Analysis of candidate genes for genotypic diagnosis in the long QT syndrome

Birgit Haack1,2, Susan Kupka1,2, Margret Ebauer1,2, Anna Siemiątkowska3, Markus Pfister2, Joanna Kwiatkowska4, Jan Ereciński3, Janusz Limon3, Karolina Ochman3, Nikolaus Blin1

1 Institute of Anthropology and Human Genetics, Department of Molecular Genetics, University of Tübingen, Germany
2 Department of Otolaryngology, University of Tübingen, Germany
3 Department of Biology and Genetics, Medical University of Gdañsk, Poland
4 Department of Paediatric Cardiology and Congenital Heart Disease, Medical University of Gdañsk, Poland

Abstract. Patients with the long QT syndrome (LQTS) suffer from cardiac arrhythmias that can lead to abrupt loss of consciousness and sudden death, already in young individuals. Thus, an early diagnosis of LQTS is essential for patients and their family members. So far, six genes (KCNQ1, HERG, SCN5A, ANK2, KCNE1, KCNE2) have been demonstrated to be involved in the development of LQTS. Since this syndrome is genetically heterogeneous and large-sized families are often not available for linkage analysis, alternative tools are required for a genetic diagnosis. To investigate genes with numerous exons, like KCNQ1, HERG, SCN5A and ANK2, segregation analysis of a Polish Romano-Ward family with eight members was performed as a reliable method faster than linkage analysis or direct sequencing. To test these four LQT loci, an appropriate selection of microsatellite markers covering different chromosomal regions was applied. Furthermore, two small genes KCNE1 and KCNE2 (at the LQT5 and LQT6 loci), and the SGK1 gene (encoding a kinase regulating KCNE1 and SCN5A channels) were sequenced. All six LQT loci and the SGK1 gene were excluded by these analyses, thus a different pathogenetic mechanism of LQT syndromes can be presumed.

Key words: candidate genes, microsatellite markers, LQTS, Romano-Ward syndrome, segregation analysis.

Introduction

The congenital long QT syndrome (LQTS) is a disorder characterised by cardiac abnormalities and other symptoms, like hearing impairment (OMIM 192500). These dysfunctions are mainly based on changes in ion channels or accessory proteins, leading to prolongation of the QT interval visible on an electrocardiogram and to disturbance of the inner ear homeostasis. In addition to variable hearing impairment, patients are prone to syncope, seizures and sudden death.

The Romano-Ward syndrome (RWS) is the most common form of LQTS, with usually dominant inheritance, but there are also some cases of autosomal recessive-inheritance (Priori et al. 1998; Larsen et al. 1999). The duration of the QT interval varies individually in patients with LQTS and the hearing rate of RWS patients ranges from normal to a moderate hearing impairment. In general, these symptoms are based on mutations in genes encoding potassium and sodium ion channels that are responsible for normal cardiac repolarisation and inner ear function. Six genes are known to be modified in LQTS (for review, see Vatta and Towbin 2000; Mohler et al. 2003). An overview of the localization and structure of the genes is shown in Table 1. The genes KCNQ1 and HERG are the most frequently involved in pathogenesis of the disease, while SCN5A, ANK2, KCNE1 and KCNE2 are rare (Priori et al. 1999; Schott et al. 1995; Splawski et al. 2000).

The proteins encoded by KCNQ1 and HERG represent subunits forming potassium channels.
These are associated with regulatory beta-subunits of the KCNE family, encoded by genes \textit{KCNE1} and \textit{KCNE2}. The mode of binding, stoichiometry and tissue-specific differences of these complexes are still unknown.

The LQT4 locus has been identified by linkage analysis in one family (Schott et al. 1995). Recently, the gene \textit{Ankyrin-2} (\textit{ANK2}) has been identified as the mutated gene in this family (Mohler et al. 2003). Ankyrin-2 is the first identified protein implicated in LQTS that is not an ion channel or channel subunit. Ankyrins are a family of proteins that are believed to link the integral membrane proteins to the underlying spectrin-actin cytoskeleton.

\textit{SGK1} is a serine/threonine kinase that has been proven to regulate various ion channels or channel subunits, including \textit{KCNE1}, Kv1 channels and sodium channels (\textit{SCN5A}, ENaC, ROMK1). It was previously suggested that \textit{SGK1} might have the capacity to shorten the QT interval (Lang et al. 2003). Therefore we decided to analyse also this gene for possible mutations that might contribute to LQTS.

Due to their large size and numerous exons, the genes at the loci LQT1, LQT2, LQT3 and LQT4 loci were analysed by segregation analyses in this study. With this method, the loci can be reliably screened for their pathogenic relevance in families of interest.

### Table 1. Genes involved in LQTS

<table>
<thead>
<tr>
<th>LQT locus</th>
<th>Location</th>
<th>Gene name</th>
<th>Gene structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQT1</td>
<td>11p15.5</td>
<td>KVLQT1 (\textit{KCNQ1}, \textit{KCNA9})</td>
<td>ca. 400 kb, 19 exons</td>
</tr>
<tr>
<td>LQT2</td>
<td>7q35-36</td>
<td>HERG (\textit{KCNH2})</td>
<td>ca. 19 kb, 16 exons</td>
</tr>
<tr>
<td>LQT3</td>
<td>3p21-24</td>
<td>SCN5A</td>
<td>ca. 80 kb, 28 exons</td>
</tr>
<tr>
<td>LQT4</td>
<td>4q25-27</td>
<td>ANK2</td>
<td>ca. 330 kb, 46 exons</td>
</tr>
<tr>
<td>LQT5</td>
<td>21q22.1</td>
<td>KCNE1</td>
<td>3 exons (1 coding exon)</td>
</tr>
<tr>
<td>LQT6</td>
<td>21q22.1</td>
<td>KCNE2</td>
<td>1 coding exon</td>
</tr>
</tbody>
</table>

### Segregation analysis

DNA was extracted from 10 ml of whole blood collected from eight members of the family according to standard procedures. For segregation analyses of LQT1, LQT2, LQT3 and LQT4, sets of microsatellite markers were established. The markers were selected on the basis of the following parameters: location close to or within the gene of interest, evenly covering the locus (if available), and high grade of heterozygosity. Tri- and tetranucleotide repeat markers (if available) were preferred over dinucleotide markers. For each marker set, 515 markers were chosen. PCR was performed with fluorescent-labelled primers and fragment size was analysed on an ABI377 automated sequencer after checking size and concentration on a 6% nondenaturing polyacrylamide gel. PCR conditions and primer sequences can be obtained by contacting the corresponding author. Alleles were named according to their exact fragment size obtained in the automated fragment analysis and segregation of the different marker alleles was determined manually.
Sequencing

For sequencing of KCNE1 and KCNE2, the following overlapping primers were used:

- **KCNE1_1for** ctgcagcagtggaacctt,
- **KCNE1_1rev** ttgaatgggtcgttcgagtg,
- **KCNE1_2for** tacatccgctccaagaag,
- **KCNE1_2rev** tttagccagtggtggggtt,
- **KCNE2_1for** cgcctattttattatttaaattgca,
- **KCNE2_1rev** tcacagtgctcaccaggatg,
- **KCNE2_2for** aacttctactatgtcatcctgt,
- **KCNE2_2rev** tttcttctaagcaaagacaattt.

PCR reaction conditions can be obtained by contacting the corresponding author and cycle sequencing was performed according to the instructions of the Promega kit.

For sequencing 12 exons of the SGK1 gene, the primers included in Table 2 were used.

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**Table 2. SGK1 primers**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Exon</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>CCCTCTCAATGGGGACAGAAC</td>
<td>TTGGGTCTCTGGAGCAGAAG</td>
</tr>
<tr>
<td>2</td>
<td>2, 3</td>
<td>CGTTATGAAGCCGCTCAAAC</td>
<td>CCTACGGTTCCTCCACAGATG</td>
</tr>
<tr>
<td>3</td>
<td>4, 5, 6, 7</td>
<td>TGTCTCCTTTGAGCAATGG</td>
<td>TGTACGGTTCCTTTATACC</td>
</tr>
<tr>
<td>3a/b</td>
<td>4, 5, 6, 7</td>
<td>CTGAACTGTCATTTGTATGG</td>
<td>TCACTCTGCCACATTAG</td>
</tr>
<tr>
<td>4</td>
<td>8, 9</td>
<td>CTAAATGCCTCAATACCTTAGC</td>
<td>TTTGCTGTGTGAGCAATATG</td>
</tr>
<tr>
<td>4a/b</td>
<td>8, 9</td>
<td>TTTGTGATGACAGAAATCCC</td>
<td>GGCACCTGATACCTCTTAG</td>
</tr>
<tr>
<td>5</td>
<td>10, 11</td>
<td>AATGTGGCCCATAGAAAATCCCC</td>
<td>ACTTTGACAGAGACAGATACTC</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>TGTCTAGTGGTGGTCTCTTGAC</td>
<td>CAAAATCCATCGTGATCAGG</td>
</tr>
</tbody>
</table>

* sequencing primers (inside amplified sequence) used in combination with primers yielding fragment 3 and 4, respectively.

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Results

A sequencing gel is required for separation of fragments that usually differ only in 2-4 base pairs, but analysis can also be performed on a conventional sequencing gel using nonlabelled primers and silver staining. In some cases fragments from the different individuals showed greater variations in size so that different alleles were visible even on a 6% polyacrylamide gel.

Results of LQT1 segregation analysis for the family are shown in Figure 1. The different alleles are displayed as bars with the linked marker alleles, named by fragment size, next to them. The markers including their genomic intervals used in this case are also shown in this figure. Despite the high grade of heterozygosity, some markers yielded homozygous genotypes in many individuals. In this case, two identical alleles cannot be distinguished in maternal or paternal origin, therefore providing noninformative results. Due to the fact that not all family members took part in this study, the genotype of some individuals had to be reconstructed (genotypes given in parentheses). In case of dominant inheritance, there should be the same allele occurring in all affected family members and this allele should not appear in healthy individuals. In case of recessive inheritance, there should be one combination of two alleles appearing in all affected individuals but in none of the unaffected. As both cases did not apply to the segregation in our family, this locus can be considered irrelevant for development of the RWS in this family.

The segregation analysis of LQT2 again showed no co-segregation of this locus with the disease phenotype (Figure 2). Remarkably, many recombinations in different individuals occurred (individuals II:3, III:2, III:3 and III:4), but they had no effect on pathogenesis.

LQT3 segregation analysis, which once more yielded an exclusion of pathogenic relevance regarding this locus, so all the three genes most commonly causing LQTS proved to be nonpathogenic in this family (Figure 3).

Also LQT4 segregation analysis revealed segregation showing no coincidence with the disease phenotype, hence excluding that this locus is linked to the disease (Figure 4). Again the analysis showed frequent occurrence of recombinations.

The two small genes **KCNE1** (LQT5) and **KCNE2** (LQT6) were investigated by direct sequencing. While **KCNE2** showed no mutations, a base substitution was detected in **KCNE1**. An exchange from A to G was identified at position 112 of the cDNA sequence (NCBI ACC No: M26685, gi:186569), leading to an amino acid substitution from serine to glycine (p.S38G). This heterozygous substitution was observed in both affected and unaffected family members, so it does no seem to be involved in development of the disease.
Figure 1. (A) Set of microsatellite markers used for analysis of LQT1. Markers showing heterozygosity within the studied family are indicated by an asterisk. (B) Results of segregation analysis of LQT1 in the family. Alleles in parentheses were reconstructed on the basis of children’s genotypes.

Figure 2. (A) Set of microsatellite markers used for analysis of LQT2. Markers showing heterozygosity within the studied family are indicated by an asterisk. (B) Results of segregation analysis of LQT2 in the family. Alleles in parentheses were reconstructed on the basis of children’s genotypes.
Figure 3. (A) Set of microsatellite markers used for analysis of LQT3. Markers showing heterozygosity within the studied family are indicated by an asterisk. (B) Results of segregation analysis of LQT3 in the family. Alleles in parentheses were reconstructed on the basis of children’s genotypes.

Figure 4. (A) Set of microsatellite markers used for analysis of LQT4. Markers showing heterozygosity within the studied family are indicated by an asterisk. (B) Results of segregation analysis of LQT4 in the family. Alleles in parentheses were reconstructed on the basis of children’s genotypes.
The serum/glucocorticoid-inducible kinase 1 gene (SGK1) was also analysed by direct sequencing. No DNA changes were detected in the SGK1 gene, except one nonpathogenic silent c.777T>C polymorphism and four intronic.

Consequently, in this family all known loci and genes related to LQTS, along with the candidate gene SGK1, were excluded from being involved in the pathogenesis of Romano-Ward syndrome, by either segregation analysis or sequencing.

Discussion

Cardiac arrhythmias occurring in LQTS often lead to syncope or sudden death, already in young individuals. The cardiac symptoms usually begin in puberty. It is estimated that four out of ten affected children and young people die with their first symptom (http://www.sads.ca/frameset1.html). Thus, early diagnosis and medical intervention is crucial for preventing death.

The Romano-Ward syndrome (the major form of LQTS) is inherited in the autosomal dominant pattern, with normal to moderate hearing impairment, and can involve mutations in six different genes (KCNQ1, HERG, SCN5A, ANK2, KCNE1 and KCNE2). The LQT4 locus was described in one family by Schott et al. (1995). Recently, the underlying genetic defect in this family was ascribed to a mutation in the ANK2 gene (Mohler et al. 2003). To investigate the molecular genetic basis of a disease, genes with numerous exons like KCNQ1, HERG, ANK2 and SCN5A can be screened for their pathogenic effects by segregation analyses. In case of no co-segregation, laborious sequencing can be avoided. For loci with no associated gene, segregation analyses are an alternative to more extensive linkage analysis.

Marker analyses in general provide information about the complete gene locus, thus also including inherited regulatory defects. After having a proof of involvement in pathogenesis, the gene of interest can be searched for disease-causing mutations by sequencing.

Compared to linkage analysis, segregation analysis requires fewer individuals in a family. Further studies of additional families demonstrated that at least 3 affected and 3 healthy family members (depending on family structure) are essential to obtain informative results (data not shown). By using 315 microsatellite markers with suitable spacing between each other, this method provides a reliable tool for verification of pathogenic mutations.

For the RWS family presented here, the LQT1, LQT2, LQT3 and LQT4 loci were excluded from being involved in pathogenesis by segregation analyses. Furthermore, the genes KCNE1 (LQT5) and KCNE2 (LQT6) were excluded, as no diagnostic mutation was detected in the coding region of these genes. However, regulatory defects of these two genes, like promoter mutations or methylation, can only be excluded by further experiments. As both forms of LQTS (Romano-Ward syndrome and Jervell and Lange-Nielsen syndrome) are heterogeneous diseases with variable phenotypes, the involvement of other genes can be presumed.

The serine and threonine kinase, SGK1, which plays an important role in regulation of ion channels involved in pathogenesis of LQTS (KCNE1 and SCN5A) were suggested to influence the QT interval (Lang et al. 2003). Therefore after excluding all known LQT loci by either direct sequencing or segregation analysis, we screened all exons of the SGK1 gene. Only nonpathogenic, known polymorphisms were detected in all 12 exons of the SGK1 gene, thus excluding it as a candidate gene for this case of LQTS, too.

In conclusion, segregation analysis enables reliable genetic diagnosis and is a fast method after a required marker set has been established. Basing on the results of such studies, genetic counselling can be offered to affected and unaffected family members to diagnose individuals with LQTS as early as possible in regard to an appropriate therapy.

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REFERENCES

http://www.sads.ca/frameset1.html


