Background: The voltage-gated, rapid-delayed rectifier current (I_{Kr}) is important for repolarization of the heart, and mutations in the genes coding for the K^+ ion channel conducting this current, i.e., KCNH2 for the α-subunit HERG and KCNE2 for the β-subunit MiRP1, cause acquired and congenital long Q-T syndrome (LQTS) and other cardiac arrhythmias.

Methods: We developed a robust single-strand conformation polymorphism–heteroduplex screening analysis, with identical thermocycling conditions for all PCR reactions, covering all of the coding exons in KCNH2 and KCNE2. The method was used to screen 40 unrelated LQTS patients.

Results: Eleven mutations, of which six were novel, were found in KCNH2. Interestingly, six mutations were found in the region of the gene coding for the Per-Arnt-Sim (PAS) and PAS-S1 regions of the HERG protein, stressing the need to examine the entire gene when screening for mutations. No mutations were found in KCNE2, suggesting that direct involvement of MiRP1 in LQTS is rare. Furthermore, four novel single-nucleotide polymorphisms (SNPs) and one amino acid polymorphism (R1047L) were identified in KCNH2, and one novel SNP and one previously known amino acid polymorphism (T8A) were found in KCNE2.

Conclusions: The potential role of rare polymorphisms in the HERG/MiRP1 K^+ channel should be clarified with respect to drug interactions and susceptibility to arrhythmia and sudden death.

The action potential of the heart is the result of the concerted and well-orchestrated action of many ion channels conducting currents with different biophysical characteristics (1). The slow (I_{Ks}) and rapid (I_{Kr}) components of the voltage-gated delayed rectifier K^+ current are important for repolarization of the heart in the third phase of the action potential (2).

I_{Kr} current is conducted through an ion channel most likely composed of four HERG subunits (3) in combination with an unknown number of MiRP1 subunits (4). The I_{Ks} current is likewise conducted through an ion channel composed of four KvLQT1 α subunits and an unknown number of minK β subunits (5). If the ion channels responsible for I_{Kr} or I_{Ks} are dysfunctional because of mutations or pharmacological interferences, the result may be long Q-T syndrome (LQTS), a condition characterized by prolonged cardiac repolarization, and, in consequence, a prolonged Q-T interval on the electrocardiogram, a propensity for ventricular arrhythmias of the
torsades de pointes type, causing syncopes or ventricular fibrillation and death (6).

Approximately 50% of cases with clinically diagnosed LQTS can be genotyped at present (6). Among these patients, 20–40% may have mutations in KCNH2 encoding the HERG α-subunit of the HERG/MiRP1 channel conducting the I_{Kr} current, and 30–50% may have mutations in KCNQ1 encoding the KvLQT1 α-subunit of the KvLQT1/minK channel conducting the I_{Ks} current (7).

The remainder may have mutations in KCNE1 or KCNE2 encoding minK (8) and MiRP1 (4), respectively, or in the SCN5A gene encoding the SCN5A Na⁺-ion channel conducting the I_{Na} current responsible for the initial depolarization of the action potential (9, 10).

The HERG/MiRP1 ion channel is of pharmacological importance because many common drugs, e.g., haloperidol, amiodarone, ketoconazole, terfenadine, class III antiarrhythmics, and others (11–15), interact with the I_{Kr} current, and in some cases cause acquired LQTS, or may precipitate prolongation of the Q-T interval and torsades de pointes in patients with LQTS. Furthermore, patients with low- or nonpenetrant genetic LQTS may become symptomatic only when taking drugs prolonging the Q-T interval (18).

The recent discovery of KCNE2 mutations among patients suffering from drug-induced, inherited, or sporadic arrhythmia (4) further corroborates the involvement of the HERG/MiRP1 ion channel in the susceptibility to arrhythmia.

Here we describe a rapid single-strand conformation polymorphism–heteroduplex (SSCP-HD) analysis method for screening for mutations in all coding regions of the KCNH2 and KCNE2 genes, and identify 11 KCNH2 mutations, 6 of which were novel, and several polymorphisms in 40 families with LQTS.

Materials and Methods

DNA samples

Blood samples were collected from 40 unrelated patients referred to Statens Serum Institut for routine analysis for the presence of mutations causing LQTS. Detailed clinical descriptions of the patients will be presented elsewhere (M. Christiansen et al., manuscript in preparation). Genomic DNA was extracted from whole blood or filter-paper blood spots using a QIAamp reagent set (Qiagen GmbH).

PCR amplification

PCR was performed in 200-μL, thin-walled PCR tubes in a total reaction volume of 50 μL, containing 0.4–5 μL of DNA template, 5 μL of 10× reaction buffer [100 mM Tris-HCl (pH 8.85), 250 mM KCl, 50 mM (NH₄)₂SO₄], 0.5–2.0 mM MgSO₄ (optimized for each primer pair; see Table 1), 200 μM dNTPs, and 1 U of Pwo DNA polymerase (Roche Molecular Biochemicals). The PCR primers are listed in Table 1. Thermal cycling was performed in a PTC200 DNA engine (MJ Research) with the following temperature profile: 94 °C for 4 min followed by 33 cycles of 94 °C for 20 s, 58 °C for 20 s, 72 °C for 40 s, and ending with a 7-min extension at 72 °C. Unincorporated primers were removed from the PCR reaction by spin columns according to the manufacturer’s instructions (Qiagen) or by digestion of 15 μL of PCR product with 5 U of exonuclease I for 1 h at 37 °C (Amersham Life Sciences).

Mutation analysis

SSCP-HD analysis was performed on purified PCR products as follows: 1–5 μL of PCR product was mixed with 15–19 μL of formamide stop solution (US70725; Amersham) in a total volume of 20 μL. The mixture was heated at 98 °C for 5 min and transferred directly to an ice-water bath. Sample/stop solution (8 μL) was loaded on a precast 12.5% polyacrylamide gel (122 × 110 × 0.5 mm; Amersham Pharmacia Biotech). Electrophoresis was performed at 5 °C and 20 °C for 90 min (600 V; 25 mA) using a GenePhor apparatus (Amersham Pharmacia Biotech). Bands were visualized by silver staining using a Hoefer automated silver stainer and a PlusOne DNA silver staining reagent set according to the manufacturer’s instructions (Pharmacia Biotech). Automated “Dye terminator” cycle sequencing (Perkin-Elmer) was performed directly on purified PCR products according to the manufacturer’s instructions using an ABI373 DNA sequencer.

Results

Intronic PCR primer sequences were designed for robust amplification of the KCNH2 and KCNE1 exons, without nesting or additives (19, 20), under identical thermocycling conditions. The sequences, optimal MgSO₄ concentrations, and optimal amounts of template are listed in Table 1. The PCR amplifications were performed using Pwo DNA polymerase, an enzyme with 3’→5’ exoactivity, to reduce the risk of polymerase-induced errors and false-positive results caused by PCR fragments with A overhangs (21). Excessive primers were removed from the PCR products before the SSCP-HD analysis by either spin columns or exonuclease I digestion. No differences in the resulting SSCP-HD band patterns were observed between the two clean-up procedures. Both led to clear, sharp bands (Fig. 1) and allowed for subsequent direct sequencing of the PCR fragments (data not shown). The use of precast gels made the analysis highly reproducible (data not shown).

Forty probands were screened for KCNH2 and KCNE2 mutations using SSCP-HD analysis. Fragments displaying abnormal conformers or heteroduplexes were sequenced using automated DNA sequencing. Examples of SSCP-HD band patterns are shown in Fig. 1. Nine missense mutations, one nonsense mutation, and a 9-bp in-frame duplication were found in KCNH2 (Table 2). Five of these mutations have been described previously (10, 17, 19, 20, 23, 25, 26, 35), whereas six of the mutations are novel (82InsIAQ, I96T, K101E, R366X, I400N, and S621N).
None of the five known mutations was found among 50 wild-type controls, and none of the six novel mutations was found among 100 wild-type controls. A rare allelic variant of \textit{KCNH2} encoding an Arg1047Leu amino acid variant was found in 3 of 80 wild-type alleles (Table 3).

The rare amino acid polymorphism, Thr8Ala, described previously (4) was not found in any of the LQTS patients. It was, however, found in 1 of 168 wild-type alleles (Table 3).

We also identified eight different single-nucleotide polymorphisms (SNPs) in \textit{KCNH2} and one SNP in \textit{KCNE2} among the patients and the wild-type controls (Table 3). Five of these SNPs have not been described previously. The 29 probands without mutations in \textit{KCNH2} were

\begin{table}[h]
\centering
\caption{Intronic PCR primers for amplification of the coding regions of \textit{KCNH2} and \textit{KCNE2}.}
\small
\begin{tabular}{lllll}
\hline
Oligonucleotide sequence, from 5' to 3' & Amplified region, exon no. & Optimal MgSO$_4$ concentration, mM & Template volume, $\mu$L & Size of amplicon, bp & Reference \\
\hline
\textit{KCNH2} & & & & & \\
GCCCACCCGAACTGACTGCT & 1 & 0.5 & 0.4 & 231 & This study \\
CAGGCCCCCATCACCAC & & & & & This study \\
GGTCCCGTCACGCCACAC & 2 & 0.75 & 4 & 312 & 20 \\
TTGACCGCCCGCATGCGTTG & & & & & 20 \\
GGGCAATATCCGCAAGCCCG & 3 & 0.75 & 2 & 213 & 20 \\
ACGCTGCCCAAGCTGACGAC & 4 & 0.75 & 2 & 230 & This study \\
GACCAGTGGGATGGATGTAC & & & & & This study \\
GTGCCGCGAGAAGTGAGT & 4 & 0.75 & 2 & 361 & This study \\
GCCAGAAATGCCGCAAGCCTG & & & & & 20 \\
GCCCTGACCAGGTGCGCTC & 5 & 1 & 2 & 293 & 20 \\
CCTCTCAAGGCTCTCCCA & & & & & 20 \\
CAGAGTGGCATGCTGAC & 6 & 2 & 2 & 295 & 20 \\
CAGGCGTGGCAACTGCGTAG & & & & & 20 \\
CGAGCTGAGGCTGAGTACAA & 6 & 0.75 & 2 & 301 & 19 \\
CACCTGCTGAGGATTGAC & & & & & 19 \\
TTCTGCTGAGGAGAGGGG & 6 & 0.75 & 2 & 296 & 19 \\
TACACCATGCTCTCCTGCTG & & & & & 19 \\
TGCCCCATCAACCGATGTG & 7 & 0.75 & 2 & 240 & 19 \\
CAGGAGCAGTGGTGGAGTCC & & & & & 19 \\
TAGCTGAGCTATGCTGAC & 7 & 0.75 & 2 & 277 & 19 \\
GCCGCCGCCTGGACACCTCA & & & & & 19 \\
TGGGGTCCCCTCAGAGGCTG & 8 & 0.75 & 2 & 289 & This study \\
CTCTGAGCCTCGCCACCA & & & & & This study \\
GGCTGGAGGTTGAGATTTC & 9 & 0.75 & 2 & 258 & This study \\
GGGCTGTGAGTGGATCTT & & & & & This study \\
GCACTGAAACCCTCTCGAG & 9 & 0.75 & 2 & 222 & This study \\
GGCTATCCGCTGCAAGTC & 9 & 0.75 & 2 & 258 & This study \\
TGAGGTTCCCTCTCTATATG & 10 & 0.75 & 2 & 243 & This study \\
CTCAGGGGAGCCAATCCACATC & & & & & This study \\
GTTGCGGAGTAGAGACAGT & 11 & 0.75 & 2 & 163 & This study \\
TCCCCCGCTCACCGTCTC & & & & & This study \\
TCTGTGCTCTCCCTCTCTCT & 12 & 1 & 5 & 331 & This study \\
GCCGCGATATCCTGACAG & & & & & This study \\
TACCCAGCTCTGCTCTGCT & 13 & 0.75 & 2 & 300 & This study \\
AGGCCCCCTCCCTCTCCAG & & & & & This study \\
ATCCGCTGACGGGCTG & 14 & 0.75 & 2 & 287 & 19 \\
GAACAGCGCGTACGGGAT & & & & & 19 \\
TCTGTGCTCTCCCTCATC & 15 & 0.75 & 2 & 281 & 19 \\
ACGTGCTGCTCCGACCTG & & & & & 19 \\
\textit{KCNE2} & & & & & \\
TCCTTGTCTCTGC & 1.5 & 1 & 255 & This study \\
GCCAGTGATGAAAGAGAGA & & & & & This study \\
GATGCTGAGAACCTCTCTATG & 1.5 & 1 & 289 & This study \\
GTCTGAGGACGAGATGT & 1.5 & 1 & This study \\
\hline
\end{tabular}
\end{table}
also screened for mutations in KCNQ1, KCNE1, and SCN5A, and several additional mutations were found (M. Christiansen et al., manuscript in preparation).

Discussion

We have developed a rapid screening method for detection of mutations in the KCNH2 and KCNE2 genes involved in congenital and acquired LQTS. The most important advantage of the method is that it is suitable for large-scale screening because the amplifications of KCNH2 and KCNE2 exons are performed under identical thermocycling conditions. This is an improvement over previously published methods, in which several annealing temperatures, additives, and/or nested PCR conditions were necessary to ensure amplification of all exons (19, 20). Furthermore, the use of exonuclease digestion eliminates the need for spin columns, making the method even more amenable for screening a large number of patients.

The combination of SSCP and HD has been shown previously (22) to increase the mutation detection rate compared with SSCP analysis alone. In this study, all mutations found were detectable by SSCP analysis alone. However, for some mutations, the difference between abnormal and normal SSCP conformers were minor compared with a clear difference in the migration of the heteroduplexes (Fig. 1, B and C). Such weak differences in the SSCP patterns are very sensitive small variations in the electrophoresis conditions and may be missed if the detection is based on SSCP alone. Thus, although it has not been examined in a controlled study, we find it likely that the combined use of SSCP and HD, as described here, is more efficient than SSCP alone for mutation screening in the KCNH2 and KCNE2 genes. Furthermore, multiplexing and detection using automated capillary electrophoresis may increase performance even more (23).

A general testing strategy for the molecular diagnosis of LQTS, based on initial mutation screening, would be the following: (a) electrocardiographic measuring of the proband; (b) SSCP-HD screening of KCNH2 and KCNQ1; and (c) sequencing of abnormal conformers. If no mutations are found, the following would be performed: (a) SSCP-HD screening of SCN5A, KCNE1, and KCNE2 and (b) sequencing of abnormal conformers. When a suspected disease-causing mutation is found, the following would be performed: (a) SSCP-HD screening of 100 wild-type controls and (b) DNA sequencing of the mutated exon in all accessible family members.

We found 11 mutations in KCNH2 in 40 families with LQTS, and this number is to be expected from the distribution of LQTS-associated mutations in the published literature as compiled in the LQTdbase (www.ssi.dk/en/forskning/lqtsdb.htm). Each mutation was found in only 1 proband and not in any of the 39 additional probands or in 50 wild-type controls. Furthermore, an additional 50 wild-type controls were screened.

![Fig. 1. Examples of SSCP-HD band patterns.](image)

(A), KCNH2, exon 7, N629S and S621N polymorphisms (20 °C). (B), KCNH2, exon 6, I400N polymorphism (20 °C). (C), A/G variant in KCNH2, intron 13 (5 °C). Abnormal conformers are indicated by arrows. The direction of electrophoresis was from top to bottom.

### Table 2. KCNH2 mutations associated with LQTS.

<table>
<thead>
<tr>
<th>Mutation no.</th>
<th>Nucleotide change*</th>
<th>Amino acid change</th>
<th>Coding effect</th>
<th>Region</th>
<th>Exon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>87 C→A</td>
<td>F29L</td>
<td>Missense</td>
<td>PAS</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>244,252ins9</td>
<td>82−84insIAQ</td>
<td>Duplication</td>
<td>PAS</td>
<td>2</td>
<td>This study</td>
</tr>
<tr>
<td>3</td>
<td>287 T→C</td>
<td>I96T</td>
<td>Missense</td>
<td>PAS</td>
<td>2</td>
<td>This study</td>
</tr>
<tr>
<td>4</td>
<td>391 A→G</td>
<td>K101E</td>
<td>Missense</td>
<td>PAS</td>
<td>2</td>
<td>This study</td>
</tr>
<tr>
<td>5</td>
<td>1096 C→T</td>
<td>R366X</td>
<td>Nonsense</td>
<td>Pre S1</td>
<td>5</td>
<td>This study</td>
</tr>
<tr>
<td>6</td>
<td>1199 T→A</td>
<td>I400N</td>
<td>Missense</td>
<td>S1</td>
<td>6</td>
<td>This study</td>
</tr>
<tr>
<td>7</td>
<td>1600 C→T</td>
<td>R534C</td>
<td>Missense</td>
<td>S4</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>1682 C→T</td>
<td>A561V</td>
<td>Missense</td>
<td>S5</td>
<td>7</td>
<td>10, 17, 35</td>
</tr>
<tr>
<td>9</td>
<td>1714 G→C</td>
<td>G572R</td>
<td>Missense</td>
<td>S5-Pore</td>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>1862 G→A</td>
<td>S621N</td>
<td>Missense</td>
<td>Pore</td>
<td>7</td>
<td>This study</td>
</tr>
<tr>
<td>11</td>
<td>1886 A→G</td>
<td>N629S</td>
<td>Missense</td>
<td>Pore</td>
<td>7</td>
<td>23, 25</td>
</tr>
</tbody>
</table>

* Numbering according to GenBank Accession No. U04270. ATG start codon was assigned to position 1.
for the presence of the six novel mutations. Thus, we find it unlikely that any of the 11 mutations listed in Table 2 are nonpathologic variants. However, the possibility of rare allelic variants cannot be fully excluded until functional analysis in a cell-based model system has been performed.

Interestingly, six of the mutations (five novel) were located N-terminally to the S1 transmembrane segment, in the PAS or PAS-S1 domains (24), making it clear that it is necessary to screen the entire gene when looking for LQT2-associated mutations. In the early days of LQTS-genotyping, most KCNH2 mutations were found in the S4-S5-pore-S6 region, and the clinical phenotype was often (10, 25–27), but not always (17, 28), characterized by a dominant mode of inheritance and high penetrance caused by a pore-associated mutation affecting the whole tetrameric HERG ion channel in a dominant-negative way and thus making it possible for the mutation to be identified by linkage studies (10, 27). Recent functional studies have disclosed that many mechanisms may cause mutations in KCNH2 to produce LQTS (29).

However, despite the screening of the entire coding sequence and the surrounding intronic regions, an unknown number of LQTS-associated mutations may still be missed by the method presented here, e.g., mutations in upstream regulatory sequences, deletions of whole exons, or rare splice-site mutations outside of the amplified intronic segments. Such mutations may be found by genetic linkage analysis in large families or from inclusion of mutation analysis based on illegitimate mRNA expression in leukocytes. The novel SNPs and amino acid polymorphisms defined here may be used for linkage studies.

The screening for mutations in the KCNE2 gene revealed only a previously published (4) rare amino acid variant, Thr8Ala. Thus, in agreement with the previous study, our data suggest that the direct involvement of MiRP1 in LQTS is rare. However, the existence of the Thr8Ala variant in MiRP1 and our discovery of a novel Arg1047Leu amino acid variant in the C-terminal region of HERG shows that rare amino acid variants exist in the population, as has been shown previously for KvLQT1 (30). Although no investigation has been performed to determine whether these allelic variants have a phenotypic effect, it is tempting to speculate that rare amino acid variants, such as Arg1047Leu, may be associated with a particular susceptibility toward certain drugs or hypokalemia, as suggested by another study showing that C-terminal mutations in HERG may become symptomatic in hypokalemic patients (28) or in patients with other known torsades de pointes-inducing factors.

Combinations of different polymorphisms in α- and β-subunits of ion channels may explain the varying penetrance of LQTS, as well as the genetic linkage in the general population between LQT-linked loci and quantitative characteristics of the electrocardiogram (31), and possibly the relationship between prolongation of neonatal Q-T time and sudden infant death syndrome (32–34). The method presented here will make it possible to conduct large studies to clarify these issues.

We would like to thank Jette Rasmussen and Mads Dahm Johansen for excellent technical assistance. We gratefully acknowledge support from The Danish Heart Foundation, The Novo Nordisk Foundation, Lægeforeningens Forskningsfond, and Kong Christian den Tiendes Fond.

References

2. Sanguinetti MC. Dysfunction of delayed rectifier potassium chan-

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**Table 3. Polymorphisms in KCNH2 and KCNE2 found in the Danish population.**

<table>
<thead>
<tr>
<th>Mutation no.</th>
<th>Allelic variant</th>
<th>Type of polymorphism</th>
<th>Frequency of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNH2</td>
<td>1 1056 C/T</td>
<td>SNP</td>
<td>C 0.995 T 0.005</td>
</tr>
<tr>
<td></td>
<td>2 1467 C/T</td>
<td>SNP</td>
<td>C 0.82 T 0.18</td>
</tr>
<tr>
<td></td>
<td>3 1539 C/T</td>
<td>SNP</td>
<td>C 0.82 T 0.18</td>
</tr>
<tr>
<td></td>
<td>4 1692 A/G</td>
<td>SNP</td>
<td>A 0.58 G 0.42</td>
</tr>
<tr>
<td></td>
<td>5 1956 T/C</td>
<td>SNP</td>
<td>C 0.62 T 0.38</td>
</tr>
<tr>
<td></td>
<td>6 IVS8 -61 A/G</td>
<td>SNP</td>
<td>A 0.50 G 0.50</td>
</tr>
<tr>
<td></td>
<td>7 IVS13 +12 C/A</td>
<td>SNP</td>
<td>A 0.01 C 0.99</td>
</tr>
<tr>
<td></td>
<td>8 IVS13 +22 A/G</td>
<td>SNP</td>
<td>A 0.10 G 0.90</td>
</tr>
<tr>
<td></td>
<td>9 Arg1047Leu</td>
<td>Amino acid</td>
<td>Arg 0.96 Leu 0.04</td>
</tr>
<tr>
<td>KCNE2</td>
<td>1 -28 A/G</td>
<td>SNP</td>
<td>A 0.982 G 0.018</td>
</tr>
<tr>
<td></td>
<td>2 Thr8Ala</td>
<td>Amino acid</td>
<td>Thr 0.994 Ala 0.006</td>
</tr>
</tbody>
</table>

*Numbering according to GenBank Accession No. U04270 (KCNH2) and NM_005136 (KCNE2). TG start codon was assigned to position 1.

Total linkage disequilibrium was observed for 1467 C/T and 1539 C/T (e.g., all individuals with the genotype 1467 CC were also 1539 CC).


