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Identification of rare variants in cardiac sodium channel β4−subunit gene *SCN4B* associated with ventricular tachycardia

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Abstract

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Ventricular tachycardia (VT) causes sudden cardiac death, however, the majority of risk genes for VT remain unknown. *SCN4B* encodes a β-subunit, $Na_v\beta4$, for the voltage-gated cardiac sodium channel complex involved in generation and conduction of the cardiac action potential. We hypothesized that genomic variants in *SCN4B* increase the risk of VT. We used high-resolution melt analysis followed by Sanger sequencing to screen 199 VT patients to identify nonsynonymous variants in *SCN4B*. Two nonsynonymous heterozygous variants in *SCN4B* were identified in VT patients, including p.Gly8Ser in four VT patients and p.Ala145Ser in one VT patient. Case–control association studies were used to assess the association between variant p.Gly8Ser and VT in two independent populations for VT (299 VT cases vs. 981 controls in population 1 and 270 VT patients vs. 639 controls in population 2). Significant association was identified between p.Gly8Ser and VT in population 1 ($P = 1.21 \times 10^{-4}$, odds ratio or OR = 11.04), and the finding was confirmed in population 2 ($P = 0.03$, OR = 3.62). The association remained highly significant in the combined population ($P = 3.09 \times 10^{-5}$, OR = 6.17). Significant association was also identified between p.Gly8Ser and idiopathic VT ($P = 1.89 \times 10^{-5}$, OR = 7.27). Functional analysis with Western blotting showed that both p.Gly8Ser and p.Ala145Ser variants significantly reduced the expression level of $Na_v\beta4$. Based on 2015 ACMG Standards and Guidelines, p.Gly8Ser and p.Ala145Ser can be classified as the pathogenic and likely pathogenic variant, respectively. Our data suggest that *SCN4B* is a susceptibility gene for common VT and idiopathic VT and link rare *SCN4B* variants with large effects (OR = 6.17–7.27) to common VT.

Keywords

Single nucleotide polymorphism (SNP); Ventricular tachycardia (VT); Cardiac sodium channel β4 subunit (*SCN4B*, $Na_v\beta4$); *SCN5A*/$Na_v1.5$; Genetics

Introduction

Cardiac arrhythmias, particularly ventricular tachycardia (VT), cause numerous sudden death each year (Israel 2014). Genetic analyses of families with inherited VT (e.g., long QT syndrome [LQTS], Brugada syndrome [BrS], and cardiac conduction disease) have identified disease-causing mutations in multiple ion channel genes and regulatory genes for ion channels (Wang et al. 1995a, b, 1996, 1997, 1998; Chen et al. 1998). For example, mutations in the cardiac sodium channel gene *SCN5A* (encoding $Na_v1.5$) were found to cause LQTS and BrS, which are associated with VT and sudden death (Wang et al. 1995a, b, 1997, 1998; Chen et al. 1998). However, these mutations account for a small portion of common VT cases, and the genetic basis for common VT is largely undetermined.

Recent genome-wide association studies (GWAS) have started to unravel novel genetic variants associated with common VT. Bezzina et al. performed a GWAS for ventricular fibrillation (VF) associated with acute myocardial infarction (MI) (Bezzina et al. 2010). A single nucleotide polymorphism (SNP) (rs2824292) on chromosome 21q21 and close to *CXADR* was found to be associated with risk of VF associated with MI (Bezzina et al. 2010). Chambers et al. performed a GWAS for electrocardiographic parameters with a discovery population of 6542 Indian Asians and two replication populations of 6243 Indian Asians and 5370 Europeans, and identified SNP rs6795970 in the *SCN10A* gene associated

with prolonged P wave, PR interval, and QRS interval (Chambers et al. 2010). The same SNP was shown to be associated with a lower risk of VF (Chambers et al. 2010). On the other hand, a recent large GWAS for sudden cardiac arrest (SCA) with 3939 cases and 25,989 controls failed to identify any variant associated with SCA at the genome-wide significance level (Ashar et al. 2018). Therefore, much more work is needed to elucidate the genetic architecture of SCA and/or VT.

One major limitation of GWAS is the identification of associations with mostly common variants (minor allele frequency or MAF > 5%) (Xu et al. 2014). Moreover, the identified common variants usually explain only a small portion of heritability (Xu et al. 2014). As such, a major portion of the missing heritability for VT remains unknown. One attractive hypothesis is the “common disease, rare variants” hypothesis, which suggests that rare variants with MAF of < 1% or low frequency variants with MAF between 1 and 5% may explain a large portion of the missing heritability (Bodmer and Bonilla 2008). Rare variants may be a major component of VT and identification of such rare variants will substantially further our understanding of the molecular mechanisms of VT. The voltage-gated cardiac sodium channel $Na_v1.5$ is responsible for initiation and propagation of the cardiac action potentials (Catterall 2000). The cardiac sodium channel complex consists of the main pore-forming α-subunit $Na_v1.5$ and one or more auxiliary β-subunits, including β1–vβ4 encoded by *SCN1B–SCN4B*, respectively (Grant 2009). Previously studies identified one functional variant of *SCN4B* (p.Lys179Phe) in a patient with LQTS (Medeiros-Domingo et al. 2007) and another functional variant, p.Ser206Lys, in a patient with sudden infant death (SCID) (Tan et al. 2010). Because LQTS and SID are associated with VT, we performed mutation screening of *SCN4B* in 199 patients with regular or common VT. We identified two rare genomic variants in the *SCN4B* gene and showed that one of the variants, p.Gly8Ser, confers a large, significant risk of VT.

Materials and methods

Study subjects and isolation of human genomic DNA

The study subjects involved in this study were selected from the GeneID database, which is one of the largest databases for cardiovascular diseases, including coronary artery disease (CAD), AF, VT/VF, hypertension, and heart failure (Shi et al. 2009; Li et al. 2011; Wang et al. 2011, 2018). All study subjects belong to the ethnic origin of Han by self-description. To minimize the stratification from geological area differences, the cases and controls for each study population were selected from study subjects recruited from the same region. Both cases and controls for VT Cohort 1 were recruited from hospitals at Wuhan, whereas both cases and controls for VT Cohort 2 were recruited from hospitals at Beijing. This study was approved by the ethics committee on human subject research at Huazhong University of Science and Technology and local hospitals. All study subjects signed written informed consent. VT was diagnosed based on the data from ECG, Holter ECG recordings, and electrophysiological studies, and on a history of the disease from the medical record (premature ventricular contractions (PVCs), non-sustained ventricular tachycardia, sustained ventricular tachycardia or VF). The idiopathic VT (IVT) was defined as VT without structural heart disease, CAD, cardiomyopathies, valvular disease, hypertension, heart

failure, and rheumatic heart disease. Controls were subjects with normal ECG and without VT/VF, atrial fibrillation (AF), superventricular tachycardia, cardiac conduction disease, valvular heart disease, cerebral infarction and cardiomyopathies as demonstrated by ECG, Holter monitoring, echocardiography, magnetic resonance imaging, and/or X-ray computed tomography (CT). The detailed information for our VT case–control study populations was described previously (Chen et al. 2015; Li et al. 2017).

Human genomic DNA was isolated from peripheral blood samples using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA).

Mutational analysis

Mutational analysis was carried out for all five exons and exon–intron boundaries of *SCN4B* using DNA samples from 199 VT patients. All exons and exon–intron boundaries were amplified using polymerase chain reactions (PCR) and subjected to high-resolution melt analysis (HRM) as described (Wang et al. 2010, 2016). The primers used to PCR and HRM analyses were designed using the primer-3 web site and listed in Supplementary Table S1. HRM analysis was carried out using a Rotor-gene 6200 System (Corbett Life Science, Concorde, NSW, Australia) according to the protocols from the manufacturer (Wang et al. 2010, 2016). The samples showing abnormal HRM peaks were subjected to Sanger sequencing to identify the exact sequence changes. The primers used for DNA sequencing are listed in Supplementary Table S2. DNA sequencing was performed using BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) by Shanghai Sangni Company. Sequencing data were directly viewed using sequencing analysis 5.1.1 (Applied Biosystems) to identify potential variants.

Exons 1 and 3 of *SCN4B*, in which nonsynonymous variants p.Gly8Ser and p.Ala145Ser were identified, were analyzed by HRM analysis in control subjects as described (Wang et al. 2010, 2016). Samples with abnormal HRM peaks were analyzed by direct DNA sequence analysis.

Construction of expression plasmids for *SCN4B* and *SCN4B* variants

The cDNA for human cardiac sodium channel gene *SCN5A* was cloned into vector pcDNA3 (pcDNA3-$Na_v1.5$) as described (Dumaine et al. 1996; Chen et al. 1998; Huang et al. 2016). Human *SCN4B* cDNA was purchased from Origene (pCMV6-XL4-*SCN4B*, NM_174934). The *SCN4B* cDNA was amplified from pCMV6-XL4-*SCN4B* by PCR, digested with restriction enzymes *EcorR 1* and *Hind III*, and sub-cloned into the eukaryotic expression vector pcDNA3.1(−) (Clontech, MountainView, CA), resulting in pcDNA3.1-*SCN4B*-WT-FLAG, an expression construct for wild-type (WT) $Na_v\beta4$ tagged with FLAG at the C-terminus. *SCN4B* variants p.Gly8Ser and p.Ala145Ser were introduced into pcDNA3.1-*SCN4B*-WT-FLAG by PCR-based-mediated mutagenesis, resulting in expression constructs for mutant *SCN4B*, pcDNA3.1-*SCN4B*-G8S-FLAG and pcDNA3.1-*SCN4B*-A145S-FLAG. All constructs were sequenced to ensure that no errors in sequences were created during PCR.

Cell culture and transfection

HEK293 cells and a HEK293 cell line with stable and constant expression of $Na_v1.5$ (HEK/$Na_v1.5$) (Wu et al. 2008) were used. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B at 37 °C under 10% CO_2. Cultured cells were co-transfected with pcDNA3-$Na_v1.5$ (0.8 μg) and a WT or mutant *SCN4B* expression plasmid (0.8 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty-eight hours later, the cells were used for further studies.

The cardiac sodium current I_{Na} was recorded and analyzed in the whole-cell configuration using the patch clamp technique and a Multipath 700B amplifier (Axon Instruments, Sunnyvale, CA) as described previously (Wan et al. 2000; Wu et al. 2008; Chakrabarti et al. 2013; Huang et al. 2016). The pipette solution contained 20 mM NaCl, 150 mM CsCl, 10 mM HEPES, and 10 mM EGTA (pH 7.2). The bath solution contained 70 mM NaCl, 80 mM CsCl, 5.4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose (pH 7.3). Currents were elicited using 100 ms step pulses in different voltages from a holding potential of − 120 mV. I_{Na} was recorded at room temperature. Currents were filtered at 5 kHz with a four-pole Bessel filter and sampled at 50 kHz. The series resistances recorded in the whole-cell configuration were compensated (80%) to minimize voltage errors. The membrane potential dependence of activation, inactivation and the recovery rate from inactivation were measured as described by us previously (Wan et al. 2000; Wu et al. 2008; Chakrabarti et al. 2013; Huang et al. 2016). The late I_{Na} was recorded as described by us (Dumaine et al. 1996; Chakrabarti et al. 2013; Huang et al. 2016).

Western blot analysis

HEK293 cells were cultured following standard protocols and transfected using Lipofectamine®2000 as described above (Life Technologies). After 48 h of transfection, the cells were washed twice with cold phosphate-buffered saline and lysed in NP40 lysis buffer (1% v/v Nonidet P-40, 10% v/v glycerol, 137 mM NaCl, 20 mM Tris–HCl, pH 7.4, and 1 × protease inhibitor cocktail from Roche). Cell lysates were centrifuged at 13,800×*g* for 10 min at 4 °C. The protein extracts in the supernatant were boiled for 5 min in the loading buffer with 2% SDS, resolved in a 10% SDS polyacrylamide gel, and transferred to a PVDF membrane.

The membrane was blocked with 5% non-fat dry milk in TBST (20 mM Tric-HCl, 150 mM NaCl, 0.05% Tween-20) for 2 h at room temperature and incubated with the primary antibody overnight at 4 °C. An anti-FLAG antibody (1:5000 dilution) (Proteintech, China) was used to recognize FLAG-tagged $Na_v\beta4$, whereas an anti-integrin α5 antibody (1:500 dilution) (Proteintech, China) and anti-Na^+−K^+ ATPase antibody (1:1000 dilution) (Cell Signaling, USA) were used as loading control (Proteintech, China). After three times of washing for 5 min in TBST, the membrane was incubated with a secondary anti-mouse antibody or anti-rabbit antibody for 2 h at room temperature. After three times of washing with TBST, the membrane was developed with a SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, Illinois, USA). The protein signal was imaged

using a ChemiDoc XRS (Bio-Rad Laboratories, Richmond, CA), and analyzed using Quantity One software (Bio-Rad Laboratories, Richmond, CA).

Analysis of cardiac sodium channels on plasma membranes

Isolation of plasma membranes was performed as described by us previously (Wu et al. 2008; Chakrabarti et al. 2013; Huang et al. 2016). In brief, cells were cultured and transfected with pcDNA3.1-*SCN4B*-WT-FLAG, pcDNA3.1-*SCN4B*-G8S-FLAG or pcDNA3.1-*SCN4B*-A145S-FLAG. After 48 h of transfection, the cells were resuspended in osmotic lysis buffer (25 mM Tris–HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, and 1× protease inhibitor cocktail from Roche). The cells were then homogenized by sonication for 2 min, and centrifuged at 2000×*g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 100,000×*g* for 1 h. The pellet containing membrane protein fractions was resuspended in NP40 lysis buffer. The membrane protein fraction was then used for Western blot analysis with an anti-$Na_v1.5$ antibody (Alomone) as described above.

Bioinformatic analysis

The I-Mutant2.0 program was used for predicting the effect of genomic variants on protein stability (Capriotti et al. 2005). The GPS2.1 program was used to predict potentially kinase-specific phosphorylation sites in $Na_v\beta4$ (Xue et al. 2011). To increase the prediction accuracy, a high threshold was selected for each kinase group in GPS (http://gps.biocuckoo.org/) (Xue et al. 2011). Each variant was also analyzed for their potential effect on the gene or gene product using eight different computational programs, including SIFT (http://provean.jcvi.org/seq_submit.php), PROVEAN (http://provean.jcvi.org/seq_submit.php), PolyPhen (http://genetics.bwh.harvard.edu/pph2/index.shtml), Mutation-Assessor (http://doro.charite.de/MutationTaster/index.html), FATHMM (http://fathmm.biocompute.org.uk/), Fathmm-MKL (http://fathmm.biocompute.org.uk/fathmmMKL.htm), MetaSVM (Kim et al. 2017), MetaLR (Dormuth et al. 2016), and CADD (https://cadd.gs.washington.edu/score).

Statistical analysis

The allelic association and genotypic association under three different models (dominant, recessive or additive) were analyzed between *SCN4B* variant p.Gly8Ser and VT using a Fisher's exact test or 2 × 2 or 2 × 3 contingence table Chi-square tests implemented in SPSS version 17.0 (Shen et al. 2007, 2008; Li et al. 2011; Wang et al. 2011, 2018; Bai et al. 2014; Chen et al. 2015; Huang et al. 2015). Multivariate logistic regression analysis was used to adjust for covariates of age and sex using SPSS v17.0 (Shen et al. 2007, 2008; Li et al. 2011; Wang et al. 2011, 2018; Bai et al. 2014; Chen et al. 2015; Huang et al. 2015). Hardy–Weinberg equilibrium tests in the control groups were performed using PLINK (version 1.06) (Shen et al. 2007, 2008; Li et al. 2011; Wang et al. 2011, 2018; Bai et al. 2014; Chen et al. 2015; Huang et al. 2015).

The electrophysiological data are shown as mean ± standard error of the mean. A student's *t* test was performed using SigmaPlot (Jandel Scientific Software, San Rafael, CA, USA). Differences were considered significant when the *P* value was < 0.05.

Results

Identification of nonsynonymous variants p.Gly8Ser and p.Ala145Ser in *SCN4B* in a VT patient population

We used HRM analysis followed by direct DNA sequence analysis to screen genomic variants in *SCN4B* (NM_174934) in 199 VT patients. All five exons and exon–intron boundaries of *SCN4B* were analyzed. The identified variants are listed in Table 1. Four heterozygous variants were identified, including p.Gly8Ser in exon 1, p.C58C in exon 2, p.Ala145Ser in exon 3, and 694A > G in the 3′-untranslated region in exon 5. Only two nonsynonymous heterozygous variants (p.Gly8Ser and p.Ala145Ser) were pursued further. HRM patterns and sequencing chromatograms for variants p.Gly8Ser and p.Ala145Ser are shown in Fig. 1a. Variant p.Gly8Ser was localized in the signal peptide sequence of $Na_V\beta4$ and occurred at a highly conserved residue across species during evolution (Fig. 1b, c). Variant p.Gly8Ser was identified in 4 of 199 VT patients (a minor allele frequency or MAF of 2%), which was higher than its frequency in the gnomAD database (0.47% for the Eastern Asian population, https://gnomad.broadinstitute.org/variant/11-118023367-C-T), the ExAC database (0.55% for the Eastern Asian population, http://exac.broadinstitute.org/variant/11-118023367-C-T) or the 1000Genomes database (0.30% for the East Asian population, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?ss=ss1343163830) (Table 2). Bioinformatic analysis with six programs suggests that the p.Gly8Ser variant is damaging or possible damaging, whereas three programs suggest that it is a neutral change (Table 2). The clinical and demographic data for the four carriers are shown in Supplementary Table S3. Two carriers, #10406 and #10024, were also diagnosed with CAD (Supplementary Table S3). Carrier 20,562 was diagnosed with an atrial septal defect, valvular disease and LQTS with a prolonged QTc of 0.497 s (Supplementary Table S3). Carrier 23,916 was diagnosed also with AF, abnormal glucose tolerance, lacunar infarction (Supplementary Table S3).

Variant p.Ala145Ser was localized to the extracellular region of $Na_V\beta4$ and occurred at a highly conserved residue among different species (Fig. 1b, c). Variant p.Ala145Ser was identified in only one patient (#76011), but not in either the gnomAD or ExAC database (Table 2). Bioinformatic analysis with nine programs all suggests that the p.Ala145Ser variant is damaging or possible damaging (Table 2). Patient 76011 was a 16-year-old female affected with VT without known family history. Her ECG parameters include a HR of 69 bpm, a PR interval of 0.166 s, a QRS interval of 0.08 s, a QT interval of 0.422 s, and QTc of 0.44 s (Supplementary Table S3). Echocardiography did not detect any abnormalities (Supplementary Table S3). This mutation was absent in 990 control Chinese Han individuals.

Significant allelic association between *SCN4B* variant p.Gly8Ser and VT in a Chinese VT population

The p.Gly8Ser variant is a rare variant with a MAF of about 2%, which makes case–control association studies possible to determine whether it confers a risk of VT. The first case–control cohort for VT included 299 cases, which included the 199 VT patients used for initial mutation screening, and 981 controls from the GeneID database (Table 3). The frequency of the A allele of SNP p.Gly8Ser was 1.84% in the VT group and 0.20% in

controls (Table 4). The genotyping data for p.Gly8Ser were in Hardy–Wein-berg equilibrium in controls ($P = 1.00$) (Table 4). Significant allelic association was detected between p.Gly8Ser and VT with an observed P_{obs} value of 5.58×10^{-5} and an OR of 9.17 (Table 4). After adjusting for covariates of age and gender, the association remained significant ($P_{adj} = 1.21 \times 10^{-4}$, OR = 11.04) (Table 4).

The novel finding of association between p.Gly8Ser and VT in cohort 1 was replicated in the second case–control cohort with 270 VT patients and 639 controls. Significant allelic association was detected between p.Gly8Ser and VT with an observed P_{obs} of 4.47×10^{-3} and an OR of 4.31 (Table 4). After adjusting for age and gender, the association remained significant ($P_{adj} = 0.033$, OR = 3.62) (Table 4).

In the combined population with both cohort 1 and cohort 2, the association between p.Gly8Ser and VT became more significant ($P_{adj} = 3.09 \times 10^{-5}$, OR = 6.17) (Table 4). Moreover, it is interesting to note that highly significant association was detected also between p.Gly8Ser and idiopathic VT (IVT) without structural heart disease and other cardiovascular diseases ($P_{adj} = 1.89 \times 10^{-5}$, OR = 7.27) (Table 4).

In addition, we also performed association analysis using the minor allele frequency (MAF) of p.Gly8Ser for the East Asian population in the public ExAC database (MAF = 0.55%, OR = 3.38, $P = 3.77 \times 10^{-4}$), gnomAD (MAF = 0.47%, OR = 3.63, $P = 5.80 \times 10^{-5}$), and 1000 Genomes (MAF = 0.30%, OR = 6.28, $P = 1.28 \times 10^{-4}$), indicating a significant association with VT.

Significant genotypic association between *SCN4B* variant p.Gly8Ser and VT in a Chinese VT population

We also analyzed the genotypic association between *SCN4B* variant p.Gly8Ser and VT. Significant genotypic association was detected between *SCN4B* variant p.Gly8Ser and VT in cohort 1 under a dominant model ($P_{adj} = 1.14 \times 10^{-4}$) (Table 5). Similar findings were observed in cohort 2 under the dominant model ($P_{adj} = 0.032$) (Table 5). In the combined population, the association between p.Gly8Ser and VT became more significant under the dominant model ($P_{adj} = 2.84 \times 10^{-5}$) (Table 5).

SCN4B variants p.Gly8Ser and p.Ala145Ser significantly reduce the expression level of the $Na_v\beta4$ protein

We used Western blot analysis to assess whether the two *SCN4B* variants affect the expression level of $Na_v\beta4$ on plasma membranes. The expression levels of mutant $Na_v\beta4$ with either p.Gly8Ser or p.Ala145Ser in membranes were significantly reduced compared with WT $Na_v\beta4$ (Fig. 2a, b). These data suggest that both p.Gly8Ser and p.Ala145Ser variants significantly reduce the expression level of $Na_v\beta4$ in membranes (Fig. 2). Moreover, the expression levels of mutant $Na_v\beta4$ with p.Gly8Ser or p.Ala145Ser in total protein extracts were also significantly reduced compared to WT $Na_v\beta4$ (Fig. 2a, c). These data suggest that variants p.Gly8Ser and p.Ala145Ser affect the stability of $Na_v\beta4$. We used I-Mutant2.0 program (Capriotti et al. 2005) to predict the effects of p.Gly8Ser and p.Ala145Ser variants on stability of $Na_v\beta4$. Changes of G8 to S or A145 to S decreased the

free energy value (DDG) of $Na_v\beta4$ by 0.81 and 0.75 kcal/mol, respectively (Supplementary Fig. 1). These data support the conclusion from Western blot analysis.

We also assessed the potential effect of variant p.Gly8Ser or p.Ala145Ser on the expression level of $Na_v1.5$. Western blot analysis showed that neither variant p.Gly8Ser nor p.Ala145Ser significantly affected the expression level of $Na_v1.5$ in membranes (Fig. 2a, d) or in total cellular protein extracts (Fig. 2a, e). Patch-clamping recordings showed that neither variant p.Gly8Ser nor p.Ala145Ser significantly affected the cardiac sodium current densities, voltage-dependent activation, inactivation, recovery from inactivation (Fig. 3 and Table 6), or late sodium currents (Fig. 4). These data suggest that *SCN4B* variants p.Gly8Ser and p.Ala145Ser do not exert their effects via $Na_v1.5$.

Discussion

In this study, we performed mutation screening for all exons and exon–intron boundaries of *SCN4B*. We identified one rare nonsynonymous heterozygous variant, p.Gly8Ser, in *SCN4B*. We showed that SNP p.Gly8Ser was significantly associated with a risk of VT in two independent populations (OR = 11.04, $P = 1.21 \times 10^{-4}$ in the first population; OR = 3.62, $P = 0.03$ in the second population and in the combined population (OR = 6.17, $P = 3.09 \times 10^{-5}$) (Table 4). The p.Gly8Ser variant was also significantly associated with idiopathic VT with an OR of 7.27 ($P = 1.89 \times 10^{-5}$) (Table 4). The high ORs suggest that the p.Gly8Ser variant has a large effect on VT. The p.Gly8Ser variant occurs at a highly conserved amino acid residue from different species (Fig. 1) and bioinformatic analysis predicted that the p.Gly8Ser variant is a damaging variant. Our functional studies demonstrated that the p.Gly8Ser variant significantly reduced the stability and expression level of $Na_v\beta4$ (Fig. 2). These results suggest that *SCN4B* is a new susceptibility gene for VT and support the hypothesis that rare variants are a significant component of common VT.

We also identified one novel non-conservative missense heterozygous variant, p.Ala145Ser, in *SCN4B* in a patient with VT. The p.Ala145Ser variant was not found in 990 controls in the Chinese Han population. Due to lack of family members, we could not perform co-segregation analysis to determine whether the p.Ala145Ser variant causes VT, but our functional studies showed that the p.Ala145Ser variant significantly reduced the stability and expression level of $Na_v\beta4$ (Fig. 2). By bioinformatics analysis with the GPS 2.1 software (Xue et al. 2011), we found that the A145S mutation creates a potentially new phosphorylation site for ROCK1/2, which was associated with protein degradation (Bauer et al. 2009). Therefore, p.Ala145Ser may lead to phosphorylation of $Na_v\beta4$ by ROCK1/2, leading to increased turnover of $Na_v\beta4$. Moreover, the p.Ala145Ser variant occurs at a highly conserved amino acid residue from different species (Fig. 1) and bioinformatic analysis predicted that the p.Gly8Ser variant is a damaging variant. These data suggest that the p.Ala145Ser variant is likely to be a causative variant to VT.

We analyzed p.Gly8Ser and p.Ala145Ser variants for their pathogenicity using the ACMG 2015 Standards and Guidelines (Richards et al. 2015). Variant p.Gly8Ser can be classified as a pathogenic variant with two strong PS1–PS4 evidence. Evidence of pathogenicity include PS3 (functional studies supportive of a damaging effect on the gene product), PS4

(significantly higher prevalence in the VT population than in controls), and PP3 (support of a deleterious effect by multiple computational programs). Variant p.Ala145Ser can be classified as a likely pathogenic variant with one strong (PS1-PS4) evidence and one moderate (PP1-PP6) evidence. Evidence of pathogenicity include PS3 (functional studies supportive of a damaging effect on the gene product) and PP3 (support of a deleterious effect by multiple computational programs). Our functional characterization of *SCN4B* variants p.Gly8Ser and p.Ala145Ser suggest that the two variants significantly reduce the expression level and stability of Navβ4 (Fig. 2). We assessed the effects of *SCN4B* variants p.Gly8Ser and p.Ala145Ser on cardiac sodium channel $Na_v1.5$, but found that neither variant affected the expression levels of total $Na_v1.5$ or plasma membrane $Na_v1.5$ (Fig. 2) nor the densities, activation, inactivation, and recovery from inactivation of the cardiac sodium current (Fig. 3 and Table 6). Therefore, it is unlikely that variants p.Gly8Ser and p.Ala145Ser increase risk of VT by affecting $Na_v1.5$ or sodium current. Nevertheless, we found that the two genomic variants have significant effect on the expression level of *SCN4B*. This conclusion was repetitively confirmed by many independent experiments. Therefore, we conclude that *SCN4B* variants are potential causes of VT via regulation of the *SCN4B* expression levels. On the other hand, *SCN4B* variants p.Gly8Ser and p.Ala145Ser may increase risk of VT by affecting other ionic currents. Although $Na_vβ4$ was not reported to affect other ionic currents to date, its homologue, $Na_vβ1$, was found to modulate the biophysical properties of K_v1 and K_v7 channels in an isoform-specific manner (Nguyen et al. 2012). It also increased the density and modified the gating of $K_v4.3$ current (Deschenes and Tomaselli 2002). Furthermore, in addition to modulating ion channels, $Na_vβ4$ is a cell adhesion molecule (Shimizu et al. 2017), which may interact with and affect the functions of other proteins. Future cell aggregation assays may be used to characterize potential effects of *SCN4B* variants on cell adhesion. Gap junctions are essential for the coordinated depolarization of cardiac muscle, and cell adhesion proteins, such as *N*-cadherin and catenins, regulate the stability of the gap junction protein connexins (Kostetskii et al. 2005; Li et al. 2005). Loss of *N*-cadherin in the heart leads to the loss of gap junctions from the cardiac intercalated disks that was associated with both spontaneous and inducible cardiac arrhythmias (Kostetskii et al. 2005; Li et al. 2005). Therefore, *SCN4B* variants p.Gly8Ser and p.Ala145Ser may increase risk of VT by impairing the function of $Na_vβ4$ in stabilization of gap junctions to maintain the electrical coupling.

Medeiros-Domingo et al. (2007) reported that overexpression of wild-type *SCN4B* did not affect the amplitude of the cardiac sodium current when co-expressed with *SCN5A*, however, it significantly increased the slope factors of both activation and inactivation, shifted the inactivation curve to a more negative potential by 3.84 mV, and reduced slow recovery from inactivation. Tan et al. (2010) later showed that overexpression of wild-type *SCN4B* did not affect the amplitude, activation or recovery from inactivation of the cardiac sodium current, but significantly shifted the inactivation curve to a more negative potential by 6.00 mV. In our study (Table 6), we did not observe any significant effect for overexpression of wild-type *SCN4B* on the amplitude, activation, inactivation or recovery from inactivation of the cardiac sodium current. For current inactivation, *SCN4B* over-expression shifted to the inactivation curve by − 0.93 mV, but the difference did not reach a

significant level. Different experimental conditions, cell types or expression constructs may account for some of the differences.

Previous studies identified two heterozygous variants in *SCN4B* in patients with LQTS (p.Lys179Phe) (Medeiros-Domingo et al. 2007) and sudden infant death (p.Ser206Lys) (Tan et al. 2010), both of which generated persistent late I_{Na} and caused a positive shift of inactivation. However, the two *SCN4B* variants identified in this study, p.Gly8Ser and p.Ala145Ser, did not generate any late persistent I_{Na} (Fig. 4). One of the carriers with variant p.Gly8Ser (#20562) showed prolonged QTc of 0.497 s (Supplementary Table S3), which may be a coincidental finding or related to valvular disease, atrial septal defects or other unknown mechanism in the patient. Moreover, two other heterozygous *SCN4B* variants, p.Val162Gly and p.Ile166Leu, were identified in patients with AF (Li et al. 2013), but the functional effects of these variants were not studied. One of the carriers with variant p.Gly8Ser (#23196) was also affected with AF (Supplementary Table S3). We recently showed that variant p.Gly8Ser was significantly associated risk of AF, although the ORs were lower than VT in this study (Xiong et al. 2019). Altogether, identification and functional characterization of two *SCN4B* variants associated with common VT, p.Gly8Ser and p.Ala145Ser, in this study substantially expand the phenotypic spectrum associated with variants in *SCN4B*.

The frequency of the p.Gly8Ser variant appeared to be population specific and was much higher in the East Asian populations, ranging from 0.30% (1000Genomes) to 0.47% (gnomAD) and 0.55% (ExAC), compared with that in the South Asian populations (0% in 1000Genomes, 0.03% in ExAC, and 0.03% in gnomAD) or the non-Finnish European populations (0% in 1000Genomes, 0.0016% in ExAC, and 0.0008% in gnomAD). Although the cases and controls from each case–control population (Table 3) were from the same recruitment site, a precaution may be taken with the interpretation of the data from the genetic association analysis because we cannot fully exclude the potential effect of hidden population structure or genetic drift.

In conclusion, we identified two novel variants in *SCN4B*, including a rare SNP p.Gly8Ser and a private p.Ala145Ser variant in patients with VT. Population-based case–control association studies using two independent VT populations demonstrated that the p.Gly8Ser variant conferred a significant risk of common VT and idiopathic VT with particular large effect (high ORs). Functionally, both p.Gly8Ser and p.Ala145Ser variants significantly reduced the stability and expression level of $Na_v\beta4$. The results identify *SCN4B* as a new susceptibility gene for VT and suggest that *SCN4B* variants increase risk of VT by reducing the expression level of $Na_v\beta4$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

VT	Ventricular tachycardia
SNP	Single nucleotide polymorphism
LQTS	Long QT syndrome
BrS	Brugada syndrome
GWAS	Genome-wide association studies
MI	Myocardial infarction
VF	Ventricular fibrillation
ECG	Electrocardiogram
AF	Atrial fibrillation
CT	Computed tomography
PCR	Polymerase chain reaction
HRM	High resolution melt analysis
OR	Odds ratio

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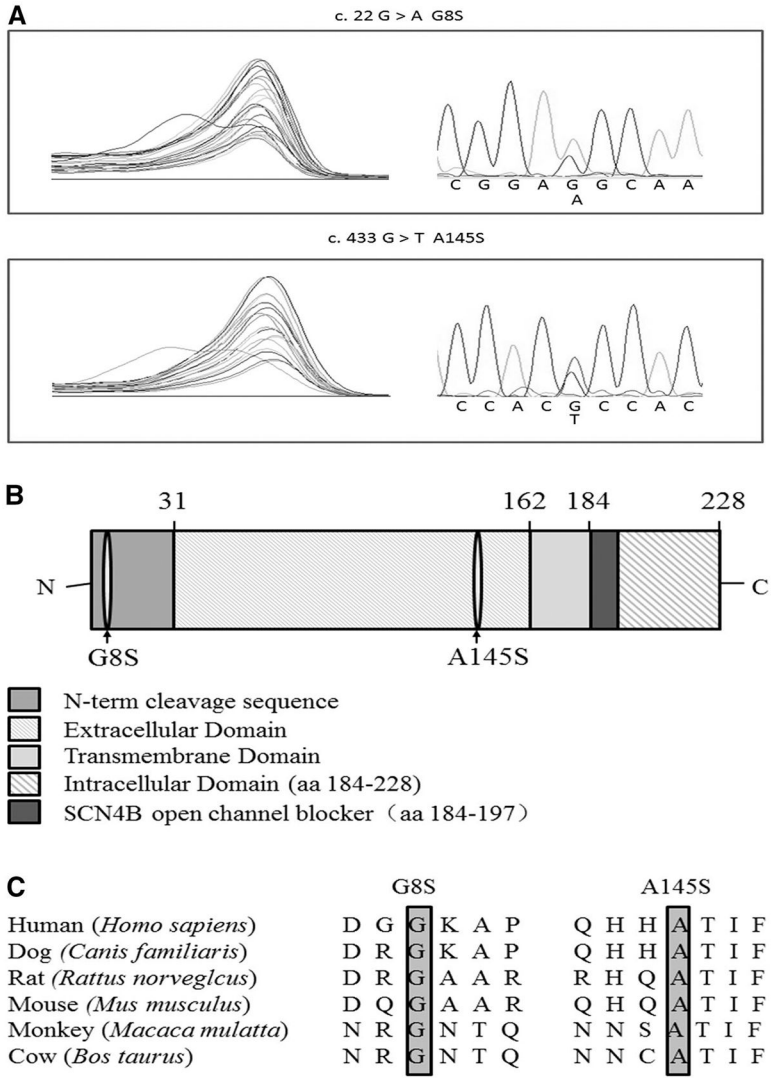

Fig. 1.

Identification of novel genomic variants p.Gly8Ser and p.Ala145Ser in *SCN4B* in VT patients. **a** Abnormal HRM patterns identified for the p.Gly8Ser variant in exon 1 and p.Ala145Ser in exon 3 of *SCN4B* (left), and sequence analyses of the two variants. **b** A schematic diagram showing functional domains of $Na_v\beta4$ and location of variants p.Gly8Ser and p.Ala145Ser. **c** Variants G8S and A145S involve residues that are highly conserved across different species during evolution

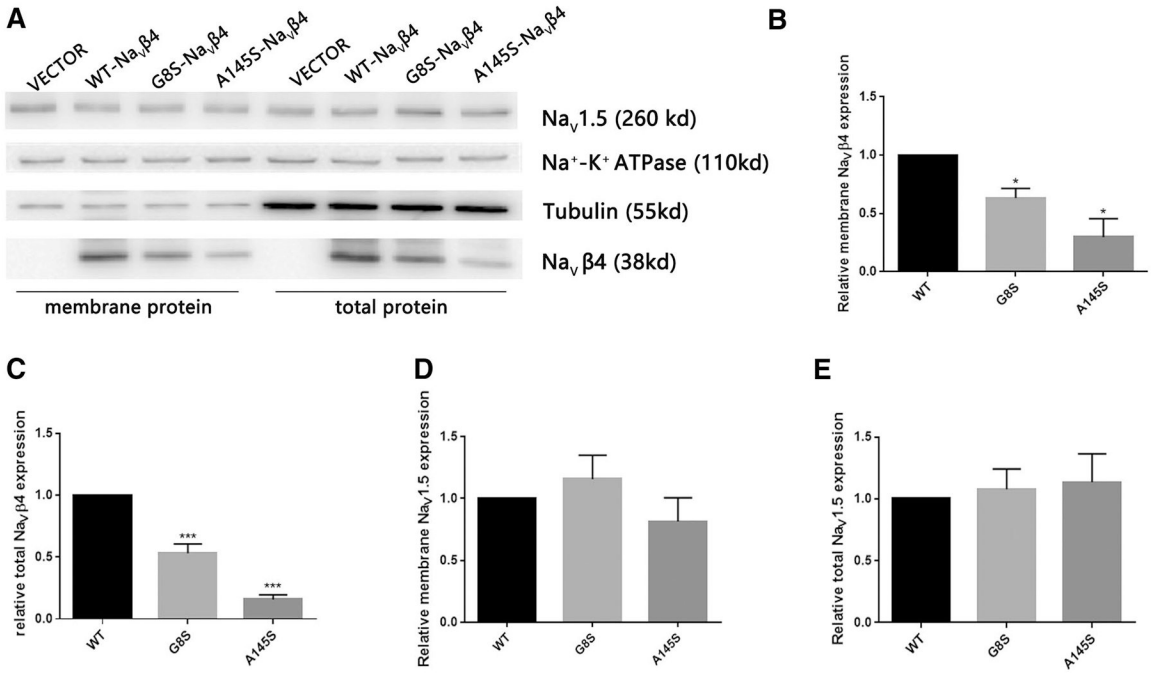

Fig. 2.

SCN4B variants p.Gly8Ser and p.Ala145Ser reduce the expression levels of total Na$_V$β4 and membrane Na$_V$β4. **a** Western blot analysis detecting FLAG-tagged Na$_V$β4 and Na$_V$1.5 in membranes protein extracts and total protein extracts. Plasma membrane protein Na^+-K^+ ATPase was used as loading control for membrane fractions and tubulin was used as loading control for total protein extracts. **b–e** Data from four independent Western blot analyses as in (**a**) were quantified and graphed. The data were quantified using Quantity One software and graphed by origin8.5. $*P < 0.05$; $***P < 0.001$ ($n = 3$)

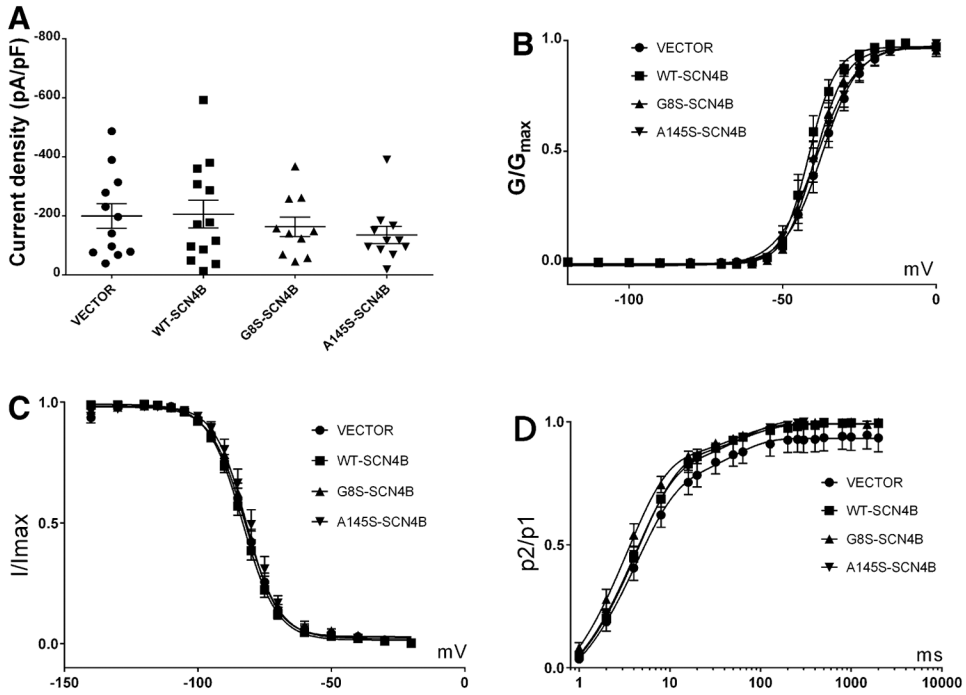

Fig. 3.

Electrophysiological characterization of *SCN4B* variants p.G8S and p.A145S for their effects on cardiac sodium currents recorded from HEK293 cells stably expressing $Na_v1.5$ with wild type (WT) or mutant $Na_v\beta4$. Cells transfected with the empty vector was used as control. **a** Peak sodium current densities at − 25 mV recorded from $Na_v1.5$ stable HEK293 cells transfected with WT $Na_v\beta4$, mutant $Na_v\beta4$ with G8S, and mutant $Na_v\beta4$ with A145S. **b** Steady-state voltage dependence of activation curves. **c** Steady-state voltage dependence of inactivation curves. **d** Steady-state time dependence of recovery from inactivation

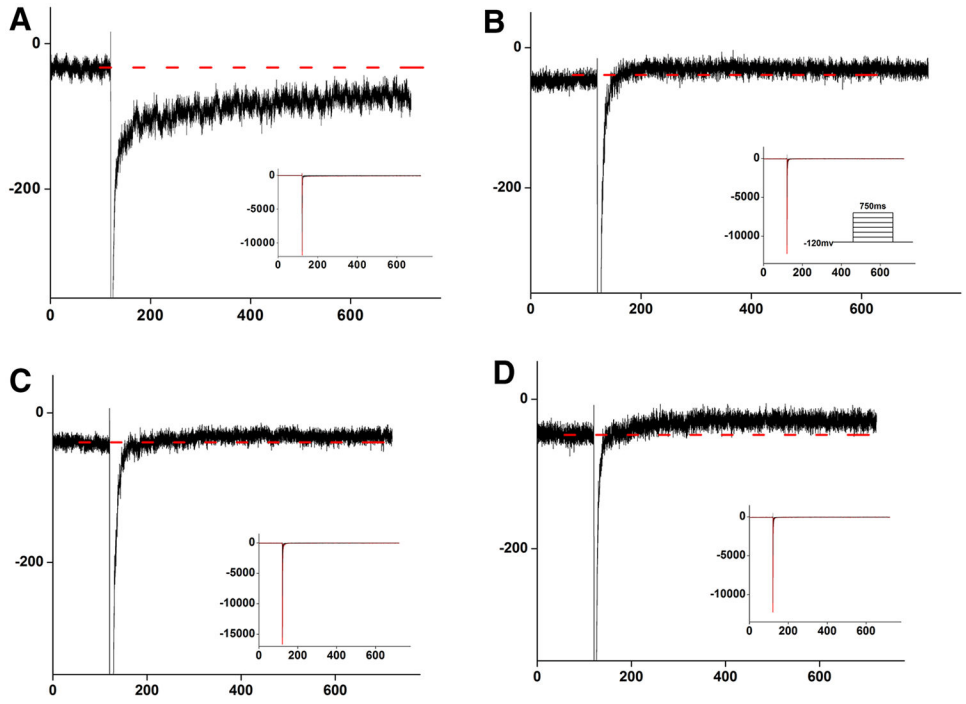

Fig. 4.

Analysis of late sodium current I_{Na} from HEK293 cells with coexpression of $Na_v1.5$ with WT $Na_v\beta4$, mutant $Na_v\beta4$ with G8S, or mutant $Na_v\beta4$ with A145S. **a** Late I_{Na} from HEK293 cells with expression of mutant $Na_v1.5$ with the ΔKPQ mutation associated with LQTS, which is known to generate late I_{Na} (positive control). **b** Late I_{Na} from HEK293 cells with coexpression of wild-type $Na_v1.5$ and WT $Na_v\beta4$. **c** Late I_{Na} from HEK293 cells with coexpression of wild type $Na_v1.5$ and mutant $Na_v\beta4$ with p.G8S. **d** Late I_{Na} from HEK293 cells with coexpression of wild type $Na_v1.5$ and mutant $Na_v\beta4$ with p.A145S. No significant differences were found between wild-type $Na_v\beta4$ and mutant $Na_v\beta4$ with p.G8S or p.A145S

Table 1

List of *SCN4B* variants identified by HRM analysis in patients with VT

Variant	Exon	Variant (nt)[a]	Variant (aa)	Mutation type	Number of carriers	DbSNP ID#
1	1	22 G > A	**Gly8Ser**	Nonsynonymous	4	rs149868494
2	2	174 A > G	Cys58Cys	Synonymous	5	rs45539032
3	3	433 G > T	**Ala145Ser**	Nonsynonymous	1	–
4	5	694 A > G	–	3'-UTR	1	rs79071006

nt nucleotide, *aa* amino acid

[a]Variant denoted at the nucleotide level

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Table 2

Bioinformatic analyses of *SCN4B* variants identified in this study

Variant	P.Gly8Ser	P.Ala145Ser
SNP ID	rs149868494	NA
Associated disease	VT	VT
Frequency in 1000 Genomes	0.30%	0
Frequency in gnomAD (east Asia)	0.47%	0
Frequency in ExAC (east Asia)	0.55%	0
SIFT_pred	T	D
PolyPhen	P	P
MutationAssessor_pred	L	M
FATHMM_pred	D	D
PROVEAN_pred	N	D
fathmm-MKL_coding_pred	D	D
MetaSVM_pred	D	D
MetaLR_pred	D	D
CADD	P	P

Data analysis was performed using VEP (Variant Effector Predictor; https://useast.ensembl.org/info/docs/tools/vep/index.html)

SNP single nucleotide polymorphism, *gnomAD* the genome aggregation database, *ExAC* the exome aggregation consortium (ExAC) database, *SIFT: D* deleterious, *T* tolerated, *Polyphen: D* probably damaging, *P* possibly damaging, *B* benign, *MutationAssessor: H* high, *M* medium, *L* low, *N* neutral, *FATHMM: D* deleterious, *T* tolerated, *PROVEAN: D* deleterious, *N* neutral, *fathmm-MKL: D* deleterious, *T* tolerated, *MetaSVM: D* deleterious, *T* tolerated, *MetaLR: D* deleterious, *T* tolerated, *CADD* combined annotation dependent depletion, *P* pathogenic, *NA* data not available

Table 3

Demographical and clinical characteristics of the study populations for VT

GeneID	VT Cohort 1		VT Cohort 2	
	Cases *n* = 299	Controls *n* = 981	Cases *n* = 270	Controls *n* = 639
Age (mean ± SD years)	52 ± 19.4	51 ± 8.4	58 ± 13.5	56 ± 11.0
Sex, male *n* (%)	142 (47.5)	604 (61.6)	204 (75.6)	544 (85.1)
Idiopathic VT (IVT)	180 (60.2)	N/A	118 (43.7)	N/A
Hypertension *n* (%)	203 (42.9)	63 (6.4)	116 (43)	247 (38.7)
CAD *n* (%)	222 (47.5)	N/A	142 (52.6)	357 (55.9)
Diabetes *n* (%)	82 (17.3)	N/A	59 (21.9)	112 (17.5)

Data are shown as mean ± standard deviation (SD) for quantitative variables and percent (%) for qualitative variables; Hypertension was defined as a systolic blood pressure of ≧ 140 mmHg or a diastolic blood pressure of ≧ 90 mmHg. Diabetes was defined as ongoing therapy of diabetes or a fasting plasma glucose level of ≥ 7.0 mmol/L; IVT, VT without any structural heart disease, coronary artery disease and myocardial infarction, cardiomyopathies, valvular disease, rheumatic heart disease, hypertension, and heart failure

CAD coronary artery disease

Table 4

Analysis of allelic association between *SCN4B* variant p.Gly8Ser and VT

Cohort	Sample size (case/control)	R.A. frequency (case/control)	P_{HWE}	Without adjustment		With adjustment[a]	
				*P*obs	OR (95% CI)	*P*adj	OR (95% CI)
VT cohort 1	299/981	1.84%/0.20%	1	5.58E – 05	9.17 (2.91–28.91)	1.21E – 04	11.04 (3.24–37.54)
VT cohort 2	270/639	1.67%/0.39%	1	4.47 E – 03	4.31 (1.44–12.94)	0.033	3.62 (1.11–18.12)
combined	569/1620	1.76%/0.28%	1	1.20E – 07	6.42 (2.92–14.15)	3.09E – 05	6.17 (2.62–14.53)
IVT	298/1620	1.85%/0.28%	1	1.04E – 06	6.75 (2.79–16.36)	1.89E – 05	7.27 (2.93–18.06)

R.A. risk allele, *IVT* idiopathic VT without structural heart disease, coronary artery disease, cardiomyopathies, valvular disease, hypertension, heart failure, and rheumatic heart disease, P_{HWE} *P* value for Hardy–Weinberg disequilibrium test, P_{obs} *P* value observed, i.e., uncorrected *P* value by a Fisher's exact test, *Padj P* value after adjustment with age and sex by multivariate logistic regression analysis for potential confounders, *OR* odds ratio, *95% CI* 95% confidence interval

[a] *P* value after adjustment of age and sex by multivariate logistic regression analysis

Table 5

Analysis of genotypic association of *SCN4B* variant G8S with VT under three different inheritance models

Cohorts (*n*, cases *vs*. controls)	Model	P_{obs}	OR (95% CI)	P_{adj}	OR (95% CI)
VT cohort 1 (299 cases vs. 981 controls)	Dominant	5.27E – 05	9.33 (2.95–29.52)	1.14E – 04	11.27 (3.29–38.57)
VT cohort 2 (270 cases vs. 639 controls)	Dominant	0.007	4.37 (1.45–13.71)	0.032	3.67 (1.12–12.04)
VT combined (569 cases vs.1620 controls)	Dominant	1.30E – 06	6.52 (2.95–14.41)	2.84E – 05	6.27 (2.65–14.81)

P_{obs} P value observed, i.e., uncorrected *P* value by a Fisher's exact test; *P_{adj} P* value after adjustment with age and sex by multivariate logistic regression analysis for potential confounders; *OR* odds ratio, *95% CI* 95% confidence interval

Table 6

Summary of electrophysiological properties of cardiac sodium channel $Na_V1.5$ when expressed together with WT $Na_V\beta4$, mutant $Na_V\beta4$ with p.A145S, mutant $Na_V\beta4$ with p.G8S or without $Na_V\beta4$ (Vector) in HEK/$Na_V1.5$ cells with constant expression of $Na_V1.5$

	Vector	WT $Na_V\beta4$	Mutant $Na_V\beta4$ (p.A145S)	Mutant $Na_V\beta4$ (p.G8S)
I_{Na} density				
(pA/pF)	199.6 ± 41.39 (n = 12)	− 205.5 ± 47.18 (n = 13)	− 135.1 ± 29.11 (n = 11)	− 162.9 ± 32.79 (n = 10)
Activation				
V1/2, (mV)	− 36.94 ± 1.80 (n = 8)	− 41.22 ± 1.45 (n = 8)	− 38.91 ± 1.37 (n = 10)	− 38.31 ± 2.14 (n = 11)
Slope factor	5.17 ± 0.45	3.95 ± 0.35	4.41 ± 0.40	4.72 ± 0.47
Inactivation				
V1/2, (mV)	− 82.24 ± 1.18 (n = 12)	− 83.17 ± 1.01 (n = 11)	− 82.09 ± 1.10 (n = 10)	− 80.49 ± 1.62 (n = 9)
Slope factor	5.93 ± 0.08	6.16 ± 0.25	5.69 ± 0.20	5.73 ± 0.23
Recovery from inactivation				
τ Fast, (ms)	4.59 ± 0.58 (n = 9)	4.46 ± 0.23 (n = 10)	3.50 ± 0.45 (n = 10)	4.01 ± 0.34 (n = 9)
τ Slow, (ms)	56.42 ± 5.60	84.47 ± 14.76	61.46 ± 10.57	73.71 ± 13.39