A novel gain-of-function KCNJ2 mutation associated with short-QT syndrome impairs inward rectification of Kir2.1 currents

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Aims
Short-QT syndrome (SQTS) is a recently recognized disorder associated with atrial fibrillation (AF) and sudden death due to ventricular arrhythmias. Mutations in several ion channel genes have been linked to SQTS; however, the mechanism remains unclear. This study describes a novel heterozygous gain-of-function mutation in the inward rectifier potassium channel gene, KCNJ2, identified in SQTS.

Methods and results
We studied an 8-year-old girl with a markedly short-QT interval (QT = 172 ms, QTc = 194 ms) who suffered from paroxysmal AF. Mutational analysis identified a novel heterozygous KCNJ2 mutation, M301K. Functional assays displayed no Kir2.1 currents when M301K channels were expressed alone. However, co-expression of wild-type (WT) with M301K resulted in larger outward currents than the WT at more than −30 mV. These results suggest a gain-of-function type modulation due to decreased inward rectification. Furthermore, we analysed the functional significance of the amino acid charge at M301 (neutral) by changing the residue. As with M301K, in M301R (positive), the homozygous channels were non-functional, whereas the heterozygous channels demonstrated decreased inward rectification. Meanwhile, the currents recorded in M301A (neutral) showed normal inward rectification under both homo- and heterozygous conditions. Heterozygous overexpression of WT and M301K in neonatal rat ventricular myocytes exhibited markedly shorter action potential durations than the WT alone.

Conclusion
In this study, we identified a novel KCNJ2 gain-of-function mutation, M301K, associated with SQTS. Functional assays revealed no functional currents in the homozygous channels, whereas impaired inward rectification demonstrated under the heterozygous condition resulted in larger outward currents, which is a novel mechanism predisposing SQTS.

Keywords
Arrhythmia (mechanisms) • Short-QT syndrome • K-channel • Atrial fibrillation • Inward rectification

1. Introduction
Short-QT syndrome (SQTS) is a recently recognized disorder, characterized by a shortened QT interval in the electrocardiogram (ECG), and associated with a high incidence of atrial fibrillation (AF), syncope, and sudden death due to ventricular tachyarrhythmias without structural cardiac abnormalities. The syndrome was first described by Gussak et al.1 in 2000 within the context of a familial AF case associated with short-QT interval. SQTS is a genetically heterogeneous disease, and five ion channel genes (SQT1-6) have been identified as causative genes thus far: KCNH2 encoding the α-subunit of the rapidly activating delayed rectifier potassium channels, Ikr (SQT1); KCNJ1 encoding the α-subunit of the slowly activating delayed rectifier potassium channels, Ik1 (SQT2); KCNJ2 encoding...
the Kir2.1 channels that underlie the inward rectifier potassium currents, \( I_{K1} \) (SQT3), \( CACNA1C, CACNB2b, \) and \( CACNA2D1, \) which encode the \( \alpha 1C, \beta 2b, \) and \( \alpha 2-b-1 \)-subunits of cardiac L-type calcium channels (SQT4, SQT5, and SQT6), respectively. SQT4 and SQT5 are considered clinical entities with the combined phenotypic characteristics of SQTS and Brugada syndrome, manifesting in a J point and ST-segment elevation in the right precordial ECG leads.

Regardless of the extensive genetic screening carried out on SQTS patients, genetic mutations have been identified in a small number of cases.1–5,7,8 In 2005, Priori et al.4 first reported that a KCNJ2 mutation was responsible for SQTS (SQT3); however, no additional SQT3 variants have been reported thus far. This lack of progress has significantly hindered our advances in understanding the mechanisms underlying this disease. In the present study, we describe a novel KCNJ2 mutation which impaired the inward rectification of Kir2.1 currents. This is a novel KCNJ2 gain-of-function mechanism leading to SQTS.

2. Methods

2.1 Genetic analysis

Genetic analysis was performed after written informed consent in accordance with the study protocol approved by the Kyoto University ethical committee. The investigation conforms to the principles outlined in the Declaration of Helsinki. Genomic DNA was isolated from blood lymphocytes and screened for the entire open-reading frames of KCNQ1, KCNH2, KCNQ1-3, KCNJ2, CACNA1C, and SCN5A by denaturing high-performance liquid chromatography using a WAVE System Model 3500 (Transgenicom, Omaha, NE, USA). Abnormal conformers were amplified by polymerase chain reaction and sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and compared with 400 Japanese control alleles.

2.2 Neonatal rat ventricular myocyte isolation

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Kyoto University Animal Experimentation Committee. A standard trypsin dissociation method was used to prepare neonatal rat ventricular myocytes (NRVMs).3 The hearts were removed from 1- to 2-day-old Wistar rats euthanized by decapitation. The ventricles were minced, and the myocytes were dissociated with trypsin. Dispersed cells were prepared on 100 mm culture dishes for 1 h at 37°C in 5% CO₂ to remove fibroblasts. Non-attached, viable myocytes were collected, and placed on 35 mm culture dishes.

2.3 Mutagenesis and transient transfection of KCNJ2 plasmids

The entire coding region of the KCNJ2 was subcloned into the pCMS-EGFP vector (Clontech, Palo Alto, CA, USA) using methods previously described.10 The mutation was introduced by site-directed mutagenesis using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA). We sequenced the entire plasmid to confirm the presence of the mutation and the absence of any unwanted variations. To assess the functional modulation of mutant channels, human embryonic kidney (HEK) 293 cells were transiently transfected with KCNJ2 WT and/or mutant plasmids using FuGENE 6 (Roche, Indianapolis, IN, USA) as directed in the manufacturer’s instructions. In order to investigate the mutant’s effects on myocyte action potentials, plasmids were transfected 1 day after plating NRVMs, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.4 Cell surface expression of KCNJ2

Immunofluorescence microscopy was used to detect the presence of KCNJ2 channels on the plasma membrane of HEK 293 cells. A haemagglutinin (HA) epitope (YPDYDVPDYA) was introduced into the pCMS-EGFP-KCNJ2 [wild-type (WT) and mutant] construct between residues Ala-115 and Ser-116 (extracellular loop between TM1 and TM2).10,12 HEK 293 cells were transfected with 1.0 µg of WT or mutant plasmids, or 0.5 µg of each WT and mutant plasmids to assess a heterozygous condition in 35 mm glass-bottom dishes. Two days later, the cells were fixed with 4% paraformaldehyde solution, and images were taken at ×40 magnification on an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

2.5 Electrophysiological analysis

For voltage-clamp experiments, a total of 0.75 µg of WT and/or mutant KCNJ2 plasmids were transfected in HEK 293 cells: 48–72 h after transfection, functional assays were conducted on GFP-positive cells by a conventional whole-cell configuration of patch-clamp techniques at 37°C, using an Axopatch 200A patch clamp amplifier and a Digidata 1322A digitizer (Axon Instruments, Foster City, CA, USA).10 Pipettes were filled with a solution (in mM): 140 KCl, 2 MgCl₂, 1 EGTA, and 10 HEPES (pH 7.3 with KOH). The bath solution was composed of (in mM): 135 NaCl, 5 KCl, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH).

In order to record action potentials on NRVMs, 3 µg of WT, or a mixture of 1.5 µg WT and 1.5 µg mutant KCNJ2 plasmids, were transfected: 48–72 h after transfection, functional assays were conducted on non-transfected or transfected cells that were recognized by their obvious green fluorescence, using a whole-cell patch-clamp technique at 37°C with the same devices. Action potentials were evoked by 2 ms supra-threshold current pulses at 10 Hz in a current-clamp mode. The pipette solution contained (in mM): KCl 140, MgCl₂ 1, MgATP 4, NaCl 10, and HEPES 10 (pH 7.2 with KOH). Tyrode solution contained (in mM): NaCl 140, KCl 4, CaCl₂ 2, MgCl₂ 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH). Action potential duration (APD) was measured as the time from the overshoot to 90% repolarization (APD₉₀).

2.6 Statistics

All the data are shown as mean ± standard error of the mean. For mean value and comparisons between two sample groups, an unpaired Student’s t-test was used to evaluate statistical significance. For comparisons between multiple groups, we applied a Steel–Dwass test. For either evaluation, a P-value <0.05 was considered significant.

3. Results

3.1 Clinical features

An 8-year-old girl with a markedly shortened QT interval (QT = 172 ms, QTc = 194 ms; Figure 1A) had been suffering from multiple disorders, such as severe mental retardation, abnormal proliferation of oesophageal blood vessels, epilepsy, and Kawasaki disease. Upon presentation during a routine check-up, her treating physician noticed an irregular heart rhythm. Her 12-lead ECG showed AF (Figure 1B), and she underwent external electrical cardioversion because intravenous infusion of procainamide (15 mg/kg) failed to recover sinus rhythm. The echocardiography revealed no significant abnormality. During further evaluation with right-heart catheterization, the Swan–Ganz catheter induced supra-ventricular tachycardia when it was inserted in the right atrium, and ventricular fibrillation occurred at the position of the right ventricular outflow tract, which suggested the presence of increased myocardial irritability.
She was diagnosed with SQTS from these clinical features (i.e. a markedly shortened QT interval, paroxysmal AF, and VF inducibility).

The proband had a family history of perinatal death in her elder sister (Figure 1C), but her family did not undergo genetic investigation or further clinical evaluation with the exception of ECGs taken for her father, elder brother, and younger sister. Genetic investigations could not be carried out due to a lack of informed consent. The ECGs for the family members displayed normal QTc intervals (410, 430, and 390 ms, respectively; Figure 1C).

3.2 Genetic analysis
In this patient, we screened for candidate cardiac ion channel genes (KCNQ1, KCNH2, KCNE1-3, KCNJ2, CACNA1C, and SCN5A). As a result of the genetic analysis, we identified a novel heterozygous mutation, a single-base substitution at nucleotide 902 (c.902T>A) in the KCNJ2 gene, resulting in an amino acid change from methionine to lysine at 301 in the Kir2.1 potassium channel (Figure 2A). The amino acid at codon 301 (methionine) is highly conserved among different species (Figure 2C). Furthermore, this mutation was absent in 400 Japanese control alleles. We failed to identify mutations in any other candidate genes.

3.3 Cell surface expression of KCNJ2 mutants
In order to investigate whether the M301K mutations affect intracellular Kir2.1 trafficking, we introduced an HA epitope into the extracellular domain of KCNJ2, and examined the subcellular distribution of channels in transfected HEK 293 cells using confocal microscopy (Figure 2D). Figure 2D illustrates the typical results of confocal imaging. HEK 293 cells were successfully transfected with either HA-KCNJ2 WT, KCNJ2 WT/HA-M301K, or HA-M301K (Figure 2D, upper panels). All types of HA-tagged Kir2.1 proteins exhibited red fluorescence at the plasma membrane (Figure 2D, middle and lower panels), indicating that both homo- and heterozygous mutant channels were trafficking-competent.
3.4 Cellular electrophysiology

We performed a functional characterization of the mutant channels in HEK 293 cells. Figure 3A shows representative current traces from cells expressing KCNJ2 WT, M301K, or WT/M301K, elicited by voltage-clamp steps (duration 400 ms) from −120 to +100 mV (10 mV step), applied from a holding potential of −60 mV. The currents were normalized to cell capacitance and were plotted as a function of test potentials (Figure 3B). As previously reported, expression of the KCNJ2 WT in HEK 293 cells resulted in normal inward rectifying potassium currents (Figure 3A left panel and blue symbols in Figure 3B). When M301K mutant channels were expressed alone, they were entirely non-functional (Figure 3A middle panel and green symbols in Figure 3B). In contrast, when cells were co-transfected with both equimolar WT and M301K, ample potassium currents showing a very weak inward rectification could be recorded (Figure 3A right panel and red symbols in Figure 3B). Average current densities were significantly smaller than those of WT Kir2.1 channels at potentials between −120 and −90 mV ($P < 0.05$), and significantly larger at potentials between −30 and +100 mV ($P < 0.05$).

3.5 Contribution of amino acid charge at residue 301 to Kir2.1 currents

Methionine at 301 is located within the G-loop that forms the narrowest segment of the cytoplasmic pathway, and negatively charged amino acids on the inner wall of the cytoplasmic pore, where the G-loop is located, are known to be important for the strength of the inward rectification. We therefore speculated that the amino acid charge at this position may be crucial for the inward rectification of Kir2.1 channels, and that its change from methionine (neutrally charged) to lysine (positively charged) may result in functional changes in Kir2.1 currents. In order to analyse the contribution of the amino acid charge at 301 to inward rectification, we changed the amino acid at M301 to another positively charged amino acid, arginine, and to another neutral amino acid, alanine, for comparison. Figure 4A illustrates the whole-cell Kir2.1 currents in homo- and heterozygous mutant conditions for M301R (left panel) and M301A (right panel). Homozygous M301R mutant channels displayed no functional currents, whereas WT/M301R attenuated the inward rectification (Figure 4A left panel). These observations suggest that the currents through the M301R channels are similar to those of the M301K channels (Figure 3) under both homo- and heterozygous conditions. On the other hand, in the M301A channels—in which the residual charge remained neutral—the currents showed normal inward rectification in both homo- and heterozygous conditions similar to those produced by WT Kir2.1 channels (Figure 4A right panel). In order to evaluate the intensity of inward rectifying properties, we assessed the rectification index, along with the ratio of the current amplitudes at 0 and −100 mV. Figure 4B shows the rectification indexes obtained from WT, M301A (0.10 ± 0.02, $n = 10$), WT/M301A (0.073 ± 0.015, $n = 11$), WT/M301K (1.12 ± 0.16, $n = 11$), and WT/M301R (0.99 ± 0.14, $n = 11$). Although the rectification indexes for WT/301A and M301A showed no significant difference, the indexes for both WT/M301K and WT/M301R were significantly increased in comparison with WT (0.061 ± 0.01, $n = 15$, $P < 0.001$, left-most bar in Figure 4B).
3.6 Action potentials recording in KCNJ2-M301K-transfected NRVMs

We investigated the impacts of M301K mutant Kir2.1 channels on NRVMs' action potentials using a transient transfection method. Figure 5A shows typical action potentials recorded for non-transfected (control) NRVMs (Figure 5A, left panel), and NRVMs transfected with KCNJ2 WT or WT/M301K (Figure 5A middle and right panels, respectively). Phase 3 repolarization was accelerated in the KCNJ2 WT-overexpressed group (Figure 5A, middle panel, bar graphs in Figure 5B). Additionally, APD90 was significantly shorter in the WT/M301K mutant-overexpressed group (9.4 ± 2.1 ms, n = 16, P < 0.001, Figure 5A, right panel; bar graph in Figure 5B) than in the WT-overexpressed group.

Figure 3 Voltage-clamp recordings from transfected HEK 293 cells. (A) Representative current traces of WT, M301K, and WT/M301K. Currents were elicited by 400 ms depolarizing voltage steps from −120 to +100 mV and from a holding potential of −60 mV. (B) Current–voltage relationships are plotted as the current. Current density was calculated by dividing the whole-cell current amplitude by cell capacitance. No functional currents were recorded in the homozygous M301K channels. On the other hand, the mean current densities of the WT/M301K channels are significantly larger than the WT (P < 0.05) at each voltage from −30 to +100 mV, and smaller at each voltage from −120 to −90 mV (P < 0.05).

4. Discussion

4.1 Major findings

In the present study, we identified a novel heterozygous KCNJ2 mutation, M301K, in a patient with a markedly shortened QT interval. The QT interval, 172 ms, of this patient is the shortest among previous SQTS reports.1–7,16 to our knowledge. The methionine at position 301 is located in the C-terminus of Kir2.1 channel, and is considered to form a pore-facing loop region.13 Functional assays using a heterologous expression system revealed that homozygous M301K Kir2.1 channels carried no currents with preserved plasma membrane expression; however, heterozygous WT/M301K Kir2.1 channels attenuated inward rectifying properties, which resulted in increased outward currents for positive voltages and negative voltages down to −30 mV. Significant increases in outward currents within the voltage range of the action potentials shortened APD by accelerating membrane repolarization as shown in Figure 5, which is implicated in increased cardiac vulnerability.

4.2 Impaired inward rectification of Kir2.1 currents: a novel mechanism predisposing SQTS

In 2005, Priori et al.4 first reported a heterozygous gain-of-function KCNJ2 mutation, D172N, in a patient with SQTS. In the report, homozygous D172N Kir2.1 channels displayed larger outward currents compared with WT Kir2.1 alone, and heterozygous channels yielded intermediate results. In both homozygous and heterozygous D172N mutant channels, the inward rectification properties of Kir2.1 currents were preserved. In heterozygous M301K mutant channels identified in our patient, however, the inward rectification was significantly reduced, allowing ample outward potassium currents at positive potentials. In addition, it should be emphasized that the homozygous M301K mutant channels were non-functional. These functional changes, such as the impaired inward rectification of the...
Kir 2.1 currents resulting in increased outward currents, are a novel KCNJ2 gain-of-function mechanism predisposing SQTS. The phenotypic characteristics of our index patient somewhat differ from those of the KCNJ2-D172N mutation carriers. No apparent arrhythmias were recorded with D172N mutation carriers. On the other hand, our M301K patient showed paroxysmal AF and multiple disorders. Additionally, mechanical stimulation by a Swan–Ganz catheter induced paroxysmal supraventricular tachycardia and VF. Moreover, the QTc interval in our patient was much shorter (QTc = 194 ms, Figure 1) than that of the D172N carriers (QTc = 315 and 320 ms). Another gain-of-function KCNJ2 mutation, V93I, was reported in a familial AF case. Their functional analysis showed a similar result with D172N, but the affected members had normal QT intervals. These diverse clinical manifestations may be related to the extent and the different gain-of-function mechanisms of the Kir2.1 currents.

### 4.3 Relationship between impaired inward rectification and charged amino acid residues at 301
Kir currents exhibit strong inward rectification, which is thought to be due to pore blocking induced by multivalent ions from intracellular Mg\(^{2+}\). Channel blockade by physiological concentrations of Mg\(^{2+}\) is influenced by the electrostatic negativity within the cytoplasmic pore. Negative charges on the inner wall of the cytoplasmic pore are therefore key determinants of the strength of the inward rectification. Many amino acid residues inside the pore demonstrate interactions with the ion over long distances, suggesting that mutations potentially affect ion or blocker energetics over the entire pore profile. The M301K mutation causes the change of the amino acid residue at 301 from a non-charged amino acid residue, methionine, to a positively charged residue, lysine. In order to evaluate the importance of the charge at 301, additional whole-cell patch-clamp recordings were carried out on M301A (remained neutral) and M301R (neutral to positive) (Figure 4). Inward rectification of Kir2.1 currents was well preserved in both homozygous and heterozygous M301A channels. Heterozygous M301R channels, however, attenuated inward rectification, and homozygous M301R channels were non-functional similar to that of the M301K channels. These electrophysiological results indicate that the neutral amino acid residue at 301 plays an important role in generating Kir2.1 inward rectification. The decrease in the net negative charge within the cytoplasmic pore may facilitate the reduction in both the susceptibility of the channel to Mg\(^{2+}\) block and the voltage dependence of the blockade. It
remains unknown why only tentative hetero-multimers of WT and M301K are active and lose their inward rectification properties. In homozygous M301K channels, all of the tetrameric subunits must have a positively charged lysine at 301, which may impair potassium ion permeation due to a conformational change in the near-pore region.

4.4 Heterozygous KCNJ2-WT/M301K overexpression shortened APD in NRVMs

In cardiomyocytes, Kir2.1, Kir2.2, and Kir2.3 channels are supposed to be able to co-assemble in order to modulate their channel properties.22 Thus, there can be a multitude of Kir2.x heteromultimers, and to date a wide range of single-channel conductances of inward rectifier channels have been reported in studies conducted on various mammalian myocytes, including human.23−25 This variety at the individual channel level may contribute to the different stoichiometry of the tetrameric channels.26 Because Kir2.1 is a major component of IK1 in the myocardium, we overexpressed the KCNJ2 M301K mutant channels in NRVMs to examine the effects of the mutation on APD. Overexpression with WT alone resulted in shorter APD in comparison with WT overexpressed alone. *P < 0.001.

4.5 Clinical features of the index patient with KCNJ2-M301K

Regarding the clinical criteria for the diagnosis of SQTS, they have yet to be defined. However, we should consider SQTS in a patient presenting with a QTc < 340 ms and other factors suggestive of arrhythmia (such as syncope or family history of sudden death).28 A prominent clinical manifestation of SQTS is arrhythmias, such as AF.
and VF. \cite{1,2,3,4,5,6} In this patient, however, additional medical histories not limited to arrhythmias, such as severe mental retardation, abnormal proliferation of the osseous skeletal muscle, disease, were also documented. Because KCNJ2 is known to be expressed in a variety of tissues, such as cardiac and skeletal muscle, the brain, arterial smooth muscle cells and developing bone structures of the craniofacial region, extremities, and vertebrae,\cite{7,8,9,10,11,12} some of her compound disorders may be attributed to the KCNJ2 mutation. In fact, loss-of-function mutations in KCNJ2 cause Andersen–Tawil syndrome, which is characterized by prolonged repolarization, dysmorphic features, and periodic paralysis.\cite{13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31} In the family of our female patient, we could not perform extensive genetic testing. We cannot exclude the possibility of the presence of other affected genes. Further analyses using knock-in mice or induced pluripotent stem cells would culminate monumental insight into the relationship between the KCNJ2 M301K mutation and the patient’s extra-cardiac phenotypes.

### 4.6 Conclusions

We described a novel KCNJ2 gain-of-function mutation, M301K, in a patient with SQTS. Functional assays revealed no functional currents in the homozygous channels, whereas impaired inward rectification in the heterozygous channels manifested in larger outward currents, which is a novel mechanism predisposing SQTS.

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