Characterization of the novel heterozygous SCN5A genetic variant Y739D associated with Brugada syndrome

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ABSTRACT

Genetic variants in SCN5A gene were identified in patients with various arrhythmogenic conditions including Brugada syndrome. Despite significant progress of last decades in studying the molecular mechanism of arrhythmia-associated SCN5A mutations, the understanding of relationship between genetics, electrophysiological consequences and clinical phenotype is lacking. We have found a novel genetic variant Y739D in the SCN5A-encoded sodium channel Na\textsubscript{1.5} of a male patient with Brugada syndrome (BrS). The objective of the study was to characterize the biophysical properties of Na\textsubscript{1.5}-Y739D and provide possible explanation of the phenotype observed in the patient. The WT and Y739D channels were heterologously expressed in the HEK-293T cells and the whole-cell sodium currents were recorded. Substitution Y739D reduced the sodium current density by 47 ± 2\% at −20 mV, positively shifted voltage-dependent activation, accelerated both fast and slow inactivation, and decelerated recovery from the slow inactivation. The Y739D loss-of-function phenotype likely causes the BrS manifestation. In the hNa\textsubscript{1.5} homology models, which are based on the cryo-EM structure of rat Na\textsubscript{1.5}, Y739 in the extracellular loop IIS1-S2 forms H-bonds with K1381 and E1435 and pi-cation contacts with K1397 (all in loop IIII5-P1). In contrast, Y739D accepts H-bonds from K1397 and Y1434. Substantially different contacts of Y739 and Y739D with loop IIII5-P1 would differently transmit allosteric signals from VSD-II to the fast-inactivation gate at the N-end of helix IIII5 and slow-inactivation gate at the C-end of helix IIII5. This may underlie the atomic mechanism of the Y739D channel dysfunction.

1. Introduction

Cardiac voltage-gated sodium channels (Na\textsubscript{1.5}) are responsible for the rapid inflow of sodium ions to cardiomyocytes during phase 0 of the action potential [1]. Genetic variants in the SCN5A gene, which encodes the pore-forming alpha subunit Na\textsubscript{1.5}, have been reported in patients with various arrhythmogenic disorders, including congenital long QT syndrome (LQT3), Brugada syndrome (BrS1), progressive cardiac conduction disease (PCCD), arrhythmogenic right ventricular cardiomyopathy (ARVC) and dilated cardiomyopathy (DCM) [1–3].

BrS is a rare congenital arrhythmic disorder with clinical manifestations varying from asymptomatic to sudden cardiac death [4]. BrS-associated genes include genes encoding sodium and calcium channels, as well as proteins affecting channel kinetics and trafficking [5]. It was shown that patients carrying SCN5A mutations have more pronounced epicardial electrical abnormalities and a more aggressive clinical presentation [6]. Moreover, the use of general anesthesia (single-bolus propofol and volatile anaesthetics) during ablation did not affect the prognostic value [7].

According to the ClinVar database, more than 900 missence genetic
variants in SCN5A gene are associated with BrS, and most of them (more than 600) is classified as the variants of unknown significance. Less than 20% of known mutations were explored in heterologously expression system or in the models with endogeneous expression of mutant SCN5A forms such as iPSC, obtained from SCN5A variants carriers. The majority of biophysically characterized SCN5A genetic variants found in BrS patients demonstrated loss-of-function phenotype via various mechanisms. These include decreased surface density of the channels, altered voltage- and time-dependent activation/inactivation, and decelerated recovery from inactivation \footnote{8}. The alpha-subunit of Na\textsubscript{v}1.5 contains four homologous repeats (I-IV). Each repeat has six transmembrane alpha-helical segments connected by extracellular and intracellular loops \footnote{9}. Segments S1-S4 in each repeat form a voltage-sensing domain (VSD), while helices S5 and S6 contribute to the pore domain. Here we report a novel genetic variant Y739D-Na\textsubscript{v}1.5 found in a male patient with BrS. The objective of the study was to explore the biophysical consequences of new variant and suggest possible mechanisms, explaining our results. We generated SCN5A-Y739D, heterologously expressed it in the HEK293T expression system, and found that the genetic variant substantially changed biophysical characteristics of the Y739D vs. the WT channel. We observed reduction of sodium current density, depolarizing shift of steady-state activation, enhanced fast and slow inactivation, and decelerated recovery from the slow inactivation. A highly conserved tyrosine Y739 is located in the VSD-II extracellular loop IIS1-S2. We have built homology models of the hNa\textsubscript{v}1.5-WT and hNa\textsubscript{v}1.5-Y739D channels, compared intersegment contacts of Y739 and Y739D, and suggested possible mechanisms by which the genetic variant may affect activation and inactivation gating.

2. Results

2.1. Patient’s phenotypic characterization and identification of the genetic variant

A 4-year-old male patient was diagnosed with Brugada syndrome based on typical ECG pattern manifesting during the fever episode – 38.4 °C during 4 h (Fig. 1A, B) and syncopy anamnesis. No familial history of sudden cardiac death was registered in the family and none of the siblings revealed similar ECG-pattern or syncopies. Genetic analysis using a panel of 108 genes associated with cardiac inherited disorders revealed a novel SCN5A genetic variant Y739D (chr3: 38639267:A\textsuperscript{>}C NM_001160161.2:, c.2215T\textsuperscript{>}G, p.Y739D) located in the extracellular loop IIS1-S2. None of the relatives carried the identified variant, thus it was considered to be \textit{de novo} \footnote{Fig. 1C and D}. This variant is not reported in gnomAD database, ClinVar or any other publicly available data bases, and was classified as pathogenic/damaging by most of the in silico prediction tools such as Mutation Tester, Mutation assessor, Provean, FIFT and LRT.

This variant is not reported in gnomAD database, ClinVar or any other databases. Based on ACMG guideline the variant was classified a variant of unknown significance. The variant was not identified neither in the parental nor in siblings, thus, being the most probably \textit{de novo}.

2.2. Biophysical characterization of the Na\textsubscript{v}1.5-Y739D channel expressed in HEK293T cells

To elucidate the functional characteristics of the Y739D genetic variant we generated a plasmid vector with the corresponding substitution, expressed the SCN5A-WT or SCN5A-Y739D channels in the HEK293T cells, and recorded sodium currents (Fig. 2A, B). Mutant Y739D exhibited a typical sodium current, resembling that for the WT channel (Fig. 2C). The Y739D channel Na\textsubscript{v}1.5 density measured at –20 mV from the holding potential of –100 mV was significantly smaller than that in WT (Fig. 2D, Table 1). We also observed in the genetic variant a mild depolarizing shift of the activation voltage-dependence and increased slope factor (Fig. 2E, Table 1). Thus, Y739D channels activation requires more depolarizing potentials compare with that for WT channels.

To explore the impact of substitution Y739D on sodium channel function, we accessed the voltage-dependence of steady-state inactivation. We observed a negative shift of steady-state inactivation with no alterations in the slope factors (Fig. 3A, Table 1). However, this protocol did not allow us to distinguish whether the fast inactivation, slow inactivation or both were affected by Y739D. Therefore, we performed additional experiments and observed enhanced voltage-dependencies of

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig1.png}
\caption{A. ECG of the patient at normal temperature. B. ECG of the patient at fever. Fever lead to marked ST segment elevation. C. The pedigree of the proband. Parents and sister of the proband were unaffected. D. Results of Sanger sequencing of proband and his family.}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Variant & Description & Implication \\
\hline
Y739D & Pathogenic & \textit{de novo} \footnote{Fig. 1C and D} \\
\hline
\end{tabular}
\caption{Summary of genetic variant classification}
\end{table}
both fast and slow inactivation (Fig. 3B and C, Table 1). Next, we analyzed the time courses of recovery from inactivation, fast inactivation and slow inactivation (Fig. 3D–F). The Y739D variant did not affect recovery from inactivation and fast inactivation, but impaired recovery from slow inactivation (Table 1).

We also found a significant change of the time constant of intermediate inactivation (Fig. 4A, Table 1), but no alterations in the onset kinetics of slow inactivation (Fig. 4B). We further analyzed activity-dependent loss of channel availability at various frequencies. A very small difference was found between WT and Y739D at the 5 Hz 100th pulse (WT: 94.6 ± 1.9%, n = 10; Y739D: 88.8 ± 3.0%, n = 14, p = 0.135) (Fig. 4C). In contrast, the Y739D I_{Na} evoked at the 10 Hz 100th pulse was significantly lower than that of WT (WT: 94.9 ± 1.7%, n = 8; Y739D: 77.5 ± 7.1%, n = 6, p = 0.008) (Fig. 4D). The normalized residual current levels recorded at the 0.66 Hz 30th pulse were 99.0 ± 1.3% for WT and 97 ± 1.4% for Y739D (p = 0.26) (Fig. 4E). The normalized residual current levels recorded at the 1.92 Hz 100th pulse were 44 ± 4.2% (n = 8) for WT and 27 ± 2.7% for Y739D (n = 6) (Fig. 4E).

2.3. Contacts of Y739 and Y739D in the rNav1.4-based models of hNav1.5

In the cryo-EM structure of the rNa\textsubscript{v}1.5 channel (10), Y739 (hNa\textsubscript{v}1.5 numbers) forms several contacts with the large extracellular loop IIIS5-P1. These include H-bonds with salt-bridged K1381 and E1435, as well as pi-cation interaction with K1397, which does not form other strong contacts (Fig. 5 A). Similar contacts are seen in the Monte Carlo-minimized model of hNa\textsubscript{v}1.5. In the Monte Carlo-minimized model of hNa\textsubscript{v}1.5\_Y739D, aspartate D739 accepted an H-bond from Y1434 and formed a salt bridge with K1397 (Fig. 5 B). Thus, substitution Y739D significantly changed contacts between extracellular loops in VSD-II (IIS1-S2) and the pore domain (IIIS5-P1).

3. Discussion

Brugada syndrome is a rare inherited cardiac disorder. According to the recent meta-analysis the worldwide pooled prevalence of BrS is 0.5 per 1000 [11]. The hallmark of BrS is the ST-segment elevation in the right precordial leads V1-V3 and right bundle branch block [4]. BrS is responsible for 20% of all sudden cardiac death cases without structural myocardial abnormalities [12]. This syndrome is associated with genetic variants in proteins, which control ionic balance in cardiomyocytes. These proteins are encoded by SCN5A, GPD1L, CACNA1C, KCNE3, SCN3B and other genes [4]. The majority of genetic variants observed in patients with BrS are detected in gene SCN5A. Despite intense studies of biophysical mechanisms of arrhythmias, still less than a half of genetic variants described in patients with BrS are functionally characterized.

Here we reported a novel SCN5A genetic variant in a male patient with BrS. According to American College of Medical Genetics guidelines this variant is classified as variant of unknown significance. Sodium current in HEK293T cells with expressed Na\textsubscript{v}1.5\_Y739D had several abnormalities: decreased peak current density, impaired activation and enhanced inactivation. In particular, variant Y739D negatively shifted steady-state inactivation and fast steady-state inactivation. It also accelerated intermediate inactivation and slow inactivation. If mutated
channel develops a slow inactivation at the time scale of fast inactivation of the WT channel, the shift of steady-state fast inactivation will be observed [13]. Therefore, we consider the slow inactivation enhancement as the major mechanism of Y739D-dependent development of BrS. Finally, we observed a slower recovery of Na<sub>1.5</sub>-Y739D from the slow inactivation and significant decrease in activity-dependent loss of channel availability at certain frequencies. Thus, the decreased peak current density and changes in the Y739D channel gating can be due to altered Na<sub>1.5</sub> kinetics rather than abnormalities in the channel trafficking. In summary, Na<sub>1.5</sub>-Y739D demonstrated loss-of-function phenotype, which is consistent with our understanding of the BrS1 molecular mechanisms.

In the cryo-EM structure of rNa<sub>1.5</sub>, Y739 (hNav1.5 numbers) forms H-bonds with K1381 and E1435 and p-ion contacts with K1397 (Fig. 5A). The latter also donates an H-bond to the backbone carboxyl of G1358 at the C-end of IIIS5 (not shown for clarity). Very similar contacts between loops IIIS1-S2 and IIIS5-P1 are seen in the rNa<sub>1.5</sub>-based model of hNa<sub>1.5</sub>. In the hNa<sub>1.5</sub>-Y739D model, Y739D accepts H-bonds from K1397 and Y1434 (Fig. 5B), while H-bond of K1397 with G1358 is lost. Several different contacts of Y739 and Y739D with loop III-S5-P1 would differentially transmit allosteric signals from VSD-II to the fast and slow inactivation gates, respectively.

The first direction is through IIIS5 towards the fast-inactivation tripeptide IFM (Fig. 5B), whose phenylalanine forms tight contacts with V1323 and A1326 at the N-end of IIIS5. The second direction is through helix IIIP1 towards K1419 in the selectivity-filter region (Fig. 5B), where H-bond of K1397 with G1358 is lost. Very similar contacts involve G1319V [20]. Previously, we reported genetic variant A1294G in the extracellular loop IIIS3-S4, which was found in our patient with combined phenotype, and found that the mutations enhanced slow inactivation [21]. Here we demonstrated that the IIIS1-S2 loop is also involved in the process of slow inactivation.

In conclusion, here we have found a new SCN5A genetic variant Y739D in a patient with BrS1 and demonstrated that the loss-of-function of Na<sub>1.5</sub>-Y739D expressed in HEK293T cells is due to accelerated slow and fast inactivation. In morphology models of the WT and mutated channels, extracellular loops IIIS1-IIIS2 in VSD-II and IIIS5-P1 in the pore domain have substantially different contacts. The models suggest that helices IIIS5 and IIIP1 are involved in the allosteric signal transduction from VSD-II to the fast and slow inactivation gates, respectively.

4. Methods

4.1. Clinical data

The study was performed according to the Declaration of Helsinki. Approval was obtained from the Almazov National Medical Research Centre Ethical Committee. Written consent was obtained from the patient prior to investigation.

4.2. Genetic analysis, mutagenesis and heterologous expression

Target sequencing was performed on Illumina MiSeq using Haloplex enrichment kit with a panel of 108 genes associated with inherited cardiac disorders as previously described [22]. All disease-related genetic variants were confirmed by Sanger sequencing and classified according to American College of Medical Genetics guidelines [23].

The pcDNA3.1 vector with WT hNa<sub>1.5</sub> and GFP (hH1-pcDNA3.1) was kindly provided by Prof. Hugues Abriel (Institute of Biochemistry and Molecular Medicine, University of Bern, Bern, Switzerland). Site-directed mutagenesis was performed by the PCR amplification according to standard mutagenesis protocol with overlapped primers (TGTTGTGCTGCTCCAGCGCCATGAAGAGTGT; TGGAGCACGACAAATAGCAAGAAGTGT; TGGAGCACGACAAATAGCAAGAAGTGT). The study was performed according to the Declaration of Helsinki.

4.3. Electrophysiology

Sodium current (I<sub>Na</sub>) was recorded using patch-clamp method (whole-cell configuration). All measurements were performed at room temperature. The extracellular solution contained (mmol/L): 140 NaCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, 10 Glucose (pH 7.4 CO<sub>2</sub>). The intracellular solution contained (mmol/L): 130 CsCl, 10 NaCl, 10 EGTA, 10 HEPES (pH 7.3 CsOH). Microelectrodes were manufactured from the borosilicate glass using a puller (P-1000, Sutter Instrument). The electrode resistance varied from 1.8 to 2.5 MΩ. The series resistance was compensated at 75–80%. Data acquisition was performed using...
amplifier Axopatch 200B (Molecular devices). Currents were acquired at 20–50 kHz and low-pass filtered at 5 kHz using an analog-to-digital interface (Digidata 1440A acquisition system, Molecular devices). At least 3 independent transfections were used for electrophysiological recordings. \( N \) means number of cells.

4.4. Data analysis

The holding potential was –100 mV. Current-voltage (I-V) curves were recorded using protocol with depolarizing voltage steps from –80 to 60 mV during 40 ms in 5 mV steps at 1 Hz frequency. Peak \( I_{\text{Na}} \) at each voltage (V) was measured and corresponding conductance (G) was calculated by using equation: \( G = I_{\text{Na}}/(V-V_{\text{rev}}) \). Normalized conductance was plotted against voltage and G-V curves reflecting voltage-dependence of steady-state activation. These data were fitted to the Boltzmann function: \( G/G_{\text{max}} = 1/(1+\exp((V_{1/2} - V)/k)) \), where \( G_{\text{max}} \) is the maximal sodium conductance, \( V_{1/2} \) is the potential of half-maximal activation, and \( k \) is the slope factor. Current densities at each test potential were assessed by dividing \( I_{\text{Na}} \) by the cell capacitance.

Voltage-dependency of steady-state inactivation was obtained by

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**Fig. 3.** A. The voltage-dependence of steady-state inactivation. Solid lines show least-square fits to the Boltzmann function for WT and Y739D. B. The voltage-dependence of steady-state fast inactivation. Y739D channels demonstrated enhanced slow inactivation vs. WT (\( I_{\text{Na}}/I_{\text{Na, max}} \) at +20 mV: WT: 0.48 ± 0.03, \( N = 12 \); Y739D: 0.33 ± 0.03, \( N = 15 \), \( p = 0.005 \)). D. The time course of recovery from inactivation for WT (black circles, \( N = 24 \)) and Y739D (black triangles, \( N = 9 \)). E. The time course of recovery from fast inactivation for WT (black circles, \( N = 13 \)) and Y739D (black triangles, \( N = 10 \)). F. The time course of recovery from slow inactivation for WT (black circles, \( N = 13 \)) and Y739D (black triangles, \( N = 10 \)).
Fig. 4. A. The time course of onset of intermediate inactivation. Solid lines show least-squares fits to the mono-exponential function. B. Onset of the slow inactivation for WT (circles, N = 16) and Y739D (triangles, N = 10). Slow inactivation of the Y739D channel remained unchanged vs. WT (p = 0.44 for 45s). C. The normalized residual current levels at 5 Hz for WT (circles) and Y739D (triangles). D. The normalized residual current levels at 10 Hz for WT (circles) and Y739D (triangles). E. The normalized residual current levels recorded at 0.66 Hz for WT (open circles) and Y739D (open triangles) and at 1.92 Hz for WT (filled circles) and Y739D (filled triangles).
using two-step protocol with 500 ms prepulse varying from −120 to 0 mV with 5 mV increments and testing 20 ms step to −15 mV. Normalized testing $I_{\text{Na}}$ was plotted against the prepulse voltage. Steady-state inactivation curves were fitted with the Boltzmann function. Voltage-dependency of fast inactivation was evaluated in similar way, but duration of the varying prepulse was 20 ms. Voltage-dependency of steady-state slow inactivation data were recorded using 10 s prepulse followed by a 20 ms hyperpolarization to −100 mV to allow recovery from fast inactivation.

The time course of recovery from inactivation was obtained using two-step protocol. First conditioning pulse lasts 500 ms at −15 mV followed by a recovering hyperpolarization gap at −100 mV, which duration varying from 1 to 3000 ms (recovery time) and 20 ms test pulse at −15 mV. $I_{\text{Na}}$ recorded from the test pulse was normalized to that from the first conditioning pulse and plotted against the recovery time. Curves were fitted with the double-exponential function:

$$y = A_{\text{fast}} \times (1 - \exp(-t/\tau_{\text{fast}})) + A_{\text{slow}} \times (1 - \exp(-t/\tau_{\text{slow}}))$$

where $A_{\text{fast}}$ and $A_{\text{slow}}$ are fractions of the fast and slow inactivating components, respectively, and $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ are their time constants. The time course of recovery from fast and intermediate inactivation were accessed using the same protocol, but with 20 ms and 1s prepulse duration, respectively.

The development of intermediate inactivation was evaluated using double-pulse protocol. First step at −15 mV with duration from 1 to 3000 ms followed by 20 ms of hyperpolarization gap at −100 mV and second test step at −15 mV. $I_{\text{Na}}$ elicited from the testing step was normalized to that from the first step and plotted against duration of the first step. Curves were fitted with the mono-exponential function: $y = y_0 + A \times \exp(-t/\tau)$, where $\tau$ is the time constant. The development of slow inactivation was assessed using double-pulse protocol with varying duration (1–45 s) of the first step to −40 mV (P1) and testing step of 40 ms to −15 mV. $I_{\text{Na}}$ elicited from the second test pulse at −15 mV was normalized to that from the first conditioning step and plotted against the first step duration.

Activity-dependent loss of channel availability was measured by applying repetitive depolarizing pulses to −15 mV at different frequencies (50 ms pulses at 5 Hz, 25 ms pulses at 10 Hz and 500 ms pulses at 0.66 Hz and 1.92 Hz). $I_{\text{Na}}$ evoked by each pulse was normalized to the current induced by the first pulse.

4.5. Statistical analysis

All data are expressed as the mean values and standard errors (SEM). Statistical comparisons were made using the unpaired Mann-Whitney
test with $p < 0.05$ considered to be statistically significant. In some Figures, the standard error bars are smaller than the data symbols.

### 4.6. Homology modeling

Cryo-EM structure of the rat Na$_{\text{v}}$1.5 channel [10] and ZMM program [24] were used to build homology models of hNa$_{\text{v}}$1.5 and hNa$_{\text{v}}$1.4 Y739D. The models were Monte Carlo-minimized as described elsewhere [25]. In the Cryo-EM template many side chains are not resolved. Upon Monte Carlo minimization all residues adopted energetically optimal conformations, while the mainchain conformations remained practically the same as in the template.

### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

Data will be made available on request.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101249.

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