Original Paper

A Functional Assay for Sick Sinus Syndrome Genetic Variants

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Key Words

Arrhythmia • Genetics • Sick sinus syndrome • Zebrafish • Sudden cardiac death

Abstract

Background/Aims: Congenital Sick Sinus Syndrome (SSS) is a disorder associated with sudden cardiac death due to severe bradycardia and prolonged pauses. Mutations in *HCN4*, the gene encoding inward Na^+/K^+ current (I_f), have been described as a cause of congenital SSS. The objective of this study is to develop an SSS model in embryonic zebrafish, and use zebrafish as a moderate-throughput assay to functionally characterize *HCN4* variants.

Methods: To determine the function of *hcn4* in zebrafish, embryos were either bathed in the I_f -specific blocker (ZD-7288), or endogenous *hcn4* expression was knocked down using splice-blocking morpholinos. To assess whether the zebrafish model discriminates benign from pathogenic variants, we tested four *HCN4* mutations known to cause human SSS and four variants of unknown significance (VUS). **Results:** Pharmacological blockade and knockdown of *hcn4* in zebrafish phenocopied human SSS, displaying bradycardia and cardiac pauses in intact embryos and explanted hearts. The zebrafish assay correctly identified all disease-causing variants. Of the VUS, the assay predicted 2 as benign and 2 as hypomorphic variants.

Conclusions: We conclude that our embryonic zebrafish assay is a novel and effective tool to functionally characterize human *HCN4* variants, which can be translated into important clinical prognostic information.

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Introduction

The sinoatrial node, originally described by Keith and Flack in 1907, is the cardiac pacemaker that generates and maintains cardiac automaticity. Sinoatrial node dysfunction or Sick Sinus Syndrome (SSS) causes bradyarrhythmia with a broad clinical spectrum ranging from no symptoms to sudden cardiac death [1]. Mutations in *HCN4* have been shown to cause sporadic and familial SSS [1, 2]. *HCN4* encodes the potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4, which contributes to the native pacemaker currents in the heart (I_p). Traditionally the functionality of *HCN4* variants has been determined by assays such as heterologous expression systems or non-human myocytes [2]. Unfortunately these assays are too laborious to functionally characterize the many variants currently detected by [1] genetic testing. Thus, the majority of *HCN4* variants are without functional interpretation and classified as variants of unknown significance (VUS) [3].

Hcn4 knock-out mice have been extensively studied [4, 5]. However, the utility of the mouse knock-out model has been limited due to embryonic lethality in homozygous knockout mice and the absence of a phenotype in heterozygous mice [4]. Zebrafish display many similar cardiac electrophysiological characteristics as humans and are more amenable to high-throughput functional experiments [6]. We have previously developed an *in vivo* zebrafish assay to characterize the functional consequences of human Long QT Syndrome genetic variants [7]. We now present another *in vivo* cardiac assay to functionally characterize potential SSS genetic variants with the goal to provide meaningful clinical interpretation to *HCN4* variants detected by genetic testing. In this study, we show that knockdown of *hcn4* in zebrafish phenocopies human SSS. Furthermore, we show that the zebrafish assay can effectively discriminate between deleterious and benign human *HCN4* variants.

Materials and Methods

Human and animal subject research for this study was approved by the University of Utah Institutional Review Board. The human subjects gave informed consent.

Zebrafish strains and characterization of the cardiac phenotype

Wild-type (WT) or Tg (cmlc2:GFP) zebrafish (*Danio rerio*) embryos were used to functionally characterize zebrafish *hcn4* and human *HCN4* variants. Embryos were maintained in embryo water at 28°C and staged according to age and morphological criteria. Cardiac phenotypes were screened using light (WT embryos) or fluorescent (Tg cmlc2:GFP) microscopy at 36-40 hour-post-fertilization (hpf). Heart rate and rhythm were recorded. Videos obtained from the Tg embryos were analyzed using Image J to determine the frequency and duration of cardiac pauses.

Molecular biology

Human *HCN4* cDNA (original expression vector provided by Michael Sanguinetti) was subcloned into the pCS2+ expression vector. The QuikChange site-directed mutagenesis kit (Agilent Technologies) was used to introduce mutations into WT *HCN4* cDNA. The presence of mutations and absence of mutagenesis artifacts were confirmed by Sanger sequencing of selected clones at the University of Utah DNA sequencing core. Plasmids were linearized with HpaI, and cRNA was prepared using the mMessage mMachine T7 *in vitro* transcription kit (Ambion) and purified with Qiagen RNeasy kit. cRNA concentrations were measured using the NanoDrop RNA quantitation kit.

Pharmacological blockade of hcn4

Intact zebrafish embryos

ZD-7288 (Sigma) was dissolved in distilled water (stock, 10 mM). After fertilization, WT embryos were allowed to develop in standard embryo water at 28°C. At 24 hpf, embryos were transferred to 1 mM ZD-7288 solution in embryo water and incubated at 28°C for 24 hours. The heart rate of non-treated (control) and ZD-7288 treated embryos was recorded at 48 hpf.

Explanted zebrafish hearts

ZD-7288 was dissolved in external control solution containing (in mmol/L) NaCl 140, KCl 4, CaCl₂ 1.8, MgCl₂ 1, glucose 10, and HEPES 10 (pH 7.4) to a concentration of 10 μM. Hearts from WT embryos (48 hpf) were dissected from the thorax *en bloc* by using fine forceps and transferred to the recording chamber. Only spontaneously beating whole hearts were studied. All experiments were performed at 22°C to 24°C. The recording chamber was superfused with external control solution or ZD-7288 solution. Suction pipettes were made from borosilicate capillary tubes (8250 glass; A-M Systems) and fire-polished to obtain resistances of 6–9 MΩ when filled with solution containing (in mmol/L) KCl 120, EGTA 5, K₂ATP 5, MgCl₂ 5, and HEPES 10 (pH 7.2).

V_m was measured by using an AxoClamp 2A amplifier (Molecular Devices) in the bridge mode with the disrupted patch technique. The pipette was positioned adjacent to the heart and a seal was formed by application of minimal suction. Using this technique, stable spontaneous action potentials (APs) were recordable for up to 3 hours. V_m was filtered at 10 kHz and digitized at a sampling frequency of 20 kHz with a 12-bit analog-to-digital converter (Digidata 1322A Interface, Molecular Devices).

Injection of morpholinos and synthesized mRNA

Hcn4 splice-blocking morpholino (SB-MO) was designed to target the exon3-intron3 boundary of *hcn4* (5'-TTTAGACACTCACCACCATCTTGTT-3'). *Hcn4* SB-MO was injected (10 ng/embryo) alone or co-injected with wild-type (WT) or mutant *HCN4* cRNAs (300 pg/embryo) at the 1- to 2-cell stage. All SB-MO / cRNA injections were repeated at least three times.

In situ hybridization

A digoxigenin-labeled antisense RNA probe was synthesized using T3 polymerase by *in vitro* transcription of a cDNA template to *hcn4*. Whole mount *in situ* hybridization was performed using a Biolane HTI *in situ* machine (Huller and Huttner AG). Zebrafish embryos were cleared in 70% glycerol in Phosphate Buffered Saline Tween. Embryos were photographed with a Leica MZ12 stereo microscope using a Dage-MTI DC330 CCD camera.

HCN4 variant screening

DNA was isolated from the subject's peripheral blood using a Gentra Autopure LS (Qiagen, Valencia, CA) and analyzed by agarose gel electrophoresis to confirm the integrity of the DNA and quantitated using a Nanodrop. PCR primers (Table 5) were designed to amplify the exons of *HCN4* using the ExonPrimer utility in the UCSC genome browser (<http://genome.ucsc.edu/>), and used to amplify DNA from the patient. An aliquot of DNA was analyzed by agarose gel electrophoresis and then the PCR product was purified by treating with 4 μl of Exo-SAP-IT (Affymetrix) at 37°C for 2 hours and 80°C for 15 minutes. The PCR product was then submitted to the University of Utah DNA sequencing core for analysis and results compared to published sequences using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To predict deleterious effects, the genetic variants were analyzed using the algorithms SIFT [8], PolyPhen2 [9], MutationTaster [10], PROVEAN [11], FATHMM [12] and MutationAssessor [13].

Statistical analysis

pCLAMP 10 software (Molecular Devices) was used to analyze digitized data. All data are expressed as mean ± SEM (n = number of sample), and statistical significance was evaluated by a Student's *t*-test or Analysis of Variance (ANOVA) where appropriate ($P \leq 0.05$ was considered a statistically significant difference). Atrial and ventricular AP parameters, including maximum upstroke velocity (P_{max}), action potential duration (APD), maximum diastolic membrane potential (MDP), and cycle length (CL) were independently analyzed. Action potential duration from a series of five APs was calculated as the time interval between the peak maximum upstroke velocity (phase 0) and the time at 10% (APD₁₀), 50% (APD₅₀) and 90% of repolarization (APD₉₀). Values are given as mean ± SEM.

A Generalized Linear Mixed Model was performed to estimate the likelihood of a normal phenotype for the *HCN4* variant, compared to the reference normal phenotype for WT *HCN4*. A random intercept was specified in the Generalized Linear Mixed Model to account for day to day experimental variability. The response variable, cardiac pause was a binary outcome dichotomized as normal (no pause) and abnormal (with pause) phenotypes. The response variable, heart rate, was a continuous variable. The percentage of

embryos with a normal phenotype and the 95% confidence interval were calculated for each *HCN4* mutant using the binominal proportion method with the normal approximation assumption.

Results

Drug inhibition of *hcn4* in zebrafish

The zebrafish gene *hcn4* (XP_686078) encodes a protein of 1130 amino acids that by alignment appears to be the orthologue of human *HCN4*. To explore whether *hcn4* controls heart rate and cardiac automaticity in zebrafish, we analyzed the spatial and temporal distribution of *hcn4* expression during zebrafish development. We found that *hcn4* is strongly expressed in the zebrafish heart after formation of the linear heart tube (Fig. 1). Given these expression and protein alignment data we reasoned that *hcn4* is the functional orthologue of *HCN4*.

To explore the mechanism by which *hcn4* functions in zebrafish, we bathed whole embryos in 4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride (ZD-7288), a nonselective hcn blocker, and assayed the heart rate at 48 hpf. We found that embryos bathed in 1 mM ZD-7288 had significantly slower heart rates than untreated embryos (Fig. 2A). However, cardiac pauses were not observed in embryos exposed to ZD-7288.

To further characterize the drug-induced bradycardia, explanted zebrafish hearts were superfused with ZD-7288 (10 μ M) and patch-clamped to measure cardiac AP parameters. As in the intact embryos, CL was significantly prolonged in both the atrium and ventricle after the drug treatment (Table 1). Rate-dependent APD prolongation (APD₅₀ and APD₉₀)

Fig. 1. Embryonic *hcn4* expression: whole-mount in situ hybridization reveals *hcn4* expression in the heart (arrow) at (A) 18 somites, (B) 24 hpf and (C) 48 hpf.

Fig. 2. Effects of pharmacological blockade of hcn channels on *in vivo* and *ex vivo* cardiac activity. (A) The nonselective hcn blocker ZD-7288 slows heart rate in intact embryonic zebrafish. Embryos were bathed with 1 mM ZD-7288 (n = 10 paired experiments). (B and C) Histograms of diastolic intervals obtained from current clamp recordings of explanted embryonic hearts before (CTRL) and after perfusion with 10 μ M ZD-7288 (ZD). The diastolic interval is a surrogate for the rate of phase 4 diastolic depolarization. For both atrial and ventricular recordings, ZD prolonged the diastolic intervals, consistent with slowing of phase 4 depolarization rate.

Fig. 3. Effects of *hcn4* MO-knockdown on heart rate and cardiac pauses. (A) *hcn4* MO-knockdown slowed sinus heart rate in intact embryos at 36–48 hpf (n = 209), *p < 0.0001. (B) *hcn4* MO-injected embryos displayed sinus pauses that were never detected in control embryos. (C) Continuous recordings of cardiac contraction in *cmcl2*-GFP control embryos (left) and embryos injected with *hcn4* MO. Embryos injected with *hcn4* MO displayed bradycardia and prolonged pauses, reminiscent of patients with SSS.

was observed in the ventricles after ZD-7288 treatment, but the rate-dependent phenomenon was not present in the atrium. Unlike the intact embryos, cardiac pauses were observed in the explanted zebrafish heart superfused with ZD-7288.

Diastolic intervals were tabulated from the patch-clamp recordings. Treatment with ZD-7288 resulted in prolongation of diastolic intervals in both the atrium and ventricle as demonstrated by the histograms in Fig. 2B and 2C. The atrial diastolic intervals were 441 ± 20 ms and 1033 ± 130 ms for control and ZD treatment, respectively (mean $\pm$ SEM; N = 97 and 211, respectively; p < 0.005; ANOVA). Ventricular diastolic intervals were 366 ± 11 ms and 1387 ± 142 ms for control and ZD treatment, respectively (N = 143 and 158, respectively; p < 0.005; ANOVA). Taking the diastolic interval as a surrogate for phase 4 diastolic depolarization, the AP phase known to be controlled by *HCN4*, these results support the notion that a member of the *hcn* family of ion channels plays a role in controlling zebrafish heart rate.

Morpholino hcn4 knock-down produces bradyarrhythmias

To determine whether *hcn4* is one of the ion channels responsible for heart rate control in zebrafish, we performed targeted *hcn4* knock-down experiments with splice-blocking antisense morpholino oligonucleotide. The overall appearance of *hcn4* knock-down morphants was normal (data not shown). Zebrafish cardiac phenotypes, including heart rate and cardiac rhythm, were assessed between 36 and 40 hpf. The phenotype observed in zebrafish *hcn4* morphants was strikingly similar to that observed in humans SSS, and included bradycardia and prolonged sinus pauses,

Table 1. Atrial (A) and ventricular (B) action potential parameters in response to ZD-7288, measured in explanted embryonic zebrafish heart. CL: cycle length (msec); MDP: maximum diastolic potential (mV); Pmax: maximum upstroke velocity (mV/msec); AP10, AP50 or AP90: action potential duration to 10%, 50% or 90% of repolarization (msec). Values are mean $\pm$ SEM

A			
Atrium	Uninjected (n=4)	Hcn4 morph. (n=4)	P-value
CL	723.3 $\pm$ 53.7	1845 $\pm$ 443.3	0.04
MDP	-63.4 $\pm$ 1.9	-62.2 $\pm$ 2.4	0.80
Pmax	4.6 $\pm$ 0.4	6.8 $\pm$ 0.8	0.07
APD ₁₀	57.7 $\pm$ 4.2	60.6 $\pm$ 11.5	0.82
APD ₅₀	103.2 $\pm$ 6.8	110.5 $\pm$ 15.6	0.68
APD ₉₀	135.1 $\pm$ 6.3	156.5 $\pm$ 14.5	0.22
B			
Ventricle	Uninjected (n=4)	Hcn4 morph. (n=4)	P-value
CL	877.4 $\pm$ 53.2	1291 $\pm$ 154.6	0.04
MDP	-65.6 $\pm$ 2.1	-66.5 $\pm$ 1.8	0.75
Pmax	6.7 $\pm$ 0.6	5.2 $\pm$ 1.5	0.39
APD ₁₀	207.3 $\pm$ 20.8	232.4 $\pm$ 28.8	0.50
APD ₅₀	359.2 $\pm$ 23.4	383.8 $\pm$ 33.6	0.57
APD ₉₀	401.1 $\pm$ 24.4	429.4 $\pm$ 30.8	0.49

Table 2. Atrial (A) and ventricular (B) action potential parameters of uninjected and *hcn4* morphant explanted embryonic heart

A			
Atrium	Control (n=5)	ZD-7288 (n=5)	P-value
CL	724.6 $\pm$ 115.6	1481 $\pm$ 248.1	<0.01
MDP	-71.0 $\pm$ 2.5	-70.7 $\pm$ 3.5	0.85
Pmax	11.9 $\pm$ 3.0	7.5 $\pm$ 1.6	0.06
APD ₁₀	60.8 $\pm$ 9.3	61.5 $\pm$ 5.9	0.89
APD ₅₀	101.5 $\pm$ 13.0	109.1 $\pm$ 12.7	0.37
APD ₉₀	127.2 $\pm$ 17.4	150.8 $\pm$ 15.1	0.14
B			
Ventricle	Control (n=5)	ZD-7288 (n=5)	P-value
CL	786.2 $\pm$ 115.9	1504 $\pm$ 163.9	0.01
MDP	-69.8 $\pm$ 0.9	-70.3 $\pm$ 1.1	0.53
Pmax	5.2 $\pm$ 4.1	4.6 $\pm$ 2.9	0.30
APD ₁₀	256.6 $\pm$ 30.2	298.6 $\pm$ 36.3	0.02
APD ₅₀	532.6 $\pm$ 60.2	624.2 $\pm$ 66.4	<0.01
APD ₉₀	598.2 $\pm$ 115.9	713.6 $\pm$ 75.7	<0.01

Fig. 4. Specificity of *hcn4* MO. Retention of intron 3 with injection of *hcn4* splice-blocking morpholino. RNA from uninjected and morphant embryos was analyzed by RT-PCR. Primers designed to amplify the exon3-exon4 boundary produced the expected PCR product in uninjected embryos (lane 2). The PCR product is too large to amplify in morphants due to inclusion of intron 3 (lane 3). To prove inclusion of intron 3 in morphant *hcn4* mRNA, primers were designed to amplify the exon3-intron3 and intron3-exon4 boundaries. Products of the expected size were found in morphants (lanes 4 and 5, respectively).

the latter of which are thought to be the cause of syncope and sudden death in SSS. The morphant heart rate ($n = 209$) was significantly slower than in uninjected ($n = 57$) embryos (88 vs 137 beats/minute; Fig. 3A). In addition, there were intermittent periods of asystole (pauses) lasting several seconds in 74% of morphants (Fig. 3B and 3C). Such pauses were never observed in uninjected embryos. In the morphants, there were 3.6 ± 0.3 pauses/minute and the average pause duration was 2.3 ± 0.4 seconds.

Current-clamp experiments of explanted hearts revealed a significant difference in CL between morphants and uninjected embryos, but none of the other AP parameters were significantly different. Unlike the pharmacological blockade of I_f by ZD-7288, the rate-dependent APD prolongation was not observed in morphant embryos (Table 2).

Evidence supporting morpholino specificity comes from RT-PCR analysis of *hcn4* splice-blocking MO injected embryos, in which there was retention of *hcn4* intron 3, which is predicted to cause premature translation termination (Fig. 4). As additional evidence for morpholino specificity, the heart rate and pause phenotypes were partially rescued (from 87 to 111 bpm and from 74% to 25%, respectively) with co-injection of WT human *HCN4* mRNA, suggesting that *HCN4* can compensate for the loss of the zebrafish orthologue (Fig. 5).

High-throughput HCN4 variant screen

Next, we tested *HCN4* variants using the zebrafish assay to determine the functional consequences. We selected *HCN4* variants (p.P174S, p.G480R, p.A485V, p.D553N) that have been identified in patients diagnosed with congenital SSS [8, 9, 10, 11]. The p.P174S variant is a novel mutation identified in a patient from our clinic with bradycardia and resuscitated sudden cardiac death (Table 3). Compared to the WT rescue of heart rate and pause, the degree of rescue by all four putative disease-causing variants were statistically lower than the WT (Fig. 5). For example, there was no statistical difference between pause frequency or pause duration between morphants alone (3.6 ± 0.3 pauses/minute and 2.3 ± 0.4 seconds) and morphants co-injected with the *HCN4* variant p.P174S (4.8 ± 0.9 pauses/minute and 3.9 ± 0.7 seconds), suggesting that the p.P174S variant is indeed non-functional and disease-causing.

In addition to the pathogenic variants, we also tested *HCN4* variants of unknown significance that were identified through the NHLBI Exome Sequencing Project (ESP) database (Table 4). Ideally we would have liked to test *HCN4* variants with a high probability of being non-pathogenic by being common ($MAF > 0.01$) in “unaffected” individuals and predicted benign by *in silico* programs. However, there are few common non-synonymous variants within the ESP database. Therefore, we decided to focus on variants predicted to be benign by at least 3 of 6 algorithms (SIFT [8], PolyPhen2 [9], MutationTaster [10], PROVEAN [11], FATHMM [12] and MutationAssessor [13]): p.G36E (rs143090627: MAF 0.05 in European Americans), p.T766A (rs372090359: MAF 0.0001), p.R934H (rs369485237: MAF 0.0001),

Fig. 5. An *in vivo* cardiac assay to distinguish benign and pathogenic *HCN4* variants. The effects of MO-knockdown and the degree of rescue by co-injection of human WT or mutant *HCN4* are displayed for heart rate (A) and sinus pauses (B). Fertilized embryos were injected with *hcn4* MO alone or in combination with WT or mutant *HCN4* at the 1-2 cell stage and assayed for heart rate and pauses at 36-40 hpf. R934H (n = 57) and V1195L (n = 75) variants rescued both heart rate and pauses to a similar degree as WT *HCN4*, implying that these are benign variants. G36E (n = 66) did not rescue heart rate or pauses to the same level as WT; T766A (n = 59) rescued pauses, but did not fully rescue the heart rate to the same degree as WT, suggesting that G36E and T766A may function as a hypomorphic alleles. *P < 0.05 compared to WT *HCN4* co-injection. (C) A schematic diagram of *HCN4* subunit showing position of the pathogenic (●), benign (○) and intermediate (◐) variants.

Table 3. Genotype and phenotype correlation of putative disease-causing *HCN4* variants. SCD: sudden cardiac death

HCN4 variant	Occurrence	Average heart rate	Symptom
p.P174S	Individual	51 bpm	Syncope, Resuscitated SCD
p.G480R	Single Family	48 bpm	None
p.A485V	Three Families	58 bpm	Pre-syncope, Resuscitated SCD
p.D553N	Single Family, one member indexed	39 bpm	Resuscitated SCD

and p.V1195L (rs143431889: MAF 0.0002 in African Americans). Furthermore none of these variants are found within a known functional domain. The p.R934H and p.V1195L variants rescued the bradycardia and pause phenotypes to a similar level as WT *HCN4*, suggesting they are functional, benign variants. In contrast, p.G36E only partially rescued the bradycardia and pause phenotypes, while p.T766A rescued the pause phenotype but not the heart rate phenotype. There was no clear relationship between *in silico* predictions of pathogenicity and the functional rescue for the variants of unknown significance.

Discussion

Congenital SSS is a heritable disease that is associated with sudden cardiac death. HCN channels that encode *I_f* are a major determinant of diastolic depolarization in sinus node cells and play a primary role controlling cardiac automaticity. There are currently more

Table 4. Predicted consequences of HCN4 genetic variants using *in silico* prediction tools

Genomic coordinates	Consequence	Allele frequency (ExAC)	SIFT	Polyphen2	Mutation Taster	PROVEAN	FATHMM	Mutation Assessor
15:73660092 G / A	p.Pro174Ser	0,0001276	Tolerated	Benign	Polymorphism	Neutral	Damaging	Neutral
15:73622066 C / G	p.Gly480Arg	novel	Damaging	Probably Damaging	Disease Causing	Deleterious	Damaging	High
15:73622050 G / A	p.Ala485Val	novel	Damaging	Probably Damaging	Disease Causing	Deleterious	Damaging	Low
15:73617719 C / T	p.Asp553Asn	0,00002472	Tolerated	Probably Damaging	Disease Causing	Deleterious	Damaging	Low
15:73660505 C / T	p.Gly366Glu	0,06131	Damaging	Benign	Polymorphism	Neutral	Damaging	Neutral
15:73616138 T / C	p.Thr766Ala	0,000008413	Tolerated	Benign	Polymorphism	Neutral	Tolerated	Neutral
15:73615633 C / T	p.Arg934His	0,00005591	Tolerated	Benign	Polymorphism	Neutral	Damaging	Neutral
15:73614851 C / A	p.Val1195Leu	0,00001295	Damaging	Benign	Disease Causing	Neutral	Damaging	Neutral

than 150 *HCN4* variants listed in the ESP database. Nearly all of the variants have yet to be characterized functionally. In this study, we show that pharmacological and genetic perturbation of *hcn4* channels in embryonic zebrafish phenocopied human SSS. In addition to bradycardia, pharmacological blockade of I_f and genetic knock-down of *hcn4* resulted in prolonged cardiac pauses.

Our results demonstrate that zebrafish can be used as a novel *in vivo* model to assay the pathogenicity of human *HCN4* variants. By assessing both heart rate and cardiac pauses,

all four “pathogenic” variants (p.P174S, p.A480R, p.A485V and p.D553N) were accurately identified as disease-causing. Interestingly, the p.G480R variant corresponds to a family of asymptomatic young patients with extreme bradycardia (Table 3), and given the zebrafish findings, perhaps these individuals are susceptible to periods of asystole when they are older.

In contrast to the pathogenic variants, two of the VUS (p.R934H and p.V1195L) behave as WT *HCN4*. However, the two VUS, p.G36E and p.T766A, exhibit an intermediate effect on heart rate and pause and, based on the degree of rescue, appear to be functionally hypomorphic. Patients with SSS caused by *HCN4* mutations are often bradycardic at rest, and range in clinical presentation from asymptomatic to sudden cardiac death. Since the unknown variants we tested herein are from individuals who are probably “normal” or asymptomatic, the hypomorphic *HCN4* variants might have been identified in individuals with a subclinical phenotype that is commonly referred to as “asymptomatic bradycardia.”

There are several inherent limitations to the assay we developed. First, despite being an *in vivo* cardiac system, some would argue that zebrafish is not an ideal model for human arrhythmogenic disease since it is a non-mammalian organism with a 2-chamber heart. However, the electrophysiological properties of zebrafish myocardium are more similar to human myocardium than other mammalian systems, such as mice [7, 14]. Second, while the assay is not particularly quantitative, it does provide a moderate-throughput approach to characterize whether an *HCN4* variant is pathogenic, benign or hypomorphic. Third, although it would be ideal to measure I_f in isolated embryonic zebrafish cardiomyocytes, this experiment is technically challenging because embryonic myocytes cannot survive enzymatic dispersion. We contend that the combination of pharmacological *hcn4* blockade and *hcn4* morpholino knock-down experiments is a reasonable surrogate method to assay the effect of I_f perturbation. Finally, although the assay does not provide a detailed mechanism to explain disease susceptibility, it offers a relatively efficient, cost-effective approach to obtain a functional read-out of *HCN4* variants.

Table 5. Nucleotide sequences of *HCN4* sequencing primers used in variant screening

Analyzed region	Forward primer (5' to 3')	Reverse primer (3' to 5')
Exon1_1	ACTCGGAGCGGGACTAGG	GTGTCCACCGAGGGCTG
Exon1_2	AGCAGTCACGGACACCTGC	GAAAGTTAACTCCGGTGGG
Exon2	GTCCCTCAGATCTTCTCTCC	ATTATCTGAGGTTCCCTGCC
Exon3	CCAGGGCAGGGAAAGTG	GTTCCAAGTCCACATCTCGC
Exon4	AGGGCCTTTCTTGCTAGTG	CACTGGCTGGTGGCCTG
Exon5_6	AGTTTAGCCAGGAATCCCC	CTCTGTCCCTCGGTATCTC
Exon7_1	CAGTCAGGGCCAAGGTCAG	CTCACCAATGCGGTCCAG
Exon7_2	CTGAGCGTGGACAATTCAA	GGCTTAGGCATAAAGGAGCC
Exon8_1	GGCTCCTTTATGCCTAAGCC	AGCTGATGGTGTGGGAGC
Exon8_2	CCACATCCAACAGCTGGC	AGGTGGCAGGAGCAAGG
Exon8_3	TCCCCGAGGAGGTCTCAG	TCTCCTAATCACAGTTAAACCTGAA

In the current era of medicine, integration of genetic testing into clinical practice has become the norm. However, genetic information without meaningful functional data is a major obstacle that hinders advances in science and medicine. Therefore, our cost-effective and labor-efficient tools such as this HCN4 zebrafish assay should be considered, not only as a research tool, but also as a tool to bridge the gap between gene discovery and clinical practice.

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Disclosure Statement

None.

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