

Postmortem Molecular Screening in Unexplained Sudden Death

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OBJECTIVES	We examined the prevalence of defects in arrhythmia-related candidate genes among patients with unexplained sudden cardiac death (SCD).
BACKGROUND	Patients with unexplained sudden death may constitute up to 5% of overall SCD cases. For such patients, systematic postmortem genetic analysis of archived tissue, using a candidate gene approach, may identify etiologies of SCD.
METHODS	We performed analysis of <i>KCNQ1</i> (<i>KVLQT1</i>), <i>KCNH2</i> (<i>HERG</i>), <i>SCN5A</i> , <i>KCNE1</i> , and <i>KCNE2</i> defects in a subgroup of 12 adult subjects with unexplained sudden death, derived from a 13-year, 270-patient autopsy series of SCD. Archived, paraffin-embedded myocardial tissue blocks obtained at the original postmortem examination were the source of deoxyribonucleic acid for genetic analysis.
RESULTS	Two patients were found to have the same <i>HERG</i> defect, a missense mutation in exon 7 (nucleotide change G1681A, coding effect A561T). The mutation was heterozygous in Patient 1, but Patient 2 appeared to be homozygous for the defect. Patch-clamp recordings showed that the A561T mutant channel expressed in human embryonic kidney cells failed to generate <i>HERG</i> current. Western blot analysis implicated a trafficking defect in the protein, resulting in loss of post-translational processing from the immature to the mature form of <i>HERG</i> . No mutations were detected among the remaining four candidate genes.
CONCLUSIONS	In this autopsy series, only 2 of 12 patients with unexplained sudden death were observed to have a defect in <i>HERG</i> among five candidate genes tested. It is likely that elucidation of SCD mechanisms in such patients will await the discovery of multiple, novel arrhythmia-causing gene defects. (J Am Coll Cardiol 2004;43:1625–9) © 2004 by the American College of Cardiology Foundation

Patients with unexplained sudden death have no discernable cardiac or extracardiac abnormalities that may have contributed to the terminal event. Among adults, such patients may constitute approximately 5% of cases in autopsy series of sudden cardiac death (SCD) (1). These subjects are usually young, and in up to 50% of cases, sudden death is the first and only clinical manifestation (2). Inherited primary electrophysiologic disorders characterized by a structurally normal heart, such as the long QT syndrome (LQTS) and Brugada syndrome, may account for an unknown proportion of such patients (3–6). Except for a recent report on sudden infant death syndrome (7), there is limited information on postmortem genetic analysis of unexplained sudden death in large series of patients with SCD.

We have previously reported a comprehensive postmortem phenotypic evaluation of a consecutive series of 270 adult cases of SCD, accrued at a cardiovascular registry over 13 years (2). In 12 patients (4.4%), sudden death occurred in

the presence of a structurally normal heart and in the absence of comorbidities associated with sudden death. We therefore performed postmortem genetic analysis to investigate the frequency of LQTS and Brugada syndrome gene defects in this subgroup of 12 adult subjects with unexplained sudden death.

METHODS

Classification and phenotyping from autopsy series.

Detailed methods have been published previously (2). In brief, the Jesse E. Edwards Cardiovascular Registry (St. Paul, Minnesota) is a repository of over 15,000 archived hearts from cases referred over approximately 45 years. The present autopsy series of SCD included all consecutively referred cases of sudden death in subjects age ≥ 20 years during 1984 to 1996 ($n = 270$). All patients were residents of the state of Minnesota, with the majority of cases (at least 87%) referred from the medical examiners of Hennepin, Ramsey, and Anoka counties. All patients with noncardiac conditions associated with sudden death were excluded. Autopsy reports and registry data of all patients were reviewed to identify those with SCD and an apparently normal heart. Of the 14 patients identified (5%), only 12 were included in the subgroup of patients with unexplained sudden death (2 had Wolff-Parkinson-White syndrome diagnosed on the 12-lead electrocardiogram [ECG] [8]). Of

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Abbreviations and Acronyms

ECG = electrocardiogram
HEK = human embryonic kidney
LQTS = long QT syndrome
PCR = polymerase chain reaction
SCD = sudden cardiac death
SSCP = single-stranded conformational polymorphism

the 12 patients, one male patient was Native American and one female patient was Asian (Hmong descent). The remaining 10 patients were Caucasian.

Definitions. Among witnessed deaths, SCD was defined as unexpected death that occurred within 6 h of symptom onset. Among unwitnessed deaths, patients were included if they were reported to have been observed symptom-free in the previous 24 h.

Genetic analysis of *KCNQ1* and *KCNH2* defects. Archived, paraffin-embedded myocardial tissue blocks obtained at the original postmortem examination were the source of deoxyribonucleic acid (DNA) for genetic analysis. Genomic DNA was extracted using the Qiagen DNeasy kit (Qiagen, Valencia, California). Segments of *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2* were amplified as previously described (9). Screening for *KCNQ1* and *KCNH2* defects was performed using single-stranded conformational polymorphism (SSCP) (10,11). All segments displaying abnormal conformers were analyzed by automated sequencing (ABI 377, Applied Biosystems, Foster City, California). Defects in *SCN5A*, *KCNE1*, and *KCNE2* were directly analyzed using automated sequencing. For Patient 2, *KCNH2* exon 7 was amplified and subcloned into pGemTeasy (Promega, Madison, Wisconsin), and multiple independent clones were sequenced.

Site-directed mutagenesis and transfection. Complementary DNA of *KCNH2* and the GeneEditor in vitro mutagenesis system (Promega) were used. Human embryonic kidney (HEK) 293 cells were transiently transfected with wild-type and A561T mutant *KCNH2*/pcDNA3 constructs using a lipofectamine method, as previously described (12).

Patch-clamp recording and Western blot analysis. Whole-cell, patch-clamp recording and Western blotting were performed 48 h after transfection. Detailed conditions and buffer compositions for patch-clamp recordings have been described previously (12). For Western blot analysis, cell lysates were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with an anti-*KCNH2* primary antibody, then secondary-conjugated antibody, and finally detected with an enhanced chemiluminescence detection kit (12).

RESULTS

Clinical profile of patients with *KCNH2* defects. Of the 12 patients tested, two had defects in *KCNH2*. Patient 1

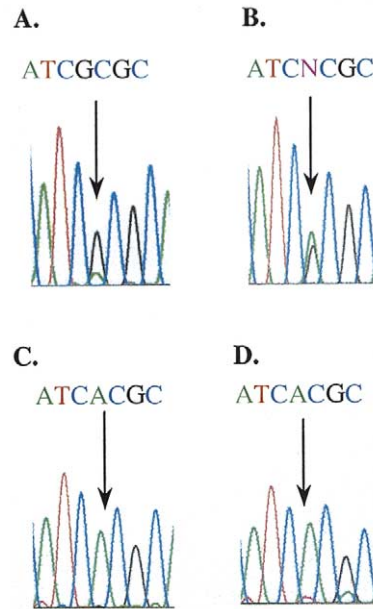


Figure 1. Partial deoxyribonucleic acid sequence of *KCNH2* exon 7 for the wild-type (A, single black peak) and mutant *KCNH2* gene demonstrating the same mutation in Patient 1 (heterozygous; B, two peaks: black and green) and Patient 2 (homozygous; C, single green peak). The latter was confirmed by PCR cloning and sequencing (D, single green peak).

was a 32-year-old white female who suffered an unwitnessed, unexpected sudden death while at home. There was a past medical history of seizure disorder, with two seizure episodes documented in seven years before death. There was no known history of cardiac disease, and a 12-lead ECG was not available in the medical records. Detailed postmortem examination revealed a structurally normal heart, and abnormalities in other organs were limited to healed, focal chronic pyelonephritis and a pleural adhesion of the right lung. Her only medication was phenobarbital sodium for treatment of seizure disorder. Findings on the postmortem serum toxicology screen were limited to phenobarbital 13.6 $\mu\text{g/ml}$ (therapeutic range 20 to 50 $\mu\text{g/ml}$). At the time of death, there was no known family history of sudden death or other cardiac disease.

Patient 2 was a 37-year-old white male who suffered a witnessed, instantaneous, out-of-hospital SCD. Immediately before collapse, the patient had complained of chest pain following moderate exercise (slow jogging for a 10-min period). A 12-lead ECG for this patient was not available. He was not taking any prescription or non-prescription drugs. Detailed autopsy and cardiac pathologic examination were normal. The postmortem serum examination was also negative. The family history was notable; the patient's mother had also succumbed to sudden death at age 33 years.

Genetic analysis of *KCNH2* defects. In both patients, SSCP revealed abnormal conformers in *KCNH2* exon 7. After automated sequencing, Patient 1 was found to have a heterozygous missense mutation in exon 7, encoding the S5 region of *KCNH2* (nucleotide change G1681A, coding effect A561T [9,13]) (Fig. 1B). The same defect was also

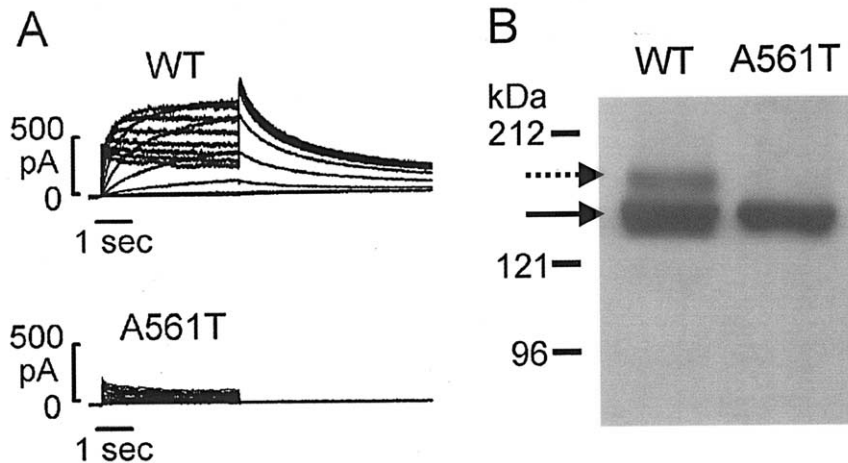


Figure 2. Patch-clamp and Western blot analyses of the *KCNH2* mutant A561T. **(A)** Representative currents recorded from HEK 293 cells transfected with wild-type (WT) *KCNH2* and A561T mutant, as indicated. The *KCNH2* currents were activated by depolarizing steps between -70 and 60 mV from a holding potential of -80 mV, and tail currents were recorded on repolarizations to -50 mV. **(B)** Western blot analysis of *KCNH2* channel proteins of wild-type *KCNH2* and A561T mutant. The **solid and dashed arrows** indicate immature and mature forms of *KCNH2* protein, respectively.

identified in Patient 2, who was homozygous for the A561T mutation (Fig. 1C). Homozygosity in this patient was again demonstrated by sequencing of multiple-cloned polymerase chain reaction (PCR) amplicons, revealing a single mutant allele (Fig. 1D).

Identification of benign polymorphisms. Among all 12 patients, only one such polymorphism was identified. A 30-year-old white female patient had a single base-pair change in *SCN5A* (nucleotide change G4650T, coding effect K1500N). This has previously been reported as a nonpathogenic variant with an estimated allele frequency of 0.9% (14).

Functional analysis of the A561T mutation. Patch-clamp recordings of wild-type *KCNH2* current showed voltage-dependent activation, with inward rectification at more positive voltages. In contrast, the A561T mutant channel did not express *KCNH2* current in transfected HEK 293 cells (6 cells). The wild-type *KCNH2* channel protein expressed two bands on Western blot analysis, a 135-kd band (immature form of *KCNH2*) and a 155-kd band (mature form of *KCNH2*) (Fig. 2B) (12). In contrast, A561T expressed only the 135-kd band.

DISCUSSION

From a 13-year autopsy series of 270 consecutive patients with SCD, 12 patients had SCD with a structurally normal heart. Postmortem genetic analysis for defects in five LQTS and Brugada syndrome genes was performed on these 12 unrelated patients, using archived myocardium, and two patients were found to have the identical mutation in exon 7 of *KCNH2* (A561T). Patient 1 was heterozygous for this mutation, but Patient 2 appeared to be homozygous for the defect. Our in vitro analysis of the *KCNH2* A561T mutant channel revealed a post-translational defect that resulted in absence of the voltage-dependent delayed rectifier potassium current. Incidentally, both patients died several years

before the gene defect for LQTS2 and the mechanistic link with *KCNH2* mutations was to be discovered. Postmortem molecular testing using a candidate gene-based approach identified gene defects in only 2 of 12 patients.

This specific missense mutation in *KCNH2* (exon 7, A561T) has previously been reported in four kindreds with chromosome 7-linked LQTS2, indicating a causal association between this defect and the increased risk of polymorphic ventricular tachycardia and SCD (9,13). Among a kindred described in detail by Dausse et al. (13), there were eight affected members spanning three generations. There was a broad spectrum of clinical manifestations, which may also have been influenced by subsequent treatment in asymptomatic individuals carrying the mutation. Three members died at ages 65, 39, and 32 years, and a fourth had aborted sudden death (alive at age 40 years). Of the remainder, one family member had a history of dizziness (age 41 years), and three were asymptomatic (age 15, 10, and 4 years). Furthermore, a finding on the 12-lead ECG was found to be common to all affected members: in the precordial leads, the T-wave was of low amplitude and had a characteristic biphasic pattern. Defects in *KCNH2* (15) have also been identified as specific targets of drugs associated with SCD (11,16). As the serum toxicology in Patient 1 was positive for phenobarbital, in addition to the inherited form of LQTS, acquired LQTS remains a possibility (11,17). The observation that Patient 1 carried a diagnosis of seizure disorder is also of interest when examined in the context of concomitant LQTS. Polymorphic tachycardia with spontaneous resolution may manifest as seizure-like activity in patients with LQTS (18). If Patient 1 did have both a seizure disorder and LQTS, this leaves open the interesting possibility that defects in *KCNH2* may have pleiotropic phenotypes, as suggested by a recent study (19).

The gene *KCNH2* encodes the alpha subunit of the potassium channel conducting the rapidly activating, de-

layed rectifier current (I_{Kr}) (15,16). The functional abnormalities of the A561T *KCNH2* mutant ion channel have not been characterized. In the present study, patch-clamp recordings showed that A561T fails to generate *KCNH2* current (Fig. 2A). In addition, Western blot analysis revealed the defect at the protein level to be a lack of post-translational processing from the immature to the mature form (Fig. 2B) (12). The wild-type *KCNH2* channel protein is initially synthesized in the endoplasmic reticulum as a core-glycosylated precursor form with a molecular mass of 135 kd. Modification occurs in the Golgi apparatus, with conversion to the mature form of the channel and a molecular mass of 155 kd. Thus, this lack of processing is due to failure of the A561T mutant channel to reach the plasma membrane (i.e., defective trafficking of *KCNH2* protein) (12). Defective protein trafficking has recently been recognized as an important mechanism for *KCNH2* channel dysfunction in LQTS2 (12,20). In fact, another LQTS2 mutation involving the same codon (A561V) also causes a protein-trafficking defect (21). Because the residue A561 is located in the S5 transmembrane domain and is highly conserved in all members of the *ether-à-go-go* family, this residue and/or domain may be a critical residue in normal folding and trafficking of the *KCNH2* channel.

All patients were referred from the same geographic area, with the majority of cases (87%) referred from the medical examiners in the Minneapolis-St. Paul tri-county region. Furthermore, the medical examiners referred all consecutive cases of sudden death in a consistent fashion (2). In the absence of existing prospective, population-based evaluations of sudden death, this methodology provides the opportunity to examine a large series of consecutive SCD cases. Although the possibility of gender bias exists, this would be unlikely, as the gender distribution in our overall series (approximately one-third were women) is consistent with previous autopsy series (22), cohort studies (23), and investigations of survivors of SCD (24). Recognizing the constraints of this population, the incidence of gene defects implicated in the LQTS and Brugada syndromes among patients with unexplained sudden death was 17% (2 of 12). However, we did not screen the present group of patients for defects in the ryanodine receptor gene (*RyR2*), which have recently been implicated in the syndrome of catecholaminergic polymorphic ventricular tachycardia (25-27).

We used archived paraffin tissue blocks as a source of genomic DNA, and the possibility of degradation of DNA and subsequent effects on genetic analysis exists. However, this approach has been used successfully by others (28) and would provide the only means to perform such an analysis from a large autopsy series spanning 13 years. Homozygous *KCNH2* mutations are rare and have not been previously described for A561T. Two previous reports in the literature (29,30) reported on three individuals with novel homozygous defects among two separate kindreds. Both kindreds had a separate mutation: L200fs/144 and L552S. All

affected individuals had a severe cardiac phenotype manifesting as torsade de pointes, 2:1 atrioventricular block, or sudden death, and no other phenotypic abnormalities. We were unable to use additional means to confirm the apparent homozygosity of the mutation in Patient 2, such as Southern blotting (the mutation does not create/destroy a restriction site) or familial segregation (other details of family history or familial samples could not be obtained). However, sequencing results were confirmed using multiple primers for amplification, as well as multiple independent PCRs.

Conclusions. In this 13-year autopsy series of 270 consecutive cases of SCD, there were 12 unrelated patients with unexplained sudden death. Molecular screening using a candidate gene approach identified gene defects in only 2 of 12 patients, both in *KCNH2*. The quest for novel, arrhythmia-causing gene defects to elucidate mechanisms of arrhythmogenesis in such patients will likely require larger, prospective, population-based investigations.

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