A Double *SCN5A* Mutation Underlying Asymptomatic Brugada Syndrome

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Abstract

Objective/Background: Patients with the Brugada syndrome who experience syncope or aborted sudden death are at high risk for recurrent lethal arrhythmias. However, the prognosis and the therapeutic approaches in asymptomatic individuals with a Brugada-type ECG (asymptomatic Brugada syndrome) are controversial.

Methods/ Results: We genetically screened 30 asymptomatic probands (male 29, female 1; mean age, 47.1 years) exhibiting a spontaneous Brugada-type ECG. Family members of patients with the Brugada syndrome were excluded. Twenty-nine of 30 patients (96.7%) remained free from symptoms for at least three years. One patient (case #1) who had a family history of sudden death died suddenly during sleep. Ventricular fibrillation was induced by programmed electrical stimulation in 14 of 18 subjects (78%), but none of these 18 subjects developed spontaneous ventricular arrhythmias. Genetic screening failed to identify *SCN5A* mutations in most cases, but demonstrated a novel double missense mutation (K1527R and A1569P) located on the same allele in another asymptomatic subject (case #2). Heterologously expressed mutant Na channels exhibited a negative shift of steady-state inactivation (9.2 mV) and enhanced slow inactivation, suggesting that this individual harbors a subclinical channel dysfunction compatible with symptomatic Brugada syndrome.

Conclusions: Asymptomatic individuals with a Brugada-type ECG generally have a better

prognosis than their symptomatic counterparts, but there may be a subgroup of these individuals with poor prognosis. Severe Na channel dysfunction due to *SCN5A* mutations may not be sufficient to cause symptoms or arrhythmias in the Brugada syndrome, suggesting some unknown factors or modifier genes influencing the arrhythmogenesis.

Key Words: Brugada syndrome, Asymptomatic mutation carrier, Patch clamp, Sodium channel, Genetics, Slow inactivation, *SCN5A*, Ventricular fibrillation

List of abbreviations used in the manuscript

VF: Ventricular fibrillation, *SCN5A*: The gene encoding human cardiac voltage-gated sodium channel α subunit (Nav1.5), SUNDS: Sudden unexplained nocturnal death syndrome, PES: Programmed electrical stimulation, PCR: Polymerase chain reaction, SSCP: Single-strand conformational polymorphism, WT: Wild-type, $V_{1/2}$: The voltages for half maximal inactivation or conductance, NS: no significant difference

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INTRODUCTION

The Brugada syndrome is a primary electrical disorder without underlying structural heart diseases characterized by the coved-type or saddle back-type ST elevation in the right precordial leads [1,2]. It predisposes affected individuals to ventricular fibrillation (VF), and patients with aborted sudden cardiac death are candidates for implantation of a defibrillator because of a high risk of recurrent ventricular arrhythmias. Mutations in the cardiac Na channel α subunit gene (*SCN5A*) are identified in some patients with the Brugada syndrome, and heterologously expressed mutant Na channels exhibit biophysical abnormalities resulting in reduced cardiac Na current [3].

Sudden unexplained nocturnal death syndrome (SUNDS) is one of the leading causes of sudden death in young or middle-aged men in Japan and Southeast Asian countries. Although SUNDS is recognized as phenotypically and genetically equivalent to the Brugada syndrome [4], its electrocardiographic manifestations, especially among those with sudden death as the first event, are often uncertain unless they have a strong family history or have been resuscitated from sudden death. Recent studies have revealed that the prevalence of a Brugada-type ECG is 0.1-0.7% in the general population in Asia and other countries [5-7]. However, the pathophysiology, prognosis, and the therapeutic approaches in asymptomatic

individuals exhibiting a Brugada-type ECG are controversial. Brugada *et al*. showed that the prognosis of this patient group is unfavorable; sixteen of the 111 (14%) asymptomatic individuals with a spontaneous abnormal ECG had arrhythmic events during the follow-up period of 27±29 months[8]. In contrast, Priori *et al.* found no episodes of malignant arrhythmias over a period of 3 years in 30 asymptomatic patients with a Brugada sign [9]. A very low rate of arrhythmic events in this patient group has been confirmed by multiple recent and larger-scale studies [7,10-13]. In one study of 14,000 individuals in Japan, the mortality rate of 98 subjects with a Brugada sign was not higher than the rest of the cohort [7]. Despite the discrepancy in prognosis among different studies, there is evidence that some asymptomatic individuals with a Brugada-type ECG tend to die suddenly during sleep, a clinical observation characteristic of SUNDS, suggesting that SUNDS may underlie at least a part of Brugada-type ECG.

In the present study, we have clinically evaluated and genetically analyzed 30 asymptomatic individuals with a Brugada-type ECG who lacked a family history of the Brugada syndrome to eliminate individuals with an apparent genetic background. Twenty-nine of 30 patients remain asymptomatic during the follow-up period with the exception of one case of sudden death. In this case, the victim had a family history of sudden death (not the Brugada syndrome). We found a double *SCN5A* mutation in another asymptomatic subject without family history of sudden death that exhibited Na channel

dysfunction characteristic for the symptomatic Brugada syndrome. At least some asymptomatic subjects with a Brugada-type ECG have severe Na channel dysfunction, but they do not necessarily manifest arrhythmias. Clinical consequence and arrhythmogenesis in the Brugada syndrome may be greatly influenced by some unknown environmental factors or modifier genes.

Methods

1. Patient population

The study population consists of 30 asymptomatic probands with Brugada-type ECG who agreed to genetic testing. The ECG criteria are (1) J wave elevation higher than 0.2 mV and ST elevation higher than 0.1 mV in V_1-V_3, (2) no demonstrable underlying heart disease evaluated by echocardiography. Family members of the Brugada syndrome, and patients that exhibited transient Brugada-type ST elevation only during drug exposure were excluded. No patient had received antiarrhythmic drugs. Programmed electrical stimulation (PES) and drug provocation tests using pilsicainide or flecainide were performed in 18 and 17 patients, respectively. Patients were followed up for 2 to 6 years.

2. PES

PES was performed in the fasting state, after obtaining written informed consent. The protocol of ventricular stimuli included up to three extra stimuli (two basic cycle lengths of 600 ms and 400 ms) with the coupling interval of the extra stimuli not shorter than 200 ms. VF was induced from the right ventricular apex or right ventricular outflow tract.

3. Genetic screening of *SCN5A*

Genomic DNA was extracted from peripheral blood by using PURGEGE DNA isolation kit (Gentra Systems). The *SCN5A* exons and flanking introns were amplified by PCR as previously described [14]. Genetic screening was performed by PCR-single-strand conformational polymorphism (PCR-SSCP) analysis [15] or direct sequencing. PCR-amplified samples were run on a non-denatured 8% polyacrylamide gel with or without 10% glycerol at 160 V for 2-3 hrs, and the gels were visualized by silver staining (Daiichi Pure Chemicals, Tokyo). DNA sequencing was performed using an ABI PRISM 310 genetic analyzer (Applied Biosystems). Genetic analysis was carried out according to the protocol approved by the ethics committee of Hokkaido University Graduate School of Medicine. Written informed consent was obtained from all subjects.

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4. Construction of the mutant Na channel plasmid

The mutant Na channel cDNA was constructed by the overlapping-extension PCR strategy [15] using the human Nav1.5 cDNA as a template. A missense mutation K1527R was introduced by two rounds of PCR between nt. 4142 and 4770 (628 bp). Similarly, another mutation A1569P was introduced between nt. 4418 and 5027 (609 bp). The PCR fragments of K1527R and A1569P were digested with *Kpn*I/*Bst*EII (403 bp) and *Bst*EII/*Bam*HI (300 bp), respectively, and assembled back into the wild-type (WT) Nav1.5 cDNA which was subcloned in the mammalian expression plasmid pRcCMV (Invitrogen). Correct assembly of the mutant channel plasmid was verified by sequencing to identify clones without polymerase errors. We constructed the plasmid for the double mutation (K1527R plus A1569P) only, because we found that the mutations of K1527R and A1569P are located on the same allele (see Results).

The human cell line tsA-201 was transiently transfected with either WT or mutant plasmids in combination with a plasmid encoding CD8 (pCD8-EBO-Leu2) to visually identify transfected cells using Dynabeads (M-450 CD8, Dynal) [16]. To evaluate the effects of β_1 subunit, the pCD8-EBO-Leu2 was replaced by a bicistronic plasmid encoding both CD8 and human β_1 subunit (pCD8-IRES-hβ_1). Na currents were recorded 24 to 48 hours after transfection using the whole-cell patch-clamp technique. The pipette solution contained 10 mM NaF, 110 mM CsF, 20 mM CsCl, 10 mM EGTA, and 10 mM HEPES (pH adjusted to

7.35 with CsOH) and the bath solution contained 145 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.35 with NaOH). The holding potential was -120 mV, and the recordings were performed at room temperature. Data acquisition and analysis were accomplished by pClamp 6 or 8 (Axon Instruments) and SigmaPlot (SPSS Science). Results are presented as mean ± SEM unless otherwise stated, and statistical comparisons were made using the unpaired Student's *t* test. Statistical significance was assumed for $p<0.05$.

Results

1. Clinical characteristics of the patients (Table 1)

Thirty asymptomatic probands with Brugada-type ECG (male 29, female 1; mean age 47.1 ± 9.5 years (mean ± SD), ranging 28 - 68) were enrolled. Family history of unexplained sudden death (but not Brugada syndrome) was documented in 2 individuals. Intravenous administration of pilsicainide or flecainide (1 mg/kg) exacerbated ST elevation (> 0.2 mV) in 15 of 17 patients (88%). VF was induced by PES in 14 of 18 patients (78%). Two patients with positive tests for both drug provocation test and PES received an implantable cardioverter defibrillator (ICD), but their discharges have not been recorded. One patient (case #1) died suddenly during sleep and an *SCN5A* mutation was identified in one patient

(case #2).

Case #1.

A 43-year-old Japanese man who was pointed out as Brugada-type ECG at a regular medical check up. He had no history of syncope or palpitation. His father had died suddenly of acute myocardial infarction at the fourth decade of life, but his clinical record is not available. Twelve-lead ECG showed coved-type ST elevation in V_{1-3} (Fig. 1A). Chest X-ray and echocardiography were normal. The patient accepted genetic testing but declined further examinations including PES or drug provocation tests. Two years later in the morning, he was found dead in bed. No *SCN5A* mutation was found in this case.

Case #2.

A 60-year-old Japanese man. Coved-type ST elevation in V_{1-3} was indicated at the preoperative ECG check-up when he was 55 years old. He had no palpitation, syncope, nor family history of sudden death. He was admitted to the hospital for further examinations. Coved-type ST elevation was evident in V_{1-3} (Fig 1B). Structural heart diseases were excluded by chest X-ray and echocardiography. Late potentials by signal-averaged ECG were positive (filtered QRS=119 ms, under 40 μV duration= 57 ms, RMS_{40}= 5.5 μV), and intravenous administration of 50 mg flecainide augmented ST elevation in V_{1-3}. VF was induced by

double extra stimulations at the right ventricular outflow tract. He was advised for implantation of an ICD, but he declined it. He has been free from symptoms for 3 years.

2. Genetic analysis of the case #2

PCR-SSCP analysis showed an aberrant conformer in exon 27 of case #2 (Fig 2A). Direct sequence confirmed two heterozygous base substitutions A4580G and G4705C, leading to amino acid substitutions of Arg for Lys-1527 (K1527R) and Pro for Ala-1569 (A1569P), respectively (Fig. 2B). His family members declined further examinations including DNA diagnostics. In order to determine whether these mutations are located on one allele (double mutation) or on different alleles (compound mutations), PCR fragment of exon 27 was subcloned into a vector pGEM-T easy (Promega), and multiple independent clones were sequenced. Approximately 50% of the clones showed wild-type sequence, and the rest of the clones showed both mutations K1527R and A1569R (data not shown), indicating that two mutations are located on the same allele, but not compound mutations. Although genomic information of his family members is not available, two mutations were most likely inherited from one of his parents. Furthermore, neither K1527R nor A1569R was observed in 500 normal chromosomes, excluding the possibility of DNA polymorphisms.

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2. Biophysical properties of the double mutant channel

Whole-cell Na currents of the K1527R+A1569P mutant channel heterologously expressed in tsA-201 cells showed current decay nearly indistinguishable from WT (Fig 3A). Persistent Na current, a biophysical property most commonly observed in mutant Na channels responsible for type-3 long QT syndrome (LQT3)[17], was not evident in the mutant channel. Conductance-voltage (GV) curve showed that the slope factor *k* of the mutant channel was significantly larger than that of WT (WT: 5.3 ± 0.3 mV, n=9; K1527R+A1569P: 7.8 ± 0.8 mV, n=8; $p<0.01$), whereas the voltages for the half maximal conductance ($V_{1/2}$) were comparable (WT: -49.8 ± 1.3 mV, K1527R+A1569P: -45.8 ± 1.6 mV; NS) (Fig 3B). The voltage-dependence of fast inactivation was significantly shifted in a hyperpolarizing direction by 9.2 mV in the mutant than WT ($V_{1/2}$; WT: -88.6 ± 1.0 mV, n=9; K1527R+A1569P: -97.8 ± 1.6 mV, n=17; $p<0.001$), while the slope factors were not significantly different (*k*; WT: -7.9 ± 0.2 mV, K1527R+A1569P: -7.4 ± 0.2 mV; NS). These data show that activation is less-voltage dependent and the steady-state channel availability at voltages near the resting potentials is reduced in the mutant channel. Recovery from inactivation of the mutant channel fit with double exponential equation was nearly indistinguishable from WT (Fig 3C).

In addition to the fast inactivation, intermediate inactivation (I_M), a distinct

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inactivation gating property with kinetics intermediate between fast and slow inactivation, has been implicated in Brugada syndrome [18,19] (Fig 4A). Voltage-dependence of I_M was evaluated by a 1 sec prepulse of various potentials followed by a 20 ms brief recovery pulse to -120 mV to remove fast inactivation, and the channel availability was assessed by a -20 mV test pulse (Fig 4B). Voltage-dependence of steady-state I_M fit with Boltzmann equation [19] showed that the magnitude of I_M is significantly larger in the mutant channel (WT= 0.20 ± 0.02, n=11; K1527R+A1569P= 0.39 ± 0.03, n=13; $p<0.01$) and the mid point of the curve was significantly shifted in the hyperpolarizing direction in the mutant channel ($V_{1/2}$; WT= -92.9 ± 1.0 mV, K1527R+A1569P= -99.2 ± 2.7 mV; $p<0.01$). Slow inactivation was elicited by various lengths of prepulses at -20 mV, and the time constant of I_M obtained by fitting with a single exponential function was comparable (τ; WT= 273 ± 64 ms, K1527R+A1569P= 220 ± 50 ms); however, magnitude of the I_M was significantly larger in the mutant channel (WT= 0.18 ± 0.02, n=11; K1527R+A1569P= 0.39 ± 0.03, n=12; $p<0.05$), showing enhanced entry to I_M in the mutant channel. Recovery from I_M was virtually identical between WT and the mutant channels, and co-expression of human Na channel β_1 subunit did not significantly change the gating properties of either WT or mutant channel channels (data not shown). These results suggest that the rate and the extent of I_M are substantially enhanced in the mutant channel.

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Discussion

Since the first identification of *SCN5A* mutations in the Brugada syndrome in 1998 [20], more than 50 distinct mutations have been reported. The functional properties of *SCN5A* mutations responsible for the Brugada syndrome show variable biophysical abnormalities including the following: (1) changes in Na channel gating properties [18], (2) defective membrane trafficking[21], or (3) a non-functional channel [22]. A common denominator of these mutations is a reduction of cardiac Na current leaving the transient outward K current (I_{to}) unopposed in phase 1, and a loss of the action potential dome in the right ventricular epicardium but not endocardium. The large transmural voltage-gradient in the right ventricle results in ST elevation in the right precordial leads and "phase 2 reentry" [23]. In contrast to the refined pathophysiology underlying the Brugada syndrome, it is still unclear whether the Brugada-type ECG is an electrocardiographic entity distinct from the Brugada syndrome, or whether it constitutes an asymptomatic subgroup of the Brugada syndrome sharing the same molecular and cellular abnormalities due to genetic defects in *SCN5A*.

In this study, we genetically screened 30 asymptomatic probands who showed a Brugada-type ECG without a family history of the disease, and identified a double *SCN5A* mutation, K1527R+A1569P. This mutation is not only a novel *SCN5A* mutation associated

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with a Brugada-type ECG, but also the first naturally occurring double mutation in *SCN5A*, although a double mutation was previously reported in the Na channel gene (*SCN4A*) of skeletal muscle [24]. The prevalence of *SCN5A* mutation in asymptomatic individuals with a Brugada-type ECG in our study was only 3.3% and substantially lower than the 22% prevalence reported by Priori *et al*. in 130 probands with the Brugada syndrome [12]. There are several possible explanations for the apparent difference: 1) asymptomatic and symptomatic Brugada syndrome have distinct genetic bases, 2) ethnic variability between study populations, or 3) technical differences in genetic screening between laboratories. Recent genetic studies in the Brugada syndrome including both symptomatic and asymptomatic subjects in the Chinese population showed four *SCN5A* mutations in 5 (14%) of 36 Brugada-syndrome probands (two symptomatic and three asymptomatic)[25], which is comparable to the recent study in Japanese population (4 out of 38; 11%, Makiyama *et al*. personal communication). Therefore, the prevalence of *SCN5A* does not seem to be greatly affected by ethnicity. However, there was an important difference in the family history of sudden death between the study population of Priori *et al.* and our study. Priori *et al.* documented a family history of sudden death in 26 (20%) of 130 probands [12], while we had only one patient (case #1) who had a family history of sudden death. The prevalence of a Brugada-type ECG in the general population is reported to be 0.1-0.7% in Japan, and the vast majority of the asymptomatic individuals exhibiting a Brugada-type ECG are sporadic[6,7,26]. These results suggest that asymptomatic individuals with

a Brugada-type ECG most likely have a genetic background distinct from symptomatic Brugada syndrome patients.

Risk stratification of asymptomatic individuals with the Brugada syndrome is controversial. Brugada *et al*. showed that the prognosis of this patient group is unfavorable, and VF-inducibility is a good predictor of lethal arrhythmias [8,27], whereas Priori *et al.* found no episodes of malignant arrhythmias over a period of 3 years in 30 asymptomatic patients with a Brugada sign [9]. We cannot precisely evaluate the prognostic value of PES or genetic testing in asymptomatic Brugada syndrome, because the case #1 subject declined further examinations including PES or provocative drug testing, and no *SCN5A* mutations were identified. However, PES in our study showed relatively high VF inducibility (14 out of 17, 78%) in asymptomatic individuals despite the fact that they remained asymptomatic, consistent with previous observations (8 out of 11; 73%)[10]. These results suggest that asymptomatic individuals with a Brugada–type ECG have a relatively benign prognosis, and that VF inducibility does not seem to be a good predictor of lethal events, at least for asymptomatic individuals without a family history of the Brugada syndrome. These results conflict with the observations of Brugada *et al*. who showed that individuals with inducible VF during PES have an elevated risk for lethal arrhythmias, and recommended prophylactic implantation of ICD for such individuals even though they are asymptomatic [8,27]. The reason

for the discrepancy between studies is not clear, but it may be attributable in part to the enrollment of asymptomatic individuals with a family history of the Brugada syndrome.

Genetic screening of *SCN5A* is the most powerful diagnostic tool for the Brugada syndrome, especially for screening individuals within a family of a proband with an identified mutation. Demonstration of a novel mutation K1527R+A1569P in our study suggests that genetic defects of *SCN5A* are at least partially responsible for a Brugada-type ECG in asymptomatic as well as symptomatic individuals. It is true that identification of a new mutation in a sporadic case is sometimes equivocal, and the existence of *SCN5A* mutations is not regarded as a reliable predicting value in the Brugada syndrome because of its substantially low sensitivity and specificity to identify patients with cardiac arrest [12]. However, it is plausible to speculate that functional evaluation of the *SCN5A* mutations may help to substantiate their pathophysiological relevance, which in turn may help stratify the risk of sudden death. Because the double mutant channel showed a negative shift of the steady-state inactivation curve and an increased proportion of Na channels that enter an intermediate state of inactivation, there is a reduction in cardiac Na current, which is characteristic of *SCN5A* mutations in those with symptomatic Brugada syndrome [18,28]. These results suggest that asymptomatic individuals with “functionally proven” *SCN5A* mutations with loss-of-function properties should be carefully followed to avoid lethal events as was observed in the SUNDS

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victim case #1. Further clinical and genetic studies with larger population and longer follow-up period are required to evaluate the predicting value of *SCN5A* mutations with loss-of-function properties in asymptomatic subjects.

Despite exhibiting typical coved-type ST elevation and severe functional defect in cardiac Na channel, the case #2 subject remains asymptomatic, but the underlying genetic, cellular, or electrophysiological mechanisms are not clear. Clinical consequences of *SCN5A* mutations are usually determined by the functional properties of each mutation, leading to multiple distinct cardiac Na channelopathies including Brugada syndrome, LQT3, and cardiac conduction defect. However, there are several lines of evidence that do not agree with the abovementioned idea. Silent *SCN5A* mutation carriers are occasionally observed in the pedigrees of in Brugada syndrome families [29] as well as in long-QT syndrome [30]. We previously found an *SCN5A* mutation R367H in a family with atrial standstill complicated with J wave elevation in the inferior leads [22], while Hong et al. found the same mutation in a typical Brugada syndrome family [31]. Moreover, a single mutation G1406R results in Brugada syndrome or cardiac conduction defect in the same family[32]. These results suggest that the clinical consequence of the some *SCN5A* mutations are occasionally determined in individual-specific or branch-specific manners, rather than mutation-specific manner. Based on these observations, it is speculated that the severe functional defects of the double

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mutation observed in asymptomatic subject #2 are not necessarily sufficient to manifest syncope or life-threatening arrhythmias, and the clinical consequence of the mutations may be greatly influenced by some unknown environmental factors or genetic modifiers.

Our clinical and genetic study enrolled only 30 individuals with a Brugada-type ECG, and further studies in a larger population with a longer evaluation period are required to draw definitive conclusions with respect to the pathogenesis and risk stratification of this disease entity. In parallel with efforts to establish new parameters with high predictive value [33,34], further genetic screening of *SCN5A* and identification of new responsible genes are required for demonstrating the molecular basis for both symptomatic and asymptomatic Brugada syndrome.

Conclusions

Asymptomatic individuals with a Brugada-type ECG generally have a better prognosis than their symptomatic counterparts, but there may be a subgroup of these individuals with poor prognosis. Na channel dysfunction due to *SCN5A* mutation may be responsible, at least in part, for a Brugada-type ECG in asymptomatic individuals. Severe functional defect of *SCN5A* mutations may not be sufficient to cause symptoms, and some environmental factors or modifier genes may play additional roles for the arrhythmogenesis. Although the efficacy

of genetic screening is not sufficiently high to use as a diagnostic tool in the presence of a Brugada-type ECG in asymptomatic individuals, further clinical and genetic studies are required for elucidating the pathophysiology of Brugada syndrome, which in turn provide more efficient treatment of family members who are still asymptomatic.

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Figure Legends

Fig. 1 Electrocardiographic findings

(A) (B) Twelve-lead ECG recording of the two cases of asymptomatic Brugada syndrome. (A) and (B) show the ECGs of case 1 (sudden death) and case 2 (double *SCN5A* mutation), respectively. Coved-type elevation (A) and saddle-back ST elevation (B) in the right precordial leads are noted (arrows).

Fig 2. Molecular genetics of the case 2

(A) Exon 27 of the *SCN5A* was amplified from the genomic DNA and was subjected to PCR-SSCP analysis. Lanes 1-3: healthy control individuals. Lane 4: case 2. An aberrant conformer is shown with arrows.

(B) Direct DNA sequencing of the exon 27. Heterozygous nucleotide changes A4580G and G4705C resulting in missense mutations K1527R and A1569P, respectively.

(C) Predicted topology of the cardiac Na channel Nav1.5 and the location of the two mutations K1527R and A1569P. Transmembrane segments (S1-S6) in each domain (D1-D4) are shown with boxes.

Fig 3. Biophysical properties of the double mutation

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(A) Representative whole-cell current traces obtained from tsA-201 transfected with WT or K1527R+A1569P Na channels. Currents were recorded from a holding potential of -120 mV and stepped from -90 mV to +90 mV during 20 ms in 10 mV increments. Currents were normalized and superimposed.

(B) Voltage-dependence of activation and the steady-state fast inactivation of WT (open circles) and K1527R+A1569P (filled squares). Current-voltage relationship was fit to the Boltzmann equation: $I/I_{max}= (V-V_{rev}) \times(1+\exp(V-V_{1/2})/k)^{-1}$, where I_{max} represents the maximum peak current, and V, V_{rev}, $V_{1/2}$ is the test pulse potential, reversal potential, and the mid point of activation, respectively. Conductance (G) was calculated by the equation $G = I \times (V-V_{rev})^{-1}$, and the normalized peak conductance was plotted as a function of membrane potential. To assess steady-state fast inactivation, the peak currents were measured during a -20 mV test potential after a series of 100 ms prepulses from -150 mV to -30 mV. Normalized peak current was plotted as a function of prepulse potential. Steady-state fast inactivation curve was fit with the Boltzmann equation: $I/I_{max}= (1 + \exp((V-V_{1/2})/k))^{-1}$. Activation of the mutant channel was significantly less voltage-dependent and the steady-state inactivation curve was significantly shifted in a negative direction ($V_{1/2}$: WT= -88.6 ± 1.0 mV, n= 9; K1527R+A1569P= -97.8 ± 1.6 mV, n= 17; $p<0.001$).

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(C) Recovery from inactivation was assessed by a standard double pulse protocol consisted of a 500 ms conditioning pulse, followed by a various length (Δt) of recovery interval at -120 mV, and a test pulse (-20 mV, 50 ms). Normalized peak current was fit to a double exponential function: $I/I_{max} = C - A_f \times \exp(-t/\tau_f) - A_s \times \exp(-t/\tau_s)$.

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Fig 4. Intermediate inactivation properties of the double mutant channel

(A) Voltage-dependence of I_M was determined by a double pulse protocol shown in the inset. Cells were depolarized for 1 s by a prepulse with various potentials ranging from -150 mV to -30 mV to elicit I_M, followed by a 20 ms repolarization at -120 mV to allow recovery from fast inactivation. The remaining Na currents were measured with a test pulse -20 mV and the normalized currents were fit with the Boltzmann equation, $I/I_{max} = A \times (1 + \exp((V-V_{1/2})/k\ ))^{-1} + C$ to determine the fraction I_M (A) and the membrane potential for half maximal inactivation ($V_{1/2}$) [19].

(B) Time course of the development of I_M. Cells were depolarized at -20 mV for a various length of time (Δt) to elicit I_M, followed by a brief repolarization to allow recovery from fast inactivation. The remaining Na currents were measured at a test pulse to -20 mV. Normalized peak current were fit with a monoexponential equation: $I/I_{max} = A \times \exp(-t/\tau) + C$, where A is the fraction of I_M, and τ is the time constant.

Magnitude of I_M was significantly enhanced in the mutant channel (WT= 0.18±0.02, n=11; K1527R+A1569P= 0.25±0.03, n=12; $p<0.05$), while the time constant for the development of I_M was comparable.

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