

A rare *HCN4* variant with combined sinus bradycardia, left atrial dilatation, and hypertrabeculation/left ventricular noncompaction phenotype

SUPPLEMENTARY DATA

24-hour Holter ECG

We recorded the mean and minimum heart rate, as well as sinus pauses longer than 3 seconds or atrioventricular block, and correlated these parameters with symptoms. The electrocardiogram (ECG) plot was analyzed by Mortara Instrument (United States). The criteria used to define sinus dysfunction were a mean heart rate lower than 60 bpm and a minimum heart rate lower than 40 bpm in adult individuals and a resting heart rate below the fifth percentile in pediatric individuals.

Echocardiography

Echocardiography studies were performed with Epiq 7 equipment (Philips Healthcare, the Netherlands) according to ASE recommendations (references 27-29 of the main text, and see below). The echocardiographic images were analyzed by an imaging-specialized cardiologist. Left ventricular noncompaction (LVNC) was defined as present, absent, or uncertain (hypertrabeculation without meeting criteria) (references 10 and 11 of the main text). Ejection fractions were calculated using the Simpson biplane method.

Cardiac magnetic resonance

Cardiac magnetic resonance (CMR) was performed with a 1.5-T scanner (Achieva DS Nova Freave 32, Philips Healthcare) equipped with a 32-element phased-array cardiac coil using gadolinium contrast in uncertain patients or those who met LVNC criteria on transthoracic echocardiography.

The study included steady-state free precession (SSFP) end-expiratory breath-hold cine sequences to assess the cardiac morphology and function and tissue characterization by late gadolinium enhancement (LGE) following the current recommendations (reference 30 of the main text). Postprocessing was performed offline using Intelli Space Portal software (Philips Healthcare).

We diagnosed LVNC in those patients who met Petersen and/or Jacquier criteria (references 12 and 13 of the main text).

The study also included novel tissue characterization sequences: native and postcontrast T1 (modified look-locker inversion recovery [MOLLI]). Mapping postprocessing was performed using Medis suite version 2.1 software (Medis Medical Imaging Systems, the Netherlands) with times calculated by drawing the “region of interest” (ROI) in the midseptum segment in the short axis of the left ventricle. We considered normal T1 times to be those extracted from controls without heart disease performed using the same technique in our center (native T1, 988 ± 63 ms; postcontrast T1, 382 ± 25 ms).

Extracellular volume was calculated according to the standard formula by adding the hematocrit value, with the normal value considered to be $25.3\% \pm 3.5\%$ (reference 31 of the main text).

Additionally, a myocardial deformation study was performed to detect subclinical ventricular dysfunction with feature-tracking using the Medis Qstrain version 2.1.12.2 software package (Medis Medical Imaging Systems). A semiautomated tracking algorithm was applied in the cine sequences throughout the cardiac cycle and was then visually reviewed and manually adjusted if necessary. We calculated the left ventricular global longitudinal strain (GLS) from the average of 3 standard long-axis cine views and the global circumferential strain (GCS) from the 3 short-axis views. Pathological values were determined by comparison to those derived from normal individuals in the literature (reference 32 of the main text).

Stress echocardiography and cardiopulmonary exercise testing

Exercise was performed on a treadmill with the Bruce protocol. Images were acquired with Epiq 7 echocardiography equipment (Philips Healthcare) and the cardiopulmonary exercise testing was performed with Ergostik equipment and Blue Cherry diagnostic software (Geratherm Medical AG, Germany).

Echocardiographic images were acquired basally, at peak exercise, and immediately after the exercise. With these images, we analyzed global and segmental systolic function, diastolic function, contractile reserve, and the induction of pulmonary hypertension with exercise. In the cardiopulmonary exercise testing, we assessed the exercise time, chronotropic response (maximum heart rate reached, chronotropic reserve, and first-minute recovery), the presence of arrhythmias, the Respiratory Exchange Ratio (RER), peak VO_2 (and percent predicted), VO_2 at the ventilatory threshold (anaerobic threshold), and the minute ventilation/carbon dioxide production (V_E/V_{CO_2}) relationship (reference 33 of the main text). We classified the patients according to New York Heart Association (NYHA), Weber, and ventilatory classes.

Genetic testing

Sample preparation was performed using the SureSelect Target Enrichment kit (Agilent, United States) for the paired-end multiplexed sequencing method (Illumina, United States). The enrichment was performed by means of a library of customized probes (SureSelect, Agilent) for the coding regions and flanking intron areas of the selected genes. After clusters were generated using the cBot (Illumina) device, the DNA libraries were sequenced on the Illumina HiSeq 1500 platform.

Chinese hamster ovary cell transfection

Human *HCN4* (NM_005477.2) cDNA subcloned into the pEGFP-C1 plasmid (Invitrogen, United States) was kindly provided by Drs Niels Decher (Philips University of Marburg, Germany) and Nikolaj Klöcker (Heinrich Heine University Düsseldorf, Germany). The p.R375C *HCN4* variant (NP_005468.1) was introduced by using the QuikChange Site-Directed Mutagenesis kit (Stratagene, United States)

and confirmed by direct DNA sequencing (Secugen SL, Spain). Chinese hamster ovary cells were cultured as previously described (references 9, 34, and 35 of the main text) and transiently transfected with cDNA encoding WT or mutated HCN4 channels (1.6 μ g) by using FUGENE XtremeGENE (Roche Diagnostics, Switzerland) following the manufacturer's instructions. HCN4 expression was identified by fluorescence detection. In some experiments, the effects of the cotransfection of WT and p.R375C HCN4 at a 1:1 ratio (0.8 μ g each) were also determined.

Patch-clamp recordings

Currents were recorded at room temperature (21-23°C) by means of the whole-cell patch-clamp technique using Axopatch-200B patch-clamp amplifiers and PCLAMP software (Molecular Devices, United States) (references 9, 34, and 35 of the main text). Recording pipettes were pulled from 1.0-mm-outside diameter borosilicate capillary tubes (GD1, Narishige Co, Ltd, Japan) using a programmable patch micropipette puller (Model P-2000 Brown-Flaming, Sutter Instruments Co, United States) and were heat-polished with a microforge (Model MF-83, Narishige). Micropipette resistance was 3 to 5 M Ω when filled with the internal solution and immersed in the external solution. In all of the experiments, we manually compensated for the series resistance by using the series resistance compensation unit of the Axopatch amplifier, and $\geq 80\%$ compensation was achieved. The remaining access resistance after compensation and the cell capacitance were 1.7 ± 0.4 M Ω and 13.9 ± 0.7 pF ($n = 61$), respectively. Therefore, under our experimental conditions, no significant voltage errors (< 5 mV) due to series resistance were expected with the micropipettes used. Currents were filtered at half the sampling frequency and stored on the hard disk of a computer for subsequent analysis.

The external solution contained (mM): NaCl 110, KCl 30, HEPES 5, MgCl₂ 0.5, CaCl₂ 1.8, and glucose 10 (pH = 7.4 with NaOH). Recording pipettes were filled with an internal solution containing (mM): K-aspartate 80, KCl 42, KH₂PO₄ 10, MgATP 5, phosphocreatine 3, HEPES 5, and EGTA 5 (pH 7.2 with KOH).

The protocol to obtain current–voltage relationships comprised 2-second steps that were imposed in 10-mV increments from a holding potential of -40 mV to potentials ranging from -140 to $+20$ mV. Current amplitude was measured at the end of the pulse and normalized in each experiment to membrane capacitance to obtain current densities. To quantify the time course of the current activation, a monoexponential function was fitted to the activation phase of currents generated by pulses to -130 mV yielding the time constant (τ_{act}) that defines the process. To analyze the voltage-dependence of HCN4 channel activation, 2-second pulses from -40 mV to potentials ranging from -140 to $+20$ mV followed by the tail currents were recorded by applying 1-second pulses to -140 mV. The tail current amplitude was normalized to the maximum value and plotted as a function of the membrane potential of the preceding pulse to construct the activation curves. Afterward, a Boltzmann function was fitted to the data to calculate the midpoint (V_h) and the slope (k) of the curves. To determine putative effects on the ion selectivity of HCN4 channels, the reversal potential was measured at extracellular and intracellular K^+ concentrations of 30 and 142 mM, respectively. To this end, 3-second pulses were applied to -120 mV followed by a 1-second pulse to membrane potentials ranging from -50 to $+20$ mV. The reversal potential was calculated in each experiment from the intersection of the linear regression to the data with the abscissas axis.

REFERENCES

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