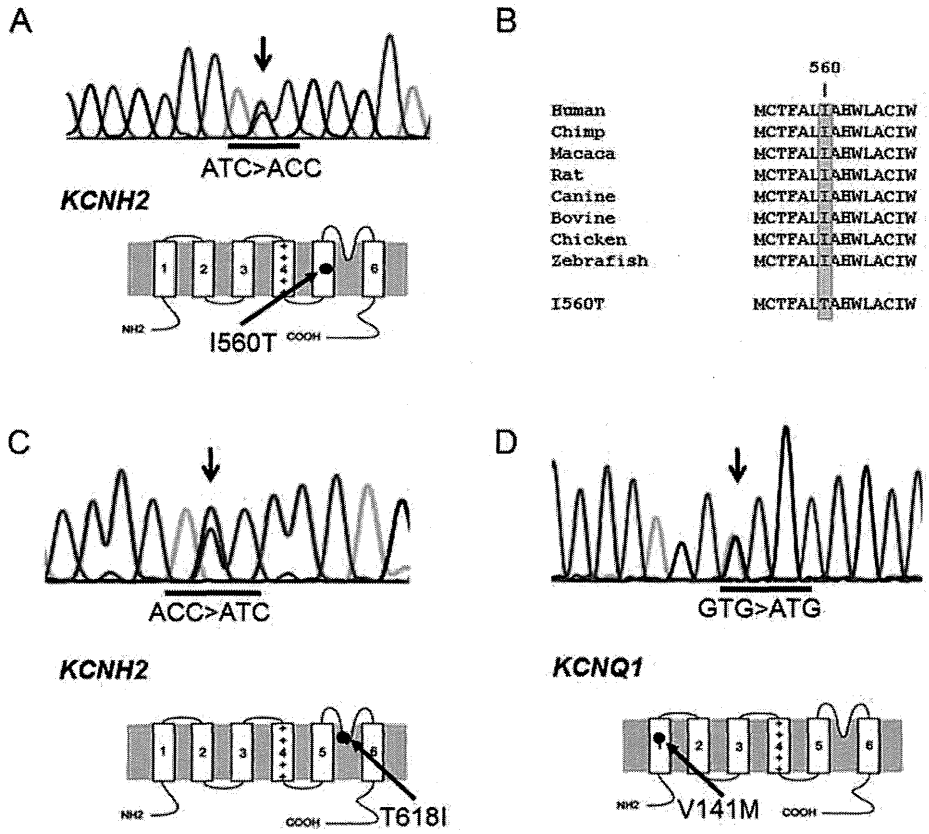

Fig. 2. Sequence analysis of the SQTS probands. (A) An electropherogram within exon 7 of *KCNH2* of the Family 1 proband. I560T is located in the S5 transmembrane segment. (B) Amino acid sequence between 554 and 568 of *KCNH2* is identical among eight species. (C) An electropherogram within exon 7 of *KCNH2* of the Family 2 proband. T168I is located in the pore region of *KCNH2*. (C) An electropherogram within exon 3 of *KCNQ1* of the Family 3 proband. V141M is located in the S1 transmembrane segment.

administration of pilsicainide, indicating a complication of Brugada syndrome (Supplemental Fig. S1, F). A programmed electrical stimulation failed to induce VF. His mother died at 43 years and an uncle died suddenly from unknown causes. Genetic testing was negative for all six SQTS genes, as well as Brugada syndrome candidate genes including *SCN5A*, *HCN4*, *KCND3*, *KCNE3*, *SCN1B*, *SCN3B*, *SCN10A*, and *TRPM4*. An ICD was implanted as a primary preventative measure.

3.2. Electrophysiological properties of KCNH2-I560T

KCNH2-I560T heterologously expressed in COS-7 cells resulted in a significant 2.5-fold increase in the peak I_{Kr} current density versus WT (I560T: 99.7 ± 10.2 pA/pF; WT: 40.6 ± 10.4 pA/pF; $p < 0.005$) (Fig. 3A,B), whereas the voltage dependence of activation was comparable (I560T: −19.7 ± 3.2 mV; WT: −18.5 ± 1.6 mV; NS) (Fig. 3C).

Table 1
Summary of clinical characteristics of SQTS families.

Family	Proband/family	Gender	Age of manifestation (year)	QTc (ms)	Mutations	Arrhythmias	Symptoms	Family history
1	Proband	M	64	319	*KCNH2*-I560T	Paroxysmal AF, AFL	Palpitation, syncope	SCD
2	Proband	F	39	322	*KCNH2*-T618I	Aborted VF	Palpitation	SCD
	Brother	M	42	330		–	–	
	Nephew	M	14	330		–	–	
3	Proband	F	4*	280	*KCNQ1*-V141M	SSS, fetal bradycardia	–	PPM
	Father	M	37	375		Chronic AF, bradycardia	–	
4	Proband	F	17	330	Negative	Aborted VF	–	–
	Sister	F	19	327		–	–	
	Grandmother	F	77	321		–	–	
5	Proband	M	42	340	Negative	BrS	–	SD

AF: atrial fibrillation, AFL: atrial flutter, VF: ventricular fibrillation, SCD: sudden cardiac death, BrS: Brugada syndrome, SD: sudden death, SSS: sick sinus syndrome, PPM: permanent pacemaker.
*: Diagnosed at 10 years. Severe short QT (QTc = 280 ms) demonstrated at 4 years.

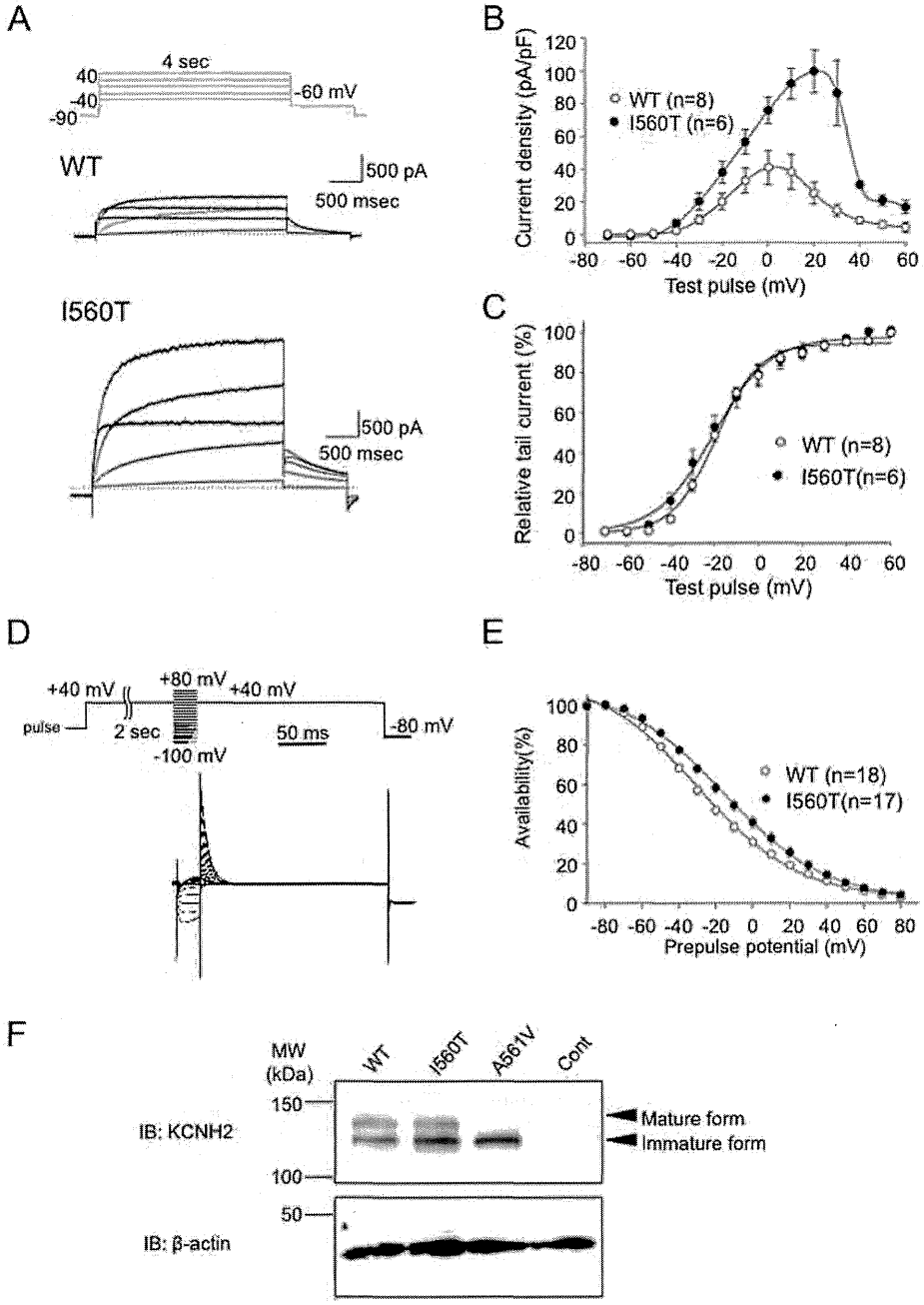

Fig. 3. Electrophysiological properties and protein expression of *KCNH2*-I560T. (A) I_{Kr} current traces of WT and *KCNH2*-I560T obtained from COS-7 cells. (B) Current–voltage relationship showing that I560T showed a 2.5-fold increase of the current density. (C) Voltage dependence of activation of WT and I560T were nearly identical. (D) Representative traces of I560T obtained with a multi-pulse protocol to determine channel availability. (E) Steady-state inactivation of I560T showed a significant 14 mV positive shift of $V_{1/2}$, but the slope factor was nearly identical. (F) I560T expressed glycosylated mature protein at similar levels to WT. The trafficking-defective LQTS mutation A561V expresses unglycosylated immature proteins.

Steady-state inactivation showed a 14 mV positive shift in the mutant channel (I560T: -13.2 ± 4.1 mV; WT: -27.3 ± 2.4 mV; $p < 0.005$). The slope factor was nearly identical (I560T: -26.5 ± 1.2 mV; WT: -25.4 ± 1.1 mV; NS) (Fig. 3E). These results suggest that the mutant channel may cause a gain of function in I_{Kr} current, which is a known trait of SQTS caused by *KCNH2* mutations.

3.3. Protein expression of KCNH2-I560T

To test if the I_{Kr} gain of function observed in the *KCNH2*-I560T channel may be attributed to increased membrane expression levels or hyperglycosylation of the mutant channel protein, we performed Western blotting using a trafficking-defective neighboring *KCNH2* mutation

A561V as a control [25]. While the A561V mutant showed only minimal expression of mature glycosylated high-molecular-weight protein, the I560T mutant showed nearly identical expression levels and pattern to the WT protein (Fig. 3F). This confirms that the gain of function in I_{Kr} observed in the I560T mutant is not due to the altered membrane expression but most likely due to the changes in channel gating properties.

3.4. In silico simulation of KCNH2-I560T

To explore whether the relatively modest gating modulation caused by the *KCNH2*-I560T mutation is sufficient to cause shortenings of APD and QT interval, we performed simulations of human ventricular action potentials with and without the *KCNH2*-I560T mutation in the 1-D myofiber model, representing the electrical behaviors of the left

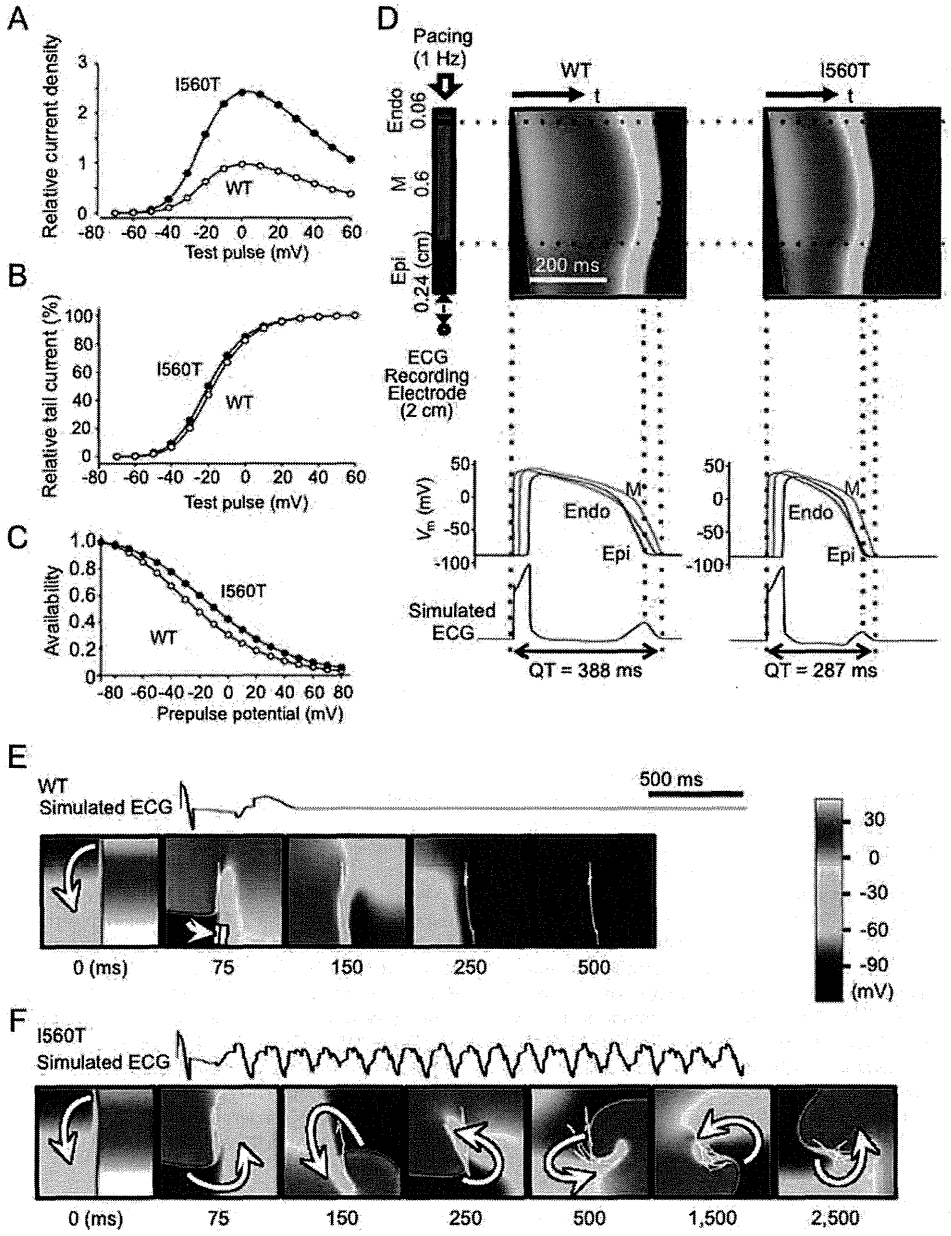

Fig. 4. Simulations of Markovian I_{Kr}, action potentials, ECGs, and effect on ventricular arrhythmogenicity of *KCNH2*-I560T. (A–C) Current–voltage relationship, voltage dependence of activation, and steady-state inactivation curves generated from the Markovian I_{Kr} model. (D) Using the myofiber model incorporating the above Markovian I_{Kr} models, transmural dispersions of action potentials with WT and I560T mutation were made. The spatial distributions of membrane potentials were color coded according to the color bar. Endo, M, and Epi denote endocardial, mid-myocardial, and epicardial layers. (E and F) Simulated ECGs and consecutive snapshots of the myocardial sheet model, incorporating the Markovian I_{Kr} models, after S1–S2 cross-field stimulation are shown. White open arrows and double short lines indicate the direction of wave front propagation and collision between wave fronts, respectively. Curved thin white lines inside the snapshots represent trajectories of the spiral wave phase singularities during the last 500 ms of activity.

ventricular free wall. Using a modified computer model of Markovian I_{Kr}, we were able to faithfully reproduce the I_{Kr} current traces and tail currents (Supplemental Fig. S2), 2.5-fold increase in current density (Fig. 4A), no significant shift in activation (Fig. 4B), and a 14 mV positive shift in inactivation (Fig. 4C), consistent with our observations in vitro. When cells were stimulated at 1 Hz, *KCNH2*-I560T showed significantly shorter APD than WT (Fig. 4D). The resulting pseudo-ECG also showed an abbreviated QT interval for the *KCNH2*-I560T mutant (287 ms) compared with WT (388 ms), which meets the diagnostic criteria of SQTS.

To explore the arrhythmogenic potency of VF in *KCNH2*-I560T, an S1–S2 cross-field protocol, with onset of S2 assumed as time zero (0 ms panel), was applied to induce a spiral wave re-entry (as a model of VF) (Fig. 4E,F). In the WT model, a counter-clockwise rotating wavefront terminated immediately (75–150 ms panels) and sustained re-entry was not induced. In contrast, the *KCNH2*-I560T model elicited a sustained meandering spiral wave re-entry with mean cycle length of ~153 ms (Supplemental videos S1 and S2).

3.5. Genotype-dependent differences in clinical characteristics of SQTS

Based on the observations in our SQTS cohort, that *KCNQ1* mutation carriers showed apparently earlier onset and more frequent bradyarrhythmia complications (Fig. 1, Table 1), we explored potential genotype-specific characteristics in the broader SQTS patient population. We combined mutation-positive SQTS patients of our cohort ($n = 6$) and those from previous publications ($n = 59$), and analyzed the clinical variables with respect to different genotypes (Tables 2 and 3). Among the SQT1, SQT2, and SQT3–6 groups, the age of manifestation was significantly later in SQT1 patients (SQT1: 35 ± 19 years, $n = 30$; SQT2: 17 ± 25, $n = 8$; SQT3–6: 19 ± 15, $n = 15$; $p = 0.011$), whereas the QTc values were comparable (Fig. 5, Table 2). Conversely, complications of SSS or bradycardia were significantly more prevalent in SQT2 patients (6/8, 75%) than non-SQT2 patients (5/57, 9%; $p < 0.001$) (Fig. 5C, Table 3), whereas there was no difference between SQT1 and non-SQT1 (Table 3). Furthermore, the prevalence of AF was also present in SQT2 patients (5/8, 63%) than non-SQT2 patients (12/57, 21%, $p = 0.012$) (Fig. 5D, Table 3). Other clinical parameters did not show significant differences between genotypes (Tables 2 and 3).

3.6. Evaluation of penetrance in 16 SQTS families

SQTS has been described as having close to complete penetrance in cohort studies, with only some exceptional cases with normal QTc [8, 9]. Despite carrying the mutation *KCNQ1*-V141M, the father of family 3 exhibited a QTc of 375 ms, which is outside the diagnostic criteria for SQTS [20], but manifested chronic AF and bradycardia, prompting us to reevaluate the genetic penetrance of SQTS (Fig. 1C, Supplemental Fig. S1, D). Among 35 SQTS families, we focused on two families from our study and 14 previously reported families [1,3,5–9,12,14, 32–36] with two or more genetically or phenotypically affected individuals (Table 4). Our family 3 was the only SQTS family in this group carrying a *KCNQ1* mutation. Among a total of 51 mutation-positive individuals, only 42 exhibited short QTc <360 ms. Therefore, the calculated overall genetic penetrance of SQTS was 82%, which was lower than previously recognized [8,9]. Furthermore, we found that the 13 families with K channel mutations (SQT1–3) showed a higher penetrance of 90%, whereas Ca channel mutations (SQT4–6) showed a much lower penetrance of 58%. Interestingly, the low penetrance observed in families with Ca channel mutations is comparable to the well-known incomplete penetrance associated with Brugada syndrome [37]. Of the nine mutation carriers who did not exhibit short QTc, four K channel mutation carriers exhibited syncope, AF, bradycardia, or instances of non-documented arrhythmia [35,36], and the five patients with Ca channel mutations remained asymptomatic [6,7].

4. Discussion

4.1. Common electrophysiological properties in KCNH2 mutations

The novel *KCNH2* mutation I560T was identified in an SQT1 patient with severe QTc shortening, and family history of sudden death. Investigation of the *KCNH2*-I560T channel expressed in COS-7 cells showed a relatively mild gain of function with a + 14 mV shift of steady-state inactivation but no activation abnormalities. The computer simulation recapitulated the APD shortening and susceptibility to ventricular reentry. Among four *KCNH2* mutations that have been functionally evaluated, N588K was associated with a severe QTc shortening and exhibited severe gain of function with a 4-fold increase in peak current density, virtually no inactivation over the physiological range, and a large positive shift (+102 mV) of steady-state inactivation [3,38]. Peak current density was increased 6-fold in both T618I and E50D, and the steady-state inactivation was shifted by +20 mV and +11.5 mV in T618I and E50D, respectively [8,14,39,40]. Taken together, these results suggest that augmented peak current density and a positive shift of steady-state inactivation are the functional channel properties commonly affected by *KCNH2* mutations responsible for SQT1. The precise mechanism for the positive shift in inactivation curve in *KCNH2*-I560T is not clear, however, it is speculated that the shift in inactivation may be due to a disruption of the hydrogen bonding between amino acid residues that span the S5 and pore helix as seen in a neighboring residue H562 [41]. These changes in inactivation may be the primary determinants for the clinical manifestations of SQT1 [40]. However, as is observed in the SQT1 case carrying *KCNH2*-I560T reported here, the degree of the gating abnormality and the clinical severity may not always correlate, suggesting the involvement of additional confounding factors. These may include a number of common genetic variations that modulate QTc, as suggested by genome-wide association studies of LQTS [42,43]. Similar mechanisms may underlie the difference between clinical severity of SQTS patients and the electrophysiological properties of the mutant channels.

4.2. Incomplete penetrance of SQTS

Penetrance in LQTS has been recognized to be as low as 25% for some mutations [44], with the latent LQTS mutant carriers still, however, at risk of lethal arrhythmias and SCD [11]. By contrast, nearly complete penetrance has been described in SQTS within cohort studies [8,9]. However, our meta-analysis of 16 SQTS families revealed an incomplete penetrance of 82%, where nine mutation-positive patients from six unrelated SQTS families exhibited longer QTc than 360 ms (Table 4). Responsible mutations for the latent SQTS cases include three K channel mutations: *KCNQ1*-V141M (family 3); *KCNH2*-E50D [8,36]; and *KCNH2*-R1135H [35]. The carriers of *KCNQ1*-V141M and *KCNH2*-R1135H showed arrhythmias in the absence of short QTc. Three other mutations

Table 2
Age of manifestation and QTc of SQTS patients.

Clinical characteristics	SQT1	SQT2	SQT3–6	p*	Non-genotyped
Age of manifestation (year)	35 ± 19 (30)	17 ± 25 (8)	19 ± 15 (15)	0.011	28 ± 18 (57)
QTc (ms)	307 ± 30 (31)	305 ± 33 (8)	329 ± 55 (21)	0.107	311 ± 27 (65)

Mean ± SD (n).
* Comparison between SQT1, SQT2, and SQT3–6.

Table 3
Genotype–phenotype comparison SQTS patients.

Clinical characteristics	SQT1 (n = 34)	non-SQT1* (n = 31)	p†	SQT2 (n = 8)	non-SQT2§ (n = 57)	p‡	Non-genotyped (n = 67)
Male	18 (53%)	12 (39%)	0.25	2 (25%)	28 (49%)	0.19	50
Syncope/palpitation	8 (24%)	3 (10%)	0.123	0 (0%)	11 (20%)	0.17	8
SCD/aborted cardiac arrest	6 (18%)	6 (19%)	0.86	2 (25%)	10 (18%)	0.6	20
AF	8 (24%)	9 (29%)	0.614	5 (63%)	12 (21%)	0.012	11
SSS/bradycardia	4 (12%)	7 (23%)	0.245	6 (75%)	5 (9%)	<0.001	7

n (%). AF: atrial fibrillation, SCD: sudden cardiac death, SSS: sick sinus syndrome.
*Non-SQT1 denotes SQT2–6.
§Non-SQT2 denotes SQT1 and SQT3–6.
†Comparison between SQT1 vs SQT2–6.
‡Comparison between SQT2 vs SQT1, 3–6.

were found in Ca channel genes: *CACNA1C*-G490R, *CACNB2*-S481L and *CACNA2D1*-S755T. The carriers were asymptomatic [6,7]. The mechanisms underlying latent SQTS have not been determined; however, coexisting common polymorphisms that prolong repolarization may be potential candidates that mask the abbreviated QT intervals. In fact, among the mutant carriers of *CACNA1C*-G490R, an individual with a well-known *KCNH2* polymorphism K897T [42] showed normal QTc, whereas the other two family members who only carry G490R manifested SQTS [6].

In SQT2, only three mutations (*KCNQ1*-V307L, -V141M, and -R259H) have to date been reported in de novo or sporadic cases [4,9,13,15,16] and our family 3 carrying V141M is the first familial instance of SQT2. In view of the fact that the proband's father has exhibited chronic AF since 3 years of age without manifesting SQTS, despite carrying the V141M mutation, there may be additional latent carriers who do not show ECG abnormalities or other arrhythmias such as AF in the absence of QT shortening. Lack of familial SQT2 may be because the phenotypic manifestations of *KCNQ1* mutations are milder than other subtypes, or because SQT2 has an extremely low penetrance. Further genetic screening of family members with non-remarkable ECG may help identify more latent *KCNQ1* carriers and better understand the natural history of SQTS.

4.3. Genotype-specific clinical characteristics in SQTS

The age of manifestation of mutation-positive SQTS patients spans from in utero to the eighth decade of life [8]. However, the age-distribution of lethal events in SQTS shows two peaks; one at the first year of life and another between 20 and 40 years of age [9]. Because we found that the age of initial clinical manifestation in SQT1 was significantly later than other subtypes, and six *KCNQ1* mutation carriers exhibited apparent early onset of bradyarrhythmia, it is speculated that two distinct peaks of the first arrhythmic events may also be attributed to two genotypes (Fig. 5A, Table 2). A similar genotype-dependent age of manifestation is well known in LQTS; the majority of LQT2 (*KCNH2*) patients manifest their first symptoms after puberty, whereas LQT1

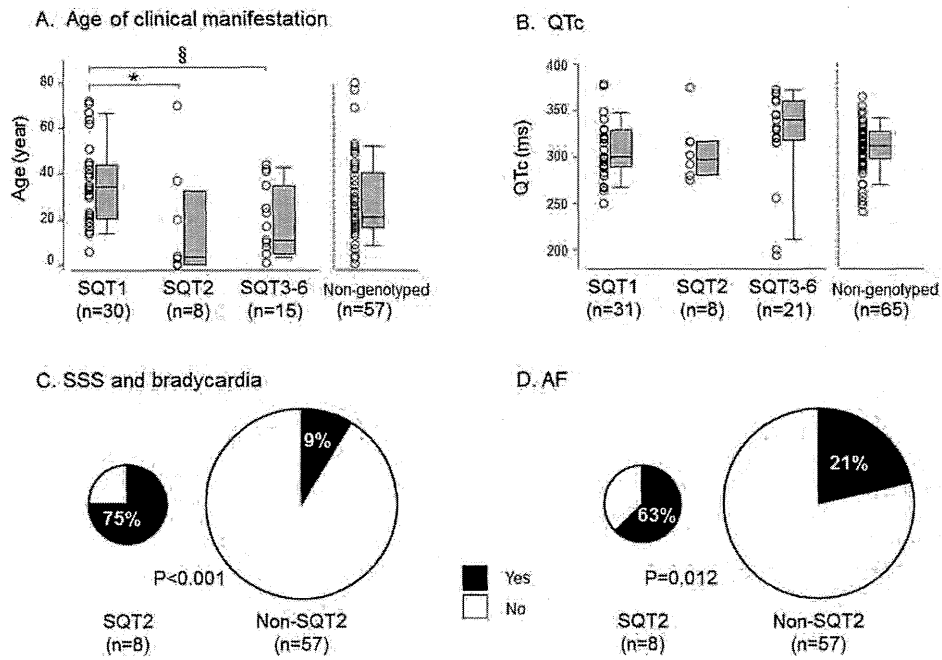

Fig. 5. Genotype-dependent clinical characteristics observed in SQTS patients. (A) Dot represents the age of manifestation of each case. SQT1 patients exhibited significantly later onset than other SQTS subgroups ($p = 0.011$). Pairwise comparison also showed significant later manifestation in SQT1 than SQT2 (*: $p = 0.043$) as well as SQT3–6 (§: $p = 0.019$). Non-genotyped SQTS, shown as a reference, exhibited a wide distribution. Boundaries of the box represent the 25th and 75th percentiles, and a line within a box marks the median. Whiskers of the box indicate the 10th and 90th percentiles. (B) QTc values were similar among SQTS subgroups. (C) Complications of SSS and bradycardia were significantly more prevalent in SQT2 than non-SQT2 subgroup ($p < 0.001$). (D) Complication of AF was significantly more prevalent in SQT2 patients than in non-SQT2 subgroup ($p = 0.012$).

Table 4
Mutations and the penetrance of 16 reported SQTS families.

Family	Genes	Mutations	Affected (n)	Mutation-positive (n)	Penetrance (%)	Other complications	References
1	*KCNH2*	N588K	3	3	100	–	[1, 32]
2	*KCNH2*	N588K	3	3	100	–	[3, 33, 34]
3	*KCNH2*	N588K	4	4	100	–	[3, 33, 34]
4	*KCNH2*	N588K	3	3	100	–	[9]
5	*KCNH2*	R1135H	1	3	33	(a), (b)	[35]
6	*KCNH2*	E50D	1	2	50	Syncope	[8, 36]
7	*KCNH2*	T618I	4	4	100	–	[14]
8	*KCNH2*	T618I	2	2	100	–	[9]
9	*KCNH2*	T618I	3	3	100	–	Current study
10	*KCNH2*	Not reported	6	6	100	–	[12]
11	*KCNQ1*	V141M	1	2	50	AF, (b)	Current study
12	*KCNJ2*	D172N	2	2	100	–	[9]
13	*KCNJ2*	D172N	2	2	100	–	[5]
14	*CACNA1C*	G490R	2	3	67	–	[6]
15	*CACNB2*	S481L	4	6	67	–	[6]
16	*CACNA2D1*	S755T	1	3	33	–	[7]
Total			42	51	82%		

(a): Non-documented arrhythmia, (b): bradycardia, AF: atrial fibrillation.

(*KCNQ1*) patients tend to become symptomatic before the age of 10 years [45].

We found a higher prevalence of bradyarrhythmia and AF in *KCNQ1* mutant carriers than for other genotypes (Fig. 5C,D, Table 3). A gain of function in I_{Ks} has not only been associated with SQTS but also with familial AF [46] and sinus bradycardia [47]. This is thought to be primarily because the APD shortening occurs in the atrium as well as in the ventricle, increasing the susceptibility of atrial tissue to sustained re-entry [48]. Furthermore, computer simulations of the SQTS mutation, *KCNQ1*-V141M, and the familial AF mutation, *KCNQ1*-V241K, demonstrated that a gain of function in I_{Ks} has been found to cause a cessation in spontaneous activity in the sinus node [15,47]. Such mechanisms may explain the observed phenotypic overlap and predominance of bradycardia and AF in SQT2.

5. Study limitations

In eight SQT2 patients we studied, six individuals carried the identical *KCNQ1* mutation V141M. Therefore, the phenotype of the V141M mutation may be over-represented in our SQT2 data. As SQTS is a rare disease, the size of the population studied is small. Further delineation of this rare lethal arrhythmic syndrome warrants more extensive genetic and population studies using larger cohorts.

6. Conclusions

In summary, our study identified two *KCNH2* mutations and one *KCNQ1* mutation in five Japanese families with SQTS. The novel *KCNH2*-I560T mutation causes severe shortening of the QT interval and can trigger VF despite only a modest shift in inactivation. Among SQTS patients, there exist latent mutation carriers with ECG abnormalities such as AF and bradycardia indicating incomplete penetrance. Furthermore, despite the limited number of reported SQTS patients, our study suggests that clinical characteristics of SQTS can differ depending on the patient genotype, as is observed in LQTS.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2015.04.090.

Conflict of Interest

None.

Acknowledgments

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Original Article

Functional Characterization of Rare Variants Implicated in Susceptibility to Lone Atrial Fibrillation

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Background—Few rare variants in atrial fibrillation (AF)–associated genes have been functionally characterized to identify a causal relationship between these variants and development of AF. We here sought to determine the clinical effect of rare variants in AF-associated genes in patients with lone AF and characterized these variants electrophysiologically and bioinformatically.

Methods and Results—We screened all coding regions in 12 AF-associated genes in 90 patients with lone AF, with an onset of 47±11 years (66 men; mean age, 56±13 years) by high-resolution melting curve analysis and DNA sequencing. The potassium and sodium currents were analyzed using whole-cell patch clamping. In addition to using 4 individual in silico prediction tools, we extended those predictions to an integrated tool (Combined Annotation Dependent Depletion). We identified 7 rare variants in *KCNA5*, *KCNQ1*, *KCNH2*, *SCN5A*, and *SCN1B* genes in 8 patients: 2 of 8 probands had a family history of AF. Electrophysiological studies revealed that 2 variants showed a loss-of-function, and 4 variants showed a gain-of-function. Five of 6 variants with electrophysiological abnormalities were predicted as pathogenic by Combined Annotation Dependent Depletion scores.

Conclusions—In our cohort of patients with lone AF, 7 rare variants in cardiac ion channels were identified in 8 probands. A combination of electrophysiological studies and in silico predictions showed that these variants could contribute to the development of lone AF, although further in vivo study is necessary to confirm these results. More than half of AF-associated rare variants showed gain-of-function behavior, which may be targeted using genotype-specific pharmacological therapy. **(*Circ Arrhythm Electrophysiol.* 2015;8:1095-1104. DOI: 10.1161/CIRCEP.114.002519.)**

Key Words: analysis of variance ■ atrial fibrillation ■ genetic association studies ■ genetic variation ■ ion channels

Atrial fibrillation (AF) is the most prevalent tachyarrhythmia, with a prevalence of 1% to 2% in the general population.[1] In most cases, AF occurs along with hypertension, mitral stenosis, ischemic heart disease, cardiomyopathy, and hyperthyroidism.[1] In addition to these underlying diseases, age, obesity, smoking, and alcohol are clinical risk factors for AF.[1] However, 11% of AF patients present with AF in the absence of predisposing factors; these are categorized as having lone AF.[2] Previous studies have shown that at least 5% of all patients with AF and 15% of those with lone AF had a positive family history.[3] Another study has shown that the risk for lone AF was 3.5× higher in those with a family history of lone AF in parents or in siblings, compared with the risk in individuals without such family history.[4] Recent studies have shown that people with certain genotypes have an increased risk for future AF.[5,6] These reports indicate that the development of AF is influenced by genetic background.

Editorial see p 1005

Genetic linkage analysis and candidate gene analysis for familial AF in 1997 indicated that a gene responsible for familial AF is located in the region of 10q22 to 10q24, and in 2003, a gain-of-function mutation in *KCNQ1* was implicated in a large Chinese kindred with autosomal dominant AF.[7] To date, many variants in genes encoding ion-channel subunits, cardiac gap junctions, and signaling molecules have been identified in monogenic AF families.[8,9] These genetic variants predispose individuals to AF by reducing the atrial refractory period as a substrate for re-entrant arrhythmias, by lengthening the atrial action potential duration, which results in ectopic activity, or by causing impaired

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WHAT IS KNOWN

- To date, many variants in genes encoding ion-channel subunits, cardiac gap junctions, and signaling molecules have been identified in monogenic families with atrial fibrillation (AF) and patients with lone AF.
- Few rare variants in AF-associated genes have been functionally characterized to identify a causal relationship between these variants and development of AF.

WHAT THE STUDY ADDS

- We identified 7 rare variants in cardiac ion channels in 8 probands from 90 patients with lone AF, indicating a prevalence of ≈9%.
- These variants were extremely rare and characterized as causing susceptibility to AF by either electrophysiological study or in silico prediction analysis including a new prediction tool, Combined Annotation Dependent Depletion scores.
- More than half of AF-associated rare variants showed gain-of-function behavior, which is likely to benefit from a drug that blocks particular ion channels.

electric cell-to-cell communication, which creates conduction heterogeneity as a substrate for the maintenance of AF.[10]

Here, we performed candidate gene studies to identify rare variants, under the hypothesis that these variants pose an AF risk in these probands. In addition, to determine the functional significance of these variants, we performed cellular electrophysiological studies and in silico prediction analysis.

Methods

Detailed description of Methods is provided in the Data Supplement.

Study Subjects

The study subjects were recruited from multiple hospitals in Japan. Lone AF was defined as AF occurring in individuals aged <65 years, who did not present with hypertension, overt structural heart disease, myocardial infarction, congestive heart failure, or thyroid dysfunction. Two hundred fifty healthy Japanese subjects with no history of the cardiovascular disease described above were also included in this study. Data from the National Heart, Lung, and Blood Institute Exome Sequencing Project Exome Variant Server (EVS)[11] and the Exome Aggregation Consortium (ExAC) data and browser[12] were used as reference groups.

The study observed the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee for Medical Research at our institution. All study patients provided written informed consent before registration.

DNA Isolation and Mutation Analysis and Genotype–Phenotype Relationships

Genomic DNA was extracted from peripheral blood leukocytes using standard methods. High-resolution melting curve analysis was used to screen *KCNA5*, *KCNQ1*, *KCNH2*, *SCN5A*, *SCN1B*, *SCN2B*, *SCN3B*, *KCNE1*, *KCNE2*, *KCNJ5*, *GJA5*, and *NPPA* using a LightScanner (BioFire Defense, Salt Lake City, UT). Samples in which the melting curve deviated from the wild-type (WT) control were subjected to DNA sequencing using an ABI PRISM 310 Genetic Analyzer. The relationship between the clinical phenotype (AF) and the genotype was determined for probands and their relatives in whom a variant was identified.

Plasmid Constructs and Electrophysiology

Mutant cDNAs were constructed using an overlap extension strategy[13] or by using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). CHO-K1 or HEK293 cells were transiently transfected with WT or mutant cDNA, using an X-tremeGENE 9 DNA Transfection Reagent (Roche Applied Science, Penzberg, Germany). Cells were cotransfected with the same amount of green fluorescent protein as each ion-channel cDNA.

Cells displaying green fluorescence at 48 to 72 hours after transfection were subjected to electrophysiological analysis. Potassium or sodium currents were studied using the whole-cell patch clamp technique with an amplifier, Axopatch-200B (Molecular Devices, Sunnyvale, CA), at room temperature. The voltage clamp protocols are described in the figures. Data were acquired using pCLAMP software (version 9; Molecular Devices, Sunnyvale, CA). Data acquisition and analysis were performed using a Digidata 1321 A/D converter and pCLAMP8.2 software (Molecular Devices, Sunnyvale, CA).

In Silico Prediction Analysis

A total of 5 prediction tools were applied to predict the pathogenicity of lone AF-associated variants: the PolyPhen algorithm,[14] Grantham chemical scores,[14] Sorting Intolerant From Tolerant analysis,[14] the Protein Variation Effect Analyzer,[15] and Combined Annotation Dependent Depletion (CADD).[16]

Statistical Analysis

Pooled electrophysiological data were expressed as mean±SE. The minor allele frequency (MAF) in the AF cohort was compared with the MAF from the EVS and the ExAC using Fisher exact test in 2×2 tables. Two-tailed Student *t* test was used for the single comparisons between the 2 groups. One-way ANOVA, followed by a Bonferroni post hoc test, was used to analyze data with unequal variance among 3 groups. Two-way repeated-measures ANOVA was used to adjust for multiple comparisons across the different values of membrane potentials. A value of $P<0.05$ was considered as statistically significant. Statistical analysis was performed using JMP Pro 11.0.0 (SAS Institute Inc, NC) and Origin 9.0 (OriginLab, Northampton, MA).

Results

Clinical Characteristics and Molecular Genetic Analysis of the Study Cohort

Of the 90 patients with lone AF that were enrolled, 26 subjects (29%) had a family history of AF in at least 1 first-degree relative (Table 1). The study subjects had a mean age of 47±11 years at the onset of AF (Table 1). Sixty-six subjects were men (73%), and 57 of the 90 patients had paroxysmal or persistent AF (63%; Table 1). Echocardiographic data of the cohort as a whole revealed a normal mean ejection fraction, with a mean left atrial dimension of 40±7 mm (Table 1). The 250 control subjects had a mean age of 39±19 years, and 157 of them were men (63%).

Screening for ion-channel variants in genomic DNA in the study cohort of 90 individuals with lone AF identified a total of 7 different variants present in *KCNA5* (H463R and T527M), *KCNQ1* (L492_E493 ins DL), *KCNH2* (T436M and T895M), *SCN5A* (R986Q), and *SCN1B* (T189M; Figure 1; Table 2). *SCN1B* T189M was detected in 2 probands (1 homozygous and 1

Table 1. Clinical Characteristics of the Lone AF Population

Total	90
Age at onset AF, y	47±11
Age at enrollment, y	56±13
Male sex (%)	66 (73)
Family history (%)	26 (29)
AF type	
Paroxysmal or persistent (%)	57 (63)
Permanent (%)	33 (37)
Complication	
Bradyarrhythmia (%)	10 (11)
Brugada syndrome (%)	3 (3)
Echocardiogram	
Left atrial size, mm	40±7
Ejection fraction (%)	65±8
Catheter ablation (%)	20 (22)

AF indicates atrial fibrillation.

heterozygous carrier). The presence of each of these variants was assessed in 250 ethnically matched population control individuals; all variants were rare (MAF, <1%; Table 2). According to the ExAC data and browser, the MAFs of *KCNA5* T527M, *KCNH2* T436M, and *SCN5A* R986Q were 0.0236%, 0.001627%, and 0.001951%, respectively, which were significantly lower compared with 0.6% in our case population (Table 2).

Clinical Characteristics and Functional Properties of *KCNA5* Variants

The proband who was heterozygous for *KCNA5* H463R was a 62-year-old woman with onset of paroxysmal AF at the age of 51 and no family history of AF. She underwent radiofrequency catheter ablation at the age of 61 (Table 3).

To define the functional effect of the H463R variant, we transiently expressed WT, H463R, and WT+H463R in cultured mammalian HEK293 cells for whole-cell voltage clamp measurements. Voltage clamp recording from cells expressing H463R alone did not exhibit any functional channels, when compared with those expressing WT alone (Figure 2A; Table 4). Coexpression of H463R with WT resulted in a significant current density, which was less than one-third of the control current observed with the expression of WT alone (Figure 2A).

The current–voltage relationships for activating currents (Figure 2B) and tail currents (Figure 2C) were recorded during depolarizing pulses. Two-way repeated-measures ANOVA revealed that there was a significant difference in the activating currents and the tail currents (Figure 2B and 2C) among these 3 channels. The amplitudes of the activating currents at 40 mV and the tail currents at 30 mV for the WT+H463R and the H463R were significantly smaller than those for WT (Figure 2B and 2C; Table I in the Data Supplement). No significant difference in activation kinetics for the WT+H463R was observed compared with WT (Figure 2D; Table I in the Data Supplement). In silico analysis predicted that H463R was pathogenic, according to 3 algorithms (Table 5).

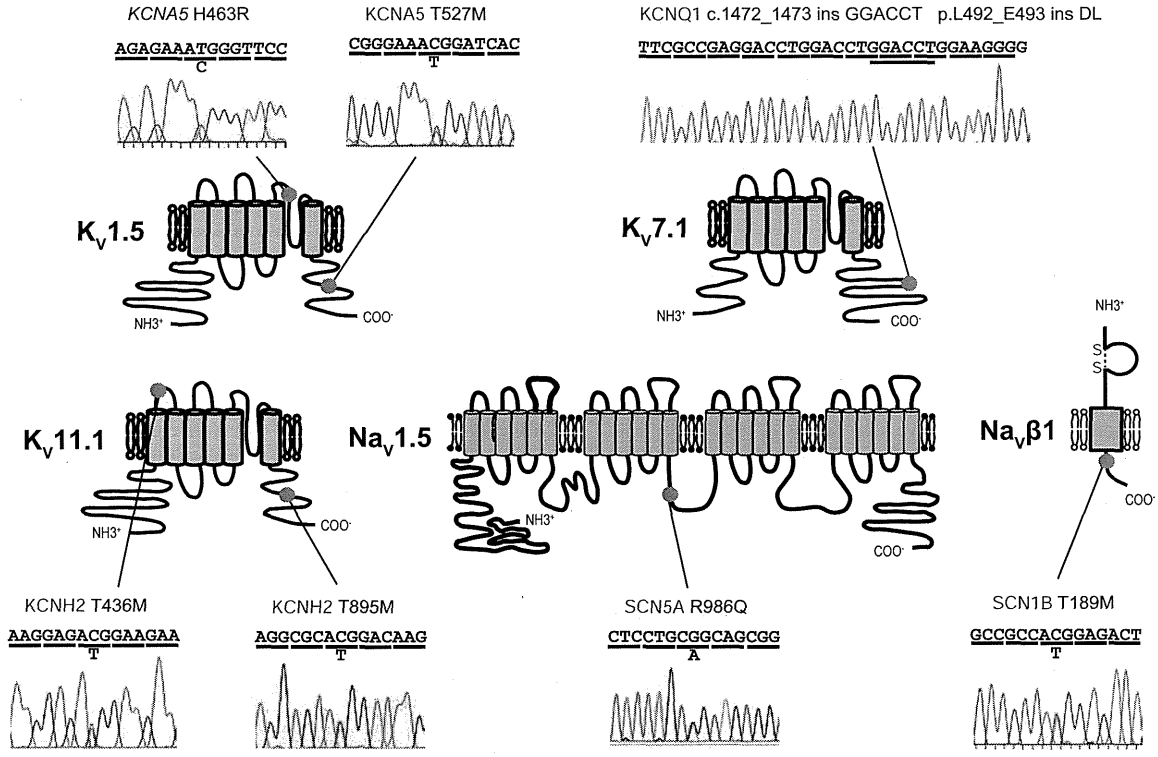

Figure 1. Sequencing of *KCNA5*, *KCNQ1*, *KCNH2*, *SCN5A*, and *SCN1B*. DNA sequencing electropherograms demonstrated 7 genetic variants. The KCNQ1 insertion variant was confirmed by sequencing the mutant allele in *KCNQ1*. The topology of the voltage-gated ion channels shows the location of the detected variants.

Table 2. Summary of Ion-Channel Rare Variants

		AF Probands, n=90			Controls, n=250			Exome Variant Server			Exome Aggregation Consortium		
Gene	Amino Acid Change	Allele Count	Allele Number	MAF	Allele Count	Allele Number	MAF	Allele Count	Allele Number	MAF	Allele count	Allele number	MAF
KCNA5	H463R	1	180	0.006	0	500	0	N/A	N/A	N/A	N/A	N/A	N/A
	T527M	1	180	0.006	1	500	0.002	N/A	N/A	N/A	29	122816	0.000236†
KCNQ1	L492_E493 ins DL	1	180	0.006	0	500	0	N/A	N/A	N/A	N/A	N/A	N/A
KCNH2	T436M	1	180	0.006	0	500	0	N/A	N/A	N/A	2	122940	1.627×10^{-5}*
	T895M	1	180	0.006	0	500	0	N/A	N/A	N/A	N/A	N/A	N/A
SCN5A	R986Q	1	180	0.006	0	500	0	1	12116	8.253×10^{-5}†	2	102516	1.951×10^{-5}*
SCN1B	T189M	3	180	0.017	1	500	0.002	N/A	N/A	N/A	N/A	N/A	N/A

AF indicates atrial fibrillation; MAF, minor allele frequency; and N/A, not available.
*$P<0.01$ vs MAF of probands.
†$P<0.05$ vs MAF of AF probands.

The proband who was heterozygous for *KCNA5* T527M was a 51-year-old man, with onset of paroxysmal AF at the age of 49 (Table 3). The ECG showed a prolonged PR interval of 220 ms and a normal QTc interval. Monitoring ECG in an outpatient clinic showed a sinus pause of 3.6 s after termination of AF. His son, who also carried the T527M variant in *KCNA5*, had never experienced AF and had a prolonged PR interval of 220 ms.

HEK293 cells expressing the T527M mutant showed a larger Kv1.5 current than did those expressing WT (Figure 3A). Cellular electrophysiological studies showed that the activating current density for T527M was significantly larger than that for WT (Figure 3B; Table I in the Data Supplement; Table 4). The T527M mutant displayed a negative voltage shift in the normalized activation curve and a significantly decreased the potential when half of the channels were activated, $V_{1/2}$ (Figure 3D; Table I in the Data Supplement). In silico analysis using 4 algorithms predicted that T527M was pathogenic (Table 5).

Clinical Characteristics and Functional Properties of *KCNQ1* Variants

The proband who was heterozygous for *KCNQ1* L492_E493insDL was a 42-year-old man who was diagnosed with AF on an ECG on routine examination (Table 3). Because he had no symptoms, the age of onset of paroxysmal AF was unknown. The ECG showed a normal QTc interval at rest and during the late recovery phase of exercise stress testing. His daughter and sons carrying the L492_E493insDL variant in *KCNQ1* had never experienced AF and had normal ECGs.

Table 3. Clinical Characteristics of Patients With Rare Variants

Proband Number	Patient Type	Age, y	Genotype	Sex	Family History	Phenotype	Onset, y	ECG, HR/ PR/QRS/QTc	Echocardiography LAD (mm)/EF (%)	Catheter Ablation
1	Proband	62	*KCNA5* H463R	F	No	Persistent	51	67/0.14/0.08/0.452	34/66	Yes
2	Proband	51	*KCNA5* T527M	M	No	Persistent	49	67/0.22/0.07/0.391	35/70	No
	Son	29	*KCNA5* T527M	M	No	AF(–)		77/0.22/0.10/0.395	...	No
3	Proband	42	*KCNQ1* L492_E493 ins DL	M	No	Paroxysmal	42	68/0.19/0.08/0.392	36/53	No
	Daughter	18	*KCNQ1* L492_E493 ins DL	F	No	AF(–)	...	58/0.144/0.09/0.387	...	No
	Son	16	*KCNQ1* L492_E493 ins DL	M	No	AF(–)	...	54/0.16/0.09/0.426	...	No
	Son	7	*KCNQ1* L492_E493 ins DL	M	No	AF(–)	...	81/0.146/0.07/0.437	...	No
4	Proband	61	*KCNH2* T436M	M	Yes	Chronic	38	73/-/0.08/0.371	49/73	No
5	Proband	58	*KCNH2* T895M	M	Yes	Persistent	40	64/0.15/0.10/0.405	42/63	Yes
	Father	87	*KCNH2* T895M	M	Yes	Paroxysmal palpitation	50s	69/0.17/0.11/0.451	...	No
	Son	37	*KCNH2* T895M	M	Yes	Paroxysmal palpitation	20s	59/0.16/0.11/0.386	...	No
6	Proband	64	*SCN5A* R986Q	M	No	Paroxysmal	58	52/0.134/0.11/0.369	37/70	No
7	Proband	59	*SCN1B* T189M (homozygous)	F	No	Paroxysmal	59	50/0.151/0.09/0.378	30/76	No
	Daughter	33	*SCN1B* T189M (heterozygous)	F	No	AF(–)	...	56/0.13/0.08/0.424	...	No
8	Proband	55	*SCN1B* T189M (heterozygous)	F	No	Paroxysmal	55	65/0.18/0.08/0.420	30/65	No

AF indicates atrial fibrillation; EF, ejection fraction; F, female; LAD, left atrial diameter; and M, male.

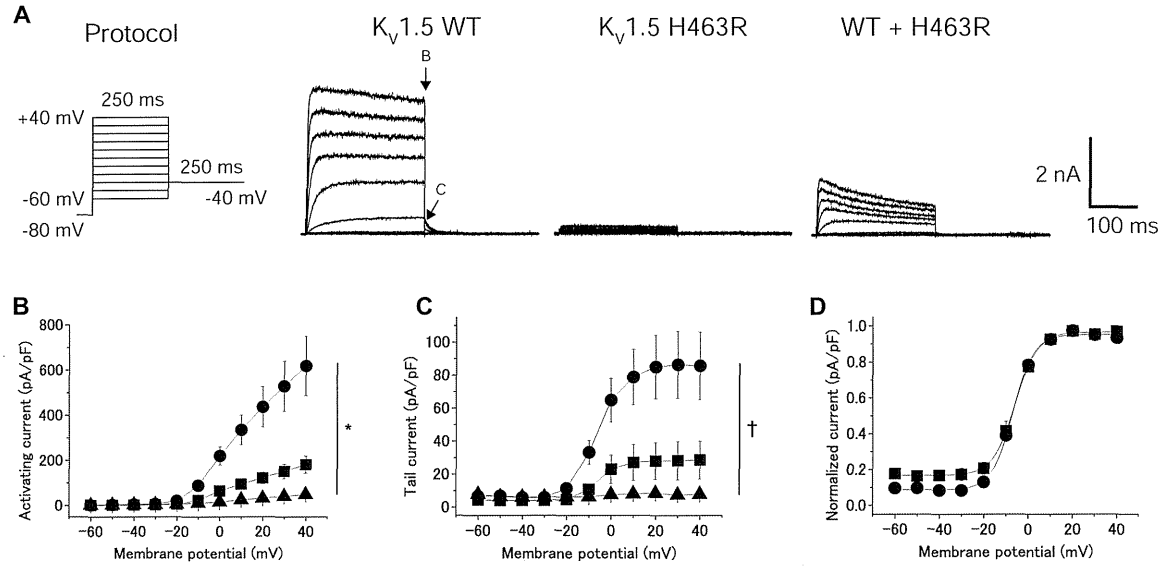

Figure 2. Functional characterization of K_V1.5 H463R in HEK293 cells. **A**, Representative currents generated in HEK293 cells that had been transfected with 0.2 μg of a vector expressing wild-type (WT) *KCNA5* alone (**left**), 0.2 μg of a vector expressing *KCNA5* H463R alone (**middle**), or 0.1 μg of each of the *KCNA5* WT and *KCNA5* H463R vectors (**right**). Pulse protocol is shown in the inset. **B** and **C**, *I–V* relationships for peak currents (**B**) and tail currents (**C**) in HEK293 cells expressing WT alone (closed circle, n=20), H463R (closed triangle, n=9), and WT+H463R (closed square, n=15). **D**, Mean amplitudes of normalized tail currents for WT alone (closed circle, n=17) and WT+H463R (closed square, n=8). **P*<0.001 or †*P*<0.01 among indicated current–voltage relationships by 2-way repeated-measures ANOVA.

Whole-cell patch clamp experiments were conducted on CHO-K1 cells transfected with vectors expressing K_V7.1 WT or K_V7.1 L492_E493insDL. The activating and tail current amplitudes of K_V7.1 WT/KCNE1 were similar to those of the K_V7.1 mutant/KCNE1 (Figure IA–IC and Table I in the Data Supplement; Table 4). The normalized tail current–voltage relationship for K_V7.1 WT/KCNE1 channels was also similar to that for K_V7.1 mutant/KCNE1 channels (Figure ID and Table I in the Data Supplement).

Clinical Characteristics and Functional Properties of *KCNH2* Variants

The proband who was heterozygous for *KCNH2* T436M was a 61-year-old man with onset of AF at the age of 38. The ECG showed chronic AF and a normal QTc interval (Table 3). His brother and sister had been affected with AF from a young age.

Table 4. In Vitro Cellular Electrophysiology

Gene	Amino Acid Change	Current Density	Channel Kinetics
KCNA5	H463R	Absent I_{kur}	No change
	T527M	Increased peak and tail currents	Faster activation
KCNQ1	L492_E493 ins DL	No change	No change
KCNH2	T436M	Increased peak currents	Slower deactivation
	T895M	Increased peak and tail currents	Slower deactivation
SCN5A	R986Q	Decreased peak currents	No change
SCN1B	T189M	Increased peak currents	Negative shift in steady-state activation

We transiently expressed WT and T436M in cultured mammalian CHO-K1 cells for whole-cell voltage clamp measurements (Figure II in the Data Supplement; Table 4). Electrophysiological studies showed that the peak current density for T436M was significantly greater than that for the WT (Figure IIB and Table II in the Data Supplement). No significant difference in activation kinetics or the steady-state inactivation kinetics was observed between the mutant and WT channels (Figure IID and IIF and Table II in the Data Supplement). In contrast, slow and fast time constants in the mutant channel were increased significantly compared with those of the WT (Figure IIE and Table II in the Data Supplement). Two-way repeated-measures ANOVA revealed that the fast time constants were significantly increased in the T436M compared with the WT (Figure IIE in the Data Supplement). In silico prediction analysis indicated that T436M was potentially benign, according to 5 algorithms (Table 5).

The proband who was heterozygous for *KCNH2* T895M was a 58-year-old man with onset of AF at the age of 40 (Table 3). The ECG showed a normal QTc interval. He underwent radiofrequency catheter ablation at the age of 59. His father, who also carried T895M in *KCNH2*, had experienced paroxysmal palpitations since his early 50s and developed a transient ischemic attack at the age of 81. The proband's son, who carried the same variant, had also experienced paroxysmal palpitations about once a week since his late 20s.

When we transiently expressed WT and T895M in cultured mammalian CHO-K1 cells for whole-cell voltage clamp

Table 5. In Silico Prediction Analysis

Gene	Amino Acid Change	Polyphen-2	Grantham	SIFT Score	PROVEAN Score	Score CADD
KCNA5	H463R	0.195	29	0.031, damaging	−3.49, deleterious	14.25, deleterious
	T527M	0.954, possibly damaging	81	0.007, damaging	−4.96, deleterious	19.35, deleterious
KCNQ1	L492_E493 ins DL	N/A	N/A	N/A	N/A	N/A
KCNH2	T436M	N/A	78	0.138	−0.42	1.52
	T895M	N/A	78	0.178	−1.57	17.78, deleterious
SCN5A	R986Q	N/A	103, radical	0.308	−0.62	11.17, deleterious
SCN1B	T189M	N/A	113, radical	0, damaging	−0.08	15.27, deleterious

CADD indicates Combined Annotation Dependent Depletion; N/A, not available; PROVEAN, Protein Variation Effect Analyzer; and SIFT, Sorting Intolerant From Tolerant.

measurements, we observed a larger current for the mutant channel than the WT channel (Figure 4). Electrophysiological studies showed that the peak and tail current densities for T895M were significantly larger than those for the WT, respectively (Figure 4B and 4C; Table II in the Data Supplement; Table 4). No significant difference in activation kinetics or the steady-state inactivation kinetics when compared with the WT was observed (Figure 4D and 4F; Table II in the Data Supplement). In contrast, both slow and fast deactivation time constants for the mutant channel were increased significantly compared with those of the WT for voltages at −40 and −30 mV (Figure 4E; Table II in the Data Supplement). In silico analysis predicted that T895M was likely to be pathogenic, although only according to the score CADD (Table 5).

Clinical Characteristics and Functional Properties of *SCN5A* and *SCN1B* Variants

The proband who was heterozygous for *SCN5A* R986Q was a 64-year-old man with onset of paroxysmal AF at the age of 58 (Table 3). None of his family members had clinical signs of AF.

CHO-K1 cells were transiently transfected with vectors expressing WT or R986Q cDNA and the human β1 subunit cDNA in combination with a bicistronic plasmid encoding green fluorescent protein. Compared with the WT, R986Q significantly reduced the peak sodium current density (; Table 4 Figure IIIB and Table III in the Data Supplement). No significant difference was observed in the voltage-dependence of steady-state activation or the voltage-dependence of steady-state fast inactivation (Figure IIIC and IIID and Table III in the Data Supplement). In silico analysis predicted that R986Q was pathogenic, according to 2 algorithms (Table 5).

SCN1B T189M was detected in 2 probands with lone AF (Table 3). One proband who was homozygous for the variant was a 59-year-old woman who showed paroxysmal AF with palpitations every few months. Her AF was always terminated soon after taking pilsicainide. Her daughter, who was heterozygous for the variant, was a 33-year-old woman and had never experienced paroxysmal palpitations. The second proband, who

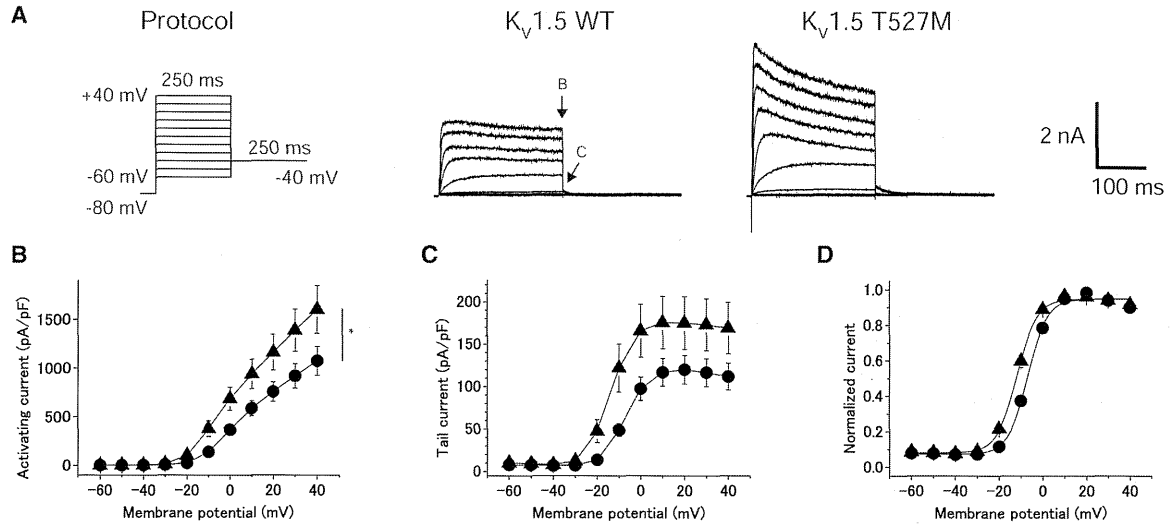

Figure 3. Functional characterization of K_V1.5 T527M in HEK293 cells. **A**, Representative currents generated in HEK293 cells, transfected with 0.2 μg of a vector expressing *KCNA5* WT alone (**left**) or 0.2 μg of a vector expressing *KCNA5* T527M alone (**right**). **B** and **C**, *I–V* relationships for peak currents (**B**) and tail currents (**C**) in HEK293 cells transfected with the wild-type (WT) vector alone (closed circle, n=33) or the T527M vector (closed triangle, n=47). **D**, Mean amplitudes of normalized tail currents for WT alone (closed circle, n=33) and T527M alone (closed triangle, n=42). *P<0.05 between the indicated current–voltage relationships by 2-way repeated-measures ANOVA.

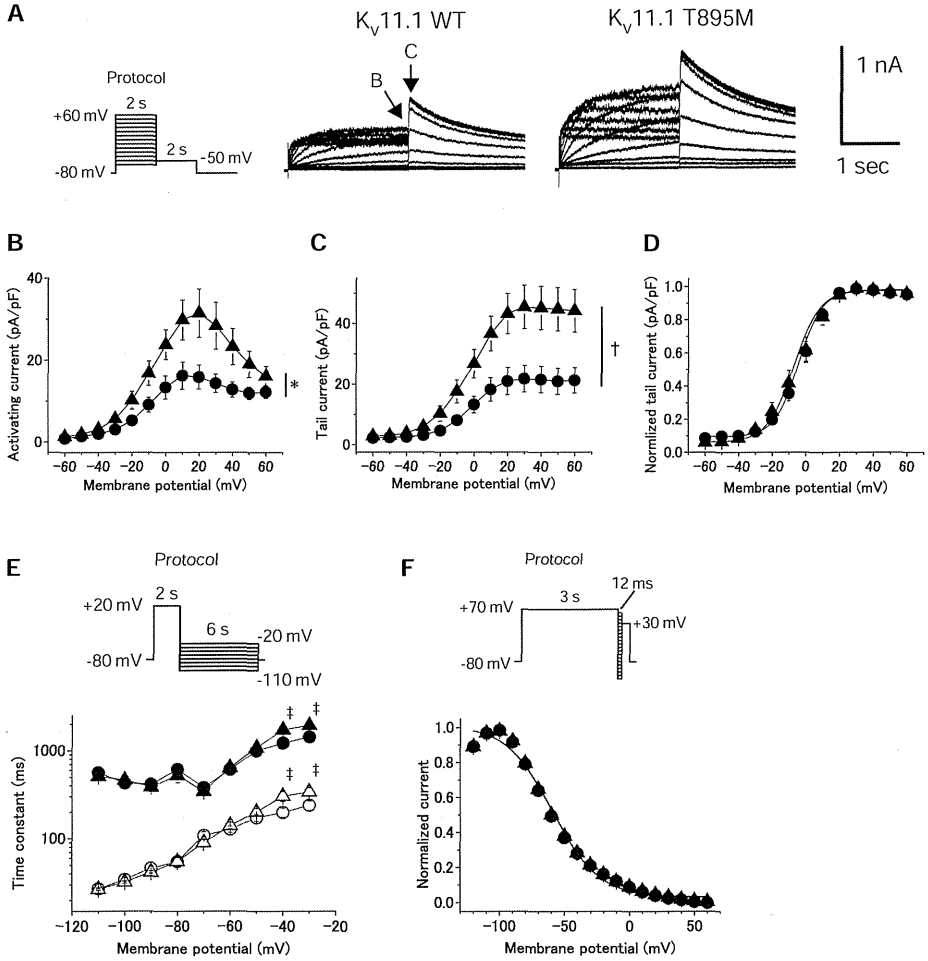

Figure 4. Functional characterization of K_V11.1 T895M in CHO-K1 cells. **A**, Representative currents generated in CHO-K1 cells, transfected with 1 μg of a vector expressing *KCNH2* wild-type (WT) alone (**left**) or 1 μg of a vector expressing *KCNH2* T895M alone (**right**). Pulse protocol is shown in the inset. **B** and **C**, *I–V* relationships for peak currents (**B**) and tail currents (**C**) in CHO-K1 cells transfected with WT alone (closed circle, n=18) and T895M (closed triangle, n=18). **D**, Mean amplitudes of normalized tail currents for WT alone (closed circle, n=10) and T895M (closed triangle, n=13). **E**, Deactivation time constants of K_V11.1 currents (protocol shown in the inset). The deactivation process was fitted to biexponential functions. Deactivation time constants of WT (fast component: open circle, n=13; slow component: closed circle, n=13) and T895M (fast component: open triangle, n=15; slow component: closed triangle, n=15) are shown. **F**, Normalized steady-state inactivation curves for WT (closed circle, n=9) and T895M (closed triangle, n=11). Pulse protocol is shown in the inset. The current amplitude at the test potential was normalized and plotted against the prepulse potential. Curves represent the best fits to a Boltzmann function. **P*<0.01 or †*P*<0.05 between the indicated current–voltage relationships by 2-way repeated-measures ANOVA. ‡*P*<0.05 vs WT by Student *t* test.

was heterozygous for the variant, was a 55-year-old woman who showed paroxysmal AF that was controlled well by pilsicainide. This variant was also detected in a control subject in his 20s.

Threonine at 189 is located in the C terminus of the $Na_V\beta1$ subunit and is highly conserved among the mammalian homologs of this protein. Thus, we performed electrophysiological analysis of the mutant $Na_V\beta1$ protein. When we coexpressed $Na_V1.5$ with the mutant $Na_V\beta1$ subunit, the expressed current density was significantly larger than that observed with the WT $Na_V\beta1$ subunit (Figure 5A; Table 4). The maximum peak current density of $Na_V1.5$ plus $Na_V\beta1$ T189M, measured at −30 mV, was −463±100 pA/pF, which was significantly smaller than the −240±34 pA/pF for $Na_V1.5$ plus $Na_V\beta1$ WT (Figure 5B; Table III in the Data Supplement). *I–V* curves of normalized peak sodium current showed that the mutant channel resulted in a significant negative voltage shift of steady-state activation when compared with the WT channel (Figure 5C; Table III in the Data Supplement). No significant difference was observed in the voltage dependence of steady-state fast inactivation (Figure 5D; Table III in the Data Supplement). In silico analysis predicted that T189M was pathogenic, according to 3 algorithms (Table 5).

In Silico Prediction Analysis of Rare Variants Associated With Lone AF

CADD is an integrated algorithm based on a total of 65 annotations; this approach predicted that both *KCNA5* variants (H463R and T527M), *KCNH2* T895M, *SCN5A* R986Q,

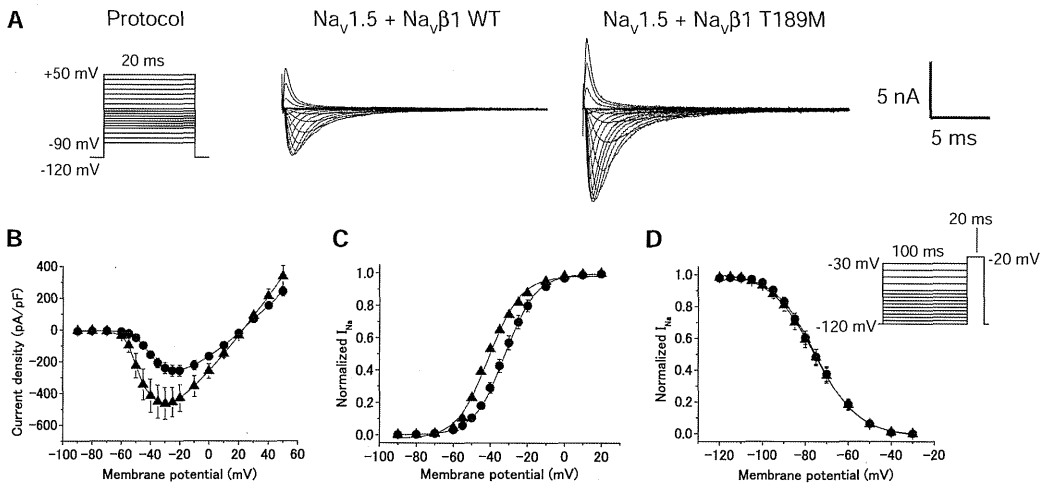

Figure 5. Functional characterization of Na$_V$1.5+Na$_V$β1 T189M in CHO-K1 cells. **A**, Representative currents generated in CHO-K1 cells that had been transfected with 0.6 μg each of a vector expressing the Na$_V$1.5 channel and wild-type (WT) Na$_V$β1 subunit (**left**) or 0.6 μg each of a vector expressing the Na$_V$1.5 channel and mutant Na$_V$β1 subunit (**right**). Pulse protocol is shown in the inset. **B**, *I–V* relationships for peak currents of Na$_V$1.5+Na$_V$β1 WT (closed circle, n=16) or Na$_V$1.5+Na$_V$β1 mutant (closed triangle, n=16). **C** and **D**, Voltage dependence of the steady-state activation and fast inactivation of Na V1.5+Na$_V$β1 WT (closed circle, n=16) or Na$_V$1.5+Na$_V$β1 mutant (closed triangle, n=16), measured using standard pulse protocols.

and *SCN1B* T189M were deleterious (CADD score, >10; Table 5). Seven variants were divided into 3 categories on the basis of their cellular electrophysiological profile and CADD scores: (1) cellular electrophysiologically abnormal and bioinformatically deleterious variants, including *KCNA5* H463R and T527M, *KCNH2* T895M, *SCN5A* R986Q, and *SCN1B* T189M; (2) cellular electrophysiologically abnormal and bioinformatically benign variant, including *KCNH2* T436M; and (3) cellular electrophysiologically normal but bioinformatically radical variant, including *KCNQ1* L492_E493 ins DL (Figure 6).

We also investigated how many rare (MAF, <1%) variants were predicted to be deleterious using an in silico prediction tool (CADD scores, >10) and how many such alleles were present in the 12 AF-associated genes, in the largest exome sequencing study to date, using 61 486 unrelated individuals (ExAC data and browser).[12] We found as many as 2255 variants (17 859 alleles) that could potentially be (mis)judged as causative based only on these frequency/in silico prediction tool strategies (Figure IV in the Data Supplement).

Discussion

In this study, we analyzed 90 patients with lone AF for rare genetic variants in 12 genes and identified 7 rare variants in 8 patients. This indicates that 8.9% of patients with lone AF carry rare genetic variants. Of these 7 variants, 2 variants were identified in the 250 control subjects (MAF, 0.2% each), 1 variant was reported in the EVS (MAF, <0.01%), and 3 variants were reported in the ExAC (MAF, <0.05% each). More variants were reported in the ExAC than the EVS, likely because the ExAC includes data of east and south Asia, whereas the EVS comprises data of black and European American individuals. Because the MAFs of 3 variants (*KCNA5* T527M, *KCNH2* T436M, and *SCN5A* R986Q) in the ExAC database were significantly smaller than those in our case population, these seem to be extremely rare variants. Recently, Olesen et al[17] reported that 29 rare variants had been identified among 192 patients with early-onset lone AF (onset of disease before the age of 40). The frequency of rare variants in our lone AF cohort (8.9%) was lower than that in Olesen's study (15.1%). There are several reasons for the difference in frequency: (1) our cohort included individuals with onset of lone AF at an

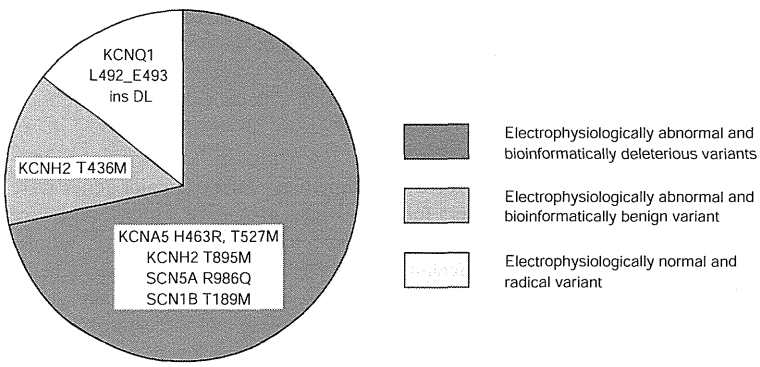

Figure 6. Classification of rare variants according to their electrophysiological and bioinformatic properties. Seven rare variants detected in this study were divided into 3 groups: cellular electrophysiologically abnormal and bioinformatically deleterious variants, cellular electrophysiologically abnormal and bioinformatically benign variants, and cellular electrophysiologically normal and bioinformatically radical variants.

older age, (2) Olesen et al[17] defined rare variants as variants with an MAF of <0.1% in the EVS, and (3) they performed screening of genomic DNA for 14 genes, whereas 12 genes were investigated in our study.

More than 100 rare variants have been reported in patients with lone AF[7–9]; however, the functional effect of most variants has not been evaluated. In this study, we carefully characterized each of the rare variants and could find that they have novel or overt abnormalities in channel function.

One of the 2 *KCNA5* variants identified in this study, H463R, was a novel mutation; the histidine at codon 463 is located in the S5-pore loop, in the vicinity of the pore of the $K_V1.5$ subunit. Our patch clamp study showed that this mutation caused dominant-negative suppression of $K_V1.5$ WT function and was considered to have a loss-of-function effect. The loss-of-function mutation in the *KCNA5* gene can lead to a decrease in I_{Kur}, action potential prolongation, and early after-depolarization. The other *KCNA5* variant, T527M, showed a gain-of-function effect with an enhanced steady-state activation, which was revealed by electrophysiological analysis. A previous study reported that 3 gain-of-function variants in *KCNA5* displayed a negative voltage shift in the steady-state activation curves.[18] The T527 variant had previously been identified in a Chinese family with AF.[19]

We identified 2 *KCNH2* variants with gain-of-function effects in patients with lone AF, although only a few *KCNH2* variants have so far been reported in patients with lone AF.[20] *KCNH2* T436M had previously been identified in a long QT syndrome family with low disease penetrance.[21] The proband in our study was affected with AF from the age of 38 and had a number of AF-affected family members. Furthermore, *KCNH2* T895M had previously been identified in a patient with sudden infant death syndrome.[22] In our study, the proband's father and son, who both carried the T895M variant, had paroxysmal palpitations. Our cellular electrophysiological studies showed that the deactivation time course in both these $K_V11.1$ channels were significantly slower than that in the WT channel, which resulted in a gain-of-function effect. A previous study of T895M in *Xenopus* oocytes showed that the deactivation time constants in the T895M channel were increased significantly compared with those of the WT,[22] which corresponded to our results. The gain-of-function *KCNH2* variants implicated in susceptibility to AF may exist more frequently than indicated in previous reports.

Several *KCNQ1* gain-of-function mutations have been identified in patients with lone AF to date.[8] We found a *KCNQ1* insertion variant (L492_E493insDL) in a lone AF proband with normal QTc intervals. This is a so-called radical variant, which is likely to be pathogenic; however, its channel properties were similar to those of the WT channel.

We found a rare *SCN5A* variant, R986Q, in patients with lone AF. In a heterologous expression study, R986Q was categorized as a loss-of-function variant and can cause shortening of the refractory period and slowing of conduction. We also identified an *SCN1B* T189M variant in 2 probands with lone AF and in 1 of 250 control subjects in this study. Previously, several *SCN1B* variants have been reported in patients with AF and had a loss-of-function effect.[8,23] Our cellular electrophysiological study showed that T189M was a gain-of-function variant, which was predicted to lower the threshold potential for cellular excitability. This is the first report on an AF-associated *SCN1B* variant that leads to an increased peak sodium current density and that changes the voltage dependence of the steady-state fast activation of the Na^+ channel. Moreover, our data indicated that the sodium-channel blocker pilsicainide was effective for AF in probands with gain-of-function variants in *SCN1B*.

Many prediction tools are available for predicting a pathogenic or benign status for rare variants identified in patients with various genetic disorders.[14,24,25] Giudicessi et al[14] tested whether 4 prediction tools—conservation analysis, Grantham values, Sorting Intolerant From Tolerant, and PolyPhen2—have the potential to enhance the classification of rare nonsynonymous single-nucleotide variants in type 1 and 2 long QT syndromes. Their findings supported the potential synergistic utility of these tools to enhance the classification of rare variants; however, their use in isolation in clinical application remains limited. CADD is an attractive prediction tool that uses a total of 65 annotations, including gene models, conservation measures, and ENCODE data summaries.[16] In this study, 5 of 6 variants exhibiting electrophysiological abnormalities were predicted to be pathogenic according to their CADD scores, reinforcing classification of these rare variants as pathogenic in patients with lone AF.

Several clinical applications can be derived from this study. First, at least 9% of patients with lone AF have a rare variant in cardiac ion-channel genes. Four of 7 rare variants implicated in susceptibility to AF showed gain-of-function effects by electrophysiological studies. Second, rare ion-channel variants identified in patients with lone AF have therapeutic implications.[10] In particular, patients with gain-of-function variants are likely to benefit from a drug that enables selective inhibition of mutant channel complexes. A previous study showed the enhanced sensitivity of *KCNQ1* gain-of-function mutations for the I_{Ks} selective blocker HMR-1556.[26] In our study, sodium-channel blockers were shown to be effective in patients harboring *SCN1B* T189M. Similarly, I_{Kur} channel blocking or I_{Kr} channel blocking may be effective for preventing the shortening of action potential duration in patients with *KCNA5* T527M or *KCNH2* T436M and T895M. Thus, genetic testing of major ion-channel genes for patients with lone AF is therefore significant. In addition, the rare variants annotated as pathogenic by CADD should be evaluated to determine whether they have loss-of-function or gain-of-function effects by electrophysiological studies. Third, several asymptomatic family members with rare variants were identified in this study and may have a risk of developing AF in future. These subjects should be advised to avoid acquired risk factors for the development of AF, and preventive care for reducing these risk factors should be provided.

Study Limitations

This study has several limitations. First, 12 major genes were examined here for rare variants associated with AF and were only a small fraction of potential genes related to AF. Whole-exome sequencing or targeted next-generation sequencing may reveal more variants associated with AF in many genes. Second, the case population (n=90) and the control population (n=250) used in this study were relatively small. Indeed,

recent reports studied 192 to 307 patients with lone AF or 216 to 240 healthy subjects as a reference.[18,27] However, we used data from the EVS[11] and the ExAC data and browser[12] as reference groups. Finally, only the individual variants identified in the cases were evaluated in the control population. It is possible that a series of rare variants may have been identified in the controls if the rest of the coding region of each gene had been fully screened in the controls. In fact, 2255 rare and deleterious variants (MAF, <1%; CADD scores, >10) in 12 AF-associated genes were found in the ExAC data and browser,[12] which comprises in excess of 60 000 unrelated individuals sequenced (Figure IV in the Data Supplement). This emphasizes the importance of both phenotype assessments and functional analyses for the determination of disease-causing variants even in this comprehensive genotyping era.

Conclusions

In our cohort of patients with lone AF, 7 rare variants in cardiac ion-channel genes were identified in 8 probands, with a prevalence of ≈9%. These variants were extremely rare and characterized as causing susceptibility to AF by either electrophysiological study or in silico prediction analysis. More than half of AF-associated rare variants showed gain-of-function behavior, which is likely to benefit from a drug that blocks particular ion channels.

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Disclosures

None.

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Functional Characterization of Rare Variants Implicated in Susceptibility to Lone Atrial Fibrillation

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Impact of Updated Diagnostic Criteria for Long QT Syndrome on Clinical Detection of Diseased Patients

Results From a Study of Patients Carrying Gene Mutations

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ABSTRACT

OBJECTIVES In this study, we scored patients with long QT syndrome (LQTS) according to the different Schwartz diagnostic criteria from 1993, 2006, and 2011, and to examine the validation of the criteria in relevance to the frequency of LQTS-related gene mutation.

BACKGROUND Although updated diagnostic criteria have been used in clinical settings, few data exist regarding their impact on the diagnosis of LQTS.

METHODS We used a cohort of 132 patients who presented with prolonged QTc intervals and/or abnormal clinical history in cardiac screening and who underwent exercise stress testing. LQTS scores of ≥3.5 points according to the 2006 and the 2011 criteria were considered to indicate a high probability of LQTS, as opposed to the 4 points used by the 1993 criteria. The 2011 criteria were updated by adding the evaluation of the recovery phase of exercise.

RESULTS The 2011 criteria significantly increased the number of high probability patients ($n = 62$) compared with the 1993 criteria ($n = 32$; $p = 0.0002$) or the 2006 criteria ($n = 36$; $p = 0.0014$). The percentage of mutation carriers in those with an intermediate score, which was rather high using the 1993 (53%) and 2006 criteria (53%), was greatly reduced with the 2011 criteria (15%, $p = 0.0014$ vs. the 1993 criteria, and $p = 0.0013$ vs. the 2006 criteria). Among 54 mutation carriers, the 1993, the 2006, and the 2011 criteria identified a high probability of carriers in 25 patients (46% sensitivity and 91% specificity), 27 patients (50% sensitivity and 88% specificity), and 48 patients (89% sensitivity and 82% specificity), respectively.

CONCLUSIONS The use of the 2011 criteria will facilitate the diagnosis of LQTS and will decrease the number of false negative results. (J Am Coll Cardiol EP 2016;■:■–■) © 2016 by the American College of Cardiology Foundation.

The long QT syndrome (LQTS) is an inherited disorder characterized by delayed cardiac repolarization with abnormal T-wave morphology and a possibility of lethal ventricular tachyarrhythmias, which result in fainting spells and sudden death (1). Mutations in various cardiac ion channel genes, including *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, *KCNJ2*, and *CACNA2*, or a membrane adaptor protein gene, *ANKB*, are known to cause this syndrome (2), and many LQTS

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ABBREVIATIONS AND ACRONYMS

AUC = area under the receiver-operating characteristic curve

CADD = combined annotation dependent depletion

dHPLC = denaturing high-performance liquid chromatography

ECG = electrocardiography

HRM = high-resolution melting

LQTS = long QT syndrome

MAF = minor allele frequency

NPV = negative predictive value

PPV = positive predictive value

ROC = receiver-operating characteristic

mutations have been identified (3,4). Sixteen genetic forms of LQTS have been described; however, the most prevalent forms are LQT1 and LQT2, which are associated with mutations in potassium channels, and LQT3, which is associated with a sodium channel mutation (2,5).

Originally, the diagnostic criteria for LQTS were proposed by Schwartz et al. in 1985 (6). Diagnoses were based on major and minor criteria, and the diagnosis could be made in the presence of either 2 major criteria or of 1 major and 2 minor criteria. These first criteria are clinically useful; however, they are nonquantitative. Subsequently, Schwartz et al. published diagnostic criteria that included points assigned to a patient's symptoms, medical and family history, and electrocardiography (ECG) findings (7). A Schwartz score of ≥4 in a patient indicated a high probability of a LQTS diagnosis. Molecular diagnosis of LQTS showed that disease penetrance was low (25%) in some families with congenital LQTS (8), and QT duration appeared normal in 10% (LQT3) to 36% (LQT1) of genotype-positive patients (9). A previous study showed that, based on the Schwartz criteria, 43% of patients with Schwartz scores of <4 were genotype-positive (10). Thus, the 1993 criteria were not helpful in identifying asymptomatic mutation carriers with a normal QT interval on a ECG at rest. In contrast, this subclinical type of LQTS is one of the risk factors for drug-induced Torsade de pointes (11). The 1993 criteria were modified by Schwartz et al. in 2006 (12). The fundamental difference was in considering a score of ≥3.5 points, rather than 4 points, as an indication of a high probability of LQTS.

Exercise testing (13-15) or an epinephrine QT stress test (16,17) can unmask patients with occult LQTS, particularly LQT1. Several studies showed that the increase in QTc during the recovery phase of exercise testing could distinguish patients with LQTS from control subjects (13-15). Although the evaluation parameters of the recovery phase of exercise were added to the LQTS diagnostic criteria in 2011 (18), little data exist regarding the clinical impact of the 2011 criteria. More recently, the Heart Rhythm Society (HRS)/European Heart Rhythm Association (EHRA)/Asia Pacific Heart Rhythm Society (APHRS) Expert Consensus Statement showed that LQTS could also be diagnosed in the presence of an unequivocally pathogenic mutation in one of the LQTS genes (19). Thus, the detection of LQTS gene mutation carriers is of clinical importance for the diagnosis of LQTS. Therefore, in our cohort of LQTS patients, we compared the LQTS scores calculated by the conventional Schwartz criteria with those calculated using the 2011 criteria (which included an evaluation of the recovery phase of exercise testing). We also performed genetic testing of the major LQTS genes, and we compared the frequency of mutation carriers in the high probability LQTS groups who were diagnosed using the 1993, 2006, and 2011 criteria.

METHODS

STUDY POPULATION. The study population consisted of 132 patients who presented with prolonged QTc intervals, and/or abnormal clinical history and familial findings during cardiac screening; all patients were referred to our hospital. These patients underwent the following examinations: ECG recording at rest, exercise stress test, LQTS score calculations, and genetic testing. A cumulative LQTS risk score was calculated using the 1993 (7), 2006 (12), and 2011 LQTS diagnostic criteria (18).

The study observed the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee for Medical Research at our institution. All study patients provided written informed consent before registration.

ECG ANALYSIS. All referred patients had standard 12-lead ECGs at rest and exercise stress tests recorded at the hospital. The QT interval was measured manually, and was defined as the time between the onset of QRS and the point at which the isoelectric line intersected a tangential line drawn at the maximal downslope of the positive T-wave. QT interval measurements were the means of 3 consecutive beats on 1 lead (lead V_5), because taking only the longest observed QTc would result in a higher rate of false positive classifications (20).

CLINICAL DIAGNOSIS OF LQTS BY SCHWARTZ SCORE. The conventional 1993 criteria score a patient's probability of LQTS based on ECG findings, clinical history, and family history (7). Patients who have never experienced the common LQTS symptoms, including recurrent syncope, seizures, and aborted cardiac death, were considered asymptomatic. The patients were divided into 3 LQTS probability categories based on the risk score: ≥4 points, high probability; 2 to 3 points, intermediate probability; and ≤1 point, low probability. The updated criteria modified by Schwartz in 2006 and 2011 categorized patients as follows: ≥3.5 points, high probability; 1.5 to 3 points, intermediate probability; and ≤1 point, low probability (12,18). The last updated 2011 criteria included an evaluation of the recovery phase of exercise testing.