Long QT syndrome with compound mutations is associated with a more severe phenotype: A Japanese multicenter study

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BACKGROUND: Long QT syndrome (LQTS) can be caused by mutations in the cardiac ion channels. Compound mutations occur at a frequency of 4% to 11% among genotyped LQTS cases.

OBJECTIVE: The purpose of this study was to determine the clinical characteristics and manner of onset of cardiac events in Japanese patients with LQTS and compound mutations.

METHODS: Six hundred three genotyped LQTS patients (310 probands and 293 family members) were divided into two groups: those with a single mutation (n = 568) and those with two mutations (n = 35). Clinical phenotypes were compared between the two groups.

RESULTS: Of 310 genotyped probands, 26 (8.4%) had two mutations in the same or different LQTS-related genes (compound mutations). Among the 603 LQTS patients, compound mutation carriers had significantly longer QTc interval (510 ± 56 ms vs 478 ± 53 ms, $P = .001$) and younger age at onset of cardiac events (10 ± 8 years vs 18 ± 16 years, $P = .043$) than did single mutation carriers. The incidence rate of cardiac events before age 40 years and use of beta-blocker therapy among compound mutation carriers also were different than in single mutation carriers. Subgroup analysis showed more cardiac events in LQTS type 1 (LQT1) and type 2 (LQT2) compound mutations compared to single LQT1 and LQT2 mutations.

CONCLUSION: Compound mutation carriers are associated with a more severe phenotype than single mutation carriers.

KEYWORDS Compound; Gene; Long QT syndrome; Mutation

ABBREVIATION QTS = long QT syndrome

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Introduction

Congenital long QT syndrome (LQTS) is a heterogeneous disease characterized by prolonged ventricular repolarization and episodes of syncope and/or life-threatening cardiac arrhythmias, particularly polymorphic ventricular tachycardia.[1] Several disease-causing genes have been identified, including genes encoding cardiac ion channel-composing proteins, namely, *KCNQ1* (LQT1), *KCNH2* (LQT2), *SCN5A* (LQT3), *KCNE1* (LQT5), *KCNE2* (LQT6), *KCNJ2* (LQT7), and *CACNA1C* (LQT8), and genes encoding a family of versatile membrane adapters, namely, *ANK2* (LQT4), *CAV3* (LQT9), *SCN4B* (LQT10), *AKAPs* (LQT11), and *SNTA1* (LQT12).[2–5] Two modes of inheritance are involved in this syndrome, which exhibits both an autosomal dominant and an autosomal recessive pattern. The majority of LQTS cases are inherited in an autosomal dominant fashion. This pattern, which has been named as Romano-Ward syndrome,[6,7] can result from a single mutation in one

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of the LQTS candidate genes. On the other hand, Jervell and Lange-Nielsen syndrome, which is inherited in an autosomal recessive fashion, is very rare,[8] affecting less than 1% of LQTS cases. It is caused by homozygous or compound heterozygous mutations of *KCNQ1* or *KCNE1*.[9,10]

Genetic analysis sometimes reveals two or more mutations in LQTS patients with clinical phenotypes of Romano-Ward syndrome. These compound mutations were shown to be associated with an increased arrhythmic risk.[11,12] However, most previous studies were conducted in Caucasian patients, and few systematic studies have involved Asian cohorts. In the present study, we analyzed the clinical characteristics of LQTS patients who were registered in a Japanese multicenter study. Analysis of the more 600 genotyped patients revealed that LQTS patients with compound mutations not only were common in Japan (8.4% among probands) but were associated with longer QTc and earlier onset of cardiac events. In patients who initially are diagnosed as LQT1 or LQT2, additional mutations may be present if patients have a more severe phenotype than expected; therefore, conducting a survey for major LQTS-related genes is critically important.

Methods

Patients and data collection

Major candidate genes were analyzed in 612 consecutive and unrelated probands with a suspected clinical diagnosis of congenital LQTS, who were referred to four centers in Japan (Shiga University of Medical Science, Otsu; Kyoto University Graduate School of Medicine, Kyoto; Kanazawa University Graduate School of Medical Science, Kanazawa; and National Cardiovascular Center, Suita) between June 1996 and January 2009. If gene mutations in LQTS-related genes were identified, further genetic analysis was conducted among family members as extensively as possible. All patients in the cohort were Japanese.

Genetic analysis

Informed consent was obtained from all individuals or their guardians according to standards established by the local institutional review boards. Genotypic and DNA sequence analyses of *KCNQ1, KCNH2, SCN5A, KCNE1,* and *KCNE2* were performed as described previously.[13] In addition, *KCNJ2* (Andersen syndrome [LQT7][14,15]) was analyzed in patients who had not only QT prolongation but also the clinical phenotype of Andersen syndrome, for example, periodic paralysis or dysmorphic features. Other candidate genes (e.g., ankyrin-B [LQT4], *CACNA1C* [Timothy syndrome, LQT8]) were not analyzed because mutations in these genes are extremely rare. Denaturing high-performance liquid chromatography was performed as described previously.[16] Abnormal conformers were amplified by polymerase chain reaction and sequenced using an ABI PRISM310 DNA sequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA, USA). "Splicing error" mutations were defined as those that occurred within three bases of the splicing sites. When mutations were detected, 200 Japanese control subjects were checked and single nucleotide polymorphisms were excluded from the study. If mutations of these genes were detected in the probands, their family members were also analyzed and genotype–phenotype correlations confirmed. Mutation-negative controls were defined as family members without mutations detected in each proband. Nonsynonymous as well as synonymous single nucleotide polymorphisms were excluded with the assistance of data from previous reports[17–19] and from the National Center for Biotechnology Information database.

Clinical characterization

Baseline clinical data were recorded for each patient and included the following: age at diagnosis, age at first cardiac event, sex, cardiac events, family history of sudden cardiac death or LQTS members, ECG measurements, and therapeutic regimens administered. Schwartz scores also were calculated.[20,21] In the analysis of triggers of arrhythmic events, triggers were divided into four categories: exercise/swimming, emotional stress/arousal stress, sleep/rest, and other conditions.

ECG parameters measured at baseline included RR, QT_{end}, QT_{peak}, and $T_{peak\text{-}end}$ ($QT_{end\text{-}peak}$) intervals. The latter is thought to reflect transmural dispersion of ventricular repolarization.[22] Measurements were the mean of at least three beats measured in lead V_5 from the 12-lead ECG during stable sinus rhythm and corrected by the Bazett formula.[23] QT_{end} was manually measured as the time interval between QRS onset (Q) and the point at which the isoelectric line intersected a tangential line drawn at the maximal downslope of the positive T wave or the maximal

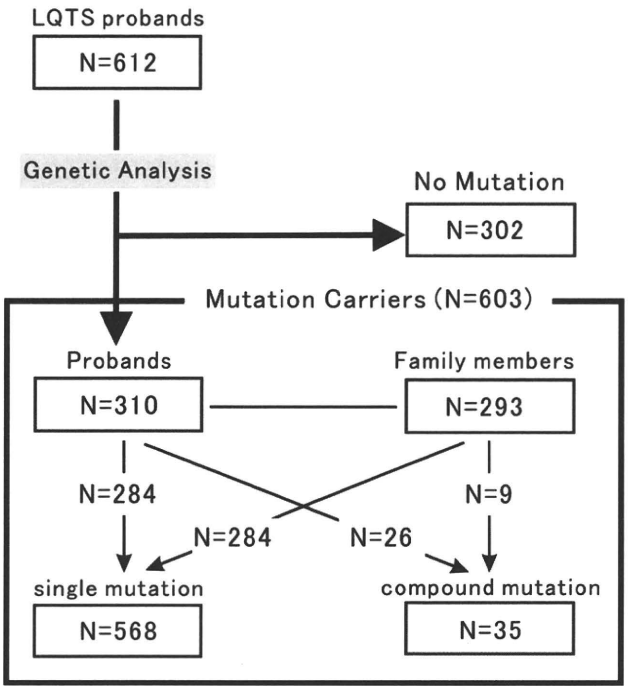

Figure 1 Schematic representation of the positive-mutation carriers in this study. LQTS = long QT syndrome.

Table 1 Overall data of patients with compound mutations

Research groups	Schwartz et al.	Westenkow et al.	Tester et al.	This study
Reported years	2003	2004	2005	2010
The corresponding number in the reference list	25	11	12	
Percentage of probands with compound mutations (probands with compound mutations/total probands) subtypes	4.6% (6/130)	5.2% (9/172*)	10.8% (29/269)	8.4% (26/310)
LQT1	7 (58%)	14 (35%)	30 (52%)	18 (35%)
LQT2	2 (17%)	10 (25%)	15 (26%)	17 (33%)
LQT3	3 (25%)	2 (5%)	13 (22%)	14 (27%)
LQT5-D85N	0 (0%)	10 (25%)	0 (0%)	0 (0%)
vs. single mutation carriers				
QTc interval	NA	prolonged	not significant	prolonged
Cardiac events	NA	frequent	not significant	not significant
Age of onset	NA	NA	younger onset	younger onset

*This table excluded probands with single nucleotide polymorphisms (SNP), NA = not available.

upslope of the negative T wave (QT_{end}). $QT_{end-peak}$ then was obtained by calculating as QT_{end} minus QT_{peak}.

Statistical analysis

All analyses were performed using the SPSS 16.0 statistical package (SPSS, Inc., Chicago, IL. USA). Data are expressed as mean ± SD. $P < 0.05$ was considered significant. Univariate comparison of parameters between groups was performed by an unpaired t-test. Differences in incidence between groups were analyzed by Chi-square test or Fisher exact probability test. The cumulative probability of a first cardiac event (syncope, torsades de pointes, ventricular fibrillation, cardiac arrest, or sudden death) occurring before age 40 years and before beta-blocker therapy or after beta-blocker therapy was determined by means of the life-table method of Kaplan-Meier, and results were compared using log rank test.[24]

Results

Genetic characteristics of mutations associated with single and compound mutations

Genetic analysis revealed gene mutations in 310 (51%) of 612 probands. The study enrolled 603 genotyped LQTS patients consisting of 310 genotyped probands and their 293 genotyped family members. A flowchart of the genetic diagnosis of the study population is shown in Figure 1.

Of the 310 genotyped probands, 26 (8.4%) had compound mutations. This rate is comparable to the rates in previous reports of Caucasian patients (Table 1). The 26 probands all had two mutations in the LQTS-related genes we examined. These 52 mutations in 26 probands consisted of 45 missense mutations, 4 frameshift mutations, 2 splice-site mutations, and 1 nonsense mutation (see Online Supplemental Data 1). The mutation types of the 284 single mutation carriers were 210 missense mutations, 34 frameshift mutations, 18 splicesite mutations, 12 deletions, 9 nonsense mutations, and 1 insertion mutation (see Online Supplemental Data 2). Therefore, the mutation types were similar between the two groups (Figure 2).

Among the 293 genotyped family members, there were 284 single mutation carriers and 9 compound mutation carriers. In total, 568 patients with a single mutation (284 probands and 284 family members) consisted of 256 with LQT1, 248 with LQT2, 62 with LQT3, and 2 with LQT5. Thirty-five compound mutation carriers (26 probands and 9 family members) consisted of 9 with LQT2 and LQT3, 7 with LQT1 and LQT2, 6 with LQT1 and LQT3, 4 with double LQT1, 3 with double LQT2 mutations, 2 with LQT1 and LQT7, 2 with LQT2 and LQT7, 1 with double LQT3, and 1 with LQT1 and LQT6.

Families associated with compound mutations

In the analysis of family members associated with compound mutations, 28 single heterozygous mutation carriers and 4 obligate single mutation carriers were identified from 9 families, and single mutation carriers had milder clinical phenotypes than compound mutation carriers (Figure 3). Only 2 (6%) of the 32 single mutation carriers had syncope but no torsades de pointes, an incidence lower than that in compound mutation carriers (54% [19/35] patients, $P < .001$). For single heterozygous mutation carriers in compound mutation families, average QTc interval was 442 ± 30 ms, which was longer than that of the 15 mutation-negative controls (408 ± 28 ms, $P = .001$) but significantly shorter than that of compound mutation carriers (510 ± 56 ms, $P < .001$).

Early onset of cardiac events and more severe QT prolongation was observed in patients with compound mutations

Table 2 compares the clinical characteristics of 35 LQTS patients with compound mutation and 568 LQTS patients with a single mutation. The female-to-male ratio was similar between the two groups. However, the incidence of family members associated with double-hit patients was significantly smaller than that with a single mutation (26% vs 50%, $P = .005$). In the ECG analysis of 496 patients with available information, corrected QT interval was significantly longer in compound mutation carriers than in single mutation carriers (510 ± 56 ms vs 478 ± 53 ms, respectively, $P = .001$), whereas other ECG findings, R-R interval, corrected QT_{peak}, corrected $QT_{peak-end}$, and rates of

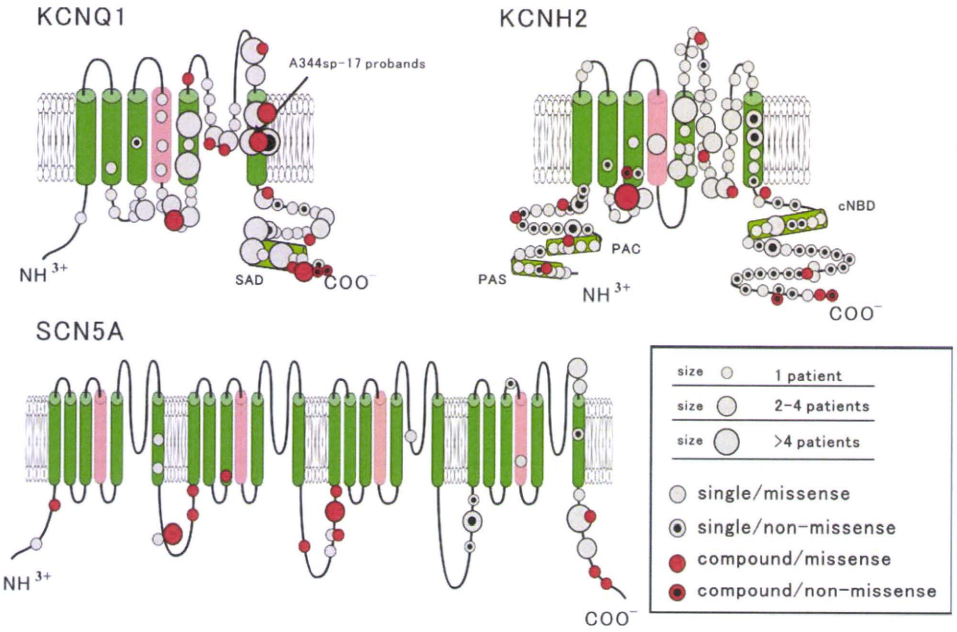

Figure 2 Conventional transmembrane topology of all mutations in the probands.

notched T wave and T-wave alternans were not different between the two groups. The frequency of patients with a normal QTc interval <440 ms was similar between the two groups, whereas the frequency of double-hit patients with QTc intervals >500 ms was significantly higher than in those with a single mutation (66% vs 26%, P <.001). Schwartz scores in the compound mutation group and the rate of patients with a score ≥4 were higher than those in the single mutation group (Schwartz score: 4.3 ± 2.1 vs 3.4 ± 1.9 points, P = .017; rates of Schwartz score ≥4 points: 70% vs 47%, P = .026). A significantly higher number of patients with compound mutations received beta-blocker therapy than did those with a single mutation (56% vs 33%, P = .006).

In the analysis of "all age groups," the frequency of cardiac events was similar between compound and single mutation groups, whereas age at first cardiac event was significantly lower in the compound mutation group (10 ± 8 years vs 18 ± 16 years, P = .043). For the occurrence of syncope or torsades de pointes before age 40 years, compound mutation carriers had significantly more events than did single mutation carriers (54% vs 37%, P = .043). The occurrence of cardiac arrest or ventricular fibrillation was similar between the two groups for patients before age 40 years. In 561 patients with available information on age at first cardiac events, Kaplan-Meier analysis showed that the cumulative rate of survival without a cardiac event before age 40 years and use of beta-blocker therapy differed significantly between compound and single mutation carriers (P = .004 by log rank test; Figure 4A) and between compound mutation carriers and each subgroup of single mutation carriers (P = .004 vs LQT1, P = .018 vs LQT2, P = .001 vs LQT3, by log rank test; Figure 4B). In the analysis of matched subtypes between single and compound mutation carriers, patients with additional mutations in an LQTS subtype had a significantly poorer prognosis than LQT1 alone (P = .001; Figure 5) and LQT2 alone (P = .035) but not LQT3 alone (P = .06).

Discussion

In this multicenter study, the major findings were as follows. (1) LQTS-associated compound mutations in the Japanese population were as common as previously reported in studies of Caucasian patient cohorts. (2) Patients with compound mutations displayed longer QTc and earlier onset of cardiac events. (3) Patients with compound mutations had more cardiac events before age 40 years and more beta-blocker therapy. (4) Subgroup analysis showed more cardiac events in LQT1 and LQT2 compound mutations compared to single LQT1 and LQT2 mutations.

Twenty-six probands (8.4% of genotyped LQTS) were found to have two variants in genes encoding ion channels (*KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2,* or *KCNJ2*). This incidence rate is in general agreement with other studies that reported a prevalence of compound or multiple mutations of 5% to 11% of genotyped LQTS (Table 1).[11,18,25]

Table 1 summarizes the genetic and clinical characteristics of patients enrolled in previous studies and compares them with the characteristics of patients enrolled in the present study. Sanguinetti and colleagues reported that patients with compound mutations not only had longer QT intervals than single mutation carriers but also had more frequent cardiac events.[11] However, Ackerman and colleagues demonstrated that, although compound mutation carriers were diagnosed at a younger age than single mutation carriers, they did not have significantly longer QT intervals.[12] The difference between these results might be explained by half of the 20 compound probands in the cohort of Sanguinetti et al possessing the common *KCNE1-*

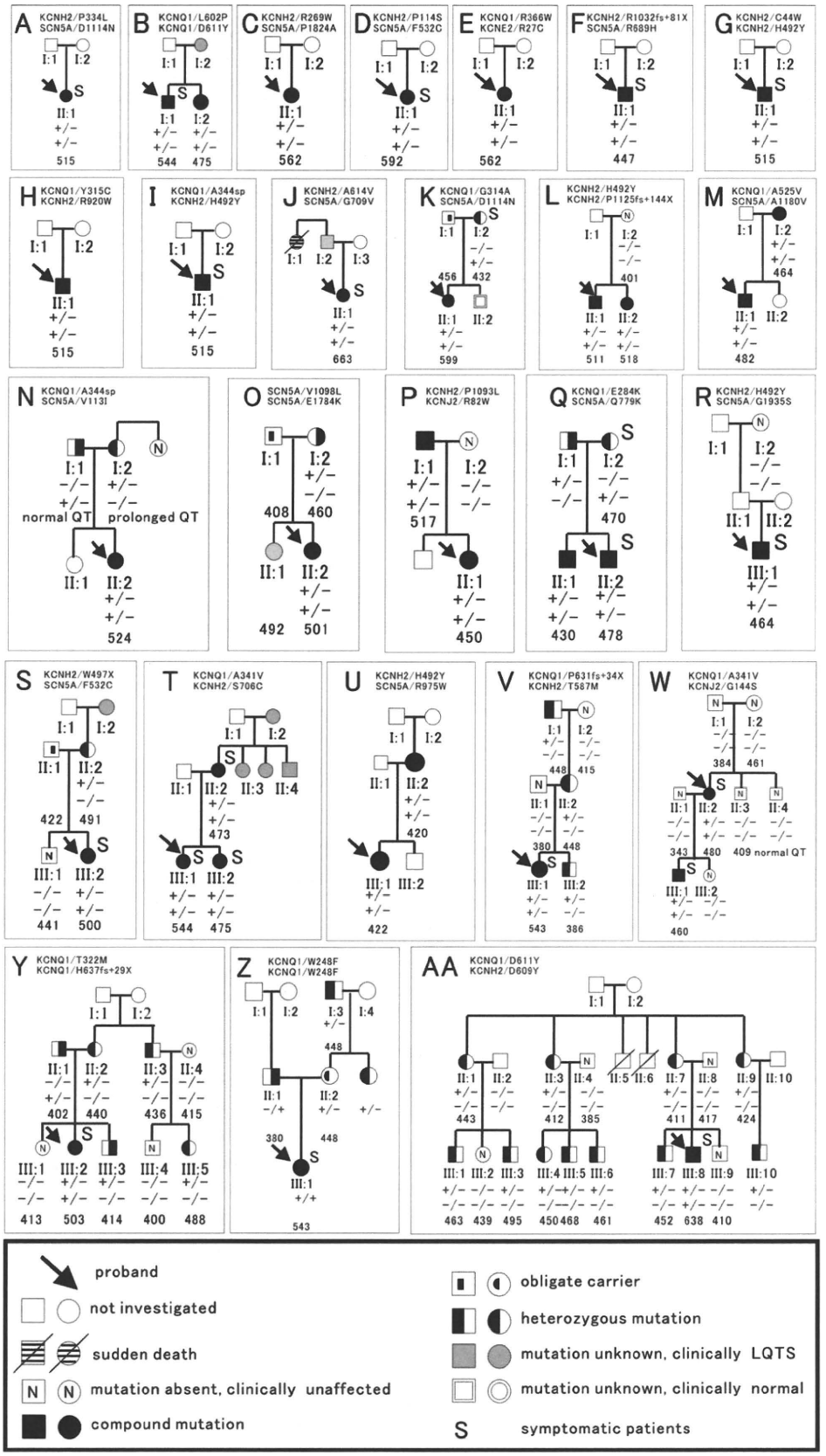

Figure 3 Pedigrees of the families associated with compound mutation probands.

Table 2 Clinical characteristics of LQTS patients with gene mutations

	Compound mutations (N=35)	Single mutations (N=568)	p value
Demographic			
Age at diagnosis (yrs)	19 ± 14 [15, 9-27]	28 ± 19 [22, 12-42]	0.001
Female gender	23 (66%)	330 (58%)	0.394
Proband	26 (74%)	284 (50%)	0.005
Family members	9 (26%)	284 (50%)	0.005
Cardiac events			
cardiac events in all age groups			
Age at first cardiac event (yrs)	10 ± 8 [11, 3.5-13.5]	18 ± 16 [12, 7-19]	0.043
syncope	19 (54%)	235 (41%)	0.161
TdP	10 (29%)	102 (18%)	0.136
cardiac arrest or VF	3 (9%)	44 (8%)	0.748
sudden death	0 (0%)	4 (1%)	1.000
cardiac events before 40 yrs			
syncope or TdP	19 (54%)	205 (37%)	0.043
cardiac arrest or VF	3 (9%)	37 (7%)	0.500
ECG measurements			
RR interval (ms)	866 ± 210	914 ± 174	0.252
corrected QT (ms)	510 ± 56	478 ± 53	0.001
corrected QT >500 ms (%)	23 (66%)	122 (26%)	<0.001
corrected QT <440 ms (%)	3 (9%)	91 (20%)	0.351
corrected QT peak (ms)	385 ± 70	384 ± 50	0.906
corrected QT peak-end (ms)	121 ± 73	95 ± 41	0.081
notched T wave	11 (31%)	200 (37%)	0.540
T-wave alternans	0 (0%)	30 (5%)	0.246
Diagnosis			
Schwartz score	4.2 ± 2.1	3.4 ± 1.9	0.017
Schwartz score ≧4	21 (70%)	219 (47%)	0.026
Therapy			
β-blocker	10 (56%)	175 (33%)	0.006
class Ib antiarrhythmic drugs	3 (9%)	53 (10%)	1.000
pacemaker	1 (3%)	15 (3%)	1.000
sympathectomy	1 (3%)	3 (1%)	0.218
defibrillator	1 (3%)	32 (6%)	0.712

TdP = torsades de pointes, VF = ventricular fibrillation, NS = not significant, corrected QT = QT interval corrected for heart rate with Bazett formula [A, B], A = median, B-C = first interquartile range-third interquartile range.

D85N polymorphism as the "second hit" (Table 1).[11,26] In all age groups of this study, the incidence of cardiac events, such as torsades de pointes or syncope, was similar between single and compound mutation carriers; however, the clinical phenotypes of those with compound mutations before 40 years of age were more serious than in those with a single mutation (Table 2). Thus, phenotypes with compound mutations appear to be more serious than single mutation carriers, regardless of race.

Beta-blocker therapy is first-line treatment for the prevention of cardiac events in LQTS. Beta-blockers have been shown to significantly reduce cardiac events in LQTS patients, especially LQT1 type.[27–29] However, patients with LQT2 or LQT3 have been reported to be less responsive to beta-blocker therapy[27,30] and may require additional therapy, such as pacemaker implantation for LQT2 or a Class Ib antiarrhythmic drug for LQT3. It may be recommended that patients with compound mutations receive additional individual therapy based on their LQTS subtype, for example, the combination of beta-blocker and Class Ib antiarrhythmic drugs for patients with LQT1 and LQT3. In patients who were first diagnosed as LQT1, Kobori et al[31] reported that additional mutations in different LQTS-related genes influenced phenotype severity and reduced beta-blocker effectiveness. Previous reports showed that approximately 20% of LQT1 patients were resistant to beta-blocker therapy. Additional or "latent" mutations may be present in these patients, and conducting a survey for major all LQTS-related genes, even after a possible mutation is identified, is critically important.

Family study analyses are of enormous importance because single mutation carriers in this study tended to have mild phenotypes. Most of the single mutation carriers in families of compound probands remained asymptomatic. However, double hits of these "latent" gene carriers could cause more serious phenotypes.[32,33] Jervell and Lange-Nielsen syndrome is a well-documented LQTS phenotype with an autosomal recessive pattern. The loss of function of I_{Ks} on both alleles generally causes not only more severe clinical phenotypes but also deafness.[9,10] In our study, two of three probands with double *KCNQ1* mutations had no deafness. We speculate that these mutations would functionally cause mild changes without complete loss of I_{Ks}. Westenskow et al[11] reported the molecular mechanism of

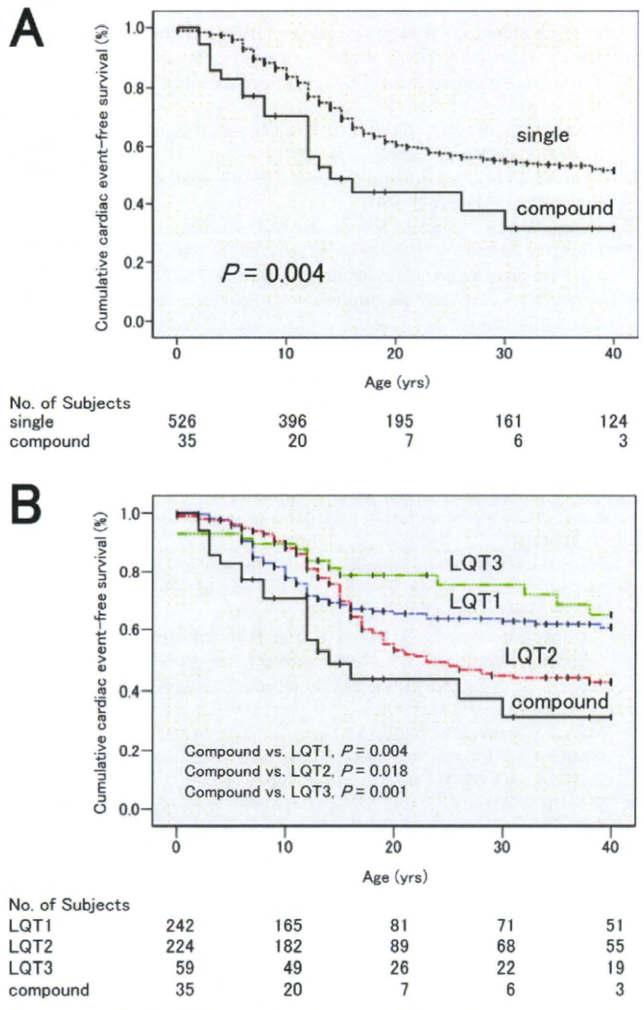

Figure 4 Kaplan-Meier cumulative probability of cardiac event-free survival from birth to age 40 years and before therapy. **A:** Comparison between patients with a single mutation and compound mutations. **B:** Comparison among patients with long QT syndrome type 1 (LQT1), type 2 (LQT2), type 3 (LQT3), and compound mutations.

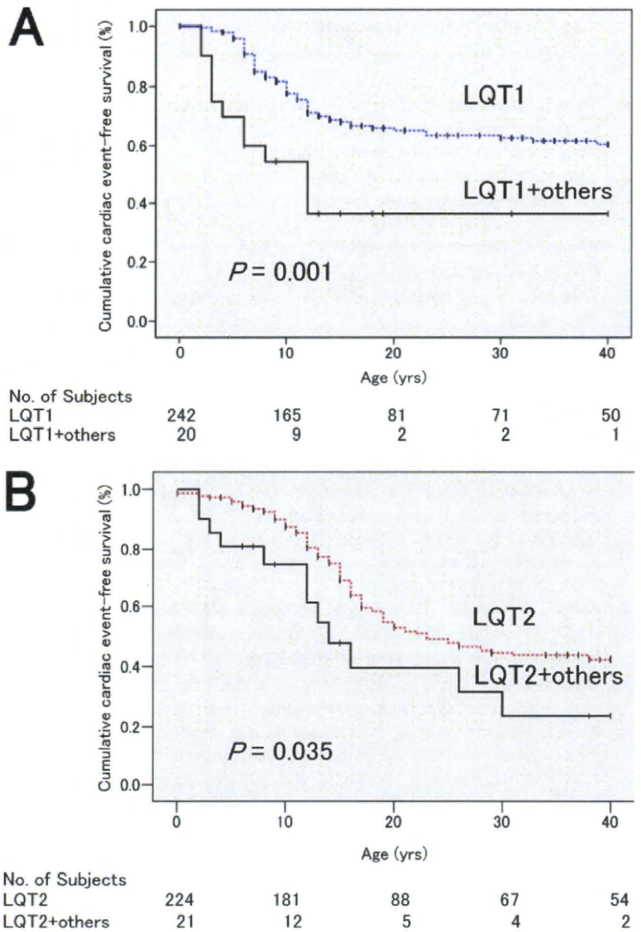

Figure 5 Kaplan-Meier cumulative probability of cardiac event-free survival from birth to 40 years of age and before therapy. **A:** Comparison between patients with long QT type 1 (LQT1_ subtype and compound mutation carriers with LQT1 plus other mutations. **B:** Comparison between patients with long QT syndrome type 2 (LQT2) and those with LQT2 plus other mutations.

increased risk through compound mutations using heterologous expressions in *Xenopus* oocytes. When wild-type and variant subunits were coexpressed in appropriate ratios to mimic the genotype of the probands with mutations, the reduction in current density was equivalent to the additive effects of the single mutations. Coexpression of two mutant subunits caused a significant but incomplete reduction. Thus, either compound mutation seems to be associated with mild functional damage. It is necessary to have "double hits" of these mild mutations in order to produce symptoms.

Study limitations

This study has several limitations. First, six major LQTS candidate genes were examined, but not for minor genes encoding a family of versatile membrane adapters. However, excluding these minor genes from our investigations would not have affected the overall study results, largely because the incidence of these minor gene mutations reportedly is ≤1%. Second, analysis of single mutation carriers in compound mutation families is dominated by their presence in only 35% (9/26) of families. Therefore, there might be a statistical bias due to a mutation-specific effect. Third, Kapa et al[19] reported the need for further studies on whether regions such as the interdomain linker of *SCN5A* could affect the clinical phenotypes of LQTS. In this study, we were able to distinguish mutations from these "genetic noises," especially in the *SCN5A* gene.

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Appendix

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hrthm.2010.06.013.

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KCNE2 modulation of Kv4.3 current and its potential role in fatal rhythm disorders

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BACKGROUND The transient outward current I_{to} is of critical importance in regulating myocardial electrical properties during the very early phase of the action potential. The auxiliary β subunit *KCNE2* recently was shown to modulate I_{to}.

OBJECTIVE The purpose of this study was to examine the contributions of *KCNE2* and its two published variants (M54T, I57T) to I_{to}.

METHODS The functional interaction between Kv4.3 (α subunit of human I_{to}) and wild-type (WT), M54T, and I57T *KCNE2*, expressed in a heterologous cell line, was studied using patch-clamp techniques.

RESULTS Compared to expression of Kv4.3 alone, co-expression of WT *KCNE2* significantly reduced peak current density, slowed the rate of inactivation, and caused a positive shift of voltage dependence of steady-state inactivation curve. These modifications rendered Kv4.3 channels more similar to native cardiac I_{to}. Both M54T and I57T variants significantly increased I_{to} current density and slowed the inactivation rate compared with WT *KCNE2*. Moreover, both variants accelerated the recovery from inactivation.

CONCLUSION The study results suggest that *KCNE2* plays a critical role in the normal function of the native I_{to} channel complex in human heart and that M54T and I57T variants lead to a gain of function of I_{to}, which may contribute to generating potential arrhythmogeneity and pathogenesis for inherited fatal rhythm disorders.

KEYWORDS Cardiac arrhythmia; M54T variation; I57T variation; *KCNE2*; Kv4.3; Sudden cardiac death

ABBREVIATIONS CHO = Chinese hamster ovary; **HERG** = human ether-a-go-go related gene; **WT** = wild type

Introduction

Classic voltage-gated K^+ channels consist of four pore-forming (α) subunits that contain the voltage sensor and ion selectivity filter[1,2] and accessory regulating (β) subunits.[3] *KCNE* family genes encode several kinds of β subunits consisting of single transmembrane-domain peptides that co-assemble with α subunits to modulate ion selectivity, gating kinetics, second messenger regulation, and the pharmacology of K^+ channels. Association of the *KCNE1* product minK with the α subunit Kv7.1 encoding *KCNQ1* forms the slowly activating delayed rectifier K^+ current I_{Ks} in the heart.[4,5] In contrast, association of the *KCNE2* product MiRP1 with the human ether-a-go-go related gene (HERG) forms the cardiac rapid delayed rectifier K^+ current I_{Kr}.[6]

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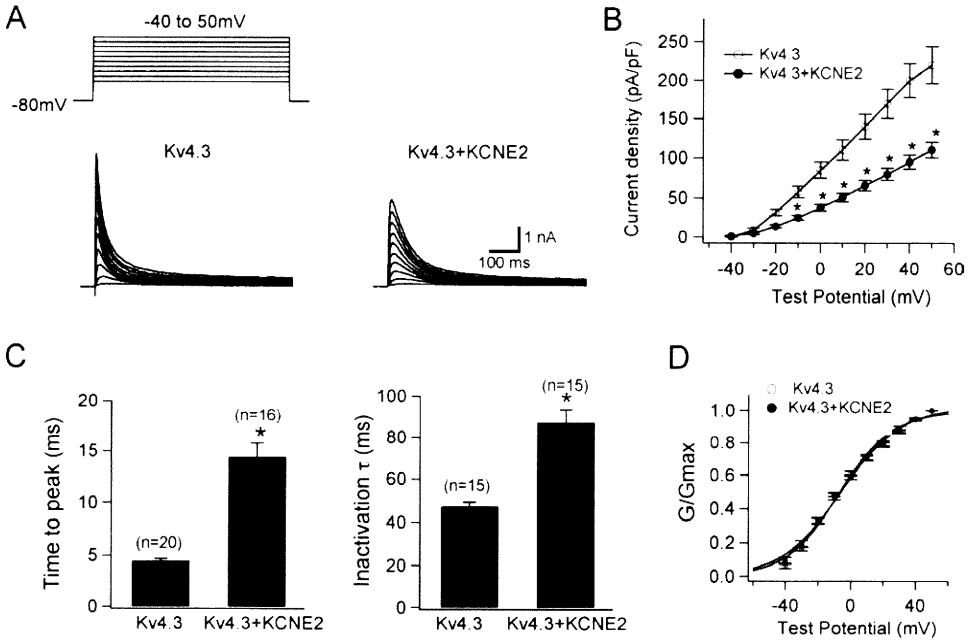

Figure 1 *KCNE2* co-expression with Kv4.3 produces smaller I_{to}-like currents with slower activation/inactivation kinetics. **A:** Representative current traces recorded from Chinese hamster ovary (CHO) cells expressing Kv4.3 **(left)** and Kv4.3 + *KCNE2* **(right).** As shown in the **inset** in panel A, depolarizing step pulses of 1-second duration were introduced from a holding potential of −80 mV to potentials ranging from −40 to +50 mV in 10-mV increments. **B:** Current–voltage relationship curve showing peak current densities in the absence and presence of co-transfected *KCNE2* (*$P < .05$ vs Kv4.3). **C:** Bar graphs showing the kinetic properties of reconstituted channel currents: time to peak of activation course **(left)** and inactivation time constants **(right)** measured using test potential to +20 mV (*$P < .05$ vs Kv4.3). Numbers in parentheses indicate numbers of experiments. **D:** Normalized conductance–voltage relationship for peak outward current of Kv4.3 and Kv4.3 + *KCNE2* channels.

Abbott et al reported that three *KCNE2* variants (Q9E, M54T, I57T) caused a loss of function in I_{Kr} and thereby were associated with the congenital or drug-induced long QT syndrome.[6,7] However, the reported QTc values in two index patients with M54T and I57T variants, both located in the transmembrane segment of MiRP1, were only mildly prolonged (390–500 ms and 470 ms).[6] We recently identified the same missense *KCNE2* variant, I57T, in which isoleucine was replaced by threonine at codon 57, in three unrelated probands showing a Brugada type 1 ECG. These findings are difficult to explain on the basis of a loss of function in I_{Kr}, thus leading us to explore other mechanisms.

Recent studies have demonstrated that interaction between α and β subunits (*KCNE*s) of voltage-gated K^+ channel is more promiscuous; for example, MiRP1 has been shown to interact with Kv7.1,[8–10] HCN1,[11] Kv2.1,[12] and Kv4.2.[13] These studies suggest that MiRP1 may also co-associate with Kv4.3 and contribute to the function of transient outward current (I_{to}) channels.[14] Indeed, a recent study reported that I_{to} is diminished in *kcne2* (−/−) mice.[15]

In the human heart, I_{to} currents are of critical importance in regulating myocardial electrical properties during the very early phase of the action potential and are thought to be central to the pathogenesis of Brugada-type ECG manifestations.[16] Antzelevitch et al demonstrated that a gain of function in I_{to} secondary to a mutation in *KCNE3* contributes to a Brugada phenotype by interacting with Kv4.3 and thereby promoting arrhythmogenicity.[14]

We hypothesized that mutations in *KCNE2* may have similar actions and characterize the functional consequences of interaction of wild-type (WT) and two mutant (I57T, M54T) MiRP1 with Kv4.3[17,18] using heterologous co-expression of these α and β subunits in Chinese hamster ovary (CHO) cells.

Methods

Heterologous expression of hKv4.3 and β subunits in CHO cells

Full-length cDNA fragment of *KCNE2* in pCR3.1 vector[10] was subcloned into pIRES-CD8 vector. This expression vector is useful in cell selection for later electrophysiologic study (see below). Two *KCNE2* mutants (M54T, I57T) were constructed using a Quick Change II XL site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA) and subcloned to the same vector. Two *KCNE2* mutants were fully sequenced (ABI3100x, Applied Biosystems, Foster City, CA, USA) to ensure fidelity. Full-length cDNA encoding the short isoform of human Kv4.3 subcloned into the pIRES-GFP (Clontech, Palo Alto, CA, USA) expression vector was kindly provided by Dr. G.F. Tomaselli (Johns Hopkins University). Full-length cDNA encoding Kv channel-interacting protein (*KCNIP2*) subcloned into the PCMV-IRS expression vector was a kind gift from Dr. G.-N. Tseng (Virginia Commonwealth University). *KCND3* was transiently transfected into CHO cells together with *KCNE2* (or M54T or I57T) cDNA at equimolar ratio (*KCND3* 1.5 μg,

Table 1 Effects of *KCNE2* on Kv4.3 and Kv4.3 + KChIP2b

Parameter	Kv4.3	Kv4.3 *KCNE2*	Kv4.3 KChIP2b	Kv4.3 KChIP2b *KCNE2*
Current density at +20 mV (pA/pF)	142.0 ± 16.0 (n = 12)	66.0 ± 6.6* (n = 12)	191.5 ± 33.8 (n = 15)	77.8 ± 5.9† (n = 20)
Steady-state activation ($V_{0.5}$ in mV)	−6.5 ± 2.1 (n = 9)	−5.5 ± 1.7 (n = 11)	−7.5 ± 1.7 (n = 8)	−7.4 ± 1.4 (n = 8)
Steady-state inactivation ($V_{0.5}$ in mV)	−46.0 ± 1.3 (n = 10)	−40.8 ± 1.7* (n = 8)	−49.8 ± 1.4 (n = 7)	−44.5 ± 1.9† (n = 7)
τ of inactivation at +20 mV (τ_{inact} in ms)	47.3 ± 2.0 (n = 15)	87.2 ± 6.2* (n = 15)	47.5 ± 2.2 (n = 15)	66.6 ± 3.5† (n = 15)
Time to peak at +50 mV (TtP in ms)	4.5 ± 0.2 (n = 20)	14.4 ± 1.4* (n = 16)	4.1 ± 0.2 (n = 15)	6.1 ± 0.5† (n = 21)
τ of recovery from inactivation (ms)	419.6 ± 18.8 (n = 6)	485.6 ± 74.8 (n = 6)	89.2 ± 5.3 (n = 6)	60.2 ± 6.9† (n = 6)

*Significantly different from Kv4.3.
†Significantly different from Kv4.3 + KChIP2b.

KCNE2 1.5 μg) using Lipofectamine (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. In one set of experiments, we also co-transfected equimolar levels of KChIP2b (*KCND3* 1.5 μg, *KCNE2* 1.5 μg, *KCNIP2* 1.5 μg). The transfected cells were then cultured in Ham's F-12 medium (Nakalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Inc., Lenexa, KS, USA) and antibiotics (100 international units per milliliter penicillin and 100 μg/mL streptomycin) in a humidified incubator gassed with 5% CO_2 and 95% air at 37°C. The cultures were passaged every 4 to 5 days using a brief trypsin-EDTA treatment. The trypsin-EDTA treated cells were seeded onto glass coverslips in a Petri dish for later patch-clamp experiments.

Electrophysiologic recordings and data analysis

After 48 hours of transfection, a coverslip with cells was transferred to a 0.5-mL bath chamber at 25°C on an inverted microscope stage and perfused at 1 to 2 mL/min with extracellular solution containing the following (in mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 5.5 glucose, and 5.0 HEPES; pH 7.4 with NaOH. Cells that emitted green fluorescence were chosen for patch-clamp experiments. If co-expressed with *KCNE2* (or its mutants), the cells were incubated with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450, Dynal, Norway) for 15 minutes. In these cases, cells that emitted green fluorescence and had attached beads were chosen for electrophysiologic recording. Whole-cell membrane currents were recorded with an EPC-8 patch-clamp amplifier (HEKA, Lambrecht, Germany), and data were low-pass filtered at 1 kHz, acquired at 5 kHz through an LIH-1600 analog-to-digital converter (HEKA), and stored on hard disk using PulseFit software (HEKA). Patch pipettes were fabricated from borosilicate glass capillaries (Narishige, Tokyo, Japan) using a horizontal microelectrode puller (P-97, Sutter Instruments, Novato, CA, USA) and the pipette tips fire-polished using a microforge. Patch pipettes had a resistance of 2.5 to 5.0 MΩ when filled with the following pipette solution (in mM): 70 potassium aspartate, 50 KCl, 10 KH_2PO_4, 1 $MgSO_4$, 3 Na_2-ATP (Sigma, Japan, Tokyo), 0.1 Li_2-GTP (Roche Diagnostics GmbH, Mannheim, Germany), 5 EGTA, and 5 HEPES (pH 7.2).

Cell membrane capacitance (C_m) was calculated from 5 mV-hyperpolarizing and depolarizing steps (20 ms) applied from a holding potential of −80 mV according to Equation 1[19]:

$$C_m = \tau_c I_0 / \Delta V_m (1 - I_\infty / I_0), \quad (1)$$

where τ_c = time constant of capacitance current relaxation, I_0 = initial peak current amplitude, ΔV_m = amplitude of voltage step, and I_∞ = steady-state current value. Whole-cell currents were elicited by a family of depolarizing voltage steps from a holding potential of −80 mV. The difference between the peak current amplitude and the current at the end of a test pulse (1-second duration) was referred to as the transient outward current. To control for cell size variability, currents were expressed as densities (pA/pF).

Steady-state activation curves were obtained by plotting the normalized conductance as a function of peak outward potentials. Steady-state inactivation curves were generated by a standard two-pulse protocol with a conditioning pulse of 500-ms duration and obtained by plotting the normalized current as a function of the test potential. Steady-state inactivation/activation kinetics were fitted to the following Boltzmann equation (Eq. 2):

$$Y(V) = 1/(1 + \exp[(V_{1/2} - V)/k]), \quad (2)$$

where Y = normalized conductance or current, $V_{1/2}$ = potential for half-maximal inactivation or activation, respectively, and k = slope factor.

Data relative to inactivation time constants, time to peak, and mean current levels were obtained by using current data recorded at +50 mV or +20 mV. Recovery from inactivation was assessed by a standard paired-pulse protocol: a 400-ms test pulse to +50 mV (P1) followed by a variable

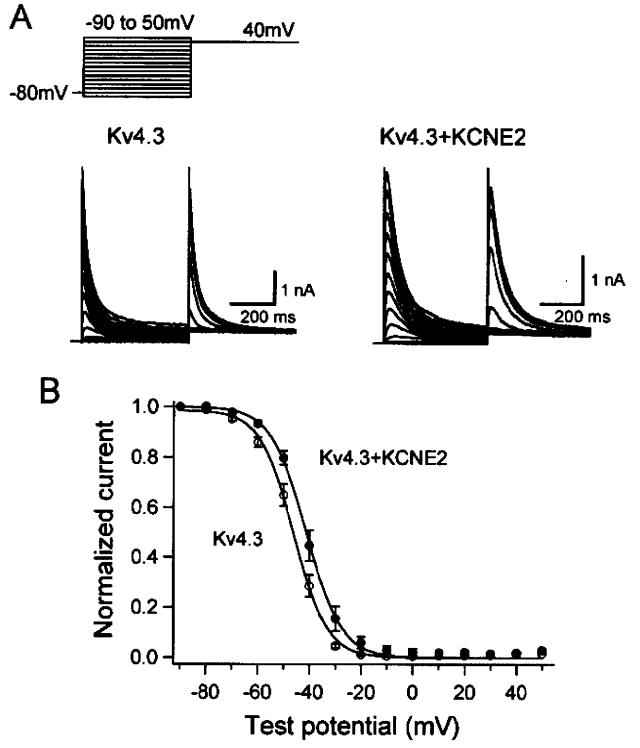

Figure 2 *KCNE2* co-expression with Kv4.3 causes a positive shift of voltage dependence of steady-state inactivation. **A:** Representative Kv4.3 and Kv4.3 + *KCNE2* current traces induced by 500-ms pulses (P1) from −90 to +50 mV applied from the holding potential −80 mV in 10-mV steps followed by a second pulse (P2) to +40 mV. **B:** Steady-state inactivation curves for Kv4.3 *(open circles)* and Kv4.3 + *KCNE2 (closed circles)* channels.

recovery interval at −80 mV and then a second test pulse to +50 mV (P2). Both the inactivation time constants and the time constant for recovery from inactivation were determined by fitting the data to a single exponential (Eq. 3):

$$I(t) \text{ (or P2/P1)} = A + B_{exp}(-t/\tau), \quad (3)$$

where I(t) = current amplitude at time t, A and B = constants, and τ = inactivation time constant or time constant for recovery from inactivation. For measurement of recovery from inactivation, the plot of P2/P1 instead of I(t) was used.

All data were given as mean ± SEM. Statistical comparisons between two groups were analyzed using Student's unpaired t-test. Comparisons among multiple groups were analyzed using analysis of variance followed by Dunnett test. $P < .05$ was considered significant.

Results

Effects of *KCNE2* on Kv4.3 currents and its gating kinetics

WT *KCNE2* initially was co-expressed with *KCND3*, the gene encoding Kv4.3, the α subunit of the I_{to} channel,[17,18] in CHO cells. Figure 1A shows representative whole-cell current traces recorded from cells transfected with *KCND3* and co-transfected with (right) or without (left) *KCNE2*. Cells expressing Kv4.3 channels alone showed rapidly activating and inactivating currents. Co-expression of *KCNE2* significantly reduced peak current densities as summarized in the current–voltage relationship curve shown in Figure 1B and slowed both activation and inactivation kinetics (Table 1). Figure 1C (left) shows mean time intervals from the onset of the pulse to maximum current (time to peak), whereas the right panel shows time constants of inactivation (at +20 mV) obtained using Equation 3. Thus, co-transfection of *KCNE2* significantly increased both the time to peak and the time constant.

In contrast, *KCNE2* did not affect the voltage dependence of steady-state activation as assessed by plotting the normalized conductance as a function of test potential (Figure 1D). Fitting to the Boltzmann equation (Eq. 2) yielded half-maximal activation potentials of −6.5 ± 2.1 mV for Kv4.3 alone (open circles) and −5.5 ± 1.7 mV for Kv4.3 + *KCNE2* channels (filled circles, P = NS; Table 1). These findings are consistent with those previously reported for studies using *Xenopus* oocytes, CHO cells, and HEK293 cells.[20,21]

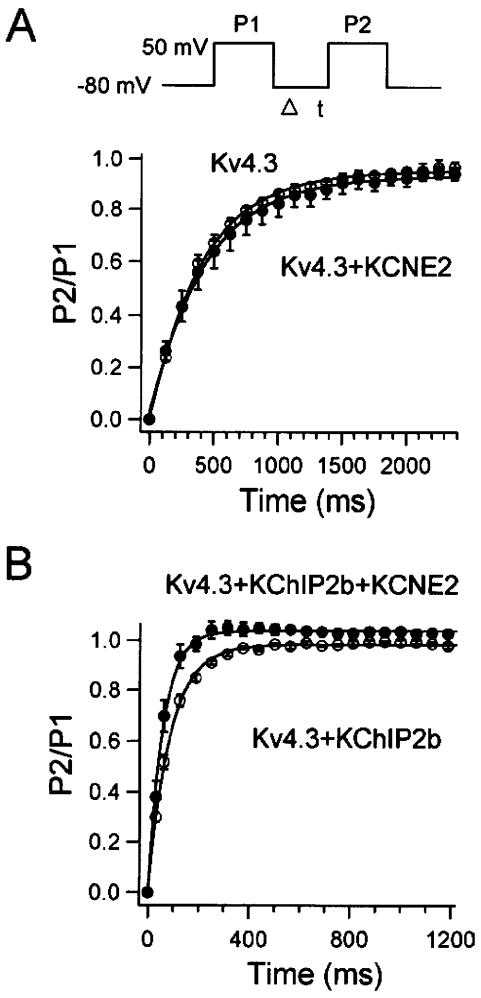

Figure 3 Effects of *KCNE2* co-expression on recovery from inactivation of Kv4.3 **(A)** and Kv4.3 + KChIP2b **(B)** currents. Recovery from inactivation was assessed by a two-pulse protocol **(A, inset):** a 400-ms test pulse to +50 mV (P1) followed by a variable interval at −80 mV, then by a second test pulse to +50 mV (P2). Data were fit to a single exponential.

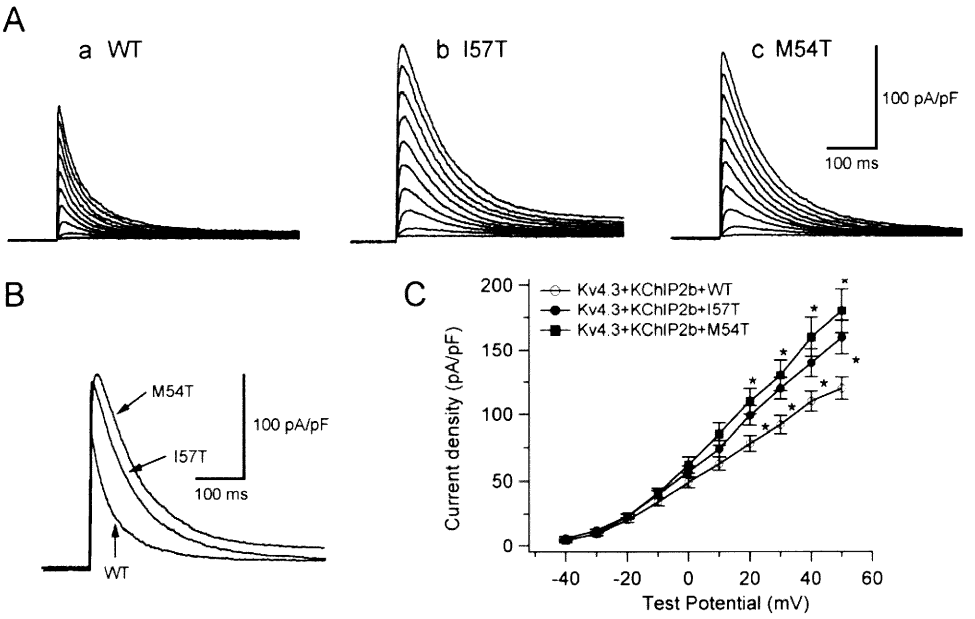

Figure 4 Two *KCNE2* transmembrane variants, I57T and M54T, increase the reconstituted Kv4.3 + KChIP2b channel current and slow its inactivation. **A:** Three sets of current traces elicited by depolarizing pulses for 500 ms from a holding potential of −80 mV to potentials ranging between −40 and +50 mV in 10-mV increments (same protocol as in experiments of Figure 1A). **B:** Superimposition of three original current traces recorded upon depolarization showing variant-related increase in peak outward current density. **C:** Current–voltage relationship curve showing average peak outward current densities (*P <.05 vs Kv4.3 + KChIP2b + WT). WT = wild type.

KCNE2 co-expression also caused a positive shift (approximately +5 mV) of voltage dependence of steady-state inactivation. Steady-state inactivation was assessed using a double-step pulse method (Figure 2A, inset). Peak outward currents recorded at various levels of prepulse (Figure 2A) were normalized by that measured after a 500-ms prepulse at −90 mV and are plotted as a function of prepulse test potentials (Figure 2B). Half-inactivation potentials of steady-state inactivation, determined by fitting data to the Boltzmann equation (Eq. 2), were −46.0 ± 1.3 mV for Kv4.3 (open circles) and −40.8 ± 1.7 mV for Kv4.3 + *KCNE2* (filled circles, P <.01), consistent with the observation of Tseng's group.[13]

A double-pulse protocol (Figure 3A, inset) was used to test the effect of *KCNE2* co-expression on the time course for recovery from inactivation. Figure 3A shows the time course of recovery of Kv4.3 alone (open circles) and Kv4.3 + *KCNE2* (filled circles). Mean time constants for recovery from inactivation were not significantly different, indicating that co-transfection of *KCNE2* did not affect the time course of recovery from inactivation.

Effects of *KCNE2* on Kv4.3 + KChIP2b current and its gating kinetics

For human native cardiac I_{to}, KChIP2 has been shown to serve as a principal β subunit.[22–25] Accordingly, in another series of experiments, we examined the effect of WT and mutant *KCNE2* on Kv4.3 + KChIP2b current. Consistent with previous reports, in the presence of KChIP2, Kv4.3 currents showed a significantly faster recovery from inactivation (Figure 3B and Table 1).[26,27] Co-expression of WT *KCNE2* produced similar changes on Kv4.3 + KChIP2b current as on Kv4.3 current (Table 1). Kv4.3 + KChIP2b current recovery from inactivation was further accelerated: average time constant was 89.2 ± 6.5 ms for Kv4.3 + KChIP2b alone (open circles) and 60.2 ± 8.4 ms for Kv4.3 + KChIP2b + *KCNE2* (filled circles, P <.05). In 16 of 21 cells transfected with *KCNE2*, we observed an "overshoot" phenomenon, which is commonly seen during recording of native I_{to} in human ventricular myocytes.[28]

KCNE2 variants increase Kv4.3 + KChIP2b current and alter its gating kinetics

The I57T variant was first identified in an asymptomatic middle-aged woman with very mild QT prolongation.[6] In addition to this variant, the authors reported another *KCNE2* variant of the transmembrane segment (M54T) that was associated with ventricular fibrillation during exercise in a middle-aged woman. This patient appeared to show a wide range of QTc interval (390–500 ms). Therefore, we tested the functional effects of these two transmembrane *KCNE2* variants on Kv4.3 + KChIP2b currents.

The three panels of Figure 4A show three sets of current traces elicited by depolarizing pulses from a holding potential of −80 mV in cells co-transfected with WT (a), I57T (b), or M54T (c) *KCNE2*. Neither variant caused a significant shift of half-maximal activation voltage: −7.4 ± 1.4 mV (n = 8) for co-expression of WT *KCNE2*, −6.1 ± 1.5 mV (n = 8) for I57T, and −6.6 ± 1.6 mV (n = 8) for M54T. Both variants significantly increased I_{to} density: 125.0 ± 10.6 pA/pF in WT *KCNE2* (n = 21), 178.1 ± 12.1 pA/pF with I57T (n = 9), and 184.3 ± 27.9 pA/pF with M54T (n = 9, Figure 4C).

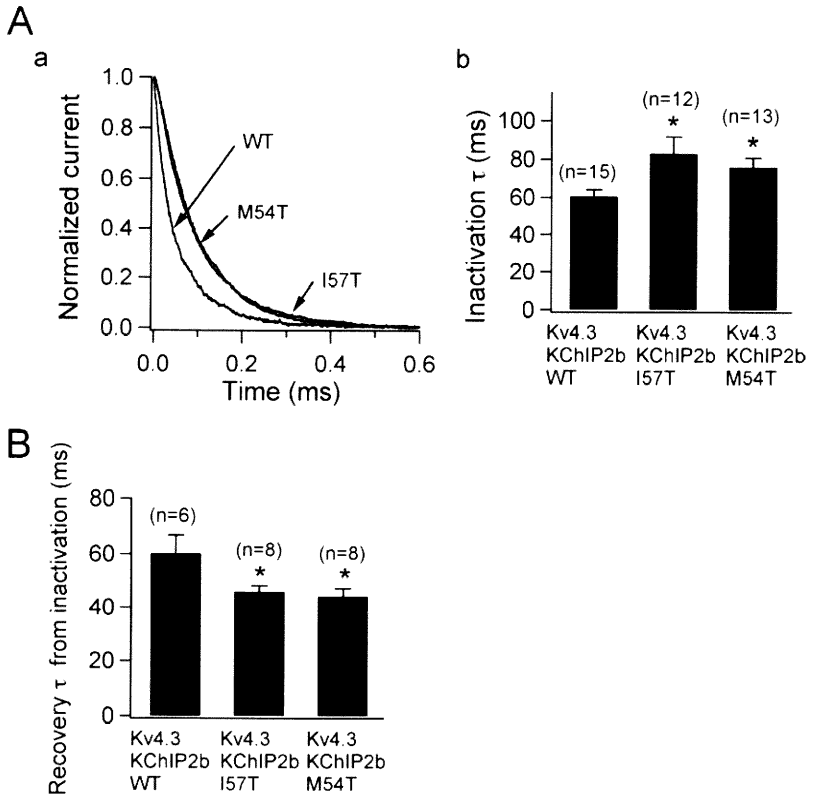

Figure 5 Two *KCNE2* variants slow inactivation kinetics and accelerate recovery from inactivation. **A, a:** Three current traces obtained from Chinese hamster ovary (CHO) cells transfected with wild-type (WT), I57T, and M54T *KCNE2* variant co-expressed with Kv4.3 and KChIP2b. Traces, which are normalized and superimposed, show that the variants slow inactivation. **A, b:** Time constants of decay at +20 mV for WT and variant *KCNE2* (*$P < .05$ vs Kv4.3 + KChIP2b + WT). Numbers in parentheses indicate numbers of observations. **B:** Time constants of recovery from inactivation recorded using a double-pulse protocol (*$P < .05$ vs Kv4.3 + KChIP2b + WT). Numbers in parentheses indicate numbers of observations.

Figure 5A shows the three traces depicted in Figure 4B normalized to their peak current level. This representation shows that the time course of inactivation of the two variant currents is slowed. The current decay was fitted by Equation 3 and the time constants (at +20 mV) summarized in Figure 5A, panel b. Finally, Figure 5B shows that the time constants of recovery of the two mutant channels from inactivation were significantly reduced. Thus, compared to WT *KCNE2*, recovery of reconstituted Kv4.3 + KChIP2b channels from inactivation was significantly accelerated with both I57T and M54T mutants.

Discussion

Kv4.3/KChIP2/MiRP1 complex can recapitulate the native I_{to}

In the present study, co-expression of WT *KCNE2* produced changes in kinetic properties (Figures 1–3 and Table 1) that led to close recapitulation of native cardiac I_{to}.[28,29] Notably, in addition to causing a positive shift of steady-state inactivation (Figure 2), *KCNE2* co-expression hastened the recovery of Kv4.3 + KChIP2b channels from inactivation (Figure 3). These modifications rendered Kv4.3 + KChIP2b channels more similar to native cardiac I_{to}, suggesting that *KCNE2* may be an important component of the native I_{to} channel complex. In contrast to a previous observation in HEK293 cells,[21] *KCNE2* co-expression decreased the current density of Kv4.3 and Kv4.3 + KChIP2b channel current in the present study, which seems to be a more reasonable result as the native I_{to} density reportedly was smaller in isolated human heart.[28] *KCNE2* co-expression has also been shown to reduce the density of Kv7.1[8,9] and HERG[6,7] channels.

Similar to the result of Deschenes and Tomaselli,[21] we failed to observe an overshoot during recovery from inactivation when *KCNE2* was co-expressed with Kv4.3 (Figure 3A), which is in contrast to the report of another group.[13] However, co-expression of *KCNE2* with Kv4.3 + KChIP2 channels produced an overshoot (Figure 3B), consistent with the report of Wettwer's group.[25] Wettwer et al also found that other *KCNE* subunits either were ineffective or induced only a small overshoot in CHO cells. Therefore, both MiRP1 and KChIP2 subunits are sufficient and necessary to recapitulate native I_{to} in the heart. Considering that the overshoot phenomenon has been described only in human ventricular I_{to} channels of the epicardial but not endocardial region,[28] these results may further implicate participation of MiRP1 and KChIP2 in the I_{to} channel complex in epicardium.

KCNE2 variants may alter the arrhythmogenic substrate by modulating I_{to}

Heterologous expression in CHO cells was conducted to examine the functional effects of I57T and M54T variants on Kv4.3 + KChIP2 channels. Both I57T and M54T

KCNE2 variants significantly (1) increased peak transient outward current density (Figure 4), (2) slowed the decay of the reconstituted I_{to} (Figure 5A), and (3) accelerated its recovery from inactivation (Figure 5B). Both variants thus caused an important gain of function in human I_{to}. These sequence changes may play a role in modulating I_{to} and thereby predispose to some inherited fatal rhythm disorders.

Functional effects on I_{to} induced by I57T and M54T resemble each other, increasing I_{to} density and accelerating its recovery from inactivation. The gain of function in I_{to} opposes the fast inward Na^+ currents during phase 0 of the action potential, leading to all or none repolarization at the end of phase 1 and loss of the epicardial action potential dome, thus promoting phase 2 reentry and fatal ventricular arrhythmias.[30]

Another *KCNE2* variant (M54T) associated with fatal arrhythmias was first identified in a woman who had a history of ventricular fibrillation and varied QT intervals.[6] It is possible that her arrhythmia was also related to a gain of function in I_{to} secondary to this variation in *KCNE2*. Interestingly, the I57T variant has been reported to produce a loss of function of HERG or Kv7.1 channels, thereby predisposing to long QT syndrome,[6,8] indicating that the same *KCNE2* variant could cause two different cardiac rhythm disorders, similar to long QT syndrome and Brugada syndrome caused by *SCN5A* mutations.[31,32]

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CLINICAL RESEARCH

Heart rate-dependent variability of cardiac events in type 2 congenital long-QT syndrome

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Aims We aimed to examine the validity of heart rate (HR) at rest before β-blocker therapy as a risk factor influencing cardiac events (ventricular fibrillation, torsades de pointes, or syncope) in long QT type 2 (LQT2) patients.

Methods and results In 110 genetically confirmed LQT2 patients (45 probands), we examined the significance of variables [HR at rest, corrected QT (QTc), female gender, age of the first cardiac event, mutation site] as a risk factor for cardiac events. We also evaluated frequency of cardiac events in four groups classified by the combination of basal HR and QTc with cutoff values of 60 b.p.m. and 500 ms to estimate if these two electrocardiographic parameters in combination could be a good predictor of outcome (mean follow-up period: 50 ± 39 months). Logistic regression analysis revealed three predictors: HR <60 b.p.m., QTc ≥500 ms, and female gender. When the study population was divided into four groups using the cutoff values of 60 b.p.m. for HR and 500 ms for QTc, the cumulative event-free survival by the Kaplan–Meier method was significantly higher in the group with HR ≥60 b.p.m. and QTc <500 ms than in the group with HR <60 b.p.m. and QTc <500 ms or that with HR <60 b.p.m. and QTc ≥500 m ($P < 0.05$). Irrespective of QTc interval, LQT2 patients with basal HR <60 b.p.m. were at significantly higher risk.

Conclusion The basal HR of <60 b.p.m. is a notable risk factor for the prediction of life-threatening arrhythmias in LQT2 patients.

Keywords Long QT syndrome • Arrhythmia • Genetics • Heart rate • Torsades de pointes

Introduction

Long QT syndrome (LQTS) is a primary electrical disease characterized by an abnormality in myocardial repolarization that leads to the prolongation of QT interval, morphological changes in T waves, and torsades de pointes (TdP) type of ventricular tachyarrhythmias on surface electrocardiogram (ECG).[1] Studies on genotype–phenotype correlation identified the clinical characteristics in each genetic subgroup, which made it possible to diagnose and introduce β-blocker therapy (BBT) appropriately in LQTS patients.[2–4] In patients with LQTS type 1 (LQT1), β-blockers are quite effective, whereas they are less effective in suppressing arrhythmic events in LQT2 and 3.[2]

Previous studies have demonstrated the importance of evaluating patients by clinical symptoms, gender, causative mutations, the type or biophysical function of mutations, and corrected QT (QTc) interval to stratify the arrhythmic risk in LQTS.[3,5–14] Heart rate (HR) has been recognized since the establishment of LQTS as a clinical entity, and a low HR for age was included in the diagnostic criteria.[15] A recent study by Schwartz *et al.*[16] demonstrated that a lower resting HR and a relatively low baroreflex sensitivity in *KCNQ1* A341V carriers are protective factors,

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whereas HR at rest in other subtypes of LQTS has not been fully investigated. In clinical practice, we have noted that in some cases of LQT2 that TdP was triggered by HR of <60 b.p.m. and suppressed by pacing at 80 b.p.m., which made us evaluate the importance of HR in arrhythmic events of LQT2 patients. For these reasons, we aimed to analyse whether HR at rest before BBT could be a novel risk factor for cardiac events besides gender, genetic locus, and prolonged QT interval in LQT2. We also evaluated the relationship between HR at rest and arrhythmic events before and after BBT through the analysis of clinical data on patients with LQT2.

Methods

Study population

From September 1996 to July 2009, 587 probands with QT prolongation underwent genetic testing in three institutes in Japan, Shiga University of Medical Science, Kyoto University Graduate School of Medicine, and the National Cardiovascular Center. One hundred and fifty-two probands (26%) were genotyped as LQT2. We also screened mutations in *KCNQ1*, *SCN5A*, *KCNE1–3*, and *KCNJ2* using the standard genetic tests[17–20] and excluded 20 probands with compound mutations and/or modifier single-nucleotide polymorphisms known to affect the QT interval (*KCNH2* K897T and *KCNE1* D85N).[21,22] The remaining 132 probands were found to have a single *KCNH2* mutation, and among them, we excluded from analyses patients under 15 years and those without detailed clinical information or with medication (except for β-blocker) which could influence baseline ECG measurements at the first medical contact and thereafter. Children <15 years old were not studied because they had relatively high basal HR. Family members of the 152 probands were recruited for the analysis if we could obtain necessary clinical information and if they were over 15 years old. As a result, the study population became 110 patients (45 probands and 65 family members) from 74 unrelated Japanese LQT2 families.

Both symptomatic and asymptomatic patients were included in the groups of probands and family members. Regardless of being probands or family members, patients were defined as symptomatic when they had a history of cardiac events (defined as ventricular fibrillation, TdP, or syncope due to ventricular arrhythmia) at the first medical contact or at the time of yearly follow-up. Patients with an apparent history of vasovagal syncope were not included in the study. The protocol for genetic analysis complied with the Declaration of Helsinki and was approved by the institutional ethics committees and performed under their guidelines. All individuals or their guardians gave written informed consent to genetic and clinical data analyses. Follow-up data were obtained from patients' regular hospitals working with the authors in case patients lived far from our institutions or hospitals and were not able to visit us.

Genetic analysis and characterization

Genomic DNA was isolated from venous blood lymphocytes using the QIAamp DNA blood midikit (Qiagen, Hilden, Germany). Established primer settings were used to amplify the entire coding regions of known LQTS genes from genomic DNA.[17–20] Denaturing high performance liquid chromatography (DHPLC) or direct sequencing techniques were employed as described elsewhere.[11] Polymerase chain reaction fragments presenting abnormal signals in DHPLC analysis were subsequently sequenced by the dideoxynucleotide chain termination method with fluorescent dideoxynucleotides on an ABI 3113xl genetic analyzer (PE Applied Biosystems).

The pore region of the *KCNH2* channel was defined as the area extending from S5 to the mid-portion of S6 involving amino acid residues from 550 through 650 according to the previous report.[10] The non-pore region included the N-terminus region, transmembrane domains apart from the pore region and the C-terminus region.

Clinical characterization

Routine demographic data and basal 12-lead ECGs were obtained from all subjects at the first medical contact as well as at yearly follow-up. In 104 patients, ECG parameters were measured before BBT was introduced. The remaining six patients, in whom BBT was started after the first cardiac event by an attending physician in other hospitals, visited a university hospital for further diagnostic confirmation of the symptoms. One of the six patients experienced aborted sudden cardiac death, four had documented TdP, and one had a syncopal attack. After obtaining informed consent, BBT was discontinued for more than five times the half life and examinations were performed, including a blood test, basal ECG, chest X ray, echocardiogram, and treadmill test for the diagnosis of congenital long QT syndrome.

Electrocardiograph parameters measured in the study were HR and QT interval. Rate-dependent QT intervals were corrected for HR using Bazett's method. QT interval was manually measured in lead V_5 using the tangent method[4] with an average of 2 or 3 consecutive beats by three investigators who were completely unaware of the patients' clinical and genetic state. There were no significant differences in the measured data among three investigators. Bifid T waves, but not U waves, were included in the QT measurements. In the presence of bifid T waves, the end of the second T wave was defined as the end of the QT interval. If ECG recordings were obtained during a cardiac event, such as the appearance of frequent ventricular tachycardia, TdP, or cardiac arrest, they were requested to perform another examination after patient's general status had improved.

Data on patients who received BBT after the initial check-up were evaluated, including the dose of each drug, HR under medication, and recurrent arrhythmic episodes. Other treatments, such as implantable cardioverter-defibrillator (ICD) implantation and surgical left cardiac sympathetic denervation, were also evaluated. Follow-up data, including the occurrence of cardiac events and therapeutic changes, were collected retrospectively.

Statistical analysis

Student's *t*-test was employed to compare continuous data. Differences in frequencies were analysed by the χ^2 test or Fisher's exact test. Analysis of variance was used to test differences of variables among more than three groups. Stepwise regression analysis was performed to determine predictors of cardiac events. Variables with $P < 0.05$ on univariate analysis were included in a logistic regression model with cardiac events as dependent variables. To determine the connection of the selected clinical variables with the occurrence of cardiac events, odds ratios for unadjusted data and their 95% confidence intervals were calculated. The cumulative probability of the first cardiac event between 15 and 50 years old was estimated using the Kaplan–Meier method. The Cox proportional-hazards survivorship model was used to investigate whether there were any prognostic factors that could influence the occurrence of cardiac events. Data are reported as the mean ± SD. Two-sided probability values <0.05 were considered significant. Statistical calculations were performed using SPSS software (version 18.0J).

Results

Clinical and genetic characteristics

The study population consisted of 110 consecutive patients from 74 unrelated Japanese LQT2 families (*Table 1*). The baseline ECG showed that the mean HR of probands tended to be lower than that of family members ($P = 0.06$).

All patients were genotyped to be a heterozygous carrier of 70 different *KCNH2* mutations (18 in the N-terminus, 15 in non-pore regions, 13 in pore regions, and 24 in the C-terminus). Forty-three mutations were missense mutations, 15 were deletion/insertions, 9 were frameshifts, and 3 were nonsense mutations.

Table 1 Basal characteristics of the study population

	All ($n = 110$)	Proband ($n = 45$)	Family member ($n = 65$)
Clinical characteristics			
Age (years)	40.8 ± 17.5 (15–87)	31.2 ± 15.6 (15–77)	47.4 ± 15.6 (16–87)**
Sex (male/female)	40/70	10/35	30/35*
Symptomatic patients [*n* (%)]	48 (44)	38 (84)	10 (15)**
Cardiac arrest (*n*)	7	4	3
Syncope (*n*)	46	38	8
Both (*n*)	5	4	1
ECG			
HR (b.p.m.)	62 ± 10	60 ± 9	63 ± 11
QTc (ms)	483 ± 58	508 ± 60	467 ± 50**

*$P < 0.05$ vs. proband.
**$P < 0.001$ vs. proband.

Factors determining cardiac events in LQT2 patients

We first evaluated whether HR and other variables (age at onset of cardiac events, female gender, site of mutation, missense mutation, and QTc) served as risk factors for cardiac events in LQT2 patients. Univariate analysis (*Table 2*) showed that HR of <60 b.p.m. *per se* was a significant risk for cardiac events ($P < 0.01$). In addition, female gender, HR as a continuous variable, a QTc interval of ≥500 ms, and pore site mutation were associated with an increased risk for cardiac events ($P < 0.05$). Other variables such as age at onset of cardiac events, sites of mutation (non-pore transmembrane, N-terminal, and C-terminal), and missense mutation were not statistically significant.

Multivariate analysis (*Table 2*) was subsequently performed using female gender, HR of <60 b.p.m., QTc of ≥500 ms, and pore site mutation. As for HR, we chose HR of <60 b.p.m. for multivariate analysis because we aimed to clarify if low HR of <60 b.p.m. was a significant risk factor for cardiac events. As shown in *Table 2*, female gender, HR <60 b.p.m., and QTc ≥500 ms were revealed to be significant risk factors for cardiac events ($P < 0.05$).

Bradycardia as an arrhythmic risk factor in LQT2 patients

We employed two ECG parameters, HR and QTc, to scrutinize who were more prone to have cardiac events in our LQT2 cohort. Using cutoff values of 60 b.p.m. for HR without β-blockers and 500 ms for QTc, we classified 110 LQT2 patients into four groups (*Figure 1*). Closed and open circles in the figure indicate symptomatic and asymptomatic patients, respectively (including both probands and family members). There were only eight symptomatic patients (23%) in the quadrant area of HR ≥60 b.p.m. and QTc <500 ms. In contrast, in the quadrant area defined as HR <60 b.p.m. and QTc ≥500 ms, 12 subjects (86%) experienced cardiac events ($P < 0.05$, vs. HR ≥60 b.p.m. and QTc <500 ms).

Table 2 Predictors of cardiac events (syncope, aborted cardiac arrest, or sudden cardiac death) in univariate and multivariate analyses

	Univariate analysis		Multivariate analysis	
	Odds ratio (95% CI)	*P*-value	Odds ratio (95% CI)	*P*-value
Age at onset	1.08 (0.78–1.49)	0.639		
Female gender	3.56 (1.51–8.38)	0.004	4.54 (1.72–12.00)	0.002
HR <60 b.p.m.	2.83 (1.30–6.16)	0.009	4.46 (1.77–11.24)	0.001
HR (continuous variable)	0.95 (0.91–0.99)	0.022		
QTc ≥500 ms	2.65 (1.18–6.00)	0.019	2.93 (1.13–7.59)	0.026
Mutation location				
Pore	2.45 (1.07–5.60)	0.034	1.77 (0.70–4.48)	0.230
Transmembrane, non-pore	0.91 (0.27–3.08)	0.914		
N-terminal	0.83 (0.33–2.04)	0.677		
C-terminal	0.57 (0.26–1.27)	0.169		
Missense mutation	2.10 (0.91–4.85)	0.081		

Table 3 summarizes the baseline characteristics of four groups divided by HR and QTc. The group of HR ≥60 b.p.m. and QTc <500 ms was defined as Group A, the group of HR <60 b.p.m. and QTc <500 ms as Group B, HR ≥60 b.p.m. and QTc ≥500 ms as Group C, and HR <60 b.p.m. and QTc ≥500 ms as Group D. There were no significant differences among four groups regarding age at baseline ECG recording, age at the first event, percentages of female gender, and BBT. In Group A, the number of proband was significantly lower than that in Groups B and D. The incidence of syncope or aborted cardiac arrest in Group A was significantly lower than in the Groups B and C. In groups of HR <60 b.p.m. (B and D), patients with QTc ≥500 ms (Group D) had more arrhythmic events than those with QTc <500 ms (Group B).

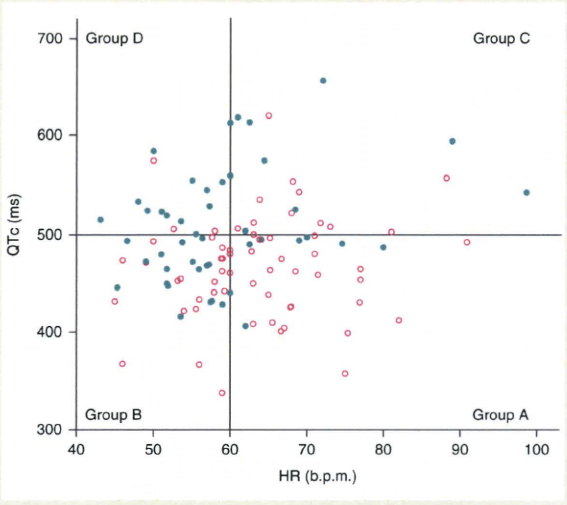

Figure 1 Distribution of KCNH2 mutation carriers according to the resting HR and QTc duration. Closed and open circles indicate symptomatic and asymptomatic patients, respectively. Two solid lines in the graph are drawn using the cutoff values of 60 b.p.m. and 500 ms. QTc was measured in lead V5. Groups A-D in the graph correspond to those in the text, *Table 3* and *Figure 2*.

We then estimated the cumulative probability of the first cardiac event between the age of 15 and 50 in four groups (Groups A–D, *Figure 2*). The Kaplan–Meier analysis of all subjects (*Figure 2A*) showed that cumulative event-free survival was significantly different ($P = 0.007$ by the log-rank test) and when adjusted for multiple comparisons, cumulative event-free survival was higher in Group A than in groups of HR <60 b.p.m. ($P = 0.014$ vs. Group B, $P = 0.001$ vs. Group D). In contrast, the survival rate was not statistically different among Groups B–D.

In *Figure 2B* and *C*, we examined the clinical course of 45 probands and 65 family members separately. The Kaplan–Meier analysis revealed no statistical difference in probands (*Figure 2B*, $P = 0.206$ by the log-rank test), whereas in family members, cumulative event-free survival was significantly different among the subgroups (*Figure 2C*, $P = 0.017$ by the log-rank test, $P = 0.058$ for Group A vs. Group B, $P = 0.002$ for Group A vs. Group D in multiple comparisons). Thus, the statistical difference in overall subjects may result from the prognosis of family members in our study population.

Finally, in order to assess the significance and independence of HR and QTc for cardiac events, we evaluated the parameters with the Cox proportional-hazards survival model (*Table 4*). The values of HR and QTc were centred at 60 b.p.m. and 500 ms for ease of interpretation. Compared with patients in Group A, patients in groups of HR <60 b.p.m. (Groups B and D) showed a higher risk for cardiac events by 2.6–4.4-fold. Although the hazard ratio in Group C was 2.16, there was no statistical difference between Groups A and C.

Table 3 Baseline clinical characteristics of four subgroups defined by QTc and basal HR

	QTc <500 ms		QTc ≥500 ms	
	Group A: HR ≥60 b.p.m. (*n* = 35)	**Group B: HR <60 b.p.m. (*n* = 39)**	**Group C: HR ≥60 b.p.m. (*n* = 22)**	**Group D: HR <60 b.p.m. (*n* = 14)**
Age (years) at ECG (range)	43 ± 18 (16–87)	39 ± 17 (15–71)	42 ± 18 (16–77)	39 ± 17 (15–64)
Age (years) at first event (range, number of patients)	25 ± 10 (13–42, *n* = 8)	27 ± 15 (15–71, *n* = 19)	26 ± 19 (15–77, *n* = 10)	26 ± 15 (13–54, *n* = 10)
Female gender [*n* (%)]	23 (66)	22 (55)	16 (73)	9 (64)
Proband [*n* (%)]	8 (23)*	18 (46)	12 (55)	7 (50)
Pore site mutation [*n* (%)]	6 (17)**	11 (28)	10 (46)	7 (50)
Schwarz score	3.1 ± 2.0§	3.6 ± 1.7§	5.5 ± 1.7	6.2 ± 1.2
Syncope or aborted cardiac arrest [*n* (%)]	8 (23)‡	19 (49)†	10 (46)	11 (79)
β-Blockers [*n* (%)]	7 (20)	13 (33)	9(41)	6 (43)

Values are given as the mean ± SD where indicated. HR = heart rate.
*$P < 0.05$ vs. Groups B and C.
**$P < 0.05$ vs. QTc ≥500 ms (Groups C and D).
§$P < 0.001$ vs. QTc ≥500 ms (Groups C and D).
†$P < 0.05$ vs. Group D.
‡$P < 0.05$ vs. HR <60 b.p.m. (Groups B and D).

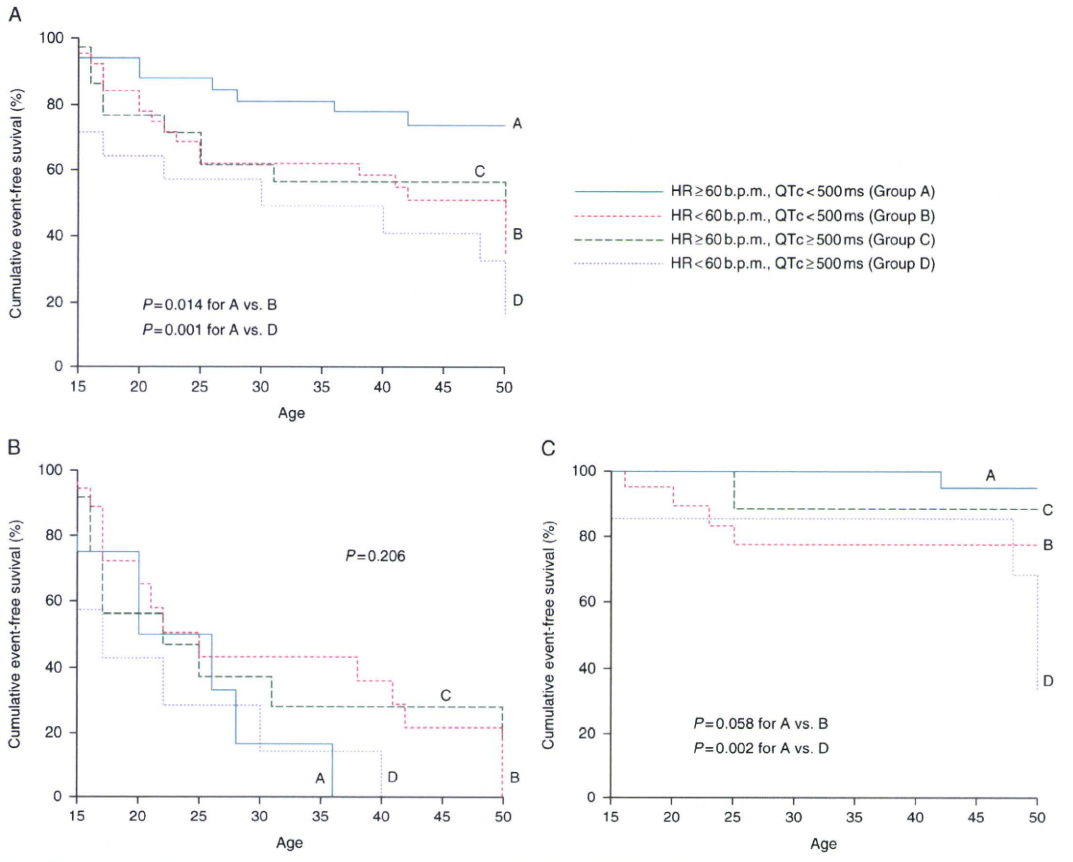

Figure 2 The Kaplan–Meier cumulative cardiac event-free survival curves from the age of 15–50 among each of four subgroups defined by cutoff values for HR of 60 b.p.m. and QTc of 500 ms. *Panels A–C* show the Kaplan–Meier curves of 110 patients, 45 probands and 65 family members, respectively. The group of HR ≥60 b.p.m. and QTc <500 ms was defined as Group A, the group of HR <60 b.p.m. and QTc <500 ms as Group B, HR ≥60 b.p.m. and QTc ≥500 ms as Group C, and HR <60 b.p.m. and QTc ≥500 ms as Group D.

Table 4 Contribution of QTc duration and HR to COX survival model

	Number of patients	Hazard ratio	95% CI	*P*-value
QTc <500 ms				
HR ≥60 b.p.m. (Group A)	35	1	–	–
HR <60 b.p.m. (Group B)	39	2.60	1.14–5.97	0.023
QTc ≥500 ms				
HR ≥60 b.p.m. (Group C)	22	2.16	0.85–5.47	0.105
HR <60 b.p.m. (Group D)	14	4.39	1.76–10.92	0.001

Treatment

β-Blocker therapy was introduced in 35 patients (29 probands) after diagnosis of LQT2 was made. Mean HR on medication was 56 ± 8 b.p.m. Metoprolol was used in 3 patients (90 ± 52 mg, 30–120), carvedilol in 3 (15 ± 9 mg, 5–20), atenolol in 4 (50 ± 0 mg, 50), propranolol in 21 (42 ± 16 mg, 30–80), and bisoprolol in 4 (4 ± 1 mg, 2.5–5).

Implantable cardioverter-defibrillator was implanted in 12 patients (VF: five patients, syncope: seven patients) during the first hospitalization or follow-up. In seven patients with a history of cardiac arrest due to VF (*Table 1*), two patients were treated with an ICD, three with both ICD and β-blocker, one with a pacemaker, and one with β-blocker alone (because the patient rejected ICD implantation). In a patient with a pacemaker, TdP was