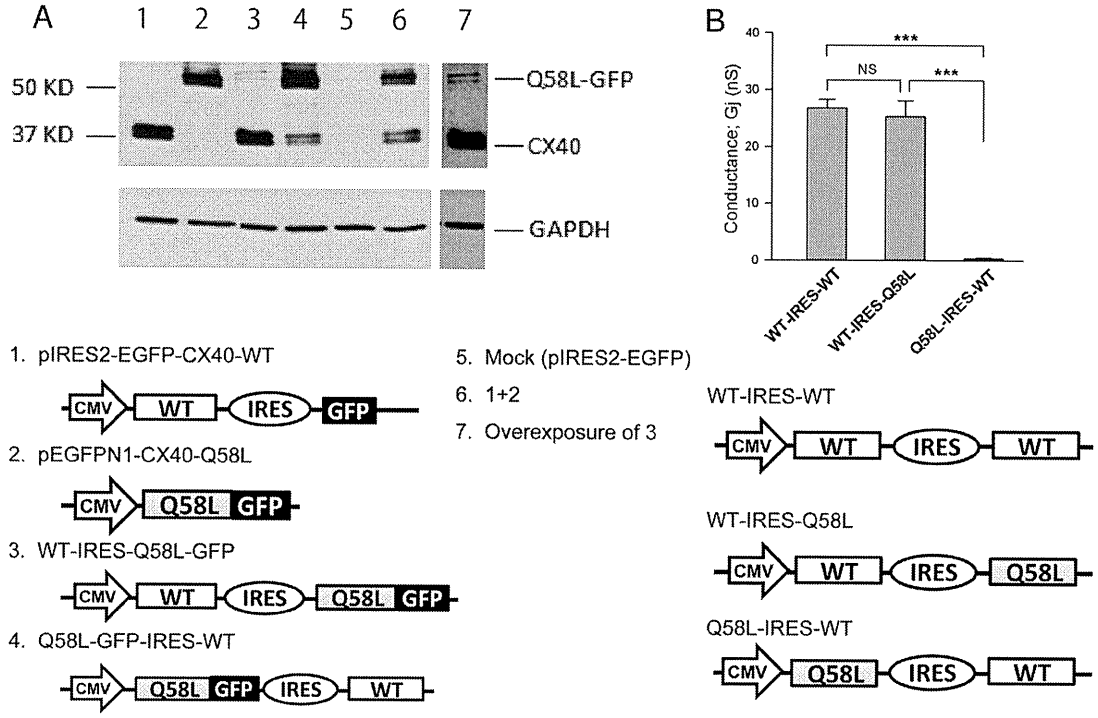

Figure 6. Mutant subunit abundance correlated with gap junction function. **A**, Neuroblastoma cells were transiently transfected with 3 μg Cx40 constructs in IRES plasmids. Cell lysates were analyzed by western blot using anti-Cx40 (top) and anti-GAPDH antibodies (bottom). The number in each lane corresponds to the plasmid noted below the image. Samples from cells cotransfected with plasmids 1 and 2 (1.5 μg each) were loaded on lane 6. Double bands of Cx40-WT (40 kDa) and Q58L-EGFP (67 kDa) are shown in lanes 3, 4, 6, and 7. Results were repeated in 3 separate experiments. Overexposure (lane 7) confirmed expression of the high-molecular-weight protein in lane 3. **B**, Junctional conductance of homomeric and heteromeric constructs (WT-IRES-Q58L and Q58L-IRES-WT). Conductance of cell pairs expressing WT-IRES-WT (n=17) was comparable to heteromeric construct WT-IRES-Q58L (n=17). However, converse heteromeric construct Q58L-IRES-WT (n=15) showed significantly reduced conductance ($P<0.001$ versus WT-IRES-WT and WT-IRES-Q58L). ***$P<0.001$. NS indicates not significant; WT, wild type.

these observations, we constructed a homomeric Cx40-WT plasmid (WT-IRES-WT) and heteromeric plasmids of Cx40-WT and Cx40-Q58L with different orientations (WT-IRES-Q58L and Q58L-IRES-WT) (Figure 6B). The junctional conductance of cell pairs expressing WT-IRES-Q58L (25.3±2.8 nS, n=17) was nearly indistinguishable from that of the homomeric plasmid WT-IRES-WT (27.8±1.4 nS, n=17, *P* not significant). By contrast, the converse heteromeric construct Q58L-IRES-WT showed substantially reduced junctional conductance (0.29±0.12 nS, n=15, $P<0.001$) comparable with that of the homomeric Q58L (0.56±0.34 nS) (Figure 3A). These results suggest that the final electrophysiological properties of the heteromeric connexons are determined predominantly by the numbers of mutant subunits in each gap junction rather than defined by a dominant-negative effect.

Discussion

Genetic screening confirmed the association of *SCN5A* and *SCN1B* with PFHB1[13–15] and revealed novel mutations within these genes (online-only Data Supplement Table I). More importantly, we identified a particularly severe, early onset case of PFHBI associated with a germ line mutation in *GJA5* in 2 blood relatives (proband and sister) given a clinical diagnosis of PFHBI. The data also indicate that the protein expressed (Cx40-Q58L) failed to form functional gap junctions in an exogenous expression system and decreased the probability of gap junction formation in cells coexpressing the WT protein.

So far, *SCN5A*, *SCB1B*, and *TRPM4* are the only genes associated with PFHB1.[11,13,14] The National Human Genome Research Institute database shows no association of *GJA5* single-nucleotide polymorphisms with arrhythmias or conduction system diseases. PR interval and QRS have been associated with several loci, including *SCN5A*, *SCN10A*, *NKX2.5*, and *TBX5*[21,22] but not *GJA5*, which is located at chromosome 1q21.1. Overall, the present results suggest that *GJA5* is a candidate gene associated with PFHBI, likely in a small fraction of the affected population. Yet, given the limited cosegregation observed in the reported family, we remain cautious in assigning a causative nature to the *GJA5* mutation. It will be of great interest to expand the screening of *GJA5* at the research level to identify other cases associated with amino acid changes in Cx40, although it may be premature to include *GJA5* as a part of the routine diagnostic screen.[17] The present results also emphasize the importance of Cx40 in the maintenance of normal cardiac rhythm.

To our knowledge, this is the first report of a germ line mutation in Cx40 associated with a high risk of ventricular arrhythmias (online-only Data Supplement Figure II). Other studies have shown somatic mutations of Cx40 or Cx43 in patients with idiopathic atrial fibrillation[5,23]; those mutations

were confined to the atria, and conduction abnormalities in the ventricles or His-Purkinje system were not observed. On the other hand, as in all cases involving identified genetic substrates for disease, the possibility of compound mutations in unexamined genes cannot be excluded. We do emphasize that the mutation led to a severe cellular phenotype in an exogenous expression system, supporting the argument that just the Q58L substitution can impair the formation of gap junctions necessary for propagation of action potentials between cells.

The results show that Cx40-Q58L was abundantly expressed in an exogenous system. The protein reached the vicinity of the cell membrane but failed to form gap junction plaques (Figure 5B). This result may be due to impaired docking of mutant hemichannels within the intercellular space because of the mutation in the extracellular loop (Figure 1C). During trafficking, connexin subunits oligomerize to form a hemichannel (or connexon). Once at the site of cell contact, connexons from apposing cells dock, sealing the hydrophilic path (the channel pore) from the extracellular space. The locking of 2 connexons into 1 gap junction channel is believed to stabilize connexin subunits in place, facilitating aggregation of other oligomers into their vicinity and eventually forming a plaque. Amino acid substitutions within the extracellular loop, as in Q58L, can prevent hemichannel docking and, thus, plaque formation.[24] The present biotinylation experiments indicate that the Q58L protein integrates into the cell membrane, supporting the notion that the inability of the Q58L mutation to form functional gap junctions is related to events that occur after the oligomer is delivered to the cell membrane and before a functional dodecamer converts into a functional channel in a gap junction plaque.

Results obtained in cells coexpressing both mutant and WT proteins clearly show that one subunit can significantly influence the fate of the other (Figure 5). This suggests that Cx40-Q58L subunits retain their ability to oligomerize not only with other mutant subunits, but also with the WT protein. The results also present an interesting paradigm in that neither the WT nor the mutant construct exerted a dominant effect over the other. After transfection with equal amounts of cDNA, we found cells where both WT and mutant proteins displayed the phenotype of the mutant construct, whereas in other cases, junctional plaques could be easily discerned (although an outline of the cell, likely resulting from the presence of the FLAG-tagged mutant protein, could still be observed [see red signal in Figure 5D]). These results can be explained if we assume that the probability of proper targeting and integration of a connexon into a plaque decreases as a function of the number of mutant subunits contained. For cotransfection, we used equal amounts of cDNA; however, it is very likely that each cell was transfected with variable amounts of each construct and, thus, expressed variable amounts of each protein. We speculate that a majority (though of unknown stoichiometry) of WT connexin subunits are required in a connexon for proper formation of functional gap junctions. Thus, if a cell captures an abundance of Q58L cDNA, most oligomers will contain an excess of mutant subunits, and gap junction formation will fail. If, on the other hand, that cell captures and expresses more of the WT cDNA, the distribution of the subunits within the oligomer will contain a majority of WT connexins, and the connexon will be properly integrated into a channel. This hypothesis will require further testing, although data presented in Figure 6 support the concept that success or failure of functional channel formation may relate to relative abundance of each protein (WT or mutant). If our hypothesis is correct, it suggests that the distribution of functional gap junctions in the His-Purkinje network of affected individuals could vary significantly among cells, depending on the extent of expression of each allele in each cell. The resulting phenotype may be that of a Purkinje network where gap junction-mediated coupling could be heterogeneous, setting the stage for local conduction block, microreentry, and ventricular arrhythmias at the Purkinje network or at the Purkinje-muscle junction.[1,2]

Overall, we show that both proband and sister have a genotype that (1) is absent in hundreds of control subjects and in the unaffected parent (the father), (2) disrupts an important functional domain of the protein, and (3) disrupts the formation of gap junction channels. The data therefore support the notion of an association between the Cx40 mutation and the clinical phenotype and emphasize the importance of future studies to assess the possible involvement of Cx40 mutations as causative of the disease.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Progressive familial heart block type I, also known as progressive cardiac conduction defect, is an inherited form of cardiac conduction system dysfunction that can lead to severe heart rhythm disturbances, including sudden cardiac death. The genetic causes of this disease are poorly understood. Here, we genetically screened 156 patients with progressive familial heart block type I. In addition to mutations in genes of the voltage-gated cardiac sodium channel complex (*SCN5A* and *SCN1B*), we found a novel germ line mutation in *GJA5,* the gene encoding the gap junction protein connexin40. The disease had an early onset and was associated with otherwise unexplained sudden cardiac death in the proband and his mother. The proband's sister is also affected. Cellular phenotype analysis revealed impaired gap junction formation at cell-cell interfaces and marked reduction of junctional conductance in cells expressing the mutated connexin40 protein. The results emphasize the importance of connexin40 in normal electrical propagation in the cardiac conduction system and open the possibility of including *GJA5* as a target gene for study in patients with progressive familial heart block type I.

REVIEW

Cardiac connexins, mutations and arrhythmias

Mario Delmar[a] *and Naomasa Makita*[b]

Purpose of review
Connexins are the pore forming subunits of gap junction channels. They are essential for cardiac action potential propagation. Connexins are modified at the transcriptional or posttranslational levels under pathological states such as cardiac hypertrophy or ischemia, thus contributing to the arrhythmogenic substrate. However, the relation between nucleotide substitutions in the connexin gene and the occurrence of cardiac arrhythmias remains largely unexplored.

Recent findings
Recent studies have reported an association between nucleotide substitutions in the connexin40 (Cx40) and connexin43 (Cx43) genes (*GJA5* and *GJA1*, respectively) and cardiac arrhythmias. Of note, however, germline mutations in Cx43 are considered causative of oculodentodigital dysplasia, a pleiotropic syndrome wherein cardiac manifestations are notoriously absent.

Summary
Here, we review some of the current knowledge on the association between cardiac connexins and inherited arrhythmias.

Keywords
arrhythmogenic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, connexin40 and connexin43, oculodentodigital dysplasia, progressive familial heart block, sudden infant death syndrome

INTRODUCTION

It is now 60 years since Silvio Weidman's classic study on 'the electrical constants of Purkinje fibers,' in which he beautifully demonstrated that electrotonic propagation in cardiac tissue extended well beyond the size of a single cell [1]. His observations provided the physiological evidence that cardiac cells are electrically coupled via low-resistive pathways. Electron microscopic observations followed, culminating with the elegant work of Revel and Karnovsky [2] showing that, at the site of close appositional membranes in the cardiac intercalated disc, the membranes were not fused but, instead, were separated by a gap with junctions that traversed it, thus leading Revel to later coin the term 'gap junctions.' Our understanding of the molecular nature of these structures, and their function in the normal heart, has expanded enormously since those (and many other) 'giants' of science first paved the way. Yet, it is fair to admit that the role of connexins and of gap junctions in cardiac arrhythmias can still be labeled, at least in some aspects, as 'controversial.' In fact, it is very likely that the role of connexins in arrhythmias is not only limited to their ability to form gap junctions (see, e.g. [3••,4]). In the present review, we focus on the association between cardiac connexins (primarily Cx43 and Cx40) and inherited diseases. We begin by describing what may be the most noticeable paradox: that patients with the only inherited disease clearly ascribed to mutations in the gene coding for connexin43 (Cx43) actually do not present with cardiac arrhythmias. We will then summarize current knowledge on the association between variants/mutations in connexin genes and arrhythmias, and will conclude with the possible implication of connexins in the phenotype of an inherited arrhythmogenic disease (arrhythmogenic cardiomyopathy) wherein loss of gap junction plaques is secondary to disruption of a closely associated protein complex, namely the cardiac desmosome.

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KEY POINTS

- Atrium-specific genetic modifications in connexin40 (Cx40) or in connexin43 (Cx43) can be a substrate for atrial fibrillation, in part by a heterogeneous decrease in myocardial electrical coupling.
- A germline mutation in *GJA5*, the gene coding for Cx40, has been reported as a likely genetic cause of progressive familial heart block type I.
- Cx43 amino acid substitutions have been associated with two isolated cases of sudden infant death syndrome, yet
- Cx43 germline mutations are known as a cause of oculodentodigital dysplasia, a pleiotropic syndrome wherein cardiac manifestations are notoriously absent.
- Cx43 deficiencies secondary to desmosomal mutations are a likely component of the arrhythmia substrate in arrhythmogenic cardiomyopathy.
- Cx43 deficiencies affect not only electrical coupling but also the function of the sodium channel. The relation between Cx43 and the sodium channel is an exciting area that deserves further investigation.

Connexin43 germline mutations and oculodentodigital dysplasia

In 2003, Paznekas *et al.* [5] reported that Cx43 mutations cause the pleiotropic phenotype of oculodentodigital dysplasia (ODDD), an autosomal dominant syndrome that presents with craniofacial (ocular, nasal, dental) and limb dysmorphisms, as well as neurologic manifestations such as spastic paraplegia and neurodegeneration. In their study, the authors reported mutations in the *GJA1* gene, coding for the gap junction protein Cx43, in all 17 families studied. In a later review, the same group compiled a summary of all 62 known *GJA1* substitutions leading to mutations in Cx43 and the ODDD phenotype [6]. From the perspective of the present article, perhaps the most striking feature of Cx43-related ODDD is the absence of a cardiac arrhythmia phenotype in the patients. In fact, the only electrocardiological phenotype reported (a first-degree arrhythmogenic cardiomyopathy block) was later found in other family members not carrying the Cx43 mutation (see supplemental information in [6]). In contrast, in-vitro studies have reported important changes in gap junction channel function consequent to the mutations (see, e.g., [7–11]), and changes in electrical properties and arrhythmias have been reported in murine models of the disease [12,13]. The reason(s) for the lack of a cardiac electrical phenotype in ODDD patients remain unclear. A compensatory increase in other cardiac connexins (Cx40 and Cx45) cannot be discarded. Of note, these results contrast with a recent report ascribing disease causality to a Cx43 amino acid substitution observed in an isolated case of sudden infant death syndrome (see [14▪▪] and also section below).

Somatic mutations of connexin40 and connexin43 and atrial fibrillation

Atrial fibrillation is the most common sustained cardiac arrhythmia, may cause significant morbidity, and is a common cause of stroke [15]. Its prevalence increases with advanced age to about 6% in individuals older than 65 years. The pathogenesis of atrial fibrillation is complex, attributed to dynamic interactions among a wide range of structural, electrophysiological, inflammatory and genetic factors. Most patients with atrial fibrillation have associated cardiovascular diseases such as valve diseases or hypertension; atrial fibrillation can be concurrent with structural alterations of the atrium. However, some atrial fibrillation patients do not have structural alterations or identifiable underlying diseases, constituting a distinct atrial fibrillation subgroup, often referred to as idiopathic or lone atrial fibrillation. Nearly 30% of patients with atrial fibrillation (with or without structural heart disease) have a family history of the disease [16]. These findings suggest that genetic factors may determine whether atrial fibrillation develops or becomes sustained, even in patients with anatomical substrates. Recent genetic studies on familial atrial fibrillation have demonstrated mutations in the genes encoding cardiac ion channels [17–21]. Most of the mutations would be expected to shorten the duration of atrial action potentials and the effective refractory period, thereby predisposing affected individuals to reentry. In addition to cardiac ion channels, gap junctions are the principal determinant of action potential propagation and conduction velocity; atrial tissue-specific mutations in genes coding for connexins have been considered a potential substrate in some cases of idiopathic atrial fibrillation. Indeed, in an initial study, Gollob *et al.* [22] identified four missense mutations in *GJA5*, the gene encoding the gap junction protein Cx40, in the genomic DNA extracted from atrial specimens. Heterologous expression assays showed that mutation P88S in the gene coding for Cx40 prevented the formation of functional Cx40-mediated gap junction channels, and the expressed protein failed to reach the cell surface and assemble gap junction plaques. Interestingly, the atrial tissue of the P88S carrier showed a mosaic pattern comprising both areas of normal expression and others with

reduced expression and intracellular accumulation of the Cx40 protein. A separate patient exhibited two nonallelic mutations, G38D and M163V. These mutations, together with P88S, were found in DNA from atrial tissue but not from lymphocytes, suggesting a somatic source. When expressed in gap junction deficient neuroblastoma cell line N2A, the G38D mutant generated only sparse gap junction plaques, whereas most of the protein remained in the intracellular space. Junctional conductance in cells expressing G38D was significantly lower than wild type. Mutant M163V formed gap junctions with nearly normal distribution and electrical conductance, suggesting that this is most likely a benign polymorphism. In contrast, A96S was a germline mutation and resulted in apparently normal junctions, but their electrical coupling was markedly reduced as compared with wild type. Furthermore, when A96S-Cx40 was coexpressed with wild-type Cx40, junctional conductance was significantly less than that observed when only the wild-type protein was expressed. The results were not different if wild-type Cx43 was also expressed. This observation suggested that mutation A96S oligomerizes with the wild-type protein and acts as a dominant-negative unit. Overall, the data suggested that somatic Cx40 mutations can predispose patients to idiopathic atrial fibrillation, likely by impairing gap junction assembly and/or electrical coupling.

In subsequent studies, Thibodeau *et al.* identified a novel single nucleotide deletion (c.932delC) of *GJA1*, the gene coding for Cx43, and studied it in the atrial tissue of a patient with idiopathic atrial fibrillation [23▪▪]. This mutation resulted in frameshift with 36 aberrant amino acids followed by a premature stop codon, leading to truncation of the C-terminal domain of Cx43. The mutation was absent from the lymphocyte DNA of the patient, indicating genetic mosaicism. Protein trafficking studies demonstrated intracellular retention of the mutant protein and a dominant-negative effect on gap junction formation of both wild-type Cx43 and Cx40. Electrophysiological studies revealed no electrical coupling of cells expressing the mutant protein alone and significant reductions in coupling when coexpressed with wild-type connexins. Atrial tissue of the affected patient showed a mosaic pattern of Cx43 immunostaining, with normal appearing intercalated disks and significant intracellular staining of Cx43 in adjacent cells. These data lead to the hypothesis that atrium-specific genetic modifications in Cx43 can be a substrate for atrial fibrillation, in part by a heterogeneous decrease in myocardial electrical coupling. Of interest, whether these Cx40 or Cx43 mutations can affect the function of other ion channels remains to be determined [3▪▪,24▪▪,25,26].

Connexin43 amino acid substitutions associated with two isolated cases of sudden infant death syndrome

Sudden infant death syndrome (SIDS) is defined as the sudden and otherwise unexplained loss of life of a child under the age of one. For the diagnosis of SIDS, the cause of death must remain unexplained after a thorough case investigation including medical autopsy, death scene investigation and detailed review of the clinical history. Although SIDS remains poorly understood and its cause is largely unknown, recent clinical and molecular evidence has implicated heritable arrhythmia syndromes due to mutations in cardiac ion channels as a cause of up to 10–15% of SIDS [27,28]. Studies in murine models have shown that complete loss of Cx43 expression leads to arrhythmias and sudden death. In a recent study, Van Norstrand *et al.* [14▪▪] reported two novel missense mutations in *GJA1* (amino acid substitutions E42K and S272P) in a group of 292 cases of SIDS. Analysis of the E42K victim's parental DNA demonstrated a de-novo mutation. Immunofluorescence demonstrated no trafficking abnormalities for either mutation and S272P demonstrated normal junctional conductance. However, junctional conductance measurements for the E42K mutation showed a loss of function that was not rescued by coexpression of the wild-type construct. Moreover, cardiac tissue obtained from the patient with the E42K mutation demonstrated a mosaic immunostaining pattern for Cx43 protein. These results open the possibility that mutations in *GJA1*, the gene coding for Cx43, may be associated with at least some cases of SIDS. It is interesting to note, however, that there are a number of reported cases of Cx43 mutations in patients afflicted with ODDD [6]. A number of those mutations are predicted to significantly affect the function of Cx43 channels [5]. Yet, cardiac arrhythmias and/or SIDS have not been reported to occur in the afflicted families. Whether the mutations reported by Van Norstrand *et al.* [14▪▪] were indeed causative of disease is an interesting question that deserves further investigation.

Connexin40 germline mutation and progressive familial heart block type-1

Although multiple mutations in genes coding for components of the voltage-gated channel complexes have been previously described in relation to arrhythmias and sudden death in the young [29], and connexin mutations have been implicated in atrial fibrillation [22,30] and in two isolated cases of SIDS [14▪▪], no study has identified an association between germline, cosegregated, inherited

mutations in gap junction proteins and inherited ventricular arrhythmias in humans.

Progressive familial heart block type I (PFHBI), also known as progressive cardiac conduction defect (PCCD) or Lenegre–Lev disease [31,32] is a dominant inherited disorder of the His–Purkinje system. Affected individuals show electrocardiographic evidence of bundle branch disease, that is, right bundle branch block, left anterior or posterior hemiblock, or complete heart block, with broad QRS complexes. The disease can progress from a normal electrocardiogram to right bundle branch block and from the latter to complete heart block. Affected individuals often present with family history of syncope, pacemaker implantation and/or sudden death [33]. Although structural abnormalities have been invoked as cause of the disease [31,32], a number of cases present with normal cardiac structure and contractile function. Mutations have been found in genes that influence cardiac excitability, such as cardiac Na channels (*SCN5A* [33] and *SCN1B* [34]) and transient receptor potential nonselective cation channel, subfamily M (*TRPM4* [35]). Recently, Makita *et al.* [36▪▪] reported that a germline mutation in *GJA5*, the gene coding for Cx40, also associated with PFHBI. Indeed, the authors screened 156 probands with diagnosis of PFHBI. In addition to 12 sodium channel mutations (*SCN5A* and *SCN1B*), they found a germline *GJA5* (Cx40) mutation (Q58L) in an afflicted family [36▪▪]. The disease had an early onset and was associated with otherwise unexplained cardiac sudden death in the proband and the proband's mother. The proband's sister is also affected. Heterologous expression of Cx40-Q58L in connexin-deficient N2A cells resulted in marked reduction of junctional conductance and diffuse localization of immunoreactive proteins in the vicinity of the plasma membrane without formation of gap junctions. Heteromeric co-transfection of Cx40-atrial fibrillation and Cx40-Q58L resulted in homogenous distribution of proteins in the plasma membrane rather than in membrane plaques in about 50% of cells; well-defined gap junctions were observed in other cells. Junctional conductance values correlated with the distribution of gap junction plaques. Mutation Cx40-Q58L impaired gap junction formation at cell–cell interfaces. Coexpression experiments indicated that Cx40-atrial fibrillation protein provided only partial rescue of the Cx40-Q58L cellular phenotype. This study represented the first demonstration of an association between germline, cosegregated (albeit in a small number of family members), inherited mutations in gap junction proteins and inherited ventricular arrhythmias in humans. The data also emphasize the importance of Cx40 in normal propagation in the specialized conduction system.

For both the relation between Cx43 and SIDS and that of Cx40 and PFHBI the challenge remains the limited (and admittedly weak) genetic evidence for the causal role of connexins in cardiac rhythm/conduction disorders. Functional studies in experimental models, though important, have a limited power to distinguish between functional polymorphisms and causal variants, even though the functional variants might contribute to the phenotype. Further research, exploring family-based associations between nucleotide substitutions in connexin genes and these or other disorders, will help clarify the role of connexin mutations as causative of arrhythmia disease.

Connexin43 deficiency secondary to mutations in desmosomal proteins: the case of arrhythmogenic cardiomyopathy

Arrhythmogenic cardiomyopathy (a term preferred to the more conventional one of 'arrhythmogenic right ventricular cardiomyopathy' or ARVC, given the common occurrence of left ventricular involvement; see [37▪▪,38]) is an inherited disease characterized by progressive myocardial loss, fibrosis, adiposis and severe ventricular arrhythmias. It is a frequent cause of sudden death in the young. Life-threatening arrhythmias often occur in the concealed phase of the disease, prior to overt structural damage [38]. Mutations in genes coding for desmosomal proteins are most commonly associated with the familial form of the disease. The question arises as to how mutations in proteins involved in mechanical coupling bring about electrical dysfunction and arrhythmias, particularly in cases wherein the lethal arrhythmias precede major disruption of the structural integrity of the heart. A first indication of involvement of ion channels as arrhythmia substrates came from the observations of Kaplan *et al.* [39,40], reporting loss of gap junction plaques in the hearts of patients affected with the disease. These results were confirmed and expanded in a follow-up study [41]. In-vitro studies have also shown that loss of expression of desmosomal proteins causes loss of gap junction plaques at sites of intercellular contact and Cx43 remodeling [42]. The intimate mechanisms leading to changes in Cx43 in arrhythmogenic cardiomyopathy remain unknown, though they may be related to the critical importance of mechanical attachment on Cx43 trafficking. Of note, however, several studies in murine models have demonstrated that a reduction in Cx43 abundance of over 50% does not lead to arrhythmias [43–46]. Additional studies have shown that loss of

desmosomal proteins also leads to a decrease in amplitude and a shift in gating kinetics of the sodium current [24▪▪,25]. The latter suggests that changes in both electrical coupling and cell excitability may provide a cellular substrate for the generation of arrhythmias in arrhythmogenic cardiomyopathy. Interestingly, recent studies have suggested a direct cross-talk between connexin and the sodium channel complex [3▪▪,24▪▪]. In fact, the loss of cell–cell coupling, as well as loss of Cx43 expression, causes a significant reduction in sodium current amplitude [3▪▪,26]. These results suggest that there is a gap junction-independent role for Cx43 in the heart, namely, the preservation of the functional integrity of the sodium channel complex, and that inherited or acquired diseases leading to Cx43 remodeling may, indirectly, disrupt the excitability of the cell (see also [47,48▪]). Whether this mechanism participates as a substrate for arrhythmias in patients with arrhythmogenic cardiomyopathy is a question for future investigation.

CONCLUSION

In summary, a series of studies have demonstrated an association between changes in the primary sequence of Cx43, or Cx40 and arrhythmias. The paradox remains, however, that patients with a variety of somatic mutations in Cx43 present a pleiotropic phenotype (ODDD) that does not include cardiac arrhythmias. Finally, Cx43 may be a critical component in the generation of arrhythmias observed in patients with desmosomal mutations, both by disrupting electrical coupling and by affecting electrical excitability. The role of desmosomes in electrical function, and the gap junction-independent functions of Cx43, are two very interesting topics that deserve future investigation.

Acknowledgements

None.

Conflicts of interest

There are no conflicts of interest.

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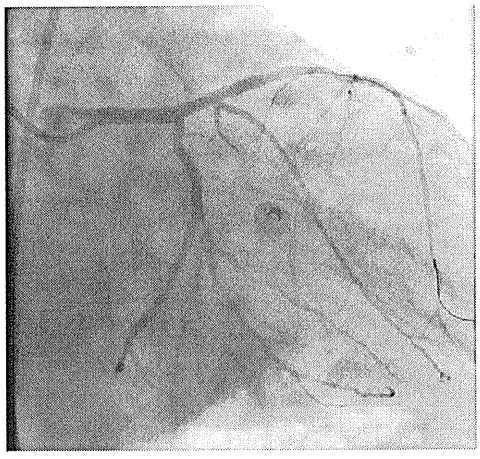

Fig. 2. Left coronary system after succesful percutanous coronary intervention.

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology (Shewan and Coats 2010; 144: 1–2).

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Electrocardiographic abnormalities and risk of complete atrioventricular block☆

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For more than a half-century, the benign prognosis of first-degree atrioventricular block has been repeatedly shown [1–5]. However, these studies have limitations, including the study populations (with respect to age and gender) and relatively small numbers of person-years of observation compared to low incidences of advanced atrioventricular block. A recent community-based cohort study showed that first-degree atrioventricular block is associated with the future need for pacemaker implantation and all-cause mortality [6]. Furthermore, the prolongation of QRS duration and bundle branch block have also been associated with cardiovascular events [7–9]. Here, we studied the association of common electrocardiographic findings frequently encountered in clinical practice with the risk of developing complete (third-degree) atrioventricular block in the general population.

This prospective, community-based, observational cohort study was based on annual health examinations in the Niigata prefecture, Japan [10,11]. In the prefecture, annual health examinations supported by the administration are available to adults, regardless of the presence or absence of illness. The annual examination consists of a face-to-face interview for a detailed medical history, physical examination, blood draw, chest X-ray, and 12-lead electrocardiogram. This study includes individuals who had an examination in 1993 as the baseline and ≥1 subsequent annual examination through 2005. Exclusion criteria included the presence of complete atrioventricular block or permanent pacemakers at the time of the initial examination. The diagnoses of electrocardiograms were made by physicians using the definitions based on Minnesota code. Complete atrioventricular block was diagnosed from medical history and a 12-lead electrocardiogram at a follow-up visit. Left ventricular hypertrophy was diagnosed according to the high amplitude R wave criteria [12]. Nonspecific ST-segment and/or T-wave (ST-T) abnormalities were defined as follows: (1) mild ST-T abnormality showing flat T-wave, or negative or diphasic T-wave (negative–positive type) with negative phase <1.0 mm; and (2) severe ST-T abnormality showing negative or

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Table 1
Baseline characteristics of study participants.

Characteristic	$n=141,798$
Age, y	60 ± 10
Women, *n* (%)	94,872 (66.9)
Antihypertensive drug, *n* (%)	27,377 (19.3)
Heart disease, *n* (%)	3711 (2.6)
Electrocardiographic abnormalities	
First-degree atrioventricular block, *n* (%)	4053 (2.9)
Right bundle branch block, *n* (%)	2490 (1.8)
Left bundle branch block, *n* (%)	234 (0.2)
Left ventricular hypertrophy, *n* (%)	12,395 (8.7)
Atrial fibrillation, *n* (%)	1023 (0.7)
ST-T abnormality	
Mild	5742 (4.0)
Severe	564 (0.4)

Plus–minus values are means ± SD.

Table 3
Electrocardiographic abnormalities and risk of developing complete atrioventricular block in all individuals[a].

Variables	HR (95% CI)	*P* value
First-degree atrioventricular block	7.61 (4.69–12.35)	<0.001
Right bundle branch block	0.50 (0.07–3.58)	0.49
Left bundle branch block	30.08 (12.90–70.12)	<0.001
Left ventricular hypertrophy	10.07 (4.69–21.61)	0.001
Atrial fibrillation	2.25 (1.37–3.69)	<0.001
ST-T abnormality		<0.001 for trend
Mild	3.66 (2.03–6.59)	<0.001
Severe	4.52 (1.09–18.7)	0.03

[a] Models are adjusted for age, gender, body mass index, hypertension, diabetes, dyslipidemia, and heart disease. HR denotes hazard ratio; CI, confidence interval.

diphasic T-wave (negative–positive type) with negative phase ≥1.0 mm, horizontal or downward sloping ST-segment depression ≥0.5 mm, or upward sloping ST depression ≥1.0 mm. Hazard ratio (HR) and 95% confidence interval (CI) were calculated from Cox proportional-hazards models adjusted for age, gender, body mass index, hypertension, diabetes, dyslipidemia, and heart disease. A two-sided $P<0.05$ was considered statistically significant.

A total of 141,798 individuals (age, 60 ± 10 years; 94,872 women) were included in this study (Table 1). During a follow-up of 8.2 ± 4.5 years (range, 1 to 12 years), 107 individuals (0.08%) developed complete atrioventricular block, and the incidence of complete atrioventricular block was 9 per 100,000 person-years. Incidences of complete atrioventricular block in individuals with electrocardiographic abnormalities are shown in Table 2. In the multivariate models, first-degree atrioventricular block, left bundle branch block, electrocardiographic left ventricular hypertrophy, atrial fibrillation, and ST-T abnormality, but not right bundle branch block, were associated with the increased risk of developing complete atrioventricular block (Table 3). As for ST-T abnormality, there was a dose-dependent association between the severity and the risk of complete atrioventricular block (P for trend<0.001).

To study the direct effects of conduction abnormities on a risk of developing complete atrioventricular block, the analyses were repeated in 96,378 individuals who did not have structural heart disease, hypertension, antihypertensive drugs, electrocardiographic left ventricular hypertrophy, atrial fibrillation, and ST-T abnormality at baseline and did not develop interim heart disease during a follow-up. We replicated similar results to the prior analyses: first-degree atrioventricular block (HR, 15.72; 95% CI, 7.62–32.45; $P<0.001$) and left bundle branch block (HR, 74.41; CI, 22.1–250.53; $P<0.001$), but not right bundle branch block (HR, 1.41; CI, 0.19–10.33; $P=0.73$) were associated with an increased risk of developing complete atrioventricular block.

Table 2
Incidence of complete atrioventricular block with electrocardiographic covariates.

Covariates	Development of complete atrioventricular block, *n* (%)	Incidence of complete atrioventricular block/100,000 person-y (95% CI)
First-degree atrioventricular block	23 (0.57)	79 (47–111)
Right bundle branch block	1 (0.04)	5 (−5–16)
Left bundle branch block	6 (2.56)	410 (83–738)
Left ventricular hypertrophy	20 (0.16)	22 (12–31)
Atrial fibrillation	9 (0.88)	150 (52–248)
ST-T abnormality		
Mild	14 (0.24)	34 (16–51)
Severe	2 (0.35)	55 (−21–132)

CI denotes confidence interval.

This study demonstrated that first-degree atrioventricular block and left bundle branch block, but not right bundle branch block, increased the risk of developing complete atrioventricular block in a large general population, consistent with the previous studies [6–9]. Structural heart disease is one of the major causes of advanced conduction disease and there is the possibility that our results were driven by concomitant heart disease [13,14]. Furthermore, left bundle branch block causes dyssynchronous contraction of the left ventricle, possibly resulting in heart failure and the increased risk of atrioventricular block [7,15]. However, the association of first-degree atrioventricular block and left bundle branch block with the increased risk of complete atrioventricular block was significant after adjustment for heart disease and other variables, and remained significant in individuals without heart disease, suggesting that these conduction abnormalities progressed to advanced atrioventricular block.

Furthermore, we identified novel electrocardiographic risk factors for complete atrioventricular block: left ventricular hypertrophy, ST-segment abnormality, and atrial fibrillation, although the mechanisms by which these abnormalities cause atrioventricular block are unknown. Hypertrophy of the interventricular septum may cause microangiopathy and degeneration of the conduction system [16–19]. However, the frequency of complete atrioventricular block is not increased in athletes, in whom left ventricular hypertrophy is a common electrocardiographic finding, and complete atrioventricular block is very rare in patients with hypertrophic cardiomyopathy [20–23]. ST-segment abnormality may have been due to a latent myocardial disorder, while ST-segment abnormality was associated with complete atrioventricular block in individuals without heart disease. Alternatively, ST-segment abnormality may have resulted from left ventricular hypertrophy, which increased the risk of atrioventricular block in this study [24]. Very rapid impulses to conduction system during atrial fibrillation may result in developing advanced atrioventricular block. Actually, atrial fibrillation with slow ventricular response accounts for 15% to 20% of indications for pacemaker therapy [25,26].

In conclusion, we identified the electrocardiographic abnormalities that increased the risk of developing complete atrioventricular block by up to 30 times. Careful follow-up for an incident event associated with bradycardia and regular electrocardiogram recordings to detect progression of conduction disease may be important in individuals with these risk factors.

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Hypertensive crisis: Comparison between diabetics and non-diabetics — Response to comment

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We would like to thank Professor Martin JF for his warm words and his valuable comments.

In our latest study we were not concerned differentiating true hypertensive crisis from pseudocrisis.

Patients with very high blood pressure, once they are symptomatic they don't belong to pseudocrisis category [1]. All our patients were symptomatic and been assessed through the emergency room, furthermore, absence of acute target organ involvement will prevent subjecting them to hazardous overtreatment [2].

We believe that defining hypertensive urgency where no acute target organ involvement under threat is so important. Such definition will guide the rate and urgency of blood pressure lowering, thus avoiding the anticipated morbidity of overtreatment. Even though, hypertensive urgency and hypertensive pseudocrisis are not synonymous but their common interception that acute blood pressure lowering is not indicated and in contrary it can be hazardous.

Concerning the relationship between the stress induced hyperglycemia and overall prognosis we agree with you that it can be a dismal sign.

It is so hard for a cardiologist to explain the pathophysiology of diabetes; however, a strong body of evidence suggests that patients admitted through the emergency with acute elevation of blood sugar warrant a formal GTT test as a follow up [3].

Unsurprisingly significant number of these patients will turn to be diabetics.

Furthermore, Gornik et al., recently has defined an elevated or alarming serum sugar of more than 7.8 mmol/L which is even a lower cutoff point for random hyperglycemia defined by other as 8 mmol/L [3,4].

In other words the threshold to define random hyperglycemia in the acute setting is lowered.

We agree with Chenug et al., that a major limitation of their study was the anticipation that some of their patients admitted with

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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, I_{Kr} (SQT1)[2]; *KCNQ1* encoding the α-subunit of the slowly activating delayed rectifier potassium channels, I_{Ks} (SQT2)[3]; *KCNJ2* encoding

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the Kir2.1 channels that underlie the inward rectifier potassium currents, I_{K1} (SQT3)[4]; *CACNA1C*, *CACNB2b*, and *CACNA2D1*, which encode the α1C, β2b, and α2δ-1-subunits of cardiac L-type calcium channels (SQT4, SQT5,[5] and SQT6[6]), 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.[2–5,7,8] 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*, *KCNE1-3*, *KCNJ2*, *CACNA1C*, and *SCN5A* by denaturing high-performance liquid chromatography using a WAVE System Model 3500 (Transgenomic, 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).[9] 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 pre-plated on 100 mm culture dishes for 1 h at 37°C in 5% CO_2 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).[11]

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 (YPYDVPDYA) 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 $MgCl_2$, 1 EGTA, and 10 HEPES (pH 7.3 with KOH). The bath solution was composed of (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 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, $MgCl_2$ 1, MgATP 4, NaCl 10, and HEPES 10 (pH 7.2 with KOH). Tyrode solution contained (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 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 (APD_{90}).

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.

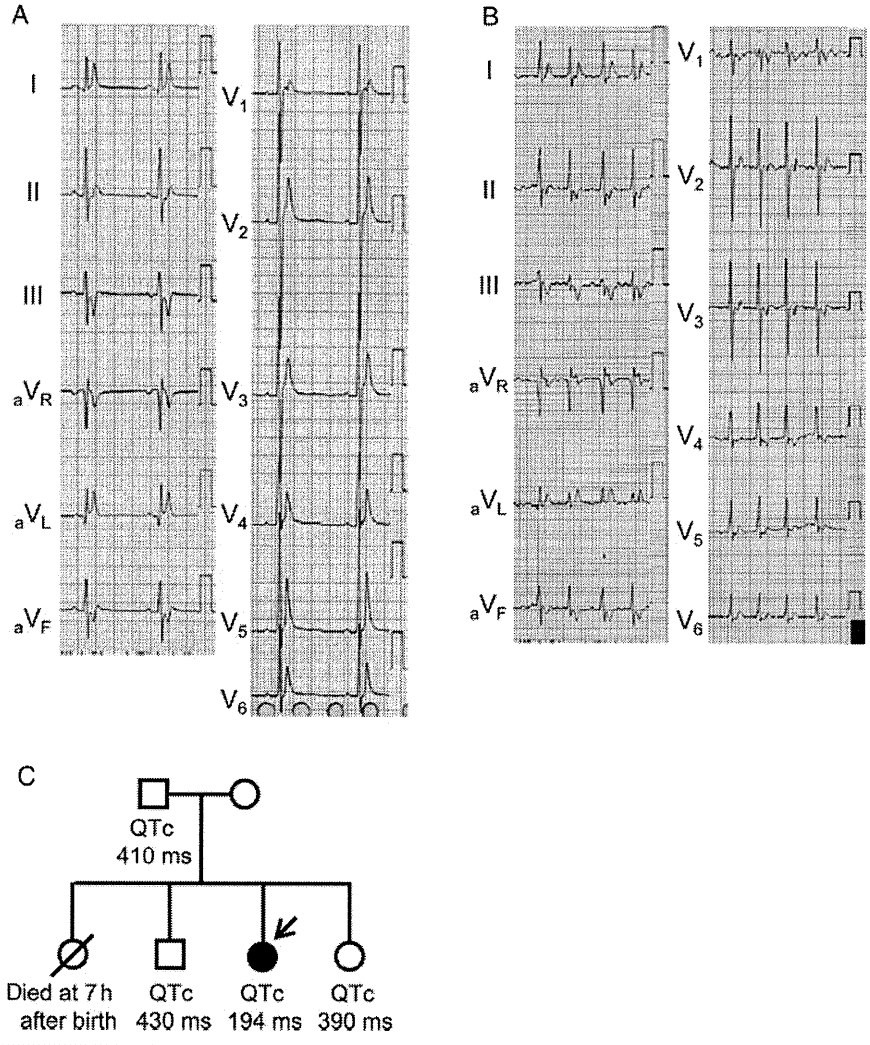

Figure 1 ECG of the proband and family pedigree. ECG shows sinus rhythm (*A*) and AF (*B*). The QT and QTc intervals were 172 and 194 ms, respectively. (*C*) Family pedigree. Arrow indicates the proband; a filled symbol indicates clinically and genetically affected individual.

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*). Met-301 is located in the C-terminal cytoplasmic domain of the channel (*Figure 2B*).[13] 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[10] (*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.

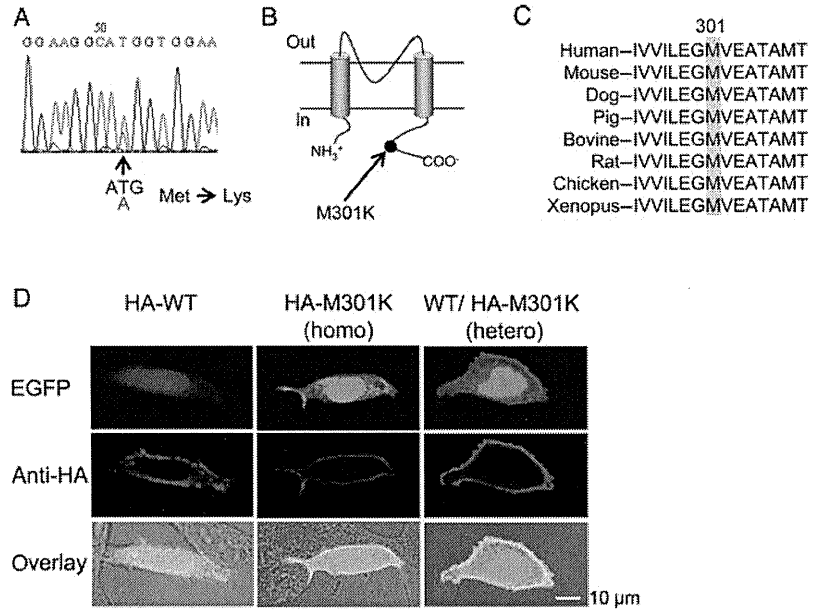

Figure 2 DNA sequence, topology, and homology. (*A*) Mutated DNA sequences derived from patient's genomic DNA. The trace shows a heterozygous substitution of thymine to adenine resulting in the amino acid change M301K. (*B*) Topology of the Kir2.1 channel showing localization of M301. (*C*) Amino acid sequence alignment of Kir2.1 channels from various species in the region surrounding codon 301 (highlighted). (*D*) Cellular localization of WT and mutant Kir2.1 channels. HA-WT indicates HA-tagged KCNJ2-WT, HA-M301K;HA-tagged KCNJ2-M301K, and WT/HA-M301K;KCNJ2-WT without HA-tagging and HA-tagged KCNJ2-M301K. The upper panel shows GFP, the middle panel shows the red fluorescence of the secondary anti-HA antibody, and the bottom panel is a mergence of the green fluorescence, red fluorescence, and transmission.

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,[13,14] 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.[13–15] 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.[15] *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*).

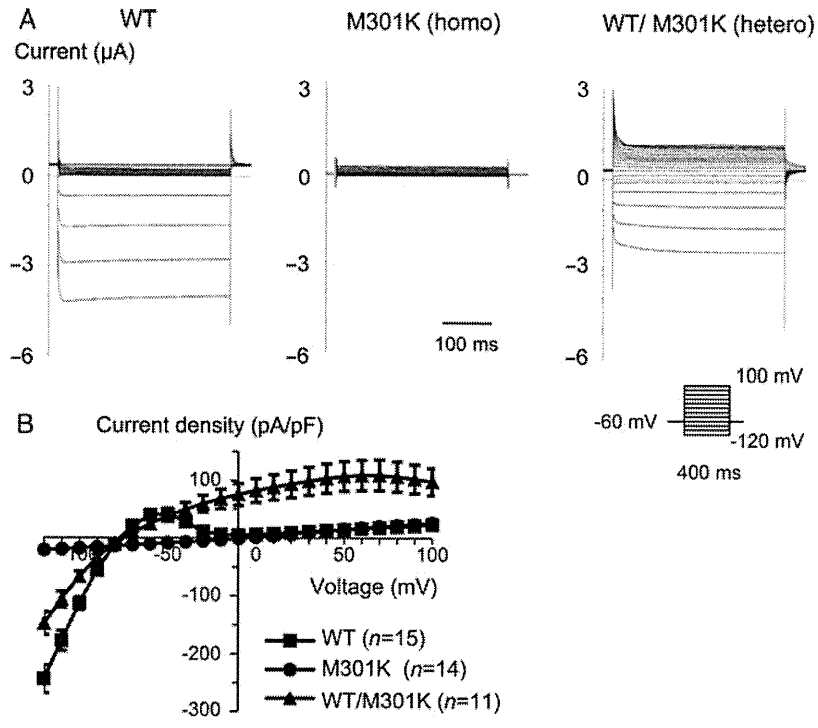

Figure 3 Voltage-clamp recordings from transfected HEK 293 cells. (*A*) Representative current traces of WT, M301K, and WT/M301K. Currents were elicited by 400 ms depolarizing voltage steps from −120 to +100 mV and from a holding potential of −60 mV. (*B*) Current–voltage relationships are plotted as 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 ($P < 0.05$).

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- and WT/M301K-overexpressed groups (*Figure 5A* middle and right panels, respectively) and we could further note that the dome is nearly lost in the WT/M301K group. APD_{90} was significantly abbreviated in the *KCNJ2* WT-overexpressed group (28.2 ± 3.4 ms, $n = 10$, $P < 0.001$, *Figure 5A*, middle panel) in comparison with the control group (123.3 ± 12.2 ms, $n = 11$, *Figure 5A*, left 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.

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,[2–7,16] 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

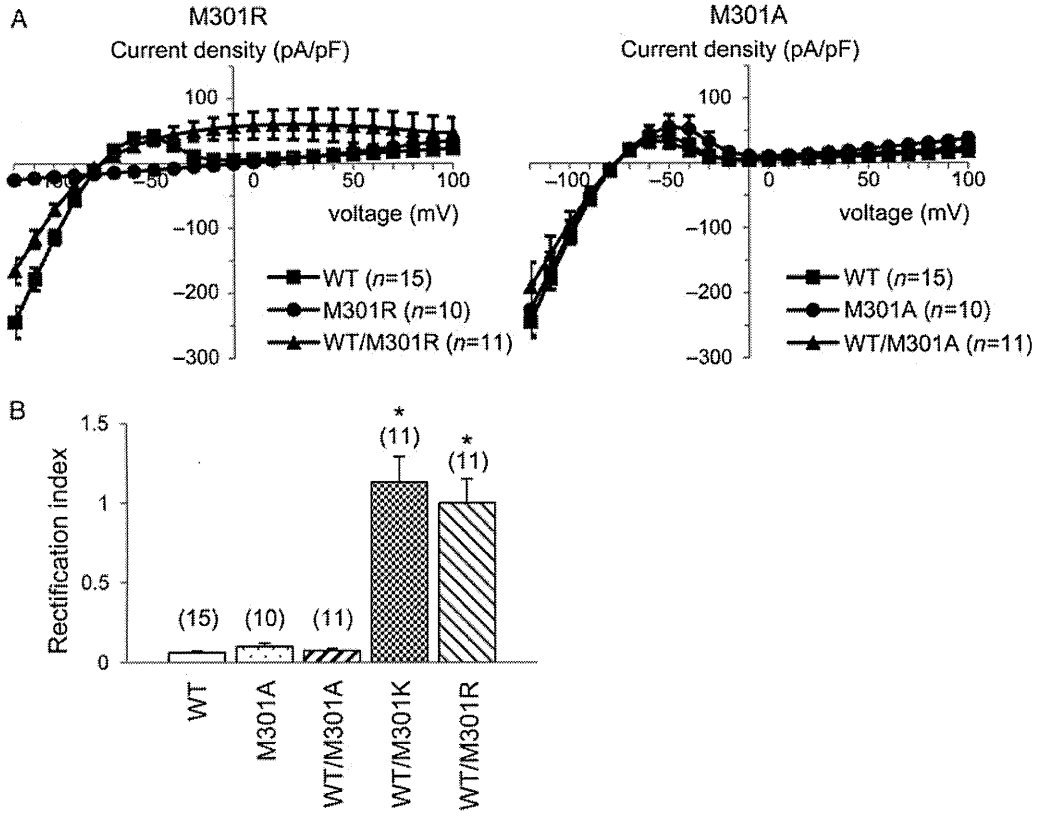

Figure 4 Comparison of macroscopic currents through WT Kir2.1 and mutants. (*A*) Current–voltage relationships for WT, M301R, and M301A are shown. M301R mutant channels displayed no functional currents and WT/M301R mutant channels displayed decreased inward rectification. On the other hand, the currents recorded in the homozygous M301A and heterozygous WT/M301A mutant channels showed no significant difference from WT. (*B*) Rectification index for WT ($n = 15$), M301A ($n = 10$), WT/M301A ($n = 11$), WT/M301K ($n = 11$), and WT/M301R ($n = 11$) channels. The rectification index was calculated by dividing the value of the outward currents measured at 0 mV by the absolute value of the inward currents measured at −100 mV. *$P < 0.001$.

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.[4] 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).[4] Another gain-of-function *KCNJ2* mutation, V93I, was reported in a familial AF case.[17] 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+}.[18–20] Channel blockade by physiological concentrations of Mg^{2+} is influenced by the electrostatic negativity within the cytoplasmic pore.[15] 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.[14,21] 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

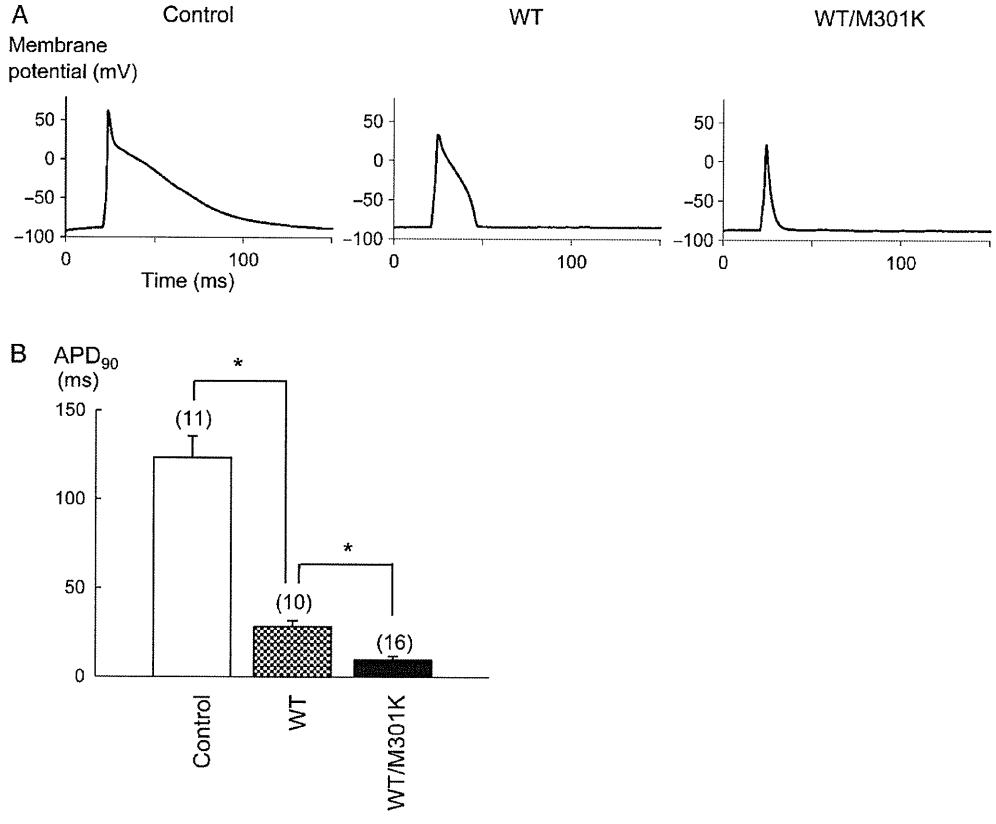

Figure 5 Effects of the M301K mutation on NRVM action potentials. Typical action potentials were demonstrated in a non-transfected cell (*A*), in a WT-overexpressed cell (*B*), and in a heterozygous overexpressed cell (*C*). Graphs show APD at 90% repolarization from the overshoot (*D*). In WT-overexpressed NRVMs, the plateau phase of the cardiac AP was markedly abbreviated, resulting in short repolarization. Under the heterozygous overexpressed condition, the results exhibited virtually no plateau phase, and the mean APD_{90} was significantly shorter in comparison with WT overexpressed alone. *$P < 0.001$.

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 non-transfected myocytes (*Figure 5B*). These results are consistent with a previously published report.[27] Notably, heterozygous overexpression with WT and M301K further amplified the shortened APD (*Figure 5C*). These results were compatible with the electrophysiological changes assessed in HEK 293 cells, because the heterozygous WT/M301K channels showed a larger outward current than WT Kir2.1 channels under the physiological range of membrane potentials (*Figure 3*). Weak inward rectification observed in the heterozygous WT/M301K channels suggests that potassium ion can get through Kir2.1 channel at depolarized potential, probably resulting in loss of the action potential dome recorded in the *KCNJ2* WT/M301K-overexpressed group. The experiments were performed using a transient overexpression system that was different from the patient's heart, and the amount of overexpressed channels was difficult to be estimated accurately. But, these results are beneficial in understanding that the heterozygous *KCNJ2* M301K mutation could abbreviate APD and cause an extremely short-QT interval in the patient's ECG.

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.[1–5,7] In this patient, however, additional medical histories not limited to arrhythmias, such as severe mental retardation, abnormal proliferation of the oesophageal blood vessels, epilepsy, and Kawasaki' 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 bony structures of the craniofacial region, extremities, and vertebrae,[29–31] 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.[10,32] 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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