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Appendix 1

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Appendix 2

The J-RHYTHM Investigators
Tabuchi T, Ogawa S, Yamashita T, Itouh H, Iinuma H, Koike A, Sagara K, Fu L-T, Watanabe E, Kumagai K, Okuyama Y, Hirayama A, Sakurai M, Komatsu T, Katoh T, Kobayashi Y, Yajima M, Kaneko Y, Okumura K, Yoshioka K, Hina K, Murakami M, Kamakura S, Shimizu W, Kurita T, Satomi K, Koretsune Y, Betsuyaku T, Satoh M, Matsumoto K, Katoh R, Aizawa Y, Chinushi M, Hanawa H, Okura Y, Washizuka T, Ajiki K, Nakajima T, Higashi Y, Togawa M, Takata Y, Goseki Y, Ishiyama T, Taniguchi Y, Nijima Y, Ando T, Furukawa T, Hamasaki S, Matsushita T, Hirai M, Inden Y, Kodama I, Miyamoto S, Atarashi H, Shimabukuro M, Ishigaki K, Anno N, Oka H, Akaishi M, Baba A, Ozaki H, Takahashi N, Hara M, Saikawa T, Noro M, Sugi K, Tsuchiya T, Tanigawa T, Fujita T, Nakayama T, Kawara T, Furukawa T, Yamada T, Takeda H, Fukunami M, Katoh M, Endou Y, Niwano S, Yonezawa K, Anzai S, Nakahara S, Fujii E, Inoue T, Yokoshiki H, Sasaki K, Abe Y, Chishaki A, Hosoda S, Kawamura Y, Kamiya N, Koga K, Masutomo K, Mikawa T, Nakagawa Y, Ngamura Y, Okishige K, Origuchi H, Yamada N, Yamanouchi Y, Yo S, Endou M, Furuno T, Haruna T, Hayano M, Hayano T, Hirabayashi T, Horie M, Hotta K, Imai S, Imamura T, Inao T, Inokubo Y, Ishikawa T, Ishizawa Y, Kanda K, Kihara H, Kimura M, Kitoh T, Kosaka T, Maruyama T, Mitani H, Morita H, Kusano K, Ohe T, Nakamura T, Nakamura Y, Nakazato Y, Nakaya Y, Nanbu T, Niho B, Ogawa O, Okazaki O, Seo K, Senoo M, Shimizu A, Shindo T, Shinozaki T, Sirayama T, Suzuki A, Takase B, Tanaka Y, Tojo H, Tsuchiya K, Tsuji S, Yamanari H, Yokozuka H, Yoshida A, Yotsukura M, Amano K, Date T, Fujiki A, Mizumaki K, Inoue H, Fukatani M, Fukushi T, Hagiwara N, Hata Y, Hattori Y, Harasawa Y, Hosoi T, Ikeda K, Ikeda T, Inoue D, Inoko M, Itouh M, Katoh N, Kawasaki K, Kawasaki N, Keida T, Kin H, Kondoh H, Yamamoto K, Kurachi Y, Kondoh H, Koura T, Kou M, Makino M, Matsumoto N, Mizobuchi S, Mizuo K, Motooka M, Nagao K, Nakamura I, Nakano A, Nirei T, Nishizaki M, Ohbayashi K, Ohkawa F, Ohki T, Ohnishi Y, Okumura K, Okumura H, Okura K, Osakada M, Sano S, Satoh T, Sawai J, Seo K, Shinagawa K, Shinoda S, Shirai T, Shiraki T, Sugano K, Suzuki M, Tachibana H, Takagi S, Takai H, Takase H, Tanno K, Tsuboi N, Ueda M, Umetani K, Uozumi H, Yamada T, Yamane T, Yamamoto K, Yamawaki K, Yasuda T, Yonezawa T, Yoshida Y, Watanabe N, Watanabe S, Nakatani H, Mitamura H

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QUARTERLY FOCUS ISSUE: HEART RHYTHM DISORDERS

D85N, a KCNE1 Polymorphism, Is a Disease-Causing Gene Variant in Long QT Syndrome

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Objectives This study aims to address whether D85N, a KCNE1 polymorphism, is a gene variant that causes long QT syndrome (LQTS) phenotype.

Background KCNE1 encodes the beta-subunit of cardiac voltage-gated K^+ channels and causes LQTS, which is characterized by the prolongation of the QT interval and torsades de pointes, a lethal arrhythmia. D85N, a KCNE1 polymorphism, is known to be a functional variant associated with drug-induced LQTS.

Methods In order to elucidate the prevalence and clinical significance of this polymorphism, we performed genetic screening in 317 LQTS probands. For comparison, we examined its presence in 496 healthy control subjects. We also conducted biophysical assays for the D85N variant in mammalian cells.

Results The allele frequency for D85N carriers was 0.81% in healthy people. In contrast, among LQTS probands, there were 1 homozygous and 23 heterozygous carriers (allele frequency 3.9%). Seven of 23 heterozygous carriers had additional mutations in LQTS-related genes, and 3 female subjects had documented factors predisposing to the symptom. After excluding these probands, the D85N prevalence was significantly higher compared with control subjects (allele frequency 2.1%, $p < 0.05$). In a heterologous expression study with Chinese hamster ovarian cells, KCNE1-D85N was found to exert significant loss-of-function effects on both KCNQ1- and KCNH2-encoded channel currents.

Conclusions The KCNE1-D85N polymorphism was significantly more frequent in our LQTS probands. The functional variant is a disease-causing gene variant of LQTS phenotype that functions by interacting with KCNH2 and KCNQ1. Since its allele frequency was ~1% among control individuals, KCNE1-D85N may be a clinically important genetic variant. (J Am Coll Cardiol 2009;54:812–9) © 2009 by the American College of Cardiology Foundation

Long QT syndrome (LQTS) is a disorder that is characterized by repolarization abnormalities in the heart, leading to torsades de pointes (TdP), syncope, and sudden death. Among the LQTS-related genes identified to date, KCNQ1 and KCNE1 are known to encode the alpha and beta subunits of voltage-gated K^+ channels, which carry I_{Ks}, a slowly activating component of delayed rectifier K^+ current (1,2). KCNE1 is also known to regulate KCNH2 (3), which encodes the Kv11.1 protein, the alpha subunit of rapidly activating delayed rectifier K^+ current (I_{Kr}) (4–6).

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A KCNE1 C-terminal polymorphism, D85N, has been found in the normal population and is known to cause a G-to-A transition at codon 253 (c.253G>A), which leads to the amino acid substitution of aspartic acid for asparagines (7). This has been shown to cause an approximately 50% reduction in KCNQ1-encoded currents in a heterologous expression system using *Xenopus* oocytes (8), although biophysical study data are not available for mammalian cells.

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The allele frequency of the polymorphism is reported to be 0.7% in apparently healthy Asians (7). Paulussen et al. (9) demonstrated in a European population that the allele frequency of D85N was 5% in acquired LQTS patients who experienced TdP as a result of drug administration, but was 0% in the control group. Iwasa et al. (10) reported that the allele frequency was 2% in 100 Japanese cases, but their cohort contained both LQTS patients and normal individuals.

In the present study, we examined the incidence rate of KCNE1-D85N polymorphisms in 317 LQTS probands from unrelated families and 496 control healthy individuals. We identified 23 heterozygous and 1 homozygous probands (allele frequency 3.9%), described the demographics of these index patients, and examined the possibility that the D85N polymorphism is an LQTS-causing genetic variant. We also conducted detailed functional assays of the variant while it was coexpressed with the 2 alpha subunits of cardiac delayed rectifier K^+ channels, KCNQ1 and KCNH2, by using a heterologous expression system involving Chinese hamster ovarian (CHO) cells.

Methods

Study subjects. Three hundred and seventeen consecutive LQTS probands who showed a prolongation of the QT interval were referred to our laboratory for genetic evaluation and were enrolled in our analysis. The electrocardiogram diagnostic criteria of Keating and Sanguinetti (11) included a corrected QT interval (QTc) of ≥470 ms in asymptomatic individuals and a QTc of >440 ms for male subjects and of >460 ms for female subjects that had 1 or more of the following: 1) stress-related syncope; 2) documented TdP; or 3) a family history of early sudden cardiac death.

The protocol for genetic analysis was approved by our institutional ethics committee and was performed under its guidelines. Informed consent was obtained from all individuals or their guardians before the analysis. The QT intervals were measured from electrocardiographic lead II or an available rhythm strip and were corrected for heart rate according to Bazett's formula. As for the control cohort, we screened the frequency of the D85N polymorphism in 496 randomly selected cases, consisting of healthy volunteers and mutation-negative family members such as probands' spouses. Their QTc were <440 ms for male subjects and <460 ms for female subjects.

Genotyping. Genomic deoxyribonucleic acid (DNA) was isolated from venous blood by use of the QIAamp DNA midikit (Qiagen, Hilden, Germany). Genetic screening for KCNE1-D85N was performed by direct polymerase chain reaction. Other LQTS-related genes, including KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2, and KCNJ2, were assayed by denaturing high-performance liquid chromatography using a WAVE System Model 3500 (Transgenomic, Omaha, Nebraska). Abnormal conformers were amplified by polymerase chain reaction. Sequencing was performed with an ABI PRISM3100 DNA sequencer (Applied Biosystems, Wellesley, Massachusetts).

Site-directed mutagenesis. Complementary deoxyribonucleic acid (cDNA) for human KCNQ1 (GenBank AF000571) and KCNE1 (GenBank M26685) were kindly provided by Dr. J. Barhanin (Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, Valbonne, France). The cDNAs were subcloned into pIRES2-EGFP (for KCNQ1) and pIRES-CD8 (for both wild-type and mutated KCNE1) vectors. cDNA for human KCNH2 (GenBank AF363636) was kindly donated by Dr. M. Sanguinetti (University of Utah, Salt Lake City, Utah). The cDNA was subcloned into a pRc-CMV vector. A KCNE1-D85N variant was constructed using a Quick Change II XL Site-Directed Mutagenesis Kit, according to the manufacturer's instructions (Stratagene, La Jolla, California). Nucleotide sequence analysis was performed on each variant construct before the expression study to confirm their sequences.

Abbreviations and Acronyms

CHO = Chinese hamster ovarian

LQTS = long QT syndrome

QTc = corrected QT interval

TdP = torsades de pointes

Cell transfection. CHO cells were maintained at 37°C in Dulbecco's modified Eagle medium and Ham's F12 nutritional mixture (Gibco-BRL, Rockville, Maryland) containing 10% fetal bovine serum supplemented with 1% penicillin and 1% streptomycin. Wild-type KCNQ1, KCNH2, and wild-type and/or variant KCNE1 clones were expressed transiently in CHO cells using the LipofectAMINE method according to the manufacturer's instructions (Invitrogen, Carlsbad, California).

To identify the cells that were positive for KCNH2 expression, CHO cells were cotransfected with 1 μg of pRc-CMV/KCNH2 vector and 0.5 μg of pEGFP-N1/CMV vector. Forty-eight to 72 h after transfection, green fluorescent protein-positive cells and anti-CD8 antibody-coated bead (Dynabeads CD8, Dynal Biotech, Oslo, Norway) decorated cells were used for the patch-clamp study.

Electrophysiological assays. Whole-cell configuration of patch-clamp techniques was employed to record membrane currents at 37°C with an EPC-8 patch-clamp amplifier (HEKA, Lambrecht, Germany). Pipette resistance ranged from 2.5 to 4 MΩ when filled with the pipette solutions described in the following text. The series resistance was electronically compensated for at 70% to 85%. The extracellular solution contained (mmol/l): 140 NaCl, 0.33 NaH_2PO_4, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, and 5 HEPES, and the pH was adjusted to 7.4 with NaOH. The internal (pipette) solution contained (mmol/l): 70 potassium aspartate, 70 KOH, 40 KCl, 10 KH_2PO_4, 1 Mg_2SO_4, 3 Na_2-ATP, 0.1 Li_2-GTP, 5 EGTA, and 5 HEPES, and the pH was adjusted to 7.2 with KOH.

KCNQ1/KCNE1-encoded currents were measured by depolarizing pulses from a holding potential of −90 mV to test potentials between −70 and +50 mV (with a 10-mV step increment), before being repolarized to −50 mV in

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order to monitor tail current amplitude. KCNH2/KCNE1-encoded currents were elicited by depolarizing pulses from a holding potential of −80 mV to test potentials between −60 to +50 mV (with a 10-mV step increment), before being repolarized to −60 mV in order to monitor tail current amplitude. Current densities (pA/pF) were calculated for each cell studied, by normalizing peak tail current amplitude to cell capacitance (Cm). The Cm was calculated by fitting a single exponential function to the decay phase of the transient capacitive current in response to ± 5 mV voltage steps (20 ms) from a holding potential of −50 mV. The liquid junction potential between the test solution and the pipette solution was measured as approximately −10 mV and was corrected. Data were collected and analyzed using the Patch master and Igor Pro (WaveMetrics, Lake Oswego, Oregon).

Data analyses. The voltage-dependence of current activation was determined by fitting the normalized tail current (Itail) versus test potential (Vtest) to Boltzmann's function, which is expressed by: Itail = $1/(1 + \exp[(V_{0.5} - Vt)/k])$, where $V_{0.5}$ is the voltage at which the current is half-activated and k is the slope factor. Time constants for deactivation (τ_{fast} and τ_{slow}) were obtained by fitting a 2-exponential function to the time course of the deactivating tail currents. All data are expressed as mean ± standard error. Statistical comparisons were made using analysis of variance, followed by a *t* test, and differences were considered significant at a value of $p < 0.05$.

Results

Clinical characteristics and genotyping. Of the 496 control volunteers, 8 (mean QTc 420.5 ± 7.5 ms) had heterozygous D85N genotypes (allele frequency 0.81%). In contrast, 23 of the 317 LQTS probands had heterozygous D85N genotypes and 1 (Table 1) (Patient #24) had a homozygous D85N genotype (allele frequency 3.9%). Table 1 and Figure 1 summarize the demographics of the 24 index patients. Their mean age was 34.8 ± 4.4 years, and their mean QTc was 507.9 ± 9.2 ms. Among the D85N-negative cases, we identified 116 probands that were positive for other LQTS-related gene mutations (Fig. 1), and their mean QTc was significantly longer (540.6 ± 6.1 ms) than those of the 24 D85N carriers ($p < 0.05$).

Seven of the 23 heterozygous probands (30%) had other LQTS-related gene variants (KCNQ1 or KCNH2), and 3 female patients (13%; Patients #1, #6, and #10) had documented predisposing factors, such as electrolyte disturbances, QT prolonging drug intake, or bradycardia (Table 1). The allele frequency of the remaining 13 patients (2.1%) was significantly higher than that in the control subjects ($p < 0.05$). Six of these 13 patients (46%) had syncope and/or TdP while 9 of 10 patients (90%) with multiple genetic variants or triggering factors were symptomatic (Fig. 1).

The mean onset age of the 6 symptomatic heterozygous D85N carriers without compromised factors to affect QT

Table 1 Clinical Characteristics of the LQTS Probands Who Carried the KCNE1-D85N Variant

Patient #	Age (F/M)	QTc (ms)	Syncope	TdP	Compound Variant	Underlying Predisposing Triggers
1	36 (F)	540	+	+		Drug (bromocriptine), hypokalemia
2	9 (M)	478	−	−		
3	21 (F)	533	−	+	KCNH2 (a, S706F)*	Drug (amphetamine), hypokalemia
4	42 (F)	650	+	+		
5	51 (F)	490	+	+	KCNH2 (a, E58K)	Sinus bradycardia
6	73 (F)	493	+	−		Drug (disopyramide), sinus bradycardia
7	30 (F)	502	+	−	KCNH2 (G745fs+55X)*	
8	17 (F)	470	−	−		
9	13 (F)	462	+	−	KCNH2 (a, S320L)	
10	41 (F)	490	−	+		Hypomagnesemia
11	73 (F)	608	+	−	KCNQ1 (a, S277L)	Sinus bradycardia
12	75 (F)	494	+	+		
13	17 (M)	500	−	−		
14	13 (F)	512	+	−		
15	57 (M)	472	−	−		
16	53 (M)	462	+	+		
17	17 (F)	520	+	−		
18	22 (F)	472	−	−		
19	13 (F)	522	−	−	KCNH2 (a, R823W)	
20	13 (M)	467	+	−		
21	52 (M)	524	+	+	KCNH2 (a, R948S)*	Drug (minor tranquilizer), hypokalemia
22	11 (M)	491	−	−		
23	51 (M)	493	−	−		
24	39 (F)	545	−	−		

*Novel variant.
LQTS = long QT syndrome; QTc = corrected QT interval; TdP = torsades de pointes.

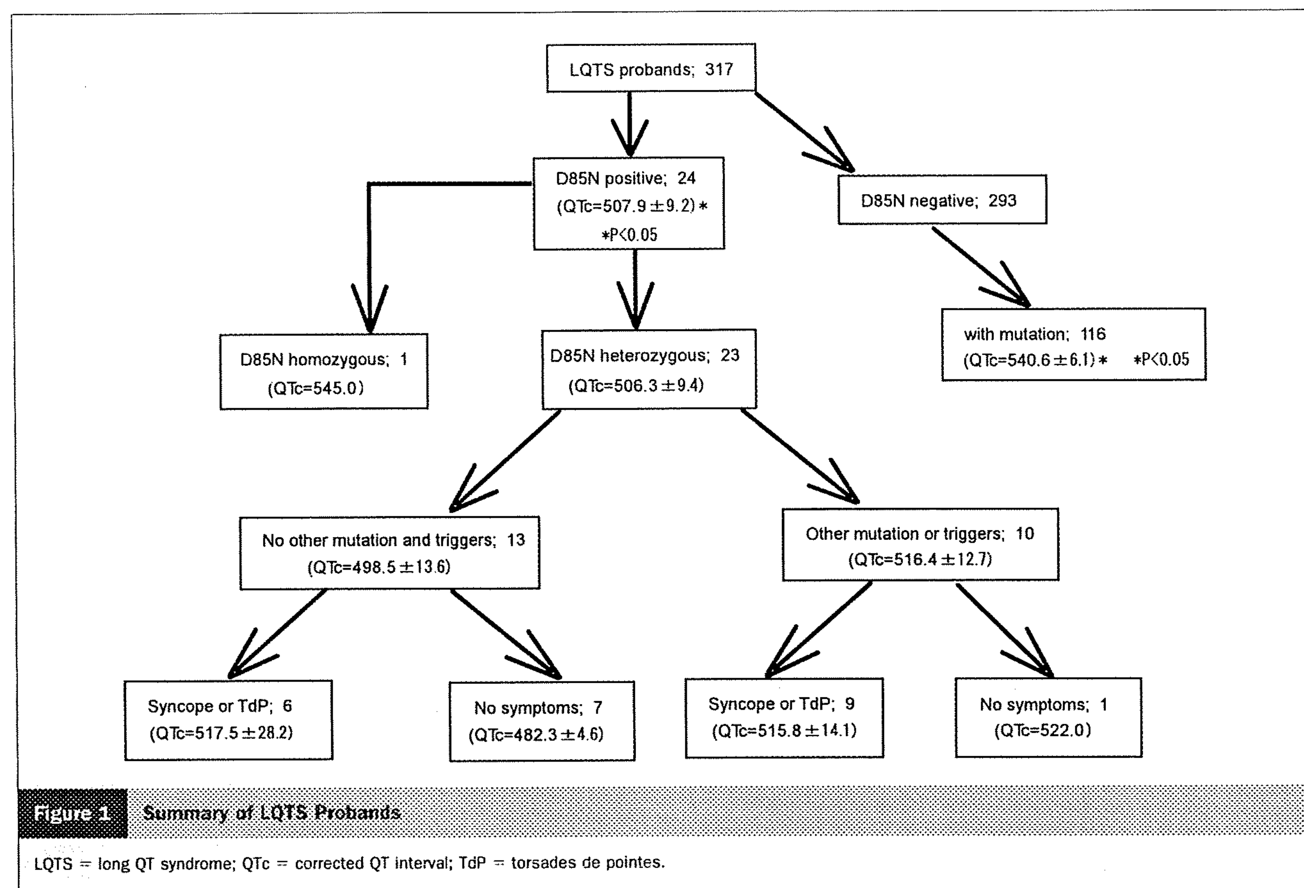

Figure 1 Summary of LQTS Probands

LQTS = long QT syndrome; QTc = corrected QT interval; TdP = torsades de pointes.

interval was 35.5 ± 10.4 years. It was significantly older than the mean onset age of the other genotyped symptomatic LQTS patients (21.0 years in our cohort of 94 genotyped LQTS) (Horie M. et al., unpublished data, September 2008). Although the clinical features of KCNE1-D85N–positive probands differed with respect to the QTc and the onset age from those of other genotyped LQTS patients, this variant appeared to be a disease-causing gene variant in congenital LQTS.

Biophysical assays of the genetic variant. KCNE1-D85N WITH KCNQ1. In order to confirm that the D85N is a disease-causing variant, we conducted functional assays using a heterologous expression system with a mammalian cell line (CHO cells). In the first line of experiments, we examined how KCNE1-D85N affected the reconstituted KCNQ1/KCNE1 currents. Figure 2 depicts representative current traces recorded from cells that coexpressed KCNQ1 and wild-type (Fig. 2A-a) or D85N (Fig. 2A-b) KCNE1 (1 μg each). Peak tail current densities measured after repolarization to −50 mV from various test pulses were calculated in individual cells and are plotted as a function of test potential in Figure 2B. Solid circles indicate the mean peak current densities from 21 cells that were transfected with KCNQ1 and wild-type KCNE1; open circles indicate the mean peak current densities from 25 cells that were transfected with KCNQ1 and D85N, and solid triangles indicate the mean peak current densities from 25 cells that were transfected with KCNQ1 alone. D85N reduced the peak tail currents of wild-type KCNQ1/KCNE1-encoded currents by 28% at 0 mV test potential ($p < 0.05$ vs. wild type).

In Figure 2C, peak tail current densities have been normalized using the current densities recorded after a test pulse to +50 mV and are plotted as a function of test potential. Fitting of data plots to Boltzmann's equation yielded $V_{0.5}$ values of −4.36 ± 1.8 mV for the wild type and 0.38 ± 1.7 mV for D85N ($p < 0.05$), suggesting that the KCNE1 variant produced a significantly positive shift in KCNQ1-encoded current activation kinetics (Table 2). The deactivation process of tail currents could be fitted by 2 exponentials, yielding fast and slow time constants. No significant difference with respect to the fast time constants was evident between the wild-type and D85N genotypes; however, slow deactivation was significantly accelerated by coexpression of D85N (Table 2).

KCNE1-D85N WITH KCNH2. In the next line of experiments, we examined how KCNE1 and its D85N variant influence KCNH2-encoded currents. Figures 3A-a and 3A-b depict 2 sets of current traces recorded from CHO cells that had been transfected with KCNH2 plus wild-type or D85N KCNE1 (1 μg each). Peak tail current densities at −60 mV were calculated in the respective cells and are plotted as a function of test potential in Figure 3B. Solid circles and open circles indicate the mean current densities calculated from 23

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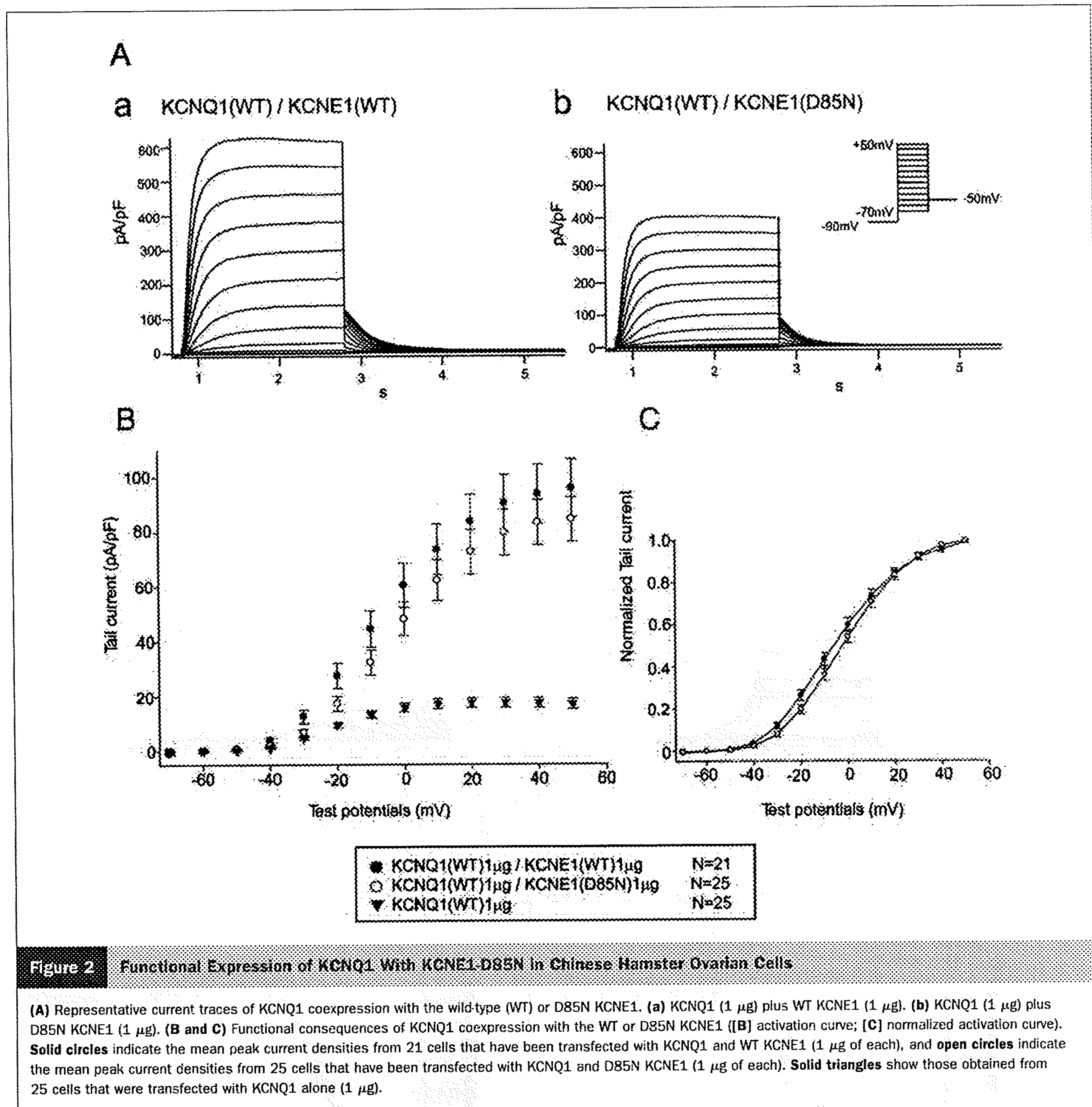

Figure 2 Functional Expression of KCNQ1 With KCNE1-D85N in Chinese Hamster Ovarian Cells

(A) Representative current traces of KCNQ1 coexpression with the wild-type (WT) or D85N KCNE1. **(a)** KCNQ1 (1 µg) plus WT KCNE1 (1 µg). **(b)** KCNQ1 (1 µg) plus D85N KCNE1 (1 µg). **(B and C)** Functional consequences of KCNQ1 coexpression with the WT or D85N KCNE1 (**[B]** activation curve; **[C]** normalized activation curve). **Solid circles** indicate the mean peak current densities from 21 cells that have been transfected with KCNQ1 and WT KCNE1 (1 µg of each), and **open circles** indicate the mean peak current densities from 25 cells that have been transfected with KCNQ1 and D85N KCNE1 (1 µg of each). **Solid triangles** show those obtained from 25 cells that were transfected with KCNQ1 alone (1 µg).

and 20 cells, respectively, which were transfected with 1 µg of KCNH2 and 1 µg of wild-type or D85N KCNE1.

D85N reduced the peak tail currents of wild-type KCNH2/KCNE1-encoded currents by 31% to 36% at test potentials between 0 and +50 mV ($p < 0.005$ vs. wild type). Fitting of normalized data to Boltzmann's equation yielded a $V_{0.5}$ of -18.33 ± 0.8 mV for the wild-type KCNH2/KCNE1 and of -22.07 ± 1.6 mV for KCNH2/KCNE1-D85N ($p < 0.05$), suggesting that the KCNE1 variant causes a significantly negative shift of KCNH2/KCNE1-encoded current activation kinetics (Fig. 3C, Table 2). Deactivation of tail currents could be fitted by 2 exponentials, yielding fast and slow time constants. The fast and slow kinetics were not significantly different between the 2 types of KCNH2 channel currents (Table 2).

Discussion

The present study demonstrates that the allele frequency of KCNE1-D85N is significantly higher in LQTS patients than in control subjects after excluding cases with compromised factors to prolong QT interval ($p < 0.05$). A biophysical assay of D85N showed that the variant affected both reconstituted I_{Ks} and I_{Kr} channel function, leading to a prolongation of the QTc with D85N working as a disease-causing variant. In a heterologous expression system with *Xenopus* oocytes (8),

Table 2 $V_{0.5}$, Slope Factor k, and τ Deactivation at −20 mV

	n	$V_{0.5}$	k	τ_{fast}	τ_{slow}
KCNQ1 (WT) 1 μg	25	−20.86 ± 1.034	8.223 ± 0.421	0.070 ± 0.005	0.136 ± 0.019
KCNQ1 (WT) 1 μg/KCNE1 (WT) 1 μg	21	−4.364 ± 1.834*	12.724 ± 0.407*	0.145 ± 0.013*	0.586 ± 0.070†
KCNQ1 (WT) 1 μg/KCNE1 (D85N) 1 μg	25	0.382 ± 1.717*‡	12.566 ± 0.429*	0.141 ± 0.013*	0.409 ± 0.050*‡
KCNH2 (WT) 1 μg/KCNE1 (WT) 1 μg	23	−18.326 ± 0.775	7.373 ± 0.289	0.183 ± 0.016	1.077 ± 0.102
KCNH2 (WT) 1 μg/KCNE1 (D85N) 1 μg	20	−22.069 ± 1.560§	7.037 ± 0.389	0.193 ± 0.013	1.258 ± 0.090

*p < 0.0001 versus KCNQ1 (wild type [WT]) 1 μg; †p = 0.0001 versus KCNQ1 (WT) 1 μg; ‡p < 0.05 versus KCNQ1 (WT) 1 μg/KCNE1 (WT) 1 μg; §p < 0.05 versus KCNH2 (WT) 1 μg/KCNE1 (WT) 1 μg.

KCNE1-D85N has been reported to cause an approximately 50% reduction in KCNQ1-encoded currents, although data for mammalian cells is not available. In our experiments using CHO cells, D85N significantly reduced KCNQ1-encoded currents by 28% ($p < 0.05$ vs. wild type), although this effect was smaller than that in *Xenopus* oocytes.

When KCNH2 was coexpressed with the wild-type or D85N variant of KCNE1, D85N significantly reduced wild-type KCNH2/KCNE1-encoded currents by 31% to 36% ($p < 0.005$ vs. wild type). Regarding the interaction between KCNE1 and KCNH2, McDonald et al. (3) demonstrated that KCNE1 forms a stable complex with KCNH2 and

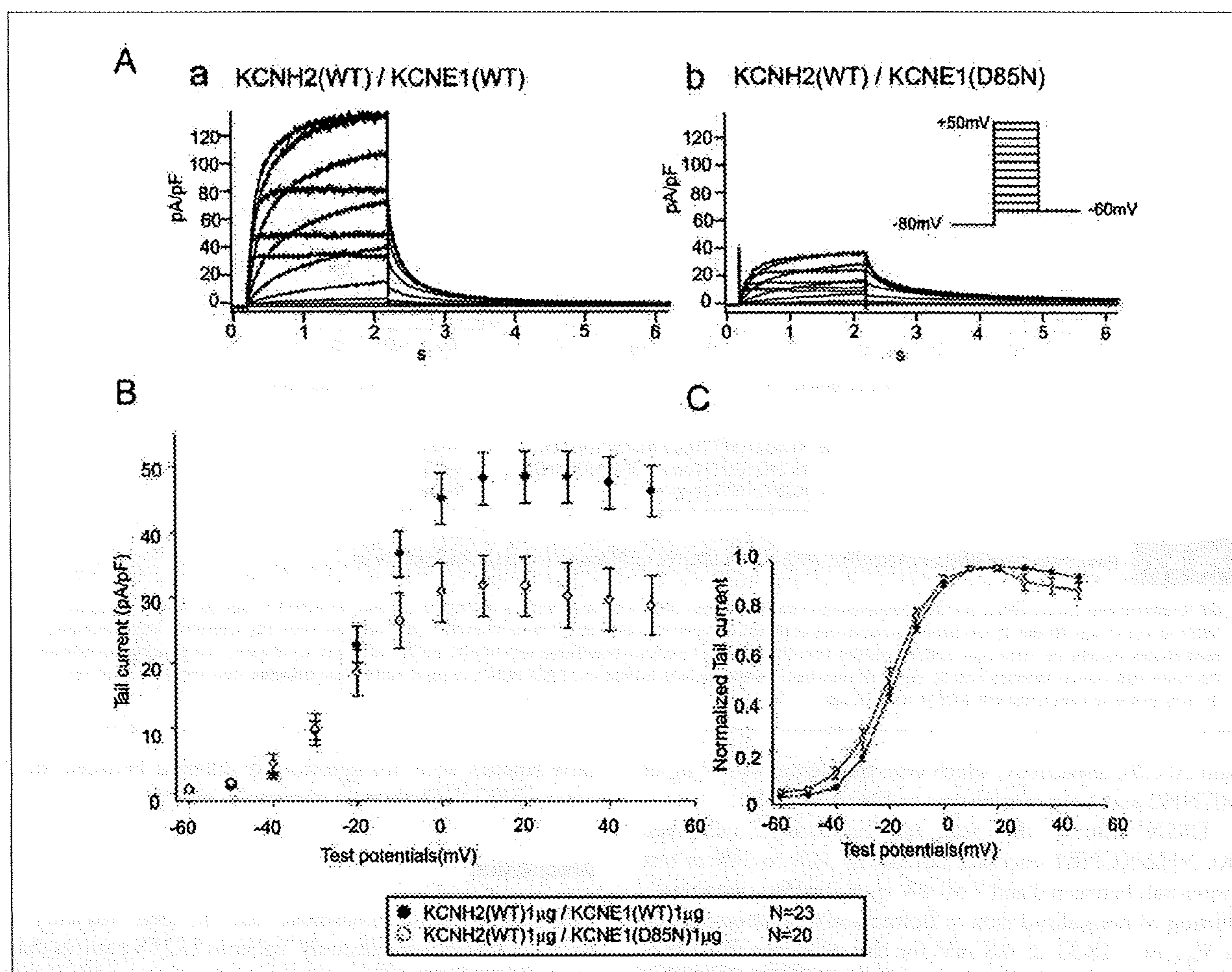

Figure 3 Functional Expression of KCNH2 With KCNE1-D85N in Chinese Hamster Ovarian Cells

(A) Representative current traces of KCNH2 coexpression with the wild-type (WT) or D85N KCNE1. **(a)** KCNH2 (1 μg) plus WT KCNE1 (1 μg). **(b)** KCNH2 (1 μg) plus D85N KCNE1 (1 μg). **(B and C)** Functional consequences of KCNH2 coexpression with the WT or D85N of KCNE1 (**[B]** activation curve; **[C]** normalized activation curve). **Solid circles** indicate data from 23 cells that were transfected with KCNH2 and WT KCNE1 (1 μg of each). **Open circles** indicate data from 20 cells that were transfected with KCNH2 and D85N KCNE1 (1 μg of each).

up-regulates I_{Kr}-like currents by 50% in CHO cells. Bianchi et al. (12) also showed interactions between the KCNE1-D76N mutation and both KCNQ1 and KCNH2 in HEK cells. In atrial tumor myocytes that expressed I_{Kr} alone, Yang et al. (13) demonstrated that antisense oligonucleotides against minK cDNA (KCNE1) significantly reduced the I_{Kr} by ~62%. More recently, Ohno et al. (14) identified a missense KCNE1 mutation, A8V, in a sporadic case of LQTS and reported that the mutation significantly reduced the magnitude of KCNH2- but not KCNQ1-encoded currents.

Collectively, it is of clinical importance that the KCNE1-D85N variant modifies not only KCNQ1- but also KCNH2-coded channel currents. Furthermore, its inhibitory action on KCNH2 was even stronger than that on KCNQ1. The KCNE1-D85N polymorphism may therefore cause phenotypes similar to those observed in type 2 LQTS such as bradycardia (15,16). The deactivation process of I_{Kr} plays a significant role in maintaining the appropriate rate of pacemakers (17) and, therefore, a decreased I_{Kr} will lead to sinus bradycardia. In the present study, 3 D85N carriers (13%) had sinus bradycardia (Table 1).

The mean onset age of 6 symptomatic heterozygous D85N carriers (Table 1) was 35.5 years, and this was significantly older than the mean age of other genotyped symptomatic LQTS patients. Shimizu et al. (18) reported that in 95 Japanese LQT1 patients with transmembrane domain mutations or C-terminal domain mutations, the mean ages of first event were 11 ± 8 years and 13 ± 9 years. Nagaoka et al. (19) also demonstrated that in 118 Japanese LQT2 patients with pore mutations or nonpore mutations, the mean ages of first event were 16 ± 10 years and 20 ± 13 years. In addition, the mean QTc of 13 D85N carriers was prolonged (498.5 ± 13.6 ms) but significantly shorter than that in 116 probands with other LQTS-related gene mutations (541 ms) (Fig. 1). These different phenotypes appear to reflect the fact that D85N causes a milder channel dysfunction than other LQTS mutations, and reveals a "forme fruste" phenotype (20).

The allele frequency of the KCNE1-D85N polymorphism was 0.81% among apparently healthy control individuals. We found only 1 report concerning D85N frequency (0.7%) (7) in control subjects, which showed equivalent results to our study. Based on 2008 healthy French individuals, Gouas et al. (21) demonstrated that the allele frequency of D85N was significantly higher in the 200 subjects with the longest QTc than in those with the shortest QTc (3.1% vs. 0.75%), suggesting that this single nucleotide polymorphism may influence the QTc length in healthy individuals.

LQTS can remain latent or subclinical because of "repolarization reserve" (22), and can become unmasked upon the intake of QT-prolonging drugs. Heterozygous D85N carriers in the control group may be at a potentially higher risk of long QT-related arrhythmias. Assuming that genetic surveys are feasible before drug therapy, D85N carriers may be recommended to avoid the secondary factors that predispose them to further QT prolongation such as QT prolonging drugs (23) and electrolyte disturbances (23–25). It is also clinically useful to search for other variants of long QT-related genes (8,26,27).

Study limitations. In the present study, we screened the mutations that are responsible for LQT1, 2, 3, 5, 6, and 7. Therefore, the comorbidity of other types of LQTS was not completely excluded, although their frequency was quite low. In general, single nucleotide polymorphisms are thought to be nonpathological although some may modify the clinical features of a disease. For example, the KCNH2-K897T polymorphism is a typical genetic modifier that aggravates LQTS phenotypes directly by reducing channel function in association with the KCNH2 mutation A1116V (28). Such a role for KCNE1-D85N was not addressed in this study and warrants further study.

Conclusions

KCNE1-D85N was a highly frequent variant in our LQTS probands and was found to cause loss-of-function effects on both I_{Kr} and I_{Ks} and work as a disease-causing variant. Since its allele frequency was 0.81% among control healthy individuals, KCNE1-D85N may be a clinically important genetic variant.

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Association between polymorphism of the AGTR1 and cardiovascular events in a Japanese general sample (The Shigaraki Study)

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Abstract

We examined whether the A1166C polymorphism of the angiotensin II type I receptor gene (AGTR1) affects cardiovascular event occurrence in a Japanese prospective cohort study. The 2212 participants who gave informed consent for genetic analysis were enrolled in this study (the Shigaraki Study). The average observation period was 1954 days. Cardiovascular events occurred in 37 individuals (1.7%). The independent factors which specified cardiovascular events were age (hazard ratio (HR)=1.13; 95% confidence interval (CI): 1.10–1.16; $p<0.0001$) and sex (HR=2.18; 95%CI: 1.23–3.85; $p=0.007$). However, the A1166C polymorphism of AGTR1 was not a predictive factor for cardiovascular events (HR=1.11; 95%CI: 0.61–2.02; $p=0.731$).
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Keywords: AGTR1; A1166C polymorphism; Cardiovascular events; Prospective cohort study

The A1166C polymorphism of AGTR1 has been reported to be associated with increased risks of hypertension [1], myocardial infarction [2], and cerebral infarction [3]. There have been no prospective cohort studies in Japanese subjects regarding the association between the polymorphism of the A1166C of AGTR1 and cardiovascular events. Thus, in this study, we examined whether the A1166C polymorphism of AGTR1 contributed to cardiovascular events in a Japanese general sample.

The study population consisted of 2902 Japanese subjects (the Shigaraki Study [4]), of which 2395 were enrolled in this genetic study after giving informed consent, and we analyzed 2212 subjects (835 men, 1377 women) who were successfully genotyped. This study was approved by the Institutional Review Board of Shiga University of Medical Science (Nos. 11–15, 1999, Nos. 8–17, 2006).

We reviewed the residence registry of all 2212 study subjects to check whether they were alive or dead. If they were alive, we checked whether they were hospitalized or not and, in case of hospitalization, their disease was obtained from their chart. If they were dead, the causes were examined. Subjects who had suffered a stroke, myocardial

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Table 1
Hazard ratio of cardiovascular event development of symptoms was calculated using the Cox proportional hazard model.

Risk characteristics	β	Hazard ratio (95%CI)	p-value
Sex, (men=1, women=0)	0.779	2.18 (1.23–3.85)	0.007
Age, years	0.121	1.13 (1.10–1.16)	<0.0001
BMI, kg/m^2	0.016	1.02 (0.95–1.09)	0.673
SBP, mm Hg	0.002	1.00 (0.99–1.01)	0.766
Alcohol, (yes=1, no=0)	−0.333	0.72 (0.44–1.17)	0.179
Smoking, (yes=1, no=0)	0.482	1.62 (0.99–2.65)	0.054
HbA1c, %	0.019	1.02 (1.00–1.04)	0.088
CHO, mg/dl	−0.001	1.00 (0.99–1.01)	0.720
UA, mg/dl	−0.006	0.99 (0.98–1.01)	0.539
1166C (yes=1, no=0)	0.105	1.11 (0.61–2.02)	0.731

The hazard ratio of event development of symptoms was calculated using the Cox proportional hazard model for sex, age, systolic blood pressure, drinking, smoking, cholesterol, HbA1c, uretic acid (UA), body mass index (BMI), and AGTR1 polymorphism.
BMI: Body mass index, SBP: systolic blood pressure, Alcohol: alcohol drinking habit, Smoking: smoking habit, CHO: cholesterol, UA: uretic acid, 1166C; A1166C polymorphism of AGTR1 (C1166C+A1166C).

infarction, or sudden death from July, 1999 to the end of December, 2004, were registered.

DNA was isolated from peripheral leukocytes and the AGTR1 genotypes were determined by a polymerase chain reaction (PCR)-based method.

The A1166C polymorphism of AGTR1 was not significantly associated with age, BMI, laboratory data, drinking and smoking habits, and cardiovascular events. Cardiovascular events occurred in 37 individuals (cardiac events 4, cerebrovascular events 33) (1.7%).

Table 1 shows the Cox proportional hazard analysis indicating that the independent factors which specified cardiovascular events were age (hazard ratio (HR)=1.13; 95% confidence interval (CI): 1.10–1.16; $p<0.0001$) and sex (HR=2.18; 95%CI: 1.23–3.85; $p=0.007$). AGTR1 gene polymorphism was not a predictive factor for cardiovascular events (HR=1.11; 95%CI: 0.61–2.02; $p=0.731$).

These results indicated that this polymorphism may have only a small effect on cardiovascular events, and this may be masked by differences in genetic background or environmental factors, including lifestyle. The A1166C polymorphism of AGTR1 may not have any functional significance itself, but may be linked to an as yet unidentified mutation in this gene. For example, Erdmann et al. reported that the AGTR1 polymorphism of A1166C showed weak but significant linkage disequilibrium with a polymorphism in the promoter region of the AGTR1, and suggested that the A1166C polymorphism may be slightly associated with the expression of AGTR1 [5].

Another possibility is that the frequency of 1166C is involved in the study results. The frequency of the 1166C allele was 7.5% in our 2212 samples. In other Japanese studies, the Suita study (3918 samples), the Ohasama study (1301 samples), and the Tanno and Sobetsu study (550 samples), reported that the frequency of the 1166C allele in a rural Japanese sample was 7.9%, 8.1% and 8.2%, respectively [6–8]. However, in Caucasian studies, the frequency of the 1166C allele was 36% in hypertensive and 28% in normotensive subjects [9]. Hindorff et al. reported that the 1166C allele was associated with an increased risk of incident ischemic stroke [10]. It appears that the 1166C allele is less frequent in Japanese than in whites. This difference of the allele frequency may have affected the different results in whites and Japanese.

In conclusion, age and sex were associated cardiovascular events; however, AGTR1 polymorphism was not a predictive factor. Follow-up study over a long period is required to confirm these findings.

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Prevalence of atrial fibrillation in the general population of Japan: An analysis based on periodic health examination

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Abstract

Background: The mortality and morbidity rates of various cardiovascular diseases differ between Western countries and Japan. The age- and gender-specific prevalence rate of atrial fibrillation (AF) in the general population of Japan was determined using the data from periodic health examinations in 2003.

Methods: Data of 630,138 subjects aged 40 years or more (47% were men and 34% were employees of companies and local governments) were collected from northern to southern Japan. The prevalence of diagnosed AF in each 10-year age group of both men and women was determined. Based on these prevalence rates and the Registry of Residents, the number of people having AF in Japan was estimated.

Results: The prevalence rate of AF increased as both male and female subjects aged, and it was 4.4% for men but only 2.2% for women aged 80 years or more ($p<0.0001$). As a whole, the AF prevalence of men was three times that of women (1.35 versus 0.43%, $p<0.0001$). There may be approximately 716,000 people (95% confidence interval (CI), 711,000–720,000) with AF in Japan, an overall prevalence of 0.56%. The number of people having AF was projected to be 1.034 (95% CI, 1.029–1.039) million, an overall prevalence of 1.09%, in 2050.

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Conclusions: The prevalence of AF increased in Japan as the population aged, as in Western countries. The overall prevalence of AF in Japan is approximately two-thirds of that in the USA. The projected increase in the number of people having AF is modest in Japan in 2050.
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Keywords: Atrial fibrillation; Epidemiology; Prevalence

1. Introduction

Atrial fibrillation (AF) is one of the common arrhythmias encountered in general practice as well as in the field of cardiology. AF could be responsible for increased mortality and various morbidities including clinical symptoms, impaired cardiac function and thromboembolism [1–5]. Several epidemiological studies from Western countries showed that prevalence of AF increased with the aging of the population and that the increase became striking after 60 years of age, affecting approximately 8% to 9% of people aged 80 years or more [6–10].

Several predisposing factors for AF other than aging are known [11,12]. Among them are hypertension, myocardial infarction, valvular heart disease, congestive heart failure, diabetes mellitus, alcohol drinking and others [11,12]. The prevalence, mortality and morbidity of various cardiovascular diseases may differ between Western countries and Japan. For example, prevalence of hypertension of Japan seems higher than that of the USA, but lower than that of England and Germany [13–16]. Another example is coronary artery disease. The incidence of myocardial infarction is lower and the prognosis after myocardial infarction is better in Japan than in Western countries [17–19]. Therefore, it seems conceivable that the epidemiology of AF could differ between Western countries and Japan. However, the epidemiological study of AF has been limited in Japan [20–22]; these epidemiological studies [20–22] showed the prevalence of AF was lower in Japan than in Western countries [6–10].

Japan has a periodic health examination system for company employees and community residents that cover the country. The Industrial Safety and Health Law decrees provision of a health examination that includes an electrocardiogram for examinees aged 40 years or more. In order to determine the prevalence of AF in the general population of Japan, we conducted a cooperative epidemiological study using the data from periodic health examinations of more than 600,000 subjects over the age of 40 years.

2. Subjects and methods

Data from periodic health examinations of community residents and employees of companies and local governments performed in 2003 were collected. After obtaining approval of the study design from the ethics committee of the participating institutes, which are distributed from the northern part of the main island of Honshu to Kyushu island of Japan, the health examination data were collected from company clinics, health screening centers, and local governments of the prefectures where the participating institutes are located.

Employees of companies and local governments undergo an annual health examination provided by their companies and governments; other people undergo a health examination provided by the local governments of their communities. Most employees of companies and governments retire at 60 years of age, and after retirement they undergo the annual health examination provided by their local governments. Health examination includes measurement of blood pressure, electrocardiogram, chest X-ray, urinalysis, and testing for blood cell count and blood chemistry. AF was diagnosed electrocardiographically by a trained doctor in each of participating centers and clinics. Conventional diagnostic criteria of AF, i.e., a grossly irregular ventricular rhythm of supraventricular origin, no visible P wave and irregular fluctuation of the baseline, were employed. In the present study, subjects were defined as having AF when their electrocardiogram showed AF at the time of health examination. Therefore, those with a history of paroxysmal AF but not having an AF episode at the time of the health examination were not counted as having AF in the following analyses.

Because the number of examinees exceeded 600,000, we collected the prevalence of AF of each 10-year age group for both men and women to calculate gender- and age-specific prevalence rates of AF. Then we estimated the absolute number of people with AF in each 10-year age group using the Japanese Registry of Residents in 2005 [23], and projected the absolute number of people having AF in the years of 2010–2050 using the medium variant estimation of the Population Projection for Japan [24].

Conventional diagnostic criteria of hypertension, hypercholesterolemia, and diabetes mellitus were employed in our health examination system. Hypertension was defined as a systolic blood pressure of 140 mmHg or higher, a diastolic blood pressure of 90 mmHg or higher, or both (Japanese Society of Hypertension, 2000). Hypercholesterolemia was defined as a total cholesterol level of 240 mg/dl or higher (Japanese Atherosclerosis Society, 2002), and diabetes mellitus, as a random blood glucose concentration of 200 mg/dl or a fasting blood glucose concentration of 126 mg/dl (Japan Diabetes Society, 1999). Additionally, in the present study, subjects were defined as having any comorbidity if they had been given drugs for hypertension, hypercholesterolemia, diabetes mellitus, or cardiac diseases.

Gender-specific prevalence of comorbidity was determined for subjects with and without AF.

2.1. Statistical analyses

Because of the number of subjects exceeding 600,000 and simplicity of data analyses, age- and gender-specific prevalence rates of AF and gender-specific prevalence of clinical characteristics were determined and compared with chi-square test. Independent predictors of AF were not determined in the present study. A p value less than 0.05 was considered statistically significant.

3. Results

Data of 630,138 subjects aged 40 years or more were collected from 25 company clinics, nine health screening centers of communities, and five local governments. Approximately 47% of the subjects were male; 31% of the subjects were aged 50–59 years, 27% were aged 40–49 years, and 23% were aged 60–69 years. Employees of companies and local governments accounted for 34% of all subjects of the study group.

3.1. Age- and gender-specific prevalence rate of AF

Since the age- and gender-specific AF prevalence of company employees and community residents was very similar, the data of both groups were pooled as a single group for the following analyses. In Table 1, age- and gender-specific prevalence rates of AF are summarized. AF prevalence increased as the subjects aged in both men and women ($p<0.0001$), and was lower in women than in men for each age group ($p<0.0001$). It was 4.4% for men aged 80 years or more, but was only 2.2% for women of the same age; as a whole, the AF prevalence of men was three times as great as that of women (1.35 versus 0.43%, $p<0.0001$).

3.2. Clinical characteristics of subjects with AF

Prevalences of clinical characteristics that could be possible risk factors for AF [11] are summarized in Table 2. Hypertension, diabetes mellitus, and cardiac diseases were present more frequently in both men and women with AF as compared with those without AF. Alcohol drinking was seen more frequently in men with AF than in those without AF. Hypercholesterolemia and current smoking were not associated with development of AF.

Table 1
Age- and gender-specific number of examinees and subjects with AF.

	Number of examinees		Number of subjects with AF	
	Men	Women	Men	Women
Age (years)				
40–49	88,156	80,432	211 (0.24%)	30 (0.04%)
50–59	96,901	97,070	760 (0.78%)	112 (0.12%)
60–69	57,998	86,156	1,126 (1.94%)	360 (0.42%)
70–79	41,770	58,296	1,436 (3.44%)	653 (1.12%)
80–	10,427	12,932	462 (4.43%)	283 (2.19%)
All ages	295,252	334,886	3,995 (1.35%)	1,438 (0.43%)

AF = atrial fibrillation.

Table 2
Clinical characteristics of subjects with and without atrial fibrillation.

	Men		Women	
	AF (+)	AF (−)	AF (+)	AF (−)
Hypertension (%)	42.4	24.2*	38.1	19.8*
Diabetes mellitus (%)	19.9	12.6*	10.4	5.0**
Hypercholesterolemia (%)	24.1	27.6	15.2	24.2**
Cardiac diseases (%)	59.1	4.6*	54.8	4.4*
Current smoker (%)	27.2	43.5*	2.1	5.2
Current drinker (%)	76.5	70.5**	15.1	22.6

AF = atrial fibrillation. *$p<0.00001$, **$p<0.05$ versus AF (+).
A complete set of data was available from 60,038 men and 45,297 women.

3.3. Estimated number of people with AF

There may be 716,000 [95% confidence interval (CI), 711,000–720,000] people having AF in Japan, an overall prevalence of 0.56%. As a whole, the absolute number of people with AF peaked at the age of 70–79 years. This was true for men. For women, however, the absolute number of people with AF was the greatest at the age of 80 or more. Eighty-five percent of the people with AF were older than 60 years, and 25% were older than 80 years.

The absolute numbers of people having AF projected into the future based on the medium variant estimates of the Population Projection for Japan [24] are summarized in Table 3. It would be 1.034 (95% CI, 1.029–1.039) million in the year of 2050, an overall prevalence of 1.09%.

4. Discussion

4.1. Major findings

The major findings of the present study were as follows. First, the prevalence rate of AF increased as people aged, and was three times greater in men than in women. Second, the estimated number of people with AF was approximately 720,000 in Japan, and the overall prevalence was 0.56%, which is two-thirds of that of the USA [8]. The number of people having AF in Japan is projected to be 1.034 million in the year of 2050, which is one fifth of that in the USA [9].

4.2. Comparison with data of previous studies

In Japan, epidemiological studies on AF have been limited in number. Nakayama et al. reported the prevalence of AF as 1.35% among 2305 residents of a community whose age was 40 years or more [20]. Other investigators [21,22] reported the AF prevalence was two to three times greater in men than in women; it was 3% for men and 1.1%

Table 3
Estimated number and prevalence of people with AF.

Year	Total population	People having AF (95% CI)	Overall prevalence (%)
	(Millions)	(Thousands)	
2005 [a]	126.87	716 (711–720)	0.56
2010	127.18	830 (824–835)	0.65
2020	122.74	974 (969–979)	0.79
2030	115.22	1049 (1044–1055)	0.91
2040	105.69	1051 (1045–1056)	0.99
2050	95.18	1034 (1029–1039)	1.09

AF = atrial fibrillation.
[a] The population numbers are based on the Japanese Registry of Residents of 2005 (23). Future population numbers were projected using the medium variant estimates of the Population Projection for Japan of the National Institute of Population and Social Security Research (24).

for women aged 60–69 years [21]. Epidemiological studies on the prevalence of AF in other Asian countries are still limited in number. Lok and Lau [25] reported that the prevalence of AF was only 1.3% of ambulatory elderly people aged 60–94 years in Hong Kong.

In Table 4, representative data of AF prevalence from Western countries and Japan are summarized. They clearly showed that the prevalence of AF increases as a population ages. In the Framingham Study reported by Wolf et al. [6], the prevalence was 0.1% for people aged 40–49 years, and increased to 8.8% for people aged 80 years or more. By contrast, based on a general practice research database in England and Wales, Majeed et al. [10] showed that prevalence of AF was more than 10% in both men and women aged 85 years or more. The prevalence of AF in the study of Ohsawa [22] as well as in the present study was much lower than the representative data reported from the Western countries [6,9,10].

Based on the data of the population-based survey, there are 2.23 million people in the USA having AF [8]. It would be 650,000 in England and Wales, an overall prevalence of 1.25% [10]. The present study estimated the number of people having AF to be 720,000 in Japan which was much lower than that reported for the USA [8].

The number of people having AF is expected to increase to 5 million in the USA in the year 2050 [9]. Other investigators estimated it to be 12–16 million in the USA by 2050 [26]. By contrast, Ohsawa et al. estimated it to be 1.08 million in Japan in 2030 [22]. Based on the present data, it would be 1.03 million in 2050, a relative increase of 40%. This modest increase in the future number of people with AF could be due to a decrease in number of people aged 50–69 in Japan, although number of people aged 80 years or more would be doubled in 2050 [24].

4.3. Clinical characteristics of subjects with AF

Hypertension is a well-known risk factor for AF [11,27]. Ohsawa et al. clearly showed that hypertension was complicated more frequently with both men and women having AF [28]. This was also true for the present study. However, hypertension itself could not account for the 3 times greater prevalence of AF in men than in women in the present study (Tables 1 and 2). Coronary artery disease is also known as a predisposing factor for AF [11,29]. Although the prevalence of coronary artery disease was not determined specifically in the present study, it was lower among patients with AF in Japan [30] as compared with those in the Western countries [9,31]. Of note is that severity of coronary artery disease is less in patients of Japan than in those of Western countries [17–19].

Diabetes mellitus is associated with AF [11,32]; this is also true for the present study. Alcohol drinking could increase the risk of AF [12]. Although Ohsawa et al. failed to show this finding [28], the present study showed prevalence of drinkers was slightly higher in men with AF than in those without AF.

Taken together, it seemed difficult to explain the difference of AF prevalence between Western countries and Japan and also between men and women in the present study only with these clinical characteristics.

4.4. Methodological considerations

The present study is limited for several reasons. First, not all community residents and company employees undergo the

Table 4
Age-specific prevalence of atrial fibrillation in epidemiological surveys expressed as a percent.

Age (years)	Wolf et al. [6] N=5070	Majeed et al. [10] N=1.4 million		Go et al. [9] N=1.89 million		Ohsawa et al. [22] N=23,713		Present study N=630,138	
	All	M	W	M	W	M	W	M	W
40–44	0.1	0.3	0.2			0.3	0.1	0.2	0.04
45–49	0.1	0.7	0.4			0.3	0.1	0.2	0.04
50–54	0.5	0.7	0.4			0.7	0.4	0.8	0.1
55–59	0.5	1.8	1.1	0.9	0.4	0.7	0.4	0.8	0.1
60–64	1.8	1.8	1.1	1.7	1.0	1.3	0.9	1.9	0.4
65–69	1.8	4.6	3.3	3.0	1.7	1.3	0.9	1.9	0.4
70–74	4.8	4.6	3.3	5.0	3.4	3.8**	2.2**	3.4	1.1
75–79	4.8	9.1	7.2	7.3	5.0	3.8**	2.2**	3.4	1.1
≥80	8.8	10.6*	10.9*	10.6	8.0	3.8**	2.2**	4.4	2.2

Age groups were in steps of 10 years starting from 40 years in the studies of Wolf et al., and Ohsawa et al., and in the present study, and starting from 35 years in the study of Majeed et al and were in steps of 5 years from 55 years in the study of Go et al.
M = men, W = women, * and ** prevalence of atrial fibrillation in subjects aged 85 years or more and in subjects aged 70 years or more, respectively.

annual health examination. In 1998, approximately 65% of Japan's population 40 years old or more underwent an annual health examination [33]. Among company employees, 73% underwent an annual health examination [33]. Some community residents who go to a hospital regularly for treatment tend not to undergo the health examination provided by their local government. This could lead to underestimation of the prevalence rate of AF for community residents. Second, the present study was cross-sectional in nature; therefore, the number of people having paroxysmal AF may be underestimated as mentioned above. Third, there could be a seasonal variation in frequency of episodes of paroxysmal AF [34]. The annual health examination is carried out through the whole year, and therefore, possible seasonal variation of AF episodes would have affected the present data. Finally, only prevalence of AF was determined for each 10-year age group, and a complete dataset of clinical characteristics was available from approximately a sixth of the subjects; therefore, detailed analyses to determine independent predictors of AF were not performed in the present study.

5. Conclusions

Although limited for these reasons, the present study, based on the data of annual health examinations of more than 600,000 people, showed the rate of AF increased as the population aged in Japan, as in Western countries. The prevalence of AF was approximately two thirds of that in the USA [8], and the projected increase in the number of people having AF in Japan is modest when compared with that of the USA [9,26] in the year of 2050.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [35].

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RESEARCH PAPER

Inhibitory actions of the phosphatidylinositol 3-kinase inhibitor LY294002 on the human Kv1.5 channel

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Background and purpose: Kv1.5 channels conduct the ultra-rapid delayed rectifier potassium current (I_{Kur}), and in humans, Kv1.5 channels are highly expressed in cardiac atria but are scarce in ventricles. Pharmacological blockade of human Kv1.5 (hKv1.5) has been regarded as effective for prevention and treatment of re-entry-based atrial tachyarrhythmias. Here we examined blockade of hKv1.5 channels by LY294002, a well-known inhibitor of phosphatidylinositol 3-kinase (PI3K).
Experimental approach: hKv1.5 channels were heterologously expressed in Chinese hamster ovary cells. Effects of LY294002 on wild-type and mutant (T462C, H463C, T480A, R487V, A501V, I502A, I508A, L510A and V516A) hKv1.5 channels were examined by using the whole-cell patch-clamp method.
Key results: LY294002 rapidly and reversibly inhibited hKv1.5 current in a concentration-dependent manner (IC_{50} of 7.9 $\mu mol \cdot L^{-1}$). In contrast, wortmannin, a structurally distinct inhibitor of PI3K, had little inhibitory effect on hKv1.5 current. LY294002 block of hKv1.5 current developed with time during depolarizing voltage-clamp steps, and this blockade was also voltage-dependent with a steep increase over the voltage range for channel openings. The apparent binding (k_{+1}) and unbinding (k_{-1}) rate constants were calculated to be 1.6 $\mu mol \cdot L^{-1-1} \cdot s^{-1}$ and 5.7 s^{-1} respectively. Inhibition by LY294002 was significantly reduced in several hKv1.5 mutant channels: T480A, R487V, I502A, I508A, L510A and V516A.
Conclusions and implications: LY294002 acts directly on hKv1.5 currents as an open channel blocker, independently of its effects on PI3K activity. Amino acid residues located in the pore region (Thr480, Arg487) and the S6 segment (Ile502, Ile508, Leu510, Val516) appear to constitute potential binding sites for LY294002.
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Keywords: Kv1.5; LY294002; PI3K; open channel block; I_{Kur}; site-directed mutagenesis; binding site

Abbreviations: AF, atrial fibrillation; APD, action potential duration; I_{Kr}, rapidly activating component of delayed rectifier potassium current; I_{Ks}, slowly activating component of delayed rectifier potassium current; I_{Kur}, ultrarapid delayed rectifier potassium current; PI3K, phosphatidylinositol 3-kinase; Kv, voltage-dependent potassium channel; CHO, Chinese hamster ovary; WT, wild-type; DMSO, dimethyl sulphoxide; GFP, green fluorescent protein; MAP kinase, mitogen-activated protein kinase; PCR, polymerase chain reaction

Introduction

Atrial fibrillation (AF) is the most frequent cardiac arrhythmia that can result in serious morbidity and a doubling of mortality in elderly persons (Chugh *et al.*, 2001). It is well known that AF or atrial flutter is caused, or at least maintained, by re-entrant wavelets. A well-established method of extinguishing or preventing such re-entries is prolongation of the myocardial refractoriness, primarily determined by the action potential duration (APD). A major determinant of APD is the amount of repolarizing outward potassium currents, particularly provided by the three (ultra-rapid, rapid and slow) delayed rectifier potassium currents (I_{Kur}, I_{Kr} and I_{Ks} respectively). Therefore, blockers of these currents can be expected to exert anti-arrhythmic actions against re-entrant-based tachyarrhythmias (Knobloch *et al.*, 2002). However, because I_{Kr} and I_{Ks} are present in both the atrium and ventricle, undesired effects such as excess prolongation of ventricular action potentials can be caused by blockers of I_{Kr} and I_{Ks}, which limits their use for the treatment of atrial arrhythmias (Bril, 2002; Knobloch *et al.*, 2004; Vos, 2004; Regan *et al.*, 2007). In

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contrast, I_{Kur} is expressed predominantly in atrial myocytes but is scarce in ventricular myocytes, in humans (Wang *et al.*, 1993; Li *et al.*, 1996). I_{Kur} may therefore be a promising molecular target for anti-arrhythmic drugs to treat re-entrant-based atrial tachyarrhythmias. Experimental evidence has also been presented to show that I_{Kur} blockade increases the plateau phase and thereby prolongs APD in atrial trabeculae obtained from patients with long-standing AF associated with electrical remodelling (Wettwer *et al.*, 2004).

The molecular component that underlies I_{Kur} in the human atrium is the Kv1.5 channel (Fedida *et al.*, 1993; Li *et al.*, 1996; Nattel *et al.*, 1999: channel nomenclature conforms to Alexander *et al.*, 2008), which is encoded by KCNA5 gene. The Kv1.5 channels belong to the super-family of voltage-gated potassium channels, comprising four pore-forming subunits, each containing six transmembrane segments (S1 to S6). The segment between S5 and S6 forms the external part of the ion conduction pathway. The flanking S5 and S6 segments may contribute to the presumably wider intracellular mouth of the ion channel (Yeola *et al.*, 1996). During the past decade, considerable efforts have been made to develop novel blockers of Kv1.5 channels (Brendel and Peukert, 2003; Varro *et al.*, 2004; Trotter *et al.*, 2006) and to characterize their binding sites in the channel. A few of the known Kv1.5 channel blockers have been tested in humans (Crijns *et al.*, 2006; Dorian *et al.*, 2007). However, newly developed compounds are not so highly selective for Kv1.5 channels and thus, currently, the clinical data to validate the effectiveness of Kv1.5 channel blockade for the treatment of AF are lacking. Mutational analyses have indicated that some residues located near the pore helix as well as in the S6 domain of Kv1.5 channels are important for the binding of the channel blockers (Decher *et al.*, 2004; 2006; Herrera *et al.*, 2005; Rezazadeh *et al.*, 2006).

The compound LY294002 is derived from the naturally occurring bioflavinoid quercetin and potently inhibits phosphatidylinositol 3-kinase (PI3K) activity (IC_{50} = 1.40 μmol·L^{-1}) via competitive inhibition of an ATP binding site on the p85α subunit (Vlahos *et al.*, 1994). This compound has been used in studies of neuronal, cardiovascular, immune and diabetes-related cellular functions for more than a decade (Knight *et al.*, 2004). In recent years, it has been reported that LY294002 can inhibit Kv channel in MIN6 insulinoma cells through a PI3K-independent mechanism (El-Kholy *et al.*, 2003).

The present study was designed to investigate the effect of LY294002 on human Kv1.5 (hKv1.5) channels, heterologously expressed in Chinese hamster ovary (CHO) cells by using the whole-cell patch-clamp technique. Our findings indicate that LY294002 interacts with hKv1.5 channels in a PI3K-independent manner and directly inhibits hKv1.5 currents as an open channel blocker. The putative binding site for this compound is found to be located at the base of the pore helix (Thr480), in the outer pore region (Arg487) and in the S6 domain (Ile502, Ile508, Leu510 and Val516) of hKv1.5 channels.

Methods

Cell preparation, site-directed mutagenesis and transfection
CHO cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum and antibiotics (100 IU·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin) under a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were passaged twice weekly by harvesting with trypsin-EDTA, and a part of treated cells were seeded onto glass coverslips (5 × 3 mm^2) for the later transfection.

The mammalian expression vector pcDNA3.1 containing hKv1.5 cDNA (kindly provided by Dr D Fedida, University of British Columbia, Vancouver, Canada) was used for expression of all constructs in this study (Eldstrom *et al.*, 2003). Polymerase chain reaction (PCR)-based site-directed mutagenesis was applied to introduce mutations into hKv1.5 cDNA by using Quikchange Kit (Stratagene, La Jolla, CA, USA). All PCR-products were fully sequenced (ABI3100, Applied Biosystems, Foster City, CA, USA) to ensure the fidelity of the PCR reactions. Wild-type (WT) hKv1.5 cDNA and hKv1.5 mutants (T462C, H463C, T480A, R487V, A501V, I502A, I508A, L510A and V516A cDNA) were transiently transfected into CHO cells together with green fluorescent protein (GFP) cDNA (0.5 μg WT or mutant hKv1.5 + 0.5 μg GFP) by using Lipofectamine (Invitrogen Life Technologies, Carlsbad, CA, USA). Patch-clamp experiments were conducted 2–3 days after transfection on GFP-positive cells.

Electrophysiological recordings and data analysis
Whole-cell membrane currents (Hamill *et al.*, 1981) 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 analogue-to-digital converter (HEKA) and stored on a hard disc drive, by using Pulse/PulseFit software (HEKA). For experiments to measure the activation time course of hKv1.5 currents, the data were low-pass filtered at 10 kHz and sampled at 50 kHz. Patch electrodes had a resistance of 2.5–3.0 MΩ when filled with the pipette solution containing (in mmol·L^{-1}) 70 potassium aspartate, 40 KCl, 10 KH_2PO_4, 1 $MgSO_4$, 3 Na_2-ATP (Sigma Chemical Co., St. Louis, MO, USA), 0.1 Li_2-GTP (Roche Diagnostics GmbH, Mannheim, Germany), 5 EGTA and 5 HEPES (pH adjusted to 7.2 with KOH). In the whole-cell configuration, average series resistances were 5.6 ± 0.1 MΩ. Because the average current was 4.9 nA at +30 mV, the voltage drops induced by the series resistances can be calculated to be 27 mV. The series resistances were usually compensated by 80%. After compensation, the voltage drops were considered to be less than 5.4 mV. Cells attached to glass coverslips were transferred to a recording chamber (0.5 mL in volume) mounted on the stage of an inverted microscope (ECLIPSE TE2000-U, Nikon, Tokyo, Japan). The chamber was maintained at 25°C and was perfused continuously at a rate of 1–2 mL·min^{-1} with Tyrode solution containing (in mmol·L^{-1}) 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 was adjusted to 7.4 with NaOH).

hKv1.5 channel currents were elicited by applying 300 ms depolarizing steps from a holding potential of −80 mV to various levels. Except for frequency-dependent experiments, the interval between voltage-clamp steps was 10 s or longer to allow channels to recover fully from possible inactivation between voltage steps. Drug-induced inhibition was measured at the end of a 300 ms depolarizing step to +30 mV unless

otherwise indicated. Voltage-dependent activation of hKv1.5 channels was assessed by fitting the normalized I–V relationship of the tail currents to a Boltzmann equation: $I_{tail} = 1/(1 + \exp((V_{1/2} - V_m)/k))$, where I_{tail} is the tail current amplitude normalized with reference to the maximum value measured at +50 mV; $V_{1/2}$ is the voltage at half-maximal activation; V_m is the test potential and k is the slope factor. The concentration–response curve for inhibition of hKv1.5 current by LY294002 was drawn by a least-squares fit of a Hill equation: $\%Control = 1/(1 + (IC_{50}/[D])^{n_H})$, where %Control represents the current in the presence of the drug normalized with reference to the control amplitude (expressed as a percentage); IC_{50} is the concentration of LY294002 causing a half-maximal inhibition; n_H is the Hill coefficient and [D] is drug concentration. A first-order blocking scheme was used to describe the drug–channel interaction (Snyders and Yeola, 1995; Yeola *et al.*, 1996). The apparent rate constants for binding (k_{+1}) and unbinding (k_{-1}) were obtained from fitting the equations: $\tau_D = 1/(k_{+1}[D] + k_{-1})$, where τ_D is the drug-induced time constant, which was calculated from single exponential fits to the traces of current decay during depolarizing step to +30 mV. The apparent dissociation constant K_D is expressed as $K_D = k_{-1}/k_{+1}$. The deactivation kinetics was determined by fitting a single exponential function to the tail current trace.

Statistical analysis

All of the averaged data are presented as mean ± s.e.mean, with the number of experiments shown in parentheses. Statistical comparisons were evaluated by using either Student's t-test or ANOVA with Dunnett's post hoc test, as appropriate. Differences were considered to be statistically significant if a P value of <0.05 was obtained.

Materials

LY294002, LY303511 (Calbiochem, San Diego, CA, USA) and wortmannin (Sigma) were dissolved in dimethyl sulphoxide (DMSO; Sigma) to yield stock solutions of 50 mmol·L^{-1}. The concentration of DMSO in the final solution was <0.1% (V/V), which had no effect on hKv1.5 currents.

Results

Inhibitory action of LY294002 on hKv1.5 current

Figure 1 demonstrates a representative experiment to examine the effects of LY294002 on the hKv1.5 channels heterologously expressed in a CHO cell. The hKv1.5 current was evoked by 300 ms depolarizing voltage-clamp steps given from a holding potential of −80 mV to various test potentials (−50 to +50 mV) with a return potential of −40 mV, before (Control, Figure 1A) and during (Figure 1B) exposure to 10 µmol·L^{-1} LY294002. In control conditions, hKv1.5 current activated rapidly upon depolarization to reach a peak and then remained stable during moderately depolarized test steps (≤+30 mV) but decayed minimally during strongly depolarized test potentials (≥+40 mV), consistent with previous studies (Snyders *et al.*, 1993; Feng *et al.*, 1998; Choi *et al.*, 2000; Herrera *et al.*, 2005; Rezazadeh *et al.*, 2006). The activation time constant of 1.46 ± 0.11 ms (n = 6) was obtained by fitting a single exponential function to the current traces during the initial 30 ms of depolarizing step to +30 mV. The decaying outward tail currents were detected on return to −40 mV.

Bath application of 10 µmol·L^{-1} LY294002 did not significantly affect the activation time constant (1.45 ± 0.16 ms at +30 mV; n = 6, P > 0.05) while modestly reducing the peak amplitude of hKv1.5 current. However, this compound caused a marked, time-dependent decline in outward currents during depolarizing test potentials, which was more prominent at more positive potentials. Figure 1C illustrates I–V relationships for late currents (measured at the end of 300 ms clamp steps) in the absence and presence of LY294002. The hKv1.5 current was blocked by LY294002 over the whole potential range for activation. The voltage-dependent activation of hKv1.5 current in the absence and presence of the drug was evaluated by fitting a Boltzmann equation to the amplitude of tail current elicited on return to −40 mV following depolarizing voltage steps to various test potentials (Figure 1D). In a total of seven cells, $V_{1/2}$ averaged −13.4 ± 1.1 mV in control and −22.6 ± 2.0 mV (P < 0.01) in the presence of LY294002, while k was 10.3 ± 0.6 mV in control and 6.2 ± 1.1 mV (P < 0.05) in the presence of the compound. Thus, LY294002 significantly shifted the voltage dependence of channel opening to more hyperpolarized potentials, as reported for the action of two other drugs (mibefradil, Perchenet and Clement-Chomienne, 2000; papaverine, Choe *et al.*, 2003) on hKv1.5 channels.

To quantify the voltage dependence of current inhibition, the relative amplitude of late currents in the presence and absence of LY294002 (% Control) was measured at each test potential and plotted, together with the activation curve obtained in control conditions, (Figure 1E). The hKv1.5 channel activated with a threshold potential of −40 mV and the channel conductance peaked near +20 mV. The current reduction steeply increased at potentials between −30 and 0 mV, which corresponded to the voltage range of channel opening. At potentials positive to +20 mV where channel conductance was nearly saturated, current reduction exhibited a shallower voltage dependence. This observation is consistent with the premise that LY294002 preferentially affected the open state of the hKv1.5 channels.

Concentration-dependent inhibition of hKv1.5 induced by LY294002

The inhibitory effect of LY294002 on hKv1.5 was examined at various concentrations between 1 and 50 µmol·L^{-1}. The hKv1.5 current was elicited every 15 s by 300 ms depolarizing step to +30 mV, before (Control) and during exposure to increasing concentrations of LY294002 in a cumulative manner after the inhibition due to the previous concentration reached a steady state (Figure 2A). It is evident that at any given concentration of LY294002, the late current level at the end of the 300 ms clamp steps was more potently reduced than initial peak current level, which supports a gradual development of channel inhibition during the open state of the channel. Figure 2B illustrates the concentration–