S6. The G480R mutation was found to be located within the transmembrane pore-forming

region²⁴ (Figure 4). Restriction digests of the DNA from all family members found the G480R substitution to fully segregate within the family. The change was not found in 100 control chromosomes from unrelated healthy persons. Glycine 480 was found to be in a conserved region.²⁵

Functional Expression of HCN4 WT and G480R Mutant in *Xenopus* Oocytes

A 2-electrode voltage clamp was used to investigate the functional change caused by the G480R mutation. G480R was expressed alone to mimic the homotetramer phenotype) or with WT (to mimic the heterotetramer phenotype). In oocytes injected with WT HCN4 RNA, 5-second hyperpolarizing steps from -40 mV elicited slowly activating inward currents at voltages below -65 mV, typical for HCN4 I_f current.²⁶ The increase in current amplitude and the time course of current activation pattern of the G480R homotetramer and heterotetramer states were strikingly different from the WT HCN4. The currents were apparent only below -90 mV and developed more slowly than in the WT (Figure 5A

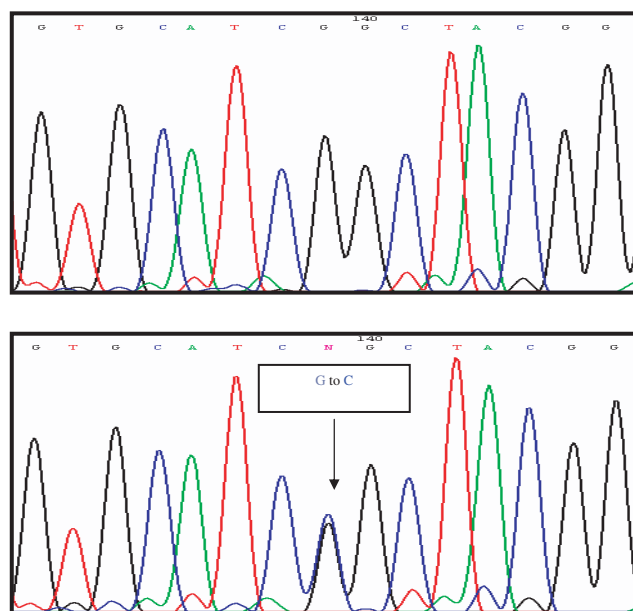


Figure 3. An electropherogram demonstrating a heterozygous mutation from GGC to CGC at position 1439.

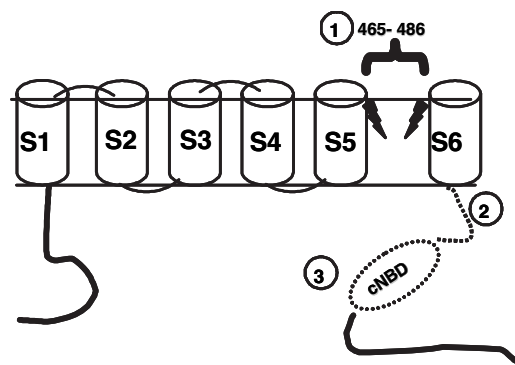


Figure 4. Schematic topology of HCN4. The amino acids in position 465 to 486 (1) encode the ion pore. Other mutations found in humans are in between the core domain and cyclic nucleic binding domain (cNBD) (2; dashed line) or in the cNBD itself (3).

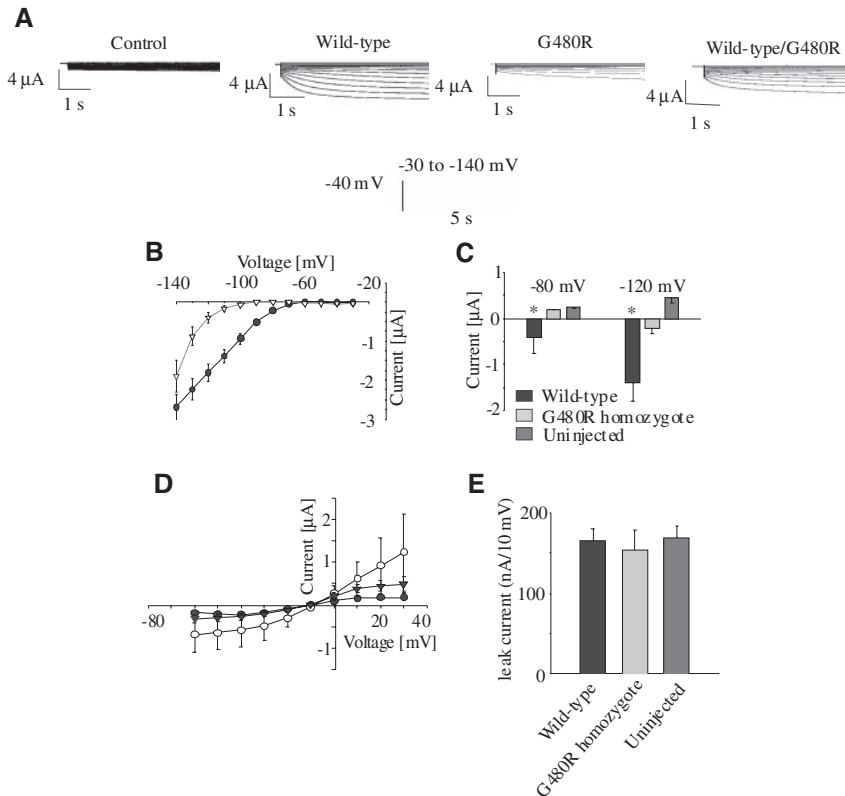


Figure 5. Expression of HCN4 WT and HCN4 G480R homotetramers in *Xenopus* oocytes. **A**, Top, Currents recorded from an uninjected oocyte (control) or oocytes injected with RNAs of HCN4 WT, G480R, or WT+G480R heterotetramer. The extracellular solution contained 24 mmol/L K^+ . Bottom, Voltage protocol. Holding potential was -40 mV, and currents were elicited by 5-second hyperpolarizing voltage steps between -30 and -140 mV in 10-mV increments. **B**, I-V relationship of WT (\bullet ; $n=4$) and G480R (∇ ; $n=6$). **C**, Histogram showing the current amplitudes recorded at the end of the 5-second voltage step to -80 and -120 mV of the WT HCN4 ($n=5$), G480R ($n=4$), and uninjected ($n=3$) oocytes. **D**, Measurement of the reversal potential of the HCN4 currents of WT (\bullet ; $n=6$), G480R (\circ ; $n=5$), and WT+G480R (∇ ; $n=4$). The measurement shows tail current amplitude at the indicated voltages after a 5-second prepulse to -130 mV. **E**, Histogram showing the leak currents (in nA per 10-mV step) of WT ($n=5$), G480R homozygote ($n=5$), and uninjected ($n=3$) oocytes. $*P<0.05$.

through 5C). No significant differences were found in the reversal potential (≈ -10 mV) (Figure 5D) or in the leak current measured from the linear part of the current-voltage (I-V) curves of WT and mutant channels, as well as uninjected oocytes (between -20 and -50 mV; Figure 5E). Attempts to observe β -adrenergic regulation of either WT or mutant channel failed; neither current amplitude nor the I-V relationship was changed by the addition of epinephrine in oocytes that expressed β_2 AR and $G\alpha_s$ (data not shown). Difficulties in reconstituting cAMP-induced modulations of heterologously expressed ion channels in oocytes and mammalian cells have been previously observed²⁷ and often are attributed to high levels of endogenous cAMP.

Functional Expression of HCN4 WT and G480R Mutant in HEK293T Cells

Whole-cell patch-clamp recording was used in HEK293T cells to investigate the functional change caused by the G480R mutation in mammalian cells. As in the oocyte expression system, the increase in current amplitude and the time course of current activation pattern of the homotetramer G480R mutated channel state were strikingly different from the WT HCN4 (Figure 6A). The I-V relationship (Figure 6B) also showed clear differences between WT and the mutant channels: WT HCN4 was activated at a voltage negative to -60 mV, whereas HEK293T cells expressing G480R alone showed a slow inward current developing at more negative voltages (below -100 mV). Cells expressing HCN4 as a "heterozygote" could be divided into 2 populations: Half of the cells were activated at voltages as the HCN4 WT, whereas half of the cells were activated as the HCN4 G480R (below

-100 mV) (Figure 6B). As a result, the current density of the G480R mutated channel at -100 mV was significantly reduced compared with the WT HCN4-transfected cells (Figure 6C).

Synthesis and Trafficking of WT and G480R HCN4 Channels

Western blot analysis of total and biotinylated HCN4 protein in HEK293T cells revealed significantly reduced expression of homomeric HCN4 G480R channels in the plasma membrane. The total amount of the mutated channel protein compared with the WT channel protein was greatly reduced (Figure 6D). The levels of calnexin, which served as a protein control for the Western blot analysis, were similar in cells expressing mutant and WT channels.

Discussion

Main Findings

We describe a family with sinus bradycardia related to a new mutation in the gene encoding the HCN4 ion channel. The mode of inheritance in the affected family, similar to other reported families with familial sinus bradycardia, is autosomal dominant.^{10–13,15}

During long-term follow-up, all affected family members were asymptomatic, suggesting the benign nature of this inherited syndrome.

To the best of our knowledge, this is the first report to describe the clinical features and natural history of familial sinus bradycardia associated with a specific related mutation in a conserved pore region. Furthermore, this is the first

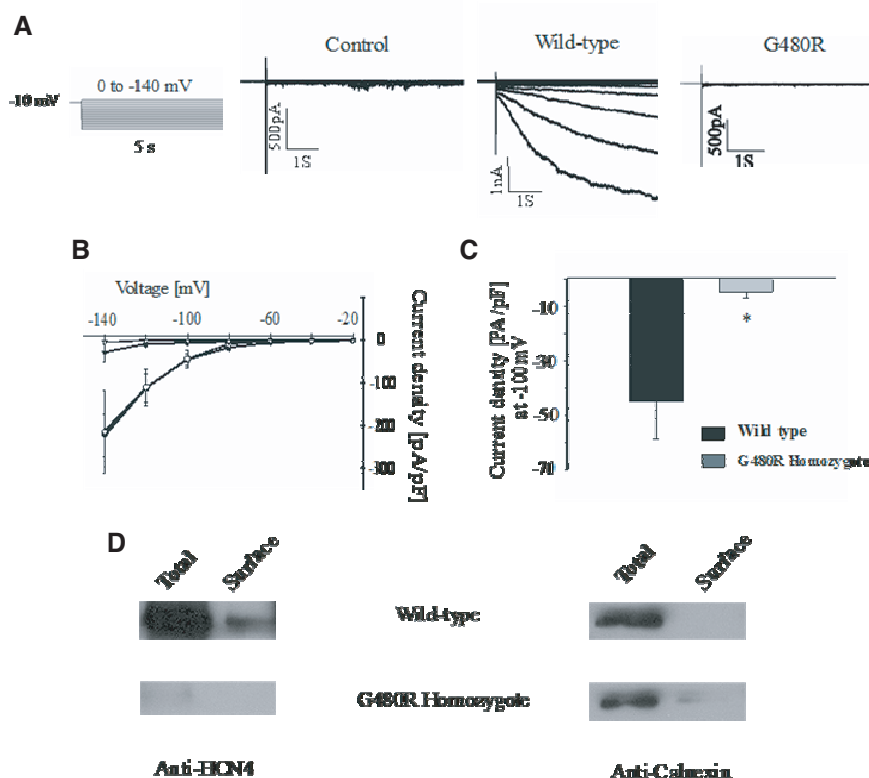


Figure 6. Expression of HCN4 WT and HCN4 G480R homotetramers in HEK293T cells. **A**, Top, Currents recorded from an untransfected cell (control) or cell transfected with DNA of HCN4 WT or G480R. The extracellular solution contained 54 mmol/L Na⁺ and 90 mmol/L K⁺. Left, Voltage protocol. Holding potential was -10 mV, and currents were elicited by 5-second hyperpolarizing voltage steps between 0 and -140 mV in 20-mV increments. **B**, I-V relationship of WT HCN4- (○; n=4), G480R- (▼; n=5), WT+G480R- (□; n=3) transfected cells containing current, and WT+G480R- (▽; n=3) transfected cells not containing current (n=3). **C**, Histogram showing the current amplitudes recorded at the end of the 5-second voltage step to -100 mV of the WT HCN4- (n=4) and G480R- (n=5) transfected cells. **D**, Western blot of total and surface protein using anti-HCN4 (left) or anti-Calnexin (right) of WT HCN4-transfected HEK293T cells or G480R HCN4-transfected cells. **P*<0.05.

description of a mutation also affecting synthesis of the HCN4 channel in humans.

Human Mutations in HCN4 Ion Cardiac Channel

Sequencing of the HCN4 gene revealed a missense substitution G480R in all affected family members. Several lines of evidence suggest that this substitution has functional consequences and is not just a silent polymorphism. First, it is segregated within the family. Second, it is not found in 100 control chromosomes. Third, the change from glycine to arginine is a major transformation from an uncharged hydrophobic amino acid to a basic, negatively charged, hydrophilic amino acid. Fourth, the change occurred in a segment highly preserved throughout evolution. Previous reports have described 3 human mutations in the HCN4 channel, all of them localized in the intramembrane C termini (Figure 4). In this report, we describe for the first time a mutation in the ion pore region of the HCN4 channel.

Clinical Features Associated With Mutations in the HCN4

The previously described mutations in HCN4 were associated with syncope, prolonged QTc, and torsade de pointes in 1 report,¹⁵ malignant syncope with intermittent bouts of atrial fibrillation in another,¹⁴ or asymptomatic sinus bradycardia.¹³ In two of these reports, the association between the mutation and the clinical bradycardia was based merely on single patients.^{14,15} The recent report of familial sinus bradycardia¹³ provided no details on the patient's cardiac evaluation (echocardiography, exercise testing, electrophysiological) or clinical follow-up. In contrast to these studies, none of our family members developed symptoms associated with low heart rate

over the years. In several reports of familial sinus bradycardia, patients suffered from additional atrial arrhythmias,^{3,6,9,14,15} which were not documented in our affected family members. Furthermore, in our study, affected family members had no evidence of chronotropic incompetence, as demonstrated by normal exercise testing. Although 24-hour ECG recording demonstrated considerable differences between G480R carriers and noncarriers (Table 2), these significant differences were mainly between minimum and average heart rates, with only minor differences between the maximal daily heart rate achieved during Holter monitoring. In 2 patients, no conduction disturbances in atrioventricular node and His-Purkinje system were found by electrophysiological testing.

Proposed Mechanism of Inherited Sinus Bradycardia in Other Studies

Normal peak sinus rate and normal PR interval during exercise testing are consistent with increased vagal tone rather than intrinsic sinus node disease.

Furthermore, the prolonged corrected SNRT, which resolved completely after atropine injection, also could be suggestive of the bradycardia being caused by high vagal

TABLE 2. Differences Between G480R Mutation Carrier and Noncarrier Family Members

	G480R Carrier Family Members	Normal Genotyped Family Members	<i>P</i>
Minimum heart rate	31±8	55±9	<0.001
Average heart rate	48±12	73±11	0.001
Maximum heart rate	101±21	126±16	0.03

tone. Previous reports of asymptomatic familial bradycardia have postulated that the mechanism responsible for the low heart rate is due to vagotonia.^{11,12} However, the mutations in HCN4 itself may mimic vagal stimulation.¹³ Mutant channels were found to be activated at more negative voltages^{13,28,29} or to have reduced ability to be activated by cAMP¹⁴ compared with the WT channels. As a result, they generated a smaller current during diastolic depolarization, resulting in a slowing of the heart rate. These changes are similar to those induced by low doses of acetylcholine.¹³

Proposed Mechanism of Inherited Sinus Bradycardia in the Affected Family

On the basis of their sequence, HCN4 channels are classified as members of the superfamily of voltage-gated K⁺ (Kv) and CNG channels; as such, they are characterized by the presence of the GYG pore sequence ("selectivity filter") typical of K⁺-permeable channels.¹⁹ However, these channels are permeable to both K⁺ and Na⁺. Furthermore, several studies have demonstrated that the mutations in the GYG sequence greatly affect the gating of HCN channels, suggesting that although this part of the pore may be involved in governing selectivity, it also substantially controls gating by an as-yet incompletely understood mechanism.^{30,31} Our results comply with these findings. Functional expression of the novel G480R mutation in the HCN4 channels in *Xenopus* oocytes has revealed that, although the mutation replaces the first glycine encompassing the selectivity filter by arginine, there is similarity in the reversal potential of HCN4 WT and G480R mutant channels, suggesting similar ion selectivity. However, the gating was greatly altered. The G480R HCN4 activation curves were shifted to more hyperpolarized potentials and comprised only 1 component of activation in both expression systems. Most important, the physiologically relevant component of activation (between -60 and -90 mV) present in the WT channels was absent in the mutant. It has been shown that HCN2 comprises the fast component of *I_f* and HCN4 underlies the slow component.^{26,32} Our results confirm the slow time course of activation of HCN4 and demonstrate that activation of the G480R mutant, alone or when coexpressed with the WT (mimicking the heterozygotic phenotype), was even slower.

The slow kinetics and the more negative range of activation in the HCN4 homozygote, as well as the heterozygote HCN4 channels, are in agreement with the reduced contribution of the mutated channel in physiological conditions to the *I_f* current.

Expression of WT and mutant HCN4 in HEK293T cells demonstrated similar results. We observed 2 populations in cells coexpressing WT and G480R HCN4. One group of cells showed currents at the same voltage range as WT cells, whereas in the other group, the I-V curves were shifted to more negative potentials as in the homozygote state. These differences possibly are due to a different stoichiometry of the channel subunits in mammalian cells compared with oocytes (ie, depending on the ratio between WT and G480R subunits in the channel protein).

Our Western blot analysis in HEK293T cells demonstrated a significant reduction in the protein expression of the HCN4

mutant channels compared with the WT. Of note, there was also a significant reduction in the ratio between total protein and surface protein in the mutant channel (data not shown), indicating that the mutation affects not only synthesis of but also trafficking to the membrane. As a consequence of this defect and the fact that channels reaching the membrane do not express currents in the physiological voltage range, these mutant channels probably do not contribute to the *I_f* current. The in vitro expression suggests that sinus bradycardia in the affected family members is due to combined synthesis and trafficking defects and the altered biophysical properties of the mutant HCN4 channels. Of note, the heterozygous expression in our patients may not confer the same properties as the heterozygous expression in heterologous cells. Therefore, the reason that our patients carry such a benign prognosis despite a mutation in such a conserved pore region may be the upregulation of the HCN4 WT protein, resulting only in a mild reduction in the *I_f* current. A different compensation mechanism may be the formation of heteromeric complexes of HCN4 and HCN2 (the dominant mRNA transcripts in atrial myocardium³²), known to be responsible for the *I_f* current in the human atrial myocytes.^{33,34} Thus, we speculate that HCN2 may underlie the benign prognosis in heterozygous family members either because of a compensatory increase in its expression or because it is generally an important contributor to *I_f* in the sinoatrial node.

The fact that all the affected family members carried only a heterozygote mutation is probably due to the severity of the homozygous phenotype. This is in agreement with a previous report¹⁶ that mice embryos lacking HCN4 channels did not survive the embryonic period.

Clinical Implications

Familial sinus bradycardia may require pacemaker implantation because of extremely low heart rate, long QT, or ventricular arrhythmia related to bradycardia.¹⁻⁸ However, the affected family members described in this study demonstrated a remarkably benign clinical pattern. They have a structurally normal heart, asymptomatic bradycardia, and excellent exercise tolerance; therefore, no pacemaker implantation was required in this family.

Genetic testing could play an important role in the differentiation between benign and malignant forms of familial sinus node dysfunction and between affected and nonaffected individuals in the same family. The underlying pathogenesis in our family and other similar previous reports may help us understand the broad phenotype spectrum of bradycardia in its inherited and sporadic forms.

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Disclosures

None.

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CLINICAL PERSPECTIVE

We describe in the present study the natural history of a family with asymptomatic sinus bradycardia related to a new mutation in the gene encoding the HCN4 ion channel pore. In people with asymptomatic sinus bradycardia, it often is difficult to distinguish between low heart rate resulting from increased vagal and decreased sympathetic tone (as in athletes) and low heart rate caused by disease of the sinus node. Genetic testing, as demonstrated in our family, may help to differentiate between these 2 conditions. Genetic testing could play an even more important role in the differentiation between benign and malignant forms of familial sinus node dysfunction and between affected and nonaffected individuals in the same family. The clinical bradycardia in our family is associated with a mutation in a highly conserved region affecting not only functional expression but also synthesis and trafficking. Understanding the pathogenesis of mutant HCN4 channels may help us understand not only the phenotype of our affected family members but also the broad phenotype spectrum of bradycardia in its inherited and sporadic forms.