Cellular Expression Studies

To study the mechanism underlying the risk for cardiac events in patients with missense C-loop mutations, we measured channel function and regulation for channels formed with wild-type (WT) subunits coexpressed with 4 mutant subunits present in C loops (G189R, R190Q, R243C, and V254M) and 4 mutant subunits present in the non–C-loop domains: 3 in the membrane-spanning domain (T312I, G168R, and S225L) and 1 in the C terminus (R555C). The mutations chosen included the most common mutations in the LQT1 registry. WT and mutant KCNQ1 subunit cDNA and KCNE1 subunit cDNA were transfected into HEK293T cells.[10] Mutant KCNQ1 cDNA was transfected in combination with WT-KCNQ1 to mimic the heterozygous nature of the disease (WT-KCNQ1:mutant KCNQ1:KCNE1=0.5:0.5:1). Fluorescence-conjugated and -tagged constructs were used to evaluate the efficiency of the cotransfection of WT and mutant subunits[11] (see the online-only Data Supplement). Of the HEK293T cells cotransfected with both WT and mutant subunits, 85% to 90% showed fluorescence of at least 1 subunit transfected, and 85% to 95% of transfected cells expressed all the subunits transfected (Figure I in the online-only Data Supplement). All electrophysiology determinations were performed with the untagged subunit. Expression of WT and mutant subunits was confirmed by Western blot (Figure II in the online-only Data Supplement). Expression levels were not significantly decreased for the mutant subunits compared with WT. We measured ion channel currents after channel depolarization to 20 mV for 4 seconds from −80-mV holding potential before and after application of forskolin, a PKA activator (10 μmol/mL), with standard electrophysiological techniques and physiological solutions. Current was normalized for all voltages to cell capacitance, and further normalization was performed between WT and mutant. The normalization to WT currents was accomplished by use of WT cell currents transfected and measured on the same day as the currents measured from mutant channel.[10] Pipettes used had resistances ranging from 2 to 6 MOhm. Series resistance compensation of >70% was used to compensate for voltage drops in the pipette. All experiments were performed at room temperature. Details of the molecular biology and electrophysiological methods are given in the Materials and Methods section of the online-only Data Supplement.

End Point

The primary end point of the study was the occurrence of a first life-threatening cardiac event, comprising ACA requiring external defibrillation as part of the resuscitation or LQTS-related SCD (abrupt in onset without evident cause if witnessed or death that was not explained by any other cause if it occurred in an unwitnessed setting) from birth through 40 years of age. Follow-up after 40 years of age was not included to minimize the influence of coronary disease on cardiac events. The consistency of the results among patients who received an implantable cardioverter-defibrillator during follow-up was evaluated in a secondary analysis that included the occurrence of a first appropriate implantable cardioverter-defibrillator shock in the composite ACA or SCD end point.

Statistical Analysis

Characteristics of the 4 subgroups of patients categorized by mutation location and type were compared by use of a 1-way ANOVA test or χ^2 and Fisher exact tests as appropriate. The probability of a first life-threatening cardiac event by the mutation location and type subgroup was graphically displayed according to the Kaplan-Meier method, with comparison of instantaneous risk by the log-rank test. The Cox proportional-hazards survivorship model was used to evaluate the independent contribution of clinical and genetic factors to the first occurrence of a life-threatening cardiac event from birth through 40 years of age. The Cox regression models, stratified by decade of birth year and allowing for time-dependent covariates, were fit to estimate the adjusted hazard ratio function of age. Therefore, to fulfill the assumption of proportional hazards for sex over the entire age range, a time-dependent covariate for sex (via an interaction with time) was incorporated, allowing for different hazard ratios by sex before and after 13 years of age. This was justified by the known higher risk of cardiac events or life-threatening cardiac events among male subjects before adolescence and a similar or higher female risk after the onset of adolescence.[12–16] Patients who did not have an ECG for QTc measurement (n=127) were identified in the Cox models as QTc missing, and all Cox models were adjusted for this QTc-missing parameter. The influence of time-dependent β-blocker therapy (the age at which β-blocker therapy was initiated) on outcome in the subgroups of patients with and without C-loop missense mutations was determined by adding a time-dependent β-blocker–by–mutation category interaction term to the multivariable Cox model. We have adjusted for the effect of potential lack of independence between subjects using the robust sandwich estimator for family membership.[17,18] This robust sandwich covariance estimator is used with correlated data. Correlations among data points in the Cox model lead to underestimation of the SE used in significance testing, whereas the robust estimator uses an inflated variance estimate, taking family membership or other clustering connection into account. All significant predictors of life-threatening event risk remained significant with or without the use of this robust measure of variance. It should be noted that there is seldom more than a single observed outcome (ACA/SCD) per family (only 7% of families had >1 event); thus, the standard model-based SEs, confidence intervals, P values, and likelihood ratio tests are valid.[16]

We have carried out the following additional secondary analyses: (1) including the biophysical function of the mutations (categorized as dominant negative, haploinsufficiency, and unknown) as a covariate in the model, (2) excluding the large subgroup of patients with V254M mutations, and (3) including appropriate implantable cardioverter-defibrillator shocks in the composite end point. In addition, to assess whether fuller adjustment for family membership was important, regression models that included shared frailty terms (ie, random effects) for family were fit.

The statistical software used for the analyses was SAS version 9.20 (SAS Institute Inc, Cary, NC). For the fitting of models with frailty terms, the software used was Splus 7.0.0 for Sun SPARC. For electrophysiology and biochemistry experiments, 1-way ANOVA followed by the Tukey post hoc test was applied to assess statistical significance for multiple-group comparisons by use of SPSS Statistics (IBM). An unpaired Student t test was used for 2-group comparisons. A 2-sided significance level of 0.05 was used for hypothesis testing.

Results

Study Sample

The spectrum of mutations as categorized by location and type and their respective number of carriers are presented in Table I-A in the online-only Data Supplement. The location and frequency of missense mutations are presented diagrammatically in Figure 1. Of the 99 total different KCNQ1 mutations identified, 77 were missense mutations and 22 were nonmissense mutations. Missense mutations were further categorized according to their location: 28 different mutations in C-terminus or N-terminus regions (26 in C terminus), 34 mutations in membrane-spanning regions, and 15 mutations in the C-loop regions (8 in S2-S3 loop and 7 in S4-S5 loop). The clinical characteristics of patients in the 4 mutation location/type subgroups are presented in Table 1. Of the 860 study subjects, 20% had C/N terminal missense mutations, 44% had membrane-spanning missense mutations, 15% had C-loop missense mutations, and 22% had nonmissense mutations. Patients with C-loop missense mutations exhibited the longest QTc interval at enrollment, were treated with β-blockers more frequently during follow-up, and had a higher frequency of cardiac events of any type, including

Table 1. Demographic and Clinical Characteristics

Parameter	Missense			Nonmissense
	C/N Terminus	Membrane Spanning	C Loops	
Patients, n (%)	172 (20.0)	376 (43.7)	125 (14.5)	187 (21.7)
Female, n (%)	94 (54.7)	221 (58.8)	70 (56.0)	119 (63.6)
Age at enrollment, median (interquartile range), y	21 (9–41)	25 (11–41)	19 (5–35)	21 (11–39)
QTc at enrollment, mean±SD, ms*	467±63	480±51	503±58	470±41
QTc at enrollment ≥500 ms, n (%)	39/154 (25.3)	94/312 (30.1)	47/102 (46.1)	41/165 (24.8)
Therapy during follow-up, n (%)				
β-blockers	64 (37.0)	167 (44.4)	63 (50.4)	74 (39.8)
Pacemaker	2 (1.2)	7 (1.9)	1 (0.8)	4 (2.2)
Defibrillator	6 (3.5)	25 (6.6)	10 (8.0)	13 (7.0)
Sympathectomy	0 (0.0)	1 (0.3)	1 (0.8)	1 (0.5)
Cardiac event during follow-up, n (%)				
Syncope	52 (30.1)	122 (32.4)	69 (55.2)	46 (24.7)
Aborted cardiac arrest	6 (3.5)	10 (2.7)	8 (6.4)	3 (1.6)
Sudden cardiac death	18 (10.4)	29 (7.7)	24 (19.2)	13 (7.0)
Any cardiac event	63 (36.4)	144 (38.3)	84 (67.2)	55 (29.6)

C loop indicates cytoplasmic loop.

*Of the 127 long-QT syndrome type 1 mutation carriers who did not have an ECG for QTc measurement, 58 (46%) died suddenly at a young age without a documented ECG.

syncope, ACA, and LQTS death, compared with the other mutation subgroups. The clinical characteristics of probands only are presented in Table I-B in the online-only Data Supplement.

Clinical Outcome of Patients According to Mutation Location and Type

There were 105 first life-threatening cardiac events (27 first ACA events and 78 first LQTS-related SCD events) among the 860 study patients. Patients were enrolled in the registry between 1978 and 2007 with follow-up through 2008; the last reported life-threatening cardiac event occurred in 2005. Figure 2 presents the cumulative probabilities of first life-threatening cardiac events in the 4 subgroups. There was a significantly higher event rate in the C-loop missense subgroup compared with the other 3 subgroups (log-rank $P<0.001$). Thus, at 40 years of age, the rate of life-threatening cardiac events was 33% in patients with C-loop missense mutations compared with ≤16% in patients with other mutations.

The findings from the multivariable analysis for the end point of a first life-threatening cardiac event are shown in Table 2. Notably, the adjusted hazard ratio for C-loop missense versus nonmissense mutation was 2.75 ($P=0.009$), and there was no statistically significant difference in the risk among the other mutation location/type subgroups.

Secondary confirmatory analyses (Table II in the online-only Data Supplement) showed that patients with C-loop missense mutations had an adjusted hazard ratio of 2.74 (95% confidence interval, 1.68–4.46; $P<0.001$) for life-threatening events compared with patients with other mutations. The results were consistent when the biophysical function of the mutations was added as a covariate to the multivariable model. To show that our results do not depend on the C-loop V254M mutation, which is the most common mutation in the C-loop subgroup (Table I in the online-only Data Supplement), accounting for 50% of C-loop patients, we

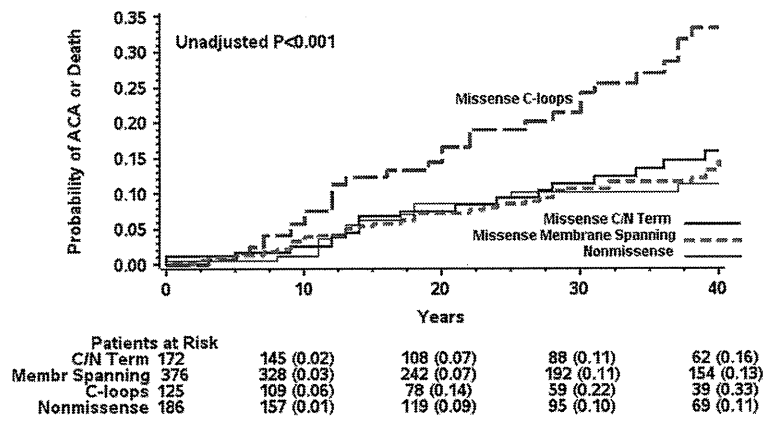

Figure 2. Kaplan-Meier estimates of cumulative probability of life-threatening cardiac events by mutation location and type. The numbers in parentheses reflect the cumulative event rate at that point in time. ACA indicates aborted cardiac arrest; LQTS, long-QT syndrome.

Table 2. Multivariable Analysis: Risk Factors for Aborted Cardiac Arrest or Sudden Cardiac Death

	Hazard Ratio	95% Confidence Interval	P
Sex/age			
Male (vs female) age <13 y	1.93	1.08–3.45	0.03
Male (vs female) age 13 to 40 y	1.13	0.67–1.91	0.65
QTc ≥500 ms (vs QTc <500 ms)	3.55	1.83–6.89	<0.001
Mutation type and location			
Cytoplasmic loops (missense) vs nonmissense	2.75	1.29–5.86	0.009
C/N terminus (missense) vs nonmissense	1.47	0.64–3.39	0.37
Membrane spanning (missense) vs nonmissense	0.85	0.41–1.78	0.67

The models are adjusted for sex by age and corrected QT category (including missing QT), mutation type and location category, and time-dependent β-blocker treatment. Of the 127 long-QT syndrome type 1 mutation carriers without available ECG data, 58 (46%) died suddenly at a young age without a documented ECG. The hazard ratio for missing QTc versus available was 10.49 (95% confidence interval, 6.61–16.66; $P<0.001$).

have carried out an additional separate analysis excluding patients who carried this mutation. Results were consistent, with patients with C-loop mutations having a greater risk for life-threatening events, demonstrating that our findings were independent of this mutation. The results were also consistent after inclusion of appropriate implantable cardioverter-defibrillator shocks in the composite end point (adjusted hazard ratio for C-loop missense mutations versus nonmissense mutations. 2.64; 95% confidence interval, 1.64–4.23; $P<0.001$) and after stratifying patients by enrolling center. To assess whether fuller adjustment for family membership was important, regression models that included frailty terms (ie, random effects) for family were fit in the multivariable Cox models. Models with gamma and gaussian frailty terms were fit, and the C-loop term had a consistent effect size with the original models while remaining statistically significant. Furthermore, in both of these models, the frailty terms were nonsignificant. The consistency of the results provides further support for the higher risk associated with C-loop mutations.

β-Blocker Therapy

In the present study, the effect of β-blocker therapy on the risk of life-threatening events among the different mutation subgroups was assessed as a time-dependent covariate (ie, β-blockers were given to patients at different time points during follow-up, and this information was taken into account in the multivariable models). Multivariable analysis showed a significant differential effect of β-blocker therapy on the outcome of patients with C-loop missense mutations compared with those who had other mutations (Table 3). β-Blocker therapy was associated with a significant 88% reduction ($P=0.02$) in the risk of life-threatening events among patients with C-loop missense mutations, whereas the benefit of β-blocker therapy was significantly attenuated among patients with other mutations in the *KCNQ1* channel

Table 3. Multivariable Analysis: Response to β-Blocker Therapy

β-Blocker vs No β-Blocker Therapy	Hazard Ratio	95% CI	P
All LQT1 patients	0.49	0.19–1.23	0.13
LQT1 patients with C-loop missense mutations*	0.12	0.02–0.73	0.02
LQT1 patients with other mutations (non–C-loop missense mutations)*	0.82	0.31–2.13	0.68

CI indicates confidence interval; LQT1, long-QT syndrome type 1; and C-loop, cytoplasmic loop. The models are adjusted for sex by age and corrected QT category (including missing QT), mutation type and location category, and time-dependent β-blocker treatment.

*P for interaction for mutation location-by–β-blocker treatment=0.04.

(adjusted hazard ratio, 0.82; $P=0.68$; P for treatment-by–mutation location/type interaction=0.04).

Consistent with those findings, the rate of ACA or SCD (Figure 3) was lowest among patients with C-loop missense mutations who were treated with β-blockers and highest among patients with C-loop missense mutations who were not treated with β-blockers (0.17 versus 1.11 per 100 patient-years, respectively), whereas patients with other mutations in the KCNQ1 channel exhibited intermediate and similar rates of life-threatening events with and without β-blocker therapy (0.36 and 0.38 per 100 patient-years, respectively; Figure 3).

In addition, we have repeated analysis for only patients who were treated by β-blockers (at any point in time) and consistently found that there is differential response to β-blocker therapy depending on mutation location (β-blocker versus no β-blocker therapy in patients with C-loop mutations: hazard ratio, 0.15; 95% confidence interval, 0.01–1.73; $P=0.13$; β-blocker versus no β-blocker therapy in patients with non–C-loop mutations: hazard ratio, 1.89; 95% confidence interval, 0.54–6.63; $P=0.32$) with a value for treatment-by–mutation location/type interaction of $P=0.028$.

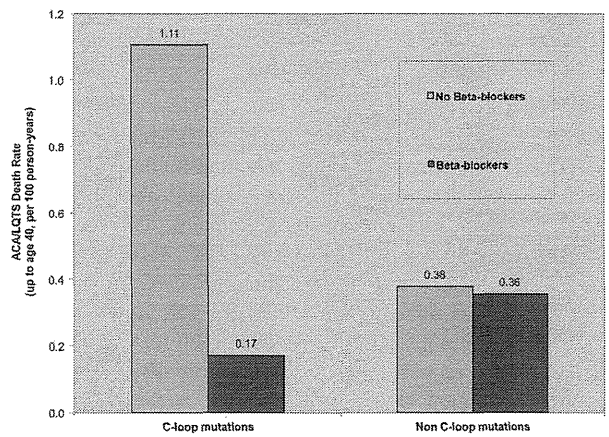

Figure 3. Risk for life-threatening cardiac events by mutation location and β-blocker treatment. Sixty-three of the 125 subjects (50%) with C-loop missense mutations were treated with β-blockers during a mean follow-up of 26.2 years; 305 of the 735 subjects (42%) with non–C-loop missense mutations were treated by β-blockers during a mean follow-up of 27.5 years. Event rates per 100 person-years were calculated by dividing the number of events during the period of β-blocker therapy or the absence of β-blocker therapy by person-years and multiplying the results by 100. ACA indicates aborted cardiac death; LQTS, long-QT syndrome.

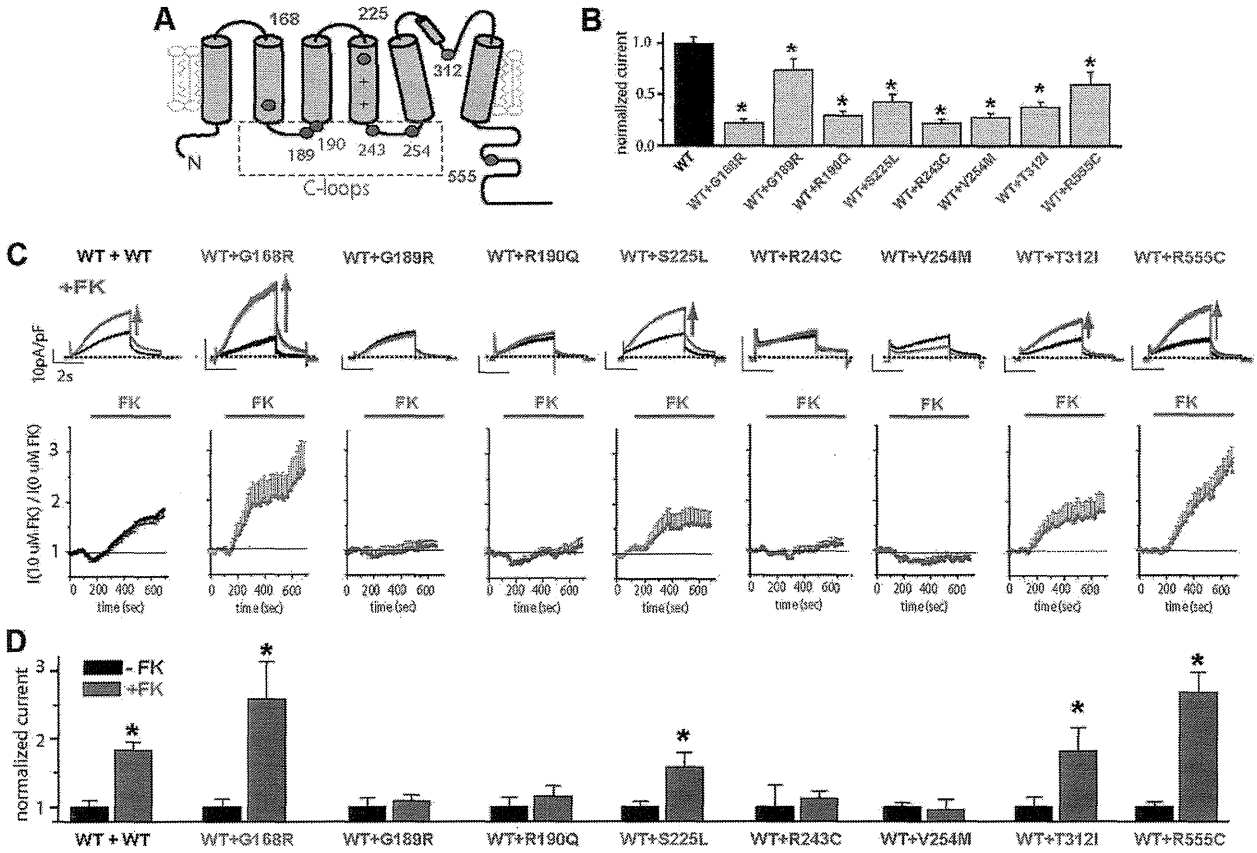

Figure 4. Regulation of LQT1 mutant channels by protein kinase A. **A**, Schematic representation of location of the mutations used in the study. **B**, Effect of each of the mutations studied in basal nonstimulated cell currents. Average current measured for cells expressing wild-type (WT) and mutant subunits measured at 40 mV after 3 seconds of depolarization. KCNQ1 and KCNE1 subunit were expressed at a ratio of 0.5 WT-KCNQ1:0.5 KCNQ1 mutant:1 KCNE1 or 0.5 WT-KCNQ1:0.5 vector:1 KCNE1 for T haploinsufficient channels. *$P<0.05$ vs WT. **C**, Top, Typical ion channel current measured before and after 10 minutes of application of the protein kinase A activator forskolin (FK; 10 μmol/L) for WT and WT and mutant subunits coexpressed. Scale bars in each panel are 10 pA/pF and 2 seconds. Scale bars are the same for all constructs. Bottom, Time course of current regulation by forskolin measured at 20 mV after 3 seconds of depolarization for channels formed by either WT or mutant coexpressed with WT subunits as indicated. Current was normalized to current in the absence of forskolin application. KCNQ1 and KCNE1 subunits were expressed at a ratio of 0.5 WT-KCNQ1:0.5 KCNQ1 mutant:1 KCNE1 or 1 WT-KCNQ1:1 KCNE1 for WT channels. Currents were activated by 4-second depolarizing steps to 20 mV from a −80-mV holding potential. These were followed by a step to −20 mV. **D**, Summary data for experiments done as in **C**. *$P<0.05$ vs the current before stimulation (black bar) in each group.

Cellular Expression Studies

To understand the mechanism underlying the increase in risk associated with C-loop mutations, we measured channel basal function and regulation in 8 mutant channels associated with LQT1: 3 in the membrane-spanning domains (T312I, G168R, and S225L), 4 located in C loops (G189R, R190Q, R243C, and V254M), and 1 in the C terminus (R555C; Figure 4A). WT and mutant subunits were coexpressed for all experiments. Basal channel current was decreased for all mutations studied compared with WT subunits (Figure 4B). In addition, because activation by PKA is thought to be particularly important for I_{Ks} function and to underlie arrhythmogenesis in LQT1,[4,19,20] we measured the effect of the PKA activator forskolin. All C-loop mutations tested showed dramatically impaired response to forskolin, whereas the other mutations showed a strong activation by forskolin, as did the WT KCNQ1 channel (Figure 4C and 4D).

Discussion

The present analysis of 860 LQT1 patients with a wide range of mutations in the *KCNQ1* channel provides several important implications regarding risk assessment and management in this study sample. First, patients with missense mutations located in the C loops exhibit the highest risk for life-threatening cardiac events independently of clinical and ECG variables. Second, β-blocker therapy is associated with a pronounced reduction in the risk of ACA or SCD among carriers of missense mutations in the C loop, whereas the benefit of this mode of medical therapy is significantly attenuated in LQT1 patients with other mutations. Third, expression studies of C-loop mutations suggest that an impaired regulation by PKA is the mechanism underlying the increased risk for cardiac events independently of patient QTc and may explain the pronounced response to medical therapy with β-blockers among patients with C-loop mutation carriers.

We have recently shown that patients with mutations located in the transmembrane region have a significantly higher rate of cardiac events than those with mutations located in the C terminus.[8] In addition, mutations in the transmembrane domain were suggested to be associated with

a greater prolongation of the QTc during exercise.[21] The present findings confirm previous work indicating that mutations in the transmembrane region are associated with higher risk but suggest that within the transmembrane region there are distinct functional domains, with the C loops (but not in the membrane-spanning domain) being associated with increased risk for life-threatening cardiac events compared with other mutations in the KCNQ1 channel. The S2-S3 and S4-S5 C loops have previously been suggested to have an important functional role in modifying the function of voltage-gated potassium channels.[22] In particular for I_{Ks}, the S4-S5 loop has been suggested to mediate a functional interaction with the auxiliary *KCNE1* subunits.[23] Most recently, LQT1 mutations in C loops, when expressed in the absence of WT subunits, were suggested to affect adrenergic channel regulation.[9] Our results showed that even when expressed in the presence of WT subunits, C-loop mutations can dramatically affect channel regulation. Consistent with our results, induced pluripotent stem cells differentiated into cardiomyocytes from a patient carrying R190Q were recently shown to lack adrenergic regulation of their I_{Ks} current.[24] Also consistent with our results, for haploinsufficient mutations, not tested here, a simple lack of mutant subunit expression is expected to maintain normal adrenergic regulation, contributing to the milder phenotype of these mutations.[8] It is conceivable that a decrease in channel regulation, as observed for the C-loop mutations, will lead to an increase in the burden of the mutation during adrenergic stimulus. The increase in cardiac risk associated with C-loop mutations is independent of traditional clinical variables; this can be explained by a blunted PKA-mediated activation because QTc is generally measured at rest. Thus, our results suggest that exercise may exacerbate the QTc prolongation for C-loop mutants. It has recently been suggested that the mutation KNCQ1(A341V) also caused an impairment in β-adrenergic activation.[25] This mutation is located at the end of the S6 domain, a region suggested to interact with the S4-S5 loop.[26] It is possible that other mutations causing functional impairment similar to that of the C-loop mutations may also carry the increased cardiac risk and β-blocker efficacy.

Current guidelines recommend empirical therapy with β-blockers in all LQTS patients.[27] The present study shows, for the first time, a mutation-specific response to β-blocker therapy in LQT1, demonstrating that β-blockers were associated with a significantly greater reduction in the risk of life-threatening cardiac events among patients with mutations located in the C loops compared with all other mutations. It is conceivable that during β-adrenergic stimulation, patients with mutations located in the C loops have an unopposed increase in inward Ca^{2+} currents and prolongation of repolarization caused by blunted PKA-mediated activation of I_{Ks}.[5] β-Blockers may decrease these unopposed inward Ca^{2+} currents, shorten repolarization, and reduce the risk of ventricular arrhythmias,[28] whereas patients with other mutations do not exhibit such an effect.

Study Limitations

Clinical history was obtained on enrollment in the registry, so follow-up data in the current study comprised historical data from birth to enrollment and prospective information collected at yearly intervals after enrollment.

The International LQTS Registry records therapies that are prescribed at the discretion of the treating physicians to enrolled subjects; therefore, β-blocker administration was not randomized. However, because the patient's physician would have been blinded to whether the patient had a C-loop mutation, the interaction of this with β-blocker therapy is still compelling. Prior studies from the International LQTS Registry have shown that β-blocker therapy is associated with a significant reduction in the risk of cardiac events in LQTS patients. However, the present study is the first to assess the benefit of β-blocker therapy for the reduction in the risk of ACA or SCD among LQT1 patients. We have shown that β-blocker therapy is associated with a significant 88% ($P=0.02$) reduction in the risk of life-threatening cardiac events among LQT1 carriers of the higher-risk C-loop mutations. Risk reduction associated with β-blocker therapy in the total study sample and among carriers of the low-risk non–C-loop mutations did not reach statistical significance. The lack of a significant β-blocker effect may be due to sample size limitation and a more limited number of events among carriers of lower risk mutations. Thus, lower-risk patients should still be treated with β-blocker therapy according to guidelines[27] because the cumulative probability of ACA or SCD from birth through 40 years of age among patients with non–C-loop missense mutations was still considerable (between 11% and 16%). These limitations also suggest that further studies in independent populations are needed before the results can be extrapolated to clinical practice.

The present results, derived from LQTS families enrolled in the registry, may be confounded by familial factors such as ethnicity. To minimize bias, we adjusted for family membership in the multivariable models and carried out a secondary analysis in which additional adjustment was made for proband status. These analyses yielded similar results, further supporting the consistency of our findings. Of the 127 LQT1 mutation carriers without available ECG data, 58 (46%) died suddenly at a young age without a documented ECG. To minimize this bias related to exclusion of higher-risk patients, all multivariable analyses included adjustment for a QTc-missing category in addition to the category of QTc >500 milliseconds.

Channel current and response to forskolin were analyzed for 8 mutations of 99 mutation types observed in this study, but the robust findings in these expression studies strongly support our suggested mechanism. Experimental data were performed at room temperature; results may be different at physiological temperature.

Conclusions

We used a combination of clinical analysis and cellular electrophysiology experiments to investigate the molecular determinants and mechanisms underlying the clinical outcomes of a large cohort of subjects having a spectrum of *KCNQ1* mutations categorized by their code type and location. Patients with *KCNQ1* missense mutations located in the cytoplasmic loops had a significantly greater risk for life-threatening cardiac events and gained grater benefit when

treated with β-blockers compared with patients having other *KCNQ1* missense or nonmissense mutations independently of clinical risk factors. We suggest that a combination of a decrease in basal function and altered adrenergic regulation of the I_{Ks} channel underlies the increased cardiac risk in this subgroup of patients. Our results highlight the importance of understanding the molecular determinants and mechanisms underlying arrhythmogenesis to identify cardiac risk factors for LQT1 patients.

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CLINICAL PERSPECTIVE

Long-QT syndrome type 1 (LQT1) arises from a decrease in repolarizing potassium current resulting from mutations in the *KCNQ1* gene. The main trigger for cardiac arrhythmic events in patients with LQT1 is activation of β1-adrenergic receptors during exercise. Despite the observed reduction in the risk of cardiac events with β-blocker therapy among LQT1 patients, there is still a considerable cardiac residual event rate, suggesting that subgroups of LQT1 patients have differential response to β-blockers. The present study of 860 patients from the International LQTS Registry shows that the presence of missense mutations in distinct functional domains of the KCNQ1 protein, the S2-S3 and S4-S5 cytoplasmic loops (C loops), is associated with a significantly increased risk for life-threatening cardiac events compared with other mutations. Furthermore, patients with missense C-loop mutations gained greater benefit when treated with β-blockers compared with patients having other *KCNQ1* mutations independently of clinical risk factors, demonstrating that LQT1 patients have differential response to β-blocker therapy depending on mutation location. Both a decrease in basal function and altered adrenergic regulation of the I_{Ks} channel underlie the increased cardiac risk and response to β-blockers in this subgroup of patients. Patients with missense C-loop mutations should be considered a high-risk group of patients but with a pronounced response to β-blockers.

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ORIGINAL ARTICLE
Arrhythmia/Electrophysiology

Seasonal and Circadian Distributions of Cardiac Events in Genotyped Patients With Congenital Long QT Syndrome

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Background: Although the incidence of ventricular tachyarrhythmias associated with structural heart disease is highest in winter and during the daytime, seasonal and circadian variations among cardiac events in patients with congenital long QT syndrome (LQTS) remain unknown. The present study aims to determine seasonal and circadian cardiac events in patients with a congenital LQTS genotype.

Methods and Results: The medical records of 196 consecutive patients with symptomatic LQTS (age, 32±19 years; female, n=133; LQT1, n=86; LQT2, n=95; LQT3, n=15) who were genotyped between 1979 and 2006 at 2 major Japanese institutions were retrospectively analyzed. The patients with LQT1, LQT2, and LQT3 developed 223,550 and 59 cardiac events during a mean follow-up of 26, 33, and 25 years, respectively. The numbers of cardiac events significantly peaked during the summer among those with LQT1 (P<0.001) and from summer to fall in those with LQT2 (P<0.001), but reached the nadir in winter among those with LQT3 (P=0.003). Cardiac events significantly peaked in the afternoon (12:00–17:59) and morning (06:00–11:59) among those with LQT1 (P<0.001) and LQT2 (P<0.001).

Conclusions: The frequency of cardiac events was specifically seasonal and circadian among patients with the 3 major genotypes of congenital LQTS. (*Circ J* 2012; **76:** 2112–2118)

Key Words: Cardiac events; Circadian; Long QT syndrome; Seasonal

Congenital long-QT syndrome (LQTS) is caused by mutations in genes encoding channels that regulate potassium, sodium, or calcium currents, and by mutations in a cytoskeletal gene that affects sodium and calcium kinetics, resulting in prolonged ventricular repolarization and an increased risk for sustained ventricular tachyarrhythmias.[1] Although the incidence of ventricular tachyarrhythmias including ventricular tachycardia (VT) and ventricular fibrillation (VF) is the highest in winter and during the daytime among patients with structural heart disease,[2–9] the seasonal and circadian occurrence of cardiac events as a result of torsade de pointes remains unknown in patients with congenital LQTS.

We analyzed the medical records of patients genotyped with congenital LQTS to determine the seasonal and circadian occurrence of cardiac events.

Methods

Study Population

The present study included 196 (age, 32±19 years; female, n=133) consecutive patients with symptomatic LQTS and detailed medical information who were genotyped at 2 major Japanese institutions (National Cerebral and Cardiovascular Center, Suita, and Shiga University of Medical Science, Ohtsu)

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Table 1. Baseline Characteristics of the Study Population (n=196)

	LQT1	LQT2	LQT3	Total
Patients	86	95	15	196
Gender, female	60 (69.8%)	67 (70.5%)	6 (40.0%)	133 (67.9%)
Age at the study (follow-up)	25.8±14.1	32.5±14.8	25.3±18.1	28.0±15.6
Age at the genetic test	15.5±15.6	26.9±13.9	18.8±13.7	20.6±15.2
β-blockers	59 (68.6%)	62 (65.3%)	6 (40.0%)	127 (64.8%)
ICD therapy	9 (10.5%)	16 (16.8%)	5 (33.3%)	30 (15.3%)
Total cardiac events	223	550	59	832
Mean events number/patient	2.6±2.7	5.8±11.5	3.9±4.9	4.2±8.5
Median/range events	2 (1–18)	3 (1–75)	2 (1–19)	2 (1–75)

LQT, Long QT; ICD, implantable cardioverter defibrillator.

Table 2. Details of Cardiac Events in Each Type of LQT Syndrome

	Presyncope	Syncope	Cardiac arrest or death	Total cardiac events
LQT1	16 (7.2%)	190 (85.2%)	17 (7.6%)	223
LQT2	220 (40.0%)	311 (56.5%)	19 (3.5%)	550
LQT3	2 (3.4%)	35 (59.3%)	22 (37.3%)	59

LQT, Long QT.

between 1979 and 2006. Eighty-six, 95, and 15 patients had LQT1, LQT2, and LQT3, respectively. The LQTS patients having compound mutations or other mutations except for LQT1, LQT2, and LQT3 were excluded from the analysis. Symptoms included presyncope, syncope, and cardiac arrest. Sudden dizziness, palpitation, and chest pain persisting for over 30 s without a complete loss of consciousness confirmed by electrocardiogram (ECG) recordings as being associated with ventricular tachyarrhythmias at least once were included as presyncope. Multiple events were defined as over 2 cardiac events per 24-h period. Written informed consent was obtained from each patient in this study to undergo genetic testing. The privacy of the patients was protected by the anonymization of all data.

Data Collection

In general, the LQTS patients were first referred to our hospital or to a local outpatient clinic for evaluation and therapy, and followed up routinely every 1–3 months. After genotyping at our hospital, they attended our outpatient clinic every 1–6 months (mean, 2.2±1.1 months; median, 2 months). The follow-up period included all periods since the first presentation at our hospital or a local outpatient clinic. A detailed history was obtained at each visit and all patients were encouraged to attend the clinic whenever they experienced palpitations, chest pain, presyncope, or syncope. Patients with cardiac arrest were usually conveyed to our hospital. We obtained as complete a medical history as possible from patients and their relatives, and retrospectively analyzed these records in detail to determine the seasonal and circadian distribution of cardiac events. Some patients were followed up at other outpatient clinics even after genetic testing when they lived far from our institutions. Local physicians or pediatric cardiologists then provided detailed information taken directly from their own medical records, as well as from the patients and their families.

Seasons are defined in the present study according to the Japanese calendar as winter, December to February; spring, March to May; summer, June to August; and fall, September to November. The time of day was classified as night-time (00:00–05:59), morning (06:00–11:59), afternoon (12:00–17:59), and evening (18:00–23:59). Triggers of cardiac events were classified as exercise, emotion, and rest or sleep without arousal according to a previous report.[10]

Statistics

Quantitative data are presented as means±SD or medians/range, and were compared using ANOVA or Kruskal-Wallis analysis. Categorical data are presented as absolute and percentage frequencies, and were analyzed using the χ^2 test. The difference in the frequency of cardiac events was analyzed using the goodness-of-fit test for multinomial distribution. A value of $P<0.05$ was considered significant.

Results

Patient Characteristics

The baseline characteristics of the study population are shown in Table 1. Females comprised about 70% of the LQT1 and LQT2 groups but only 40% of the LQT3 group. The total numbers of cardiac events were 223, 550, and 59 in the LQT1, LQT2, and LQT3 groups during a mean follow-up of 26, 33, and 25 years, respectively. The numbers of events per genotype significantly differed (P=0.007), being 2.6, 5.8, and 3.9 in the LQT1, LQT2, and LQT3 groups, respectively. Table 2 shows details of the cardiac events that occurred in each LQTS type. The frequency of more severe symptoms of cardiac events, such as syncope, cardiac arrest, and sudden death, were higher in the LQT1 and LQT3 groups than in the LQT2 group, in which such extreme symptoms accounted for 60% of the total number of events. Symptoms such as presyncope were milder in the remaining 40% of the LQT2 group.

Seasonal and Circadian Distribution of Cardiac Events in LQT1

Among a total of 223 cardiac events, details about the season and time of occurrence for 42 (18.8%) and 55 (24.7%) events, respectively, were vague. Among 181 (81.2%) events with de-

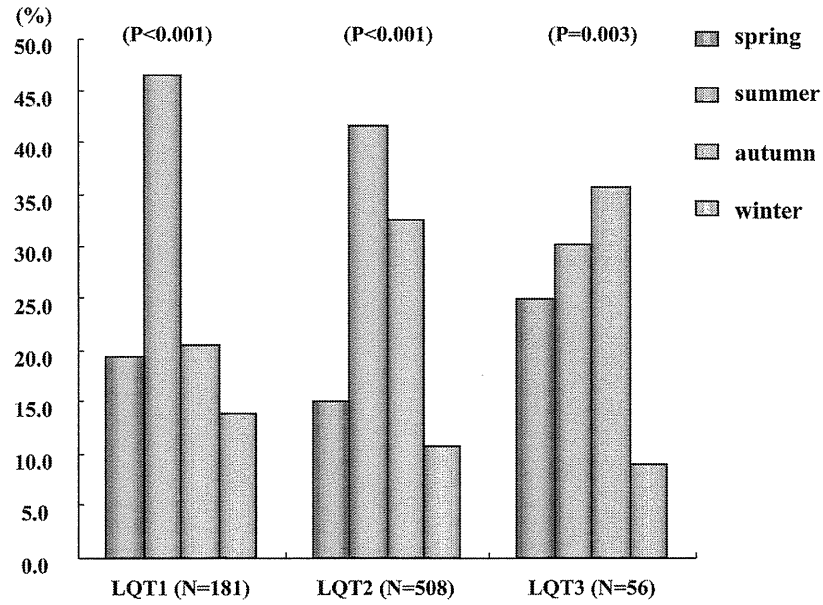

Figure 1. Bar graphs showing the seasonal distribution in occurrence of cardiac events in each genotype of congenital long QT syndrome (LQTS). Detailed seasonal information was acquired in 181/223 (81.2%), 508/550 (92.4%), and 56/59 (94.9%), in LQT1, LQT2, and LQT3, respectively.

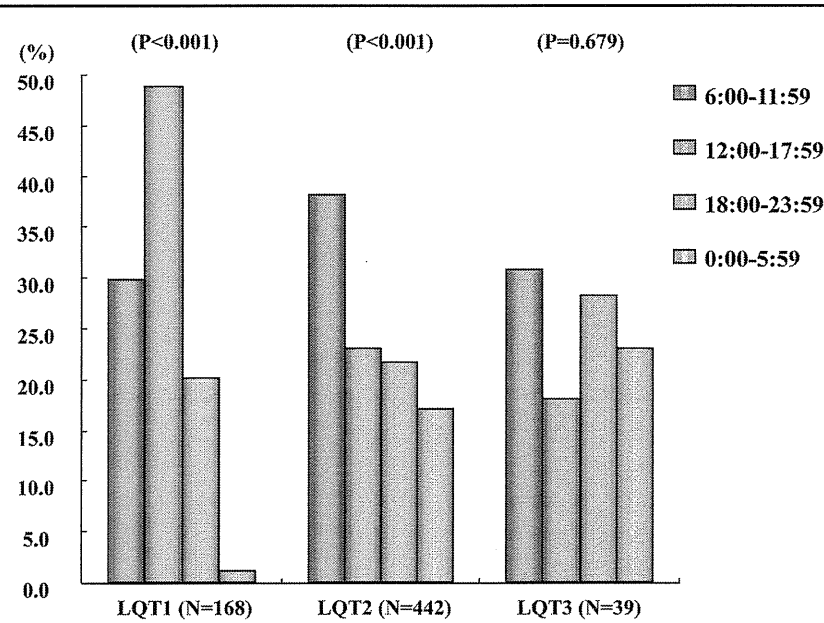

Figure 2. Bar graphs showing the circadian distribution in occurrence of cardiac events in each genotype of congenital long QT syndrome (LQTS). Detailed circadian information was acquired in 168/223 (75.3%), 442/550 (80.4%), and 39/59 (66.1%), in LQT1, LQT2, and LQT3, respectively.

tailed seasonal information, most occurred during the summer (84/181, 46.4%) and the frequency was lowest during the winter (25/181, 13.8%) (**Figure 1**). Among 168 (75.3%) events with detailed time information, the frequency was highest during the afternoon (82/168, 48.8%), followed by the morning (50/168, 29.8%), and lowest during the night-time (2/168, 1.2%) (**Figure 2**). Among the 50 events that occurred during the morning, extremely few arose at the time of awakening. Only 5 (10%) occurred during first 2 h of the morning (6:00–7:59) (P<0.001), and the remaining 45 (90%) events occurred during the late morning (8:00–11:59). Both the seasonal incidence of cardiac events among 3-month periods and the circadian incidence among 6-h periods statistically differed (both, P<0.001).

Seasonal and Circadian Distribution of Cardiac Events in LQT2

Among a total of 550 cardiac events, details about the season and time of occurrence were vague for 42 (7.6%) and 108 (19.6%) events, respectively. Among 508 (92.4%) cardiac events with detailed seasonal information, the frequency was highest during the summer (211/508, 41.5%), followed by the fall (166/508, 32.7%), and lowest during the winter (55/508, 10.8%) (**Figure 1**). Among 442 (80.4%) cardiac events with

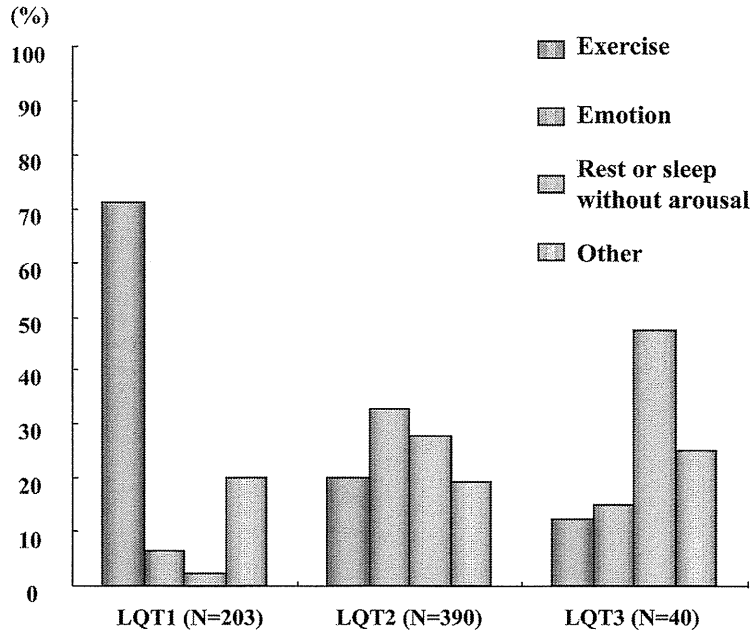

Figure 3. The frequency of 4 different factors triggering cardiac events are indicated as bar graphs. Detailed trigger was acquired in 203/223 (91.0%), 390/550 (70.9%), and 40/59 (67.8%), in LQT1, LQT2, and LQT3, respectively.

detailed time information, the frequency was highest during the morning (169/442, 38.2%), followed by the afternoon (102/442, 23.1%), and evening (96/442, 21.7%) (**Figure 2**), and lowest during the night-time (75/442, 17.0%) (**Figure 2**). Cardiac events associated with the morning were concentrated within the first 2 h of awakening (89/169, 52.7%) between 6:00 and 7:59 (P<0.001). Both the seasonal incidence of cardiac events among the 3-month periods and the circadian incidence among the 6-h periods statistically differed (both, P<0.001).

Seasonal and Circadian Distribution of Cardiac Events in LQT3

Among a total of 59 cardiac events, details about the season and time of occurrence were vague for 3 (5.1%) and 20 (33.9%) events, respectively. Among 56 (94.9%) events with detailed seasonal information, the frequency was the highest during the fall (20/56, 35.7%), followed by summer (17/56, 30.4%), and spring (14/56, 25.0%), and lowest during the winter (5/56, 8.9%) (**Figure 1**). Among 39 (66.1%) events with detailed time information, the frequency of cardiac events was highest at midnight in LQT3 compared with LQT1 and LQT2 (**Figure 2**). The seasonal incidence of cardiac events among the 3-month periods statistically differed (P=0.003), whereas the circadian incidence among the 6-h periods did not (P=0.679).

Triggers of Cardiac Events in LQTS

Figure 3 shows triggers for cardiac events. Triggers were confirmed in 203 (91.0%), 390 (70.9%), and 40 (67.8%) events in LQT1, LQT2, and LQT3, respectively. Most cardiac events in the LQT1 group developed during exercise (144/203, 70.9%), although fewer events occurred during emotional stress (13/203, 6.4%) or rest (5/203, 2.5%). Among 144 cardiac events caused by exertion, 39 (27.1%) were associated with swimming, which accounted for 52% of the triggers of cardiac events during summer exertion. No typical trigger was identified among patients with LQT2, although relatively more cardiac events developed in this group during emotional stress (128/390, 32.8%), such as arousal or being startled by the sudden ringing of a telephone or a bell, and psychological stress including fear, anxiety, and anger, and fewer developed during rest or sleep without arousal (109/390, 27.9%) or exercise (78/390, 20.0%). In addition, the features of exercise as a trigger for LQT2 were unlike those of LQT1 insofar as they were considerably milder and included routine activities such as standing from a seated position, walking, and brushing teeth. In addition, events tended to occur at the start of such activities in the LQT2 group compared with during more intense exercise in the LQT1 group. Cardiac events were more frequent during rest or sleep without arousal in the LQT3 group (19/40, 47.5%) compared with exercise (5/40, 12.5%) and emotional stress (6/40, 15.0%).

Seasonal Distribution of Multiple Events

Multiple events occurred within 24 h in 13/86 (15.1%), 30/95 (31.6%), and 4/15 (26.7%) patients with LQT1, LQT2, and LQT3, respectively. Among a total of 13 multiple events in the patients with LQT1, 2 (15.4%), 5 (38.5%), 5 (38.5%), and 1 (7.7%) occurred during the spring, summer, fall, and winter, respectively. Among a total of 55 multiple events in the LQT2 group, 3 (5.5%), 27 (49.1%), 22 (40%), and 3 (5.5%) events occurred during these respective seasons. Among a total of 4 multiple events that occurred in the LQT3 group, 2 (50.0%) occurred during the spring, 1 (25.0%) occurred during the summer, and 1 (25.0%) occurred during the winter. The seasonal distribution was significant in both the LQT1 and LQT2 groups, but was difficult to analyze in the LQT3 group because relatively few total events occurred. Over 75% and about 90% of multiple events in LQT1 and LQT2 occurred between summer and fall.

Discussion

Although several investigators have examined the seasonal or

circadian distribution of cardiac events in patients with structural heart diseases such as old myocardial infarction,[6,9] hypertrophic cardiomyopathy,[11] idiopathic dilated cardiomyopathy,[7] or non-structural heart disease such as Brugada syndrome,[12] those associated with congenital LQT syndrome have remained unknown. We therefore examined circadian and seasonal variations in patients with LQT syndrome in multiple institutions. Our findings indicated specific seasonal and circadian distributions of cardiac events in patients with congenital LQTS that are quite different from those occurring in patients with structural heart disease. **Figure 1** shows that the incidence of cardiac events significantly peaked during the summer in LQT1 and during the summer to autumn in LQT2. Although events in LQT3 did not significantly peak during any particular season, a significant nadir was reached during the winter. Cardiac events were generally associated with more severe symptoms such as syncope, cardiac arrest, and sudden death in the LQT1 and LQT3 groups, but with milder symptoms among the LQT2 group. Triggers for cardiac events among the 3 genotypes were generally similar to those reported by others.[10,13–16] The incidence of events significantly peaked between the morning and afternoon (6:00–17:59) in LQT1, and during the morning (6:00–11:59) in LQT2 (**Figure 2**). More events occurred during the late morning in LQT1 ($P<0.001$), and around the time of awakening in LQT2 ($P<0.001$). Although a significant circadian difference was not found, the frequency of cardiac events was relatively higher during the night-time to early morning in LQT3 compared with other LQT syndromes (**Figure 3**).

Possible Mechanisms for Seasonal Distribution of Cardiac Events

Although the frequency of cardiac events including VT/VF in patients with structural heart disease significantly increases during the winter,[2–4,17,18] the frequency was higher during summer to autumn in patients with LQT1 and LQT2 and lowest during the winter among those with LQT3. Several potential factors could explain the differences in seasonal distribution of cardiac events in patients with long QT syndrome compared with those having structural heart disease. The most likely reason for the highest frequency of cardiac events occurring in LQT1 during the summer is that participation in activities such as swimming and running is higher during the summer, and children might have more opportunities to play outside during the summer in Japan. Athletic and swimming meets are usually held during this season in schools. Sympathetic nerve activities and catecholamine levels are closely related to the occurrence of cardiac events in individuals with LQT1.[19–21] Most cardiac events occurred during exercise in our patients with LQT1, which supports previous findings.[10,13,22,23]

The reason why patients with LQT2 had the highest frequency of cardiac events from summer to early autumn remains unknown. However, seasonal variations in serum potassium levels could be 1 factor, as these levels are significantly lower in summer than in winter.[24] This could be a result of a loss of potassium through profuse sweating or increased water intake. A close correlation has been implied between hypokalemia and LQT2, in which the cell surface density of the voltage-gated K^+ channel, *HERG*, is regulated by a biological factor and the extracellular K^+ concentration, and the administration of oral potassium results in a greater reduction in resting corrected QT (QTc) interval.[25–27]

The frequency of cardiac events was lower in patients with LQT3 during the winter than during other seasons, which is similar to that of Brugada syndrome.[12] LQT3 and Brugada syndromes are both associated with mutations in SCN5A, the gene that encodes the α subunit of the sodium channel. Mutations in SCN5A result in an increase (gain of function) and decrease (loss of function) in the late sodium current (I_{Na}) in patients with LQT3 and Brugada syndrome, respectively, and are reportedly found in 18–30% of clinically diagnosed Brugada syndrome. Some single mutations in the SCN5A gene cause multiple phenotypes such as Brugada syndrome, sick sinus syndrome. and conduction disease in addition to the LQT3 phenotype.[22,28–30] In addition, recent evidence shows considerable clinical overlap, implying a new disease entity known as the overlap syndrome of cardiac sodium channelopathy.[31,32] The seasonal distribution of "multiple" events was similar to those of isolated episodes.

Possible Mechanisms for Circadian Distribution of Cardiac Events

One factor that might explain the varied circadian distribution of cardiac events is the effect of autonomic nervous activity. Sympathetic nerve activity is higher during the daytime and upon awakening.[33–37] Cardiac events in patients with LQT1 are closely related to sympathetic nerve activities and plasma catecholamine levels, which are also higher during the daytime. In addition, daytime provides more opportunities for physical stress, because more exercise is accomplished during the daytime than during the night-time. Thus, the circadian profiles of cardiac events are similar between LQT1 and structural heart disease.

The frequency of cardiac events was significantly higher among patients with LQT2 during the early morning, when the alarm clock rings, or when they awakened, stood upright, walked, or performed daily rituals, such as face washing or brushing teeth. The response to epinephrine test in patients with LQTS reported by Noda et al might explain this circadian profile in patients with LQT2[19] in whom the QTc duration is transiently prolonged just after starting intravenous epinephrine and returns to the baseline level at the steady state. This suggests that cardiac events tend to occur immediately after an initial increase in sympathetic nerve activities or catecholamine levels, and that cardiac responses to epinephrine and or sympathetic nerve activity might be intensified at the time of awakening.

The circadian distribution of cardiac events in patients with LQT3 is difficult to conclude because of the low incidence. However, the tendency is quite similar to that of Brugada syndrome, in which cardiac events occur during the night and while asleep. Increased vagal activity apparently plays a significant role in the genesis of VF in patients with Brugada syndrome. The hereditary association in the seasonal distribution of cardiac events in LQT3 described above might participate in the coincidence of cardiac events between LQT3 and Brugada syndromes.

Darwin et al recently uncovered molecular evidence that links circadian rhythms to vulnerability in ventricular arrhythmias in mice, in which cardiac ion-channel expression and QT-interval duration (an index of myocardial repolarization) exhibit endogenous circadian rhythmicity under the control of the clock-dependent oscillator, kruppel-like factor 15 (Klf15).[38] This factor transcriptionally controls rhythmic expression of Kv channel-interacting protein 2 (KChIP2), a critical subunit required for generating the transient outward potassium current. A deficiency or excess of Klf15 causes a loss of rhythmic QT variation, abnormal repolarization, and enhanced susceptibility to ventricular arrhythmias. These mechanisms might participate in the circadian variation of ventricular arrhythmias

associated with each type of LQT syndrome. Although gene-specific differences might be associated with a discrepancy in the occurrence of cardiac events,[39] further investigations are required.

Clinical Implications

The present results indicate a need for more specific medical therapy, although further assessments are required. For example, amounts of medication should be increased in summer and taken in the morning by patients with LQT1, increased over summer to autumn and taken before falling asleep by patients with LQT2, and increased before falling asleep for patients with LQT3.

Study Limitations

First, the timing and number of events might have been underestimated because they were based on patients' recall and medical records. Not all cardiac events were memorized like those recorded by an implantable cardioverter defibrillator. However, more extreme symptoms such as syncope and cardiac arrest or death were usually memorable and a history was taken from not only the patients but also their families. Thus, underestimation of these more disastrous events was considered to be low. In contrast, the frequency of events such as presyncope could be overestimated because they could arise as a result of causes other than ventricular tachyarrhythmias. However, we defined the symptoms of presyncope as sudden dizziness, palpitations, and chest pain persisting for over 30 s without a complete loss of consciousness that were confirmed by ECG recordings as being associated with ventricular tachyarrhythmias at least once, and we attempted to minimize false-positive cases. Second, the influence of drug therapy was not considered in this study, so the precise effect of time- or season-dependent exposure to β-blockers on the distribution of events was not analyzed. However, patients usually take medications in the morning and we did not change the medication according to the season. Third, some patients who had experienced a large number of events might have distorted the results. However, the tendencies of the seasonal and circadian distribution of cardiac events were similar, even when patients with a large number of cardiac events ($\geq$10) were excluded. In addition, the tendency remained similar regardless of the severity of cardiac events (presyncope, syncope, cardiac arrest, and death). Finally, this was a retrospective study, and the population size and the number of events was small, especially among patients with LQT3. In addition, unavoidable bias was conferred by excluding patients with LQTS whose first manifestation of illness was sudden death. Therefore, further studies of a large number of patients (with an implantable cardioverter defibrillator if possible) are required to validate the present findings and to define the underlying mechanisms.

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Disclosures

This manuscript represents original work that has not been published and is not being considered for publication elsewhere in whole or in part in any language except as an abstract. All co-authors have read and approved the submission of the manuscript. There are no financial or other relationships that could lead to a conflict of interest (Conflict of Interest: none declared).

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Disease characterization using LQTS-specific induced pluripotent stem cells

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Aims Long QT syndrome (LQTS) is an inheritable and life-threatening disease; however, it is often difficult to determine disease characteristics in sporadic cases with novel mutations, and more precise analysis is necessary for the successful development of evidence-based clinical therapies. This study thus sought to better characterize ion channel cardiac disorders using induced pluripotent stem cells (iPSCs).

Methods and results We reprogrammed somatic cells from a patient with sporadic LQTS and from controls, and differentiated them into cardiomyocytes through embryoid body (EB) formation. Electrophysiological analysis of the LQTS-iPSC-derived EBs using a multi-electrode array (MEA) system revealed a markedly prolonged field potential duration (FPD). The IKr blocker E4031 significantly prolonged FPD in control- and LQTS-iPSC-derived EBs and induced frequent severe arrhythmia only in LQTS-iPSC-derived EBs. The IKs blocker chromanol 293B did not prolong FPD in the LQTS-iPSC-derived EBs, but significantly prolonged FPD in the control EBs, suggesting the involvement of IKs disturbance in the patient. Patch-clamp analysis and immunostaining confirmed a dominant-negative role for 1893delC in IKs channels due to a trafficking deficiency in iPSC-derived cardiomyocytes and human embryonic kidney (HEK) cells.

Conclusions This study demonstrated that iPSCs could be useful to characterize LQTS disease as well as drug responses in the LQTS patient with a novel mutation. Such analyses may in turn lead to future progress in personalized medicine.

Keywords Long QT syndrome • Drug examination • iPS cells • Cardiomyocytes • Personalized medicine

1. Introduction

Sudden cardiac arrest (SCA) is a major cause of mortality in developed countries, accounting for about 10% of all deaths.[1] The majority of sudden cardiac deaths are caused by acute ventricular tachyarrhythmias,[2] which often occur in persons without known cardiac disease, structural heart disease, or coronary artery disease.[3–6] Long QT syndrome (LQTS) was initially described as a rare inherited disease causing ventricular tachyarrhythmia. Subsequently, many patients have been identified and now we know that ventricular tachyarrhythmia in LQTS is apparently common among sudden death syndromes. The reported incidence of LQTS is one in 2000, but this may underestimate the disease because many cases are not properly diagnosed because of the rarity of the condition and the wide spectrum of symptoms.[7]

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Human-induced pluripotent stem cells (hiPSCs) have become a promising tool for analysing human genetic diseases.[8,9] Many studies have already shown that apparent cellular phenotypes of familial genetic disorders are recapitulated by disease-specific iPSC-derived cells *in vitro*. In some of these, cardiomyocytes differentiated from LQTS-specific iPSCs (LQTS-iPSCs) were used to recapitulate disease phenotypes in LQTS patients who were previously characterized as having mutated channel profiles.[10–13] In reality, many patients have novel mutations and no such specific information regarding their disease phenotype is matched by the respective genotypes. To address whether iPSC technology could be used to characterize a novel mutated gene, we selected LQTS patients without family history and previous disease characterization.

2. Methods

2.1 Human iPSC generation

iPSCs were established as described previously.[8] We used lentiviral to introduce mouse solute carrier family 7, a member 1 (*Slc7a1*) gene encoding the ecotropic retrovirus receptor. Transfectants were plated at 2×10^5 cells per 60 mm dish. The next day, *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* were introduced by retroviral. Twenty four hours after transduction, aspirated off the virus-containing medium, then continued to culture under fibroblast condition. Six days later, the cells were harvested and plated at 5×10^4 cells per 100 mm dish. The cells were cultured for another 20 days. At day 25, embryonic stem cell-like colonies were mechanically dissociated and transferred to a 24-well plate on the mouse embryonic fibroblast feeders.

2.2 Patient consent

All subjects provided informed consent for blood testing for genetic abnormalities associated with hereditary LQTS. Isolation and use of patient and control fibroblasts was approved by the Ethics Committee of Keio University (20-92-5), and performed only after written consent was obtained. Our study also conforms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects.[14]

2.3 *In vitro* differentiation

Cells were harvested using 1 mg/ml collagenase IV (Invitrogen, CA, USA), and transferred to ultra-low attachment plates (Corning, NY, USA) in differentiation medium.[15] The medium was replaced every second day. The time window of differentiation for analysing the beating embryoid bodies (EBs) and cardiomyocytes was 30–60 days and 150 days from starting the differentiating conditions.

2.4 Immunofluorescence

The immunostaining was performed using the following primary antibodies and reagents: anti-OCT3/4 (sc-5279, Santa Cruz, CA, USA), anti-E-cadherin (M108, TAKARA BIO, Otsu, Japan), anti-NANOG (RCAB0003P, ReproCELL, Yokohama, Japan), anti-SSEA 1(sc-21702, Santa Cruz), anti-SSEA 3 (MAB4303, Millipore, MA, USA), anti-SSEA 4 (MAB4304, Millipore), anti-Tra1-60 (MAB4360, Millipore), anti-Tra1-81 (MAB4381, Millipore), anti-α-Actinin (A7811, Sigma-Aldrich, MO, USA), anti-ANP (sc-20158, Santa Cruz), anti-MHC (MF20, Developmental Studies Hybridoma Bank, IA, USA), anti-TNNT (13-11, Thermo Scientific, NeoMarkers, MA, USA), anti-GATA4 (sc-1237, Santa Cruz), anti-NKX2.5 (sc-8697, Santa Cruz), anti-KCNQ1 (s37A-10, ab84819, Abcam, Cambridgeshire, UK), anti-WT-KCNQ1 (APC-022, Alomone Labs, Jerusalem, Israel), fluorescent phallotoxins (A22283, Molecular Probes, OR, USA), Wheat Germ Agglutinin Conjugates[16,17] (W11262, Molecular Probes) and DAPI (Molecular Probes). Signal was detected using a conventional fluorescence laser microscope (BZ-9000, KEYENCE, Osaka, Japan) equipped with a colour charge-coupled device camera (BZ-9000, KEYENCE).

2.5 Reverse transcription–polymerase chain reaction

Total RNA samples were isolated using the TRIZOL reagent (Invitrogen) and RNase-free DNase I (Qiagen, Tokyo, Japan). cDNAs were synthesized using the Superscript First-Strand Synthesis System (Invitrogen). Real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR) was performed using 7500 Real-Time PCR System (Applied Biosystems, CA, USA), with SYBR Premix ExTaq (Takara, Otsu, Japan). The amount of mRNA was normalized to GAPDH mRNA. Primer sequences are listed in the Supplementary material online, *Table*.

2.6 Teratoma formation

The mice were anaesthetized using a mixture of ketamine (50 mg/kg), xylazine (10 mg/kg), and chlorpromazine (1.25 mg/kg). The adequacy of anaesthesia was monitored by heart rate, muscle relaxation, and the loss of sensory reflex response, i.e. non-response to tail pinching. hiPSCs (at a concentration corresponding to 25% of the cells from a confluent 150 mm dish) were injected into the testis of severe combined immunodeficiency disease (SCID) mice (CREA Japan, Tokyo, Japan). At 6–8 weeks post-injection, teratomas were dissected, fixed in 10% paraformaldehyde overnight, and embedded in paraffin. The sections were stained with haematoxylin and eosin. All experiments were performed in accordance with the Keio University animal care guidelines and approved by the Ethics Committee of Keio University (20-041-4), which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

2.7 Karyotype analysis

Karyotype analysis was performed using standard Q-banding chromosome analysis according to the Central Institute for Experimental Animals.

2.8 Genomic sequence

Genomic DNA was isolated from the patient, control volunteers, control iPSC colonies, and LQTS-iPSC colonies. The relevant *KNCQ1* gene fragment was amplified by PCR reaction using 100 ng genomic DNA. PCR products were then sequenced.

2.9 Cell culture and transient transfection

Human embryonic kidney (HEK) cells were obtained from the American Type Cell Collection and seeded in 35 mm dishes 1 day before transfection and then transfected with various plasmids using FuGENE 6 Transfection Reagent (Roche Applied Science, Penzberg, Germany). Aliquots of 1 or 0.5 μg of WT-*KCNQ1* and/or 1 or 0.5 μg of P631fs/33-*KCNQ1*, together with 1 μg of WT-*KCNE1* and 0.2 μg of GFP, were transfected into HEK cells. Cells were studied at 48–72 h after transfection.

2.10 Field potential recordings using the on-chip multi-electrode array system

Multi-electrode array (MEA) chips from Multi Channel Systems (Germany) were coated with fibronectin (F1141; Sigma-Aldrich). EBs were plated and incubated at 37°C. MEA measurements were performed at 37°C. The signals were initially processed, and the obtained data were subsequently analysed with MC_Rack (Multi Channel Systems). Data for analysis were extracted from 2–5 min of the obtained data. The recorded extracellular electrograms were used to determine local field potential duration (FPD), defined as the time interval between the initial deflection of the FP and the maximum local T wave. FPD measurements were normalized (corrected FPD: cFPD) to the activation rate using Bazett's correction formulae: $cFPD = FPD/(RR\ interval)^{1/2}$, where RR indicates the time interval (in seconds) between two consecutive beats.[18] E4031

(M5060; Sigma-Aldrich), chromanol 293B (C2615; Sigma-Aldrich), barium chloride (Fluka 34252; Sigma-Aldrich), isoproterenol hydrochloride (I6504; Sigma-Aldrich), and propranolol hydrochloride (P0884; Sigma-Aldrich) were prepared as 1 or 10 mM stock solutions. The FPs were recorded for 5 min. Drug was then added to the medium. After 5–10 min of incubation, the FPs were measured for 5–10 min. MEA recordings were performed by investigators blinded to the genotype of the cells.

2.11 Whole-cell patch-clamp electrophysiology

The external solution used to measure K^+ currents in iPSC-derived cardiomyocytes was composed of the following (in mM): *N*-methyl-D-glucamine 149, $MgCl_2$ 5, HEPES 5, and nisoldipine 0.003. IKs were separated by applying chromanol 293B. In HEK cells, Tyrode's solution used to measure KCNQ1 channel currents comprised (in mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.25, HEPES 5.0, and glucose 5.6; pH was adjusted to 7.4 with NaOH. The glass pipette had a resistance of 3–5 MΩ after filling with the internal pipette solution containing (in mM) KOH 60, KCl 80, aspartate 40, HEPES 5, EGTA 10, Mg ATP 5, sodium creatinine phosphate 5, and $CaCl_2$ 0.65; pH 7.2. KCNQ1 channel currents were recorded using Axopatch 200B, Digidata 1440A, and pClamp 10.2 (Axon Instruments, Foster City, CA, USA) for data amplification, acquisition, and analysis, respectively. For K^+ current measurement in iPSC-derived cardiomyocytes, depolarizing pulses for 3 s from −60 to 60 mV were applied from the holding potential at −60 mV at 0.1 Hz. The tail current was measured on repolarization back to −40 mV. KCNQ1 channel currents were elicited by 3 s depolarizing steps from a holding potential of −80 mV to potentials ranging from −50 to +60 mV in 10 mV increments. This was followed by a 2 s repolarization phase to −40 mV to elicit the tail current. Pulse frequency was 0.1 Hz. Whole-cell patch-clamp recordings were performed by investigators blinded to the genotype of the cells.

2.12 Statistical analysis

Data are expressed as mean ± SEM. Unless otherwise noted, statistical significance was assessed with Student's *t*-test and Fischer's exact test for simple comparisons, and ANOVA followed by Bonferroni's test for multiple comparisons. The probability level accepted for significance was $P < 0.05$ ($^*P < 0.05$, $^{**}P < 0.01$).

3. Results

A 13-year-old boy was admitted to our institution with SCA experienced during physical exercise at school. He subsequently underwent successful resuscitation using an automated external defibrillator , the data from which showed ventricular fibrillation, a fatal arrhythmic event (see Supplementary material online, *Figure S1A*). Electrocardiogram showed a significantly prolonged QT interval and QT interval corrected for heart rate, QTc (see Supplementary material online, *Figure S1B*). He had no family history of previous syncope episodes or significant QT interval abnormality (see Supplementary material online, *Figure S1C*). Since the clinical findings on syncope and the electrocardiogram morphology suggested type 1 LQTS, β-blockers were initially administered to reduce the risk of cardiac sudden death.[19] The epinephrine provocation test increases the accuracy of diagnosis of type 1 LQTS;[20] however, this test can be affected by β-blocker administration (see Supplementary material online, *Figure S1D*); thus, type 1 LQTS being the most probable diagnosis in our patient was not definitive.[21] To elucidate whether this patient is type 1 LQTS caused by a *KCNQ1* mutation, *KCNQ1* was directly sequenced. A heterozygous deletion mutant in *KCNQ1*, 1893delC (P631fs/33), was identified in our patient (see Supplementary material online, *Figure S1E* and *F*). We also confirmed that no other mutation was present in the major LQTS-related genes: *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2*. Although the *KCNQ1* 1893delC mutation was previously reported, its functional characteristics remain unknown.[22] To obtain electrophysiological properties, drug responses, and some valid data on which to base useful medical therapy, we tested the validity of iPSCs for disease characterization.

To generate iPSCs, we used dermal fibroblasts from our patient and two healthy volunteers, and reprogrammed these cells using retrovirus-mediated gene transfer of *SOX2*, *OCT3/4* (also known as *POU5F1*), *KLF4*, and *MYC*. Several clones were generated, expanded, and stored. All iPSC lines showed typical iPSC morphology and expressed human pluripotency markers (*Figure 1A* and *B*). Quantitative RT–PCR (qRT–PCR) analyses confirmed that all lines adequately expressed endogenous pluripotency markers and silenced exogenous genes (*Figure 1C* and *D*). To examine pluripotency, iPSCs were injected into SCID mice. Injected iPSC-derived teratomas contained the cell derivatives of all three germ layers, such as cartilage, intestine, muscle, and neural tissue (see Supplementary material online, *Figure S2A* and *B*). All iPSC lines maintained a normal karyotype (see Supplementary material online, *Figure S2C* and *D*). We selected two LQTS and two control iPSC lines for further characterization and cardiac differentiation.

We used an EB culture system to differentiate iPSCs into cardiomyocytes.[15,23] After 1 week of floating culture, spontaneous beating EBs were observed, and the efficiency of beating EBs showed no significant difference between control- and LQTS-iPSCs at days 30 and 60 (data not shown). Immunofluorescence staining for dissociated cardiomyocytes showed clear immunopositivity for cardiac-specific gene products in control- and LQTS-iPSC-derived cardiomyocytes (*Figure 2A* and *B*). Electron microscopy also revealed a typical cardiomyocyte structure in both control- and LQTS-iPSC-derived cardiomyocytes, including sarcomeric organization and gap junctions (see Supplementary material online, *Figure S3A* and *B*). Similarly, qRT–PCR analyses confirmed the expression of cardiac-specific genes and ion channels (*Figure 2C*). Ion channel expression in iPSCs was compatible with previous reports of multiple ion channels expressed in pluripotent stem cells.[24,25] To elucidate electrophysiological properties, we used an MEA system that enables easy measurement of the surface electrogenic activities of cell clusters and can be adapted to automatic high-throughput systems.[26] MEA analyses revealed that control- and LQTS-iPSC-derived EBs showed similar rhythmic electrical activity and spontaneous beating rate (*Figure 3A* and *B*). FPD in MEA analysis is analogous to a QT interval in an electrocardiogram.[26] The cFPD (normalized to beating frequency) of LQTS-iPSC-derived EBs was significantly longer than that of controls (*Figure 3C* and *D*), suggesting that iPSC-derived cardiomyocytes from both control and LQTS cells have cardiac-specific functional properties.

We next tested several drugs known to affect QT prolongation to elucidate the electrophysiological properties of EBs. The IKr blocker, E4031, significantly prolonged cFPD in a dose-dependent manner when added into the culture medium of control and LQTS cells (*Figure 4A* and *B*). E4031 administration induced significantly more frequent early-after depolarizations (EADs) in the LQTS-iPSC-derived beating EBs compared with the control EBs; these are spontaneous membrane depolarizations that confer risk of ventricular arrhythmias (*Figure 4C* and Supplementary material online, *Figure S4A*). In addition, higher doses of E4031 induced arrhythmic events such as

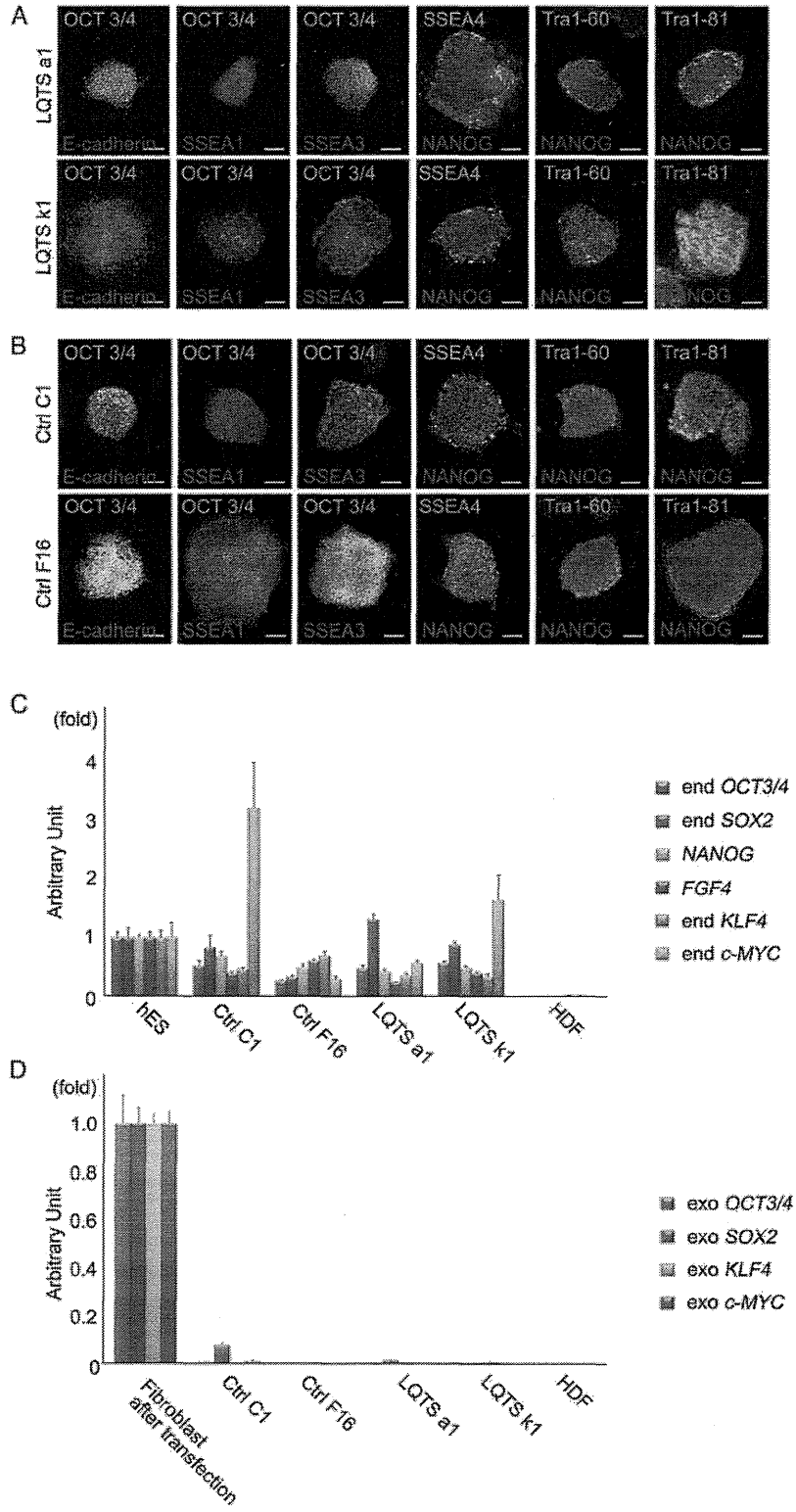

Figure 1 Generation of iPSCs from a patient with LQTS. (*A*) Immunofluorescence staining for stem cell markers (OCT3/4, E-cadherin, NANOG, SSEA3, SSEA4, Tra1-60 and Tra1-81) in LQTS-iPSC colonies. SSEA1 is not a stem cell marker in hiPSCs. Scale bar, 100 μm. (*B*) Immunofluorescence staining for stem cell markers in control-iPSC colonies. Scale bar, 100 μm. (*C*) Quantitative RT–PCR analyses for endogenous *OCT3/4*, endogenous *Sox2*, endogenous *KLF4*, endogenous *c-MYC*, and *NANOG* and *FGF4* in hES, control-iPSC, LQTS-iPSC, and human dermal fibroblasts (HDF). (*D*) Quantitative RT–PCR analyses for exogenous *OCT3/4*, exogenous *Sox2*, exogenous *KLF4* and exogenous *c-MYC* in HDF at 6 days after transfection, control-iPSC, LQTS-iPSC, and HDF.

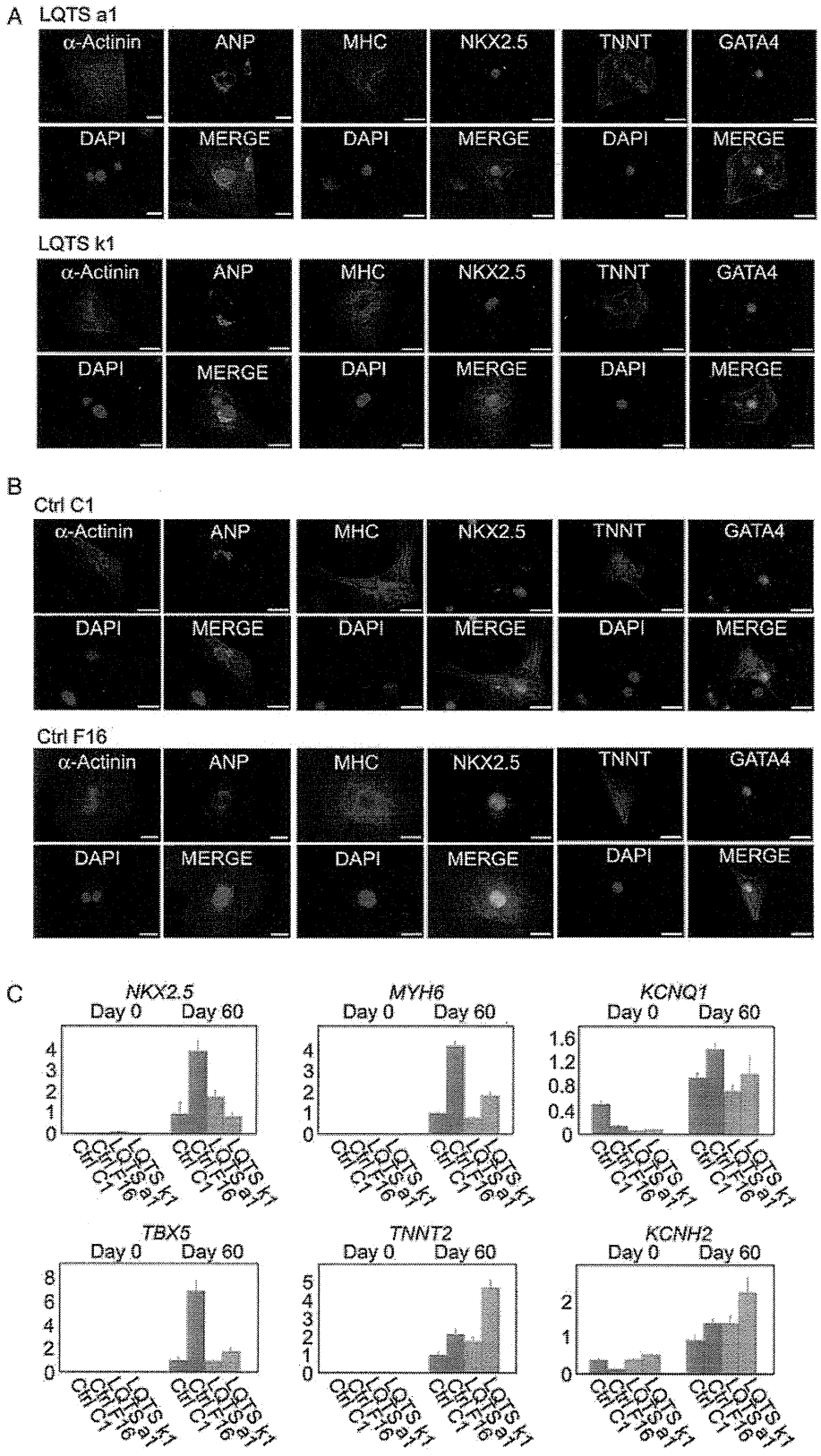

Figure 2 Cardiomyocyte generation from control- and LQTS-iPSCs. (*A*) and (*B*) Immunofluorescence staining for cardiac markers (α-Actinin, ANP, MHC, NKX2.5, GATA4, and TNNT) in the LQTS- and control-iPSC-derived cardiomyocytes. Scale bar, 20 μm. (*C*) Quantitative RT–PCR analyses for cardiac markers (*NKX2.5*, *TBX5*, *MYH6*, and *TNNT2*) and ion channels (*KCNQ1* and *KCNH2*) in the control- (Ctrl) and LQTS-iPSC, and in iPS-derived EBs at day 60.

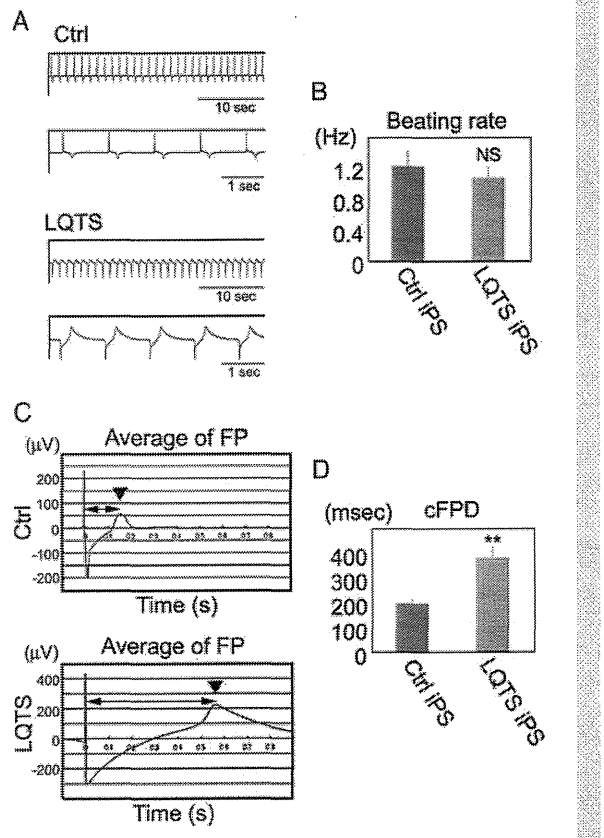

Figure 3 Electrophysiological features of LQTS-iPSC-derived cardiomyocytes. (*A*) Representative MEA recording from the control- and LQTS-iPSC-derived beating EBs. (*B*) Beating rate of the control- and LQTS-iPSC-derived EBs. (*C*) Average of field potential (FP) recorded by MEA in the control- ($n = 12$) and LQTS-iPSC-derived beating EBs ($n = 12$). Triangle indicates the peak of FP and bilateral arrow indicates the FPD. (*D*) cFPD obtained from the control- ($n = 12$) and LQTS-iPSC-derived beating EBs ($n = 12$).

polymorphic ventricular tachycardia (PVT)-like arrhythmia (*Figure 4D* and Supplementary material online, *Figure S4A*).[27] E4031-induced PVT-like arrhythmias were never observed in control-iPSC-derived beating EBs. We then found that another major repolarization potassium current relating to LQTS, IKs, was blocked by chromanol 293B, which significantly prolonged cFPD in control-iPSC-derived beating EBs, but not in LQTS-iPSC-derived beating EBs (*Figure 4E* and *F*). These data indicated that LQTS-iPSC-derived cardiomyocytes have IKs channel dysfunction and/or chromanol 293B insensitivity. We also examined the inwardly rectifying potassium current IK1 by the IK1-blocking barium administration. The application of barium prolonged FPD in both control- and LQTS-iPSC-derived cardiomyocytes (see Supplementary material online, *Figure S4B*). However, barium administration did not induce arrhythmogenic events in control- and LQTS-iPSC-derived beating EBs. These findings suggested that repolarization of LQTS-iPSC-derived cardiomyocytes would be mainly controlled by IKr. Taken together with IKr and IKs blocker administration, we proposed that IKs channels were not only genetically but functionally impaired and that IKr channels compensated for this effect in the patient-derived iPSCs, which is also known as the repolarization reserve in cardiomyocytes.[28,29] IKs channel impairment is diagnosed as type 1 LQTS. And it is well known that β-stimulant increases the risk of fatal arrhythmia and that β-blockers would effectively prevent long-QT-related arrhythmia in type1 LQTS.[30] The β-stimulant isoproterenol increased the beating rate in a dose-dependent manner in control and LQTS cells, and induced EAD and ventricular tachycardia (VT)-like arrhythmogenic events in LQTS-iPSC-derived beating EBs (see Supplementary material online, *Figure S5A* and *B* and *Figure 4G*). Interestingly, the non-selective β-blocker propranolol obviously decreased the incidence of arrhythmogenic events (*Figure 4H*). These data strongly suggested that our patient has a functional impairment in the IKs channel system. We confirmed a heterozygous deletion mutant in KCNQ1, 1893delC (P631fs/33), was identified in the LQTS-iPSCs (see Supplementary material online, *Figure S5C*).

To confirm a possible dominant-negative role of the *KCNQ1* 1893delC mutation in IKs channel function, we conducted precise electrophysiological characterizations in iPSC-derived cardiomyocytes. IKs currents can be recorded by subtraction of baseline and the IKs blocker (chromanol 293B) addition. In control, chromanol 293B (30 μM) addition apparently decreased the recorded current, and IKs current was recorded by subtraction (*Figure 5A*). In LQTS-derived cardiomyocytes, chromanol 293B addition did not show apparent differences and IKs current was subtly recorded by subtraction (*Figure 5A*). The IKs peak and tail current densities of the LQTS-derived cardiomyocytes were evidently smaller than those of control (*Figure 5B*). To clarify the mechanisms underlying such effects, we examined KCNQ1 protein expression in LQTS-iPSC-derived cardiomyocytes. We conducted immunofluorescent staining using an antibody that recognizes a C-terminal epitope on *KCNQ1* downstream of P631fs/33. Immunostaining in control showed cell peripheral expression of KCNQ1, which suggested normal shuttling of the KCNQ1 protein into the cell membrane (*Figure 5C*). In LQTS-iPSC-derived cardiomyocytes, the KCNQ1 protein was accumulated at the perinuclear cytoplasm and nucleus, instead of at the cell periphery (*Figure 5C*). These data indicated that KCNQ1 expression is downregulated at the membrane peripheral site (*Figure 5D*), which suggests that KCNQ1 1893delC has a dominant-negative effect via a trafficking deficiency.

We showed this patient has a mutation in *KCNQ1* and that LQTS-iPSC-derived cardiomyocytes have a functional disturbance in KCNQ1 channels. However, it remains unclear whether this mutation directly contributed and whether other mutations could be involved in the IKs current disturbance. To test for a pure dominant-negative role of the *KCNQ1* 1893delC mutation in IKs channel function, we also conducted electrophysiological and histochemical characterizations in HEK cells expressing exogenous wild-type and/or mutated KCNQ1. Cells with 100% incorporation of the wild-type *KCNQ1* (WT) gene recorded typical IKs currents and 50% WT *KCNQ1* gene introduction slightly reduced the IKs currents (*Figure 6A*). Introduction of 100% mutant *KNCQ1* genes (P631fs/33) (MT) significantly reduced IKs currents (*Figure 6A*). Moreover, 50% WT and 50% MT gene introductions had dominant-negative effects on IKs current (*Figure 6A*). The IKs peak and tail current densities of the 100% MT and 50/50% WT and MT were evidently smaller than those of 100% WT and 50% WT (*Figure 6B* and *C*). Then we also examined KCNQ1 protein expression in *KCNQ1*-transfected HEK cells. Cells