

**Mutation analysis of candidate genes *SCN1B*, *KCND3* and *ANK2* in patients with clinical diagnosis of long QT syndrome**

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Short title: Mutation analysis of candidate genes of long QT syndrome

### Summary:

The long QT syndrome (LQTS) is a monogenic disorder characterized by prolongation of the QT interval on electrocardiogram and syncope or sudden death caused by polymorphic ventricular tachycardia (torsades de pointes). In general, mutations in cardiac ion channel genes (*KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*) have been identified as a cause for LQTS. About 50-60% of LQTS patients have an identifiable LQTS causing mutation in one of mentioned genes. In a group of 12 LQTS patients with no identified mutations in these genes we have tested a hypothesis that other candidate genes could be involved in LQTS pathophysiology. *SCN1B* and *KCND3* genes encode ion channel proteins, *ANK2* gene encodes cytoskeletal protein interacting with ion channels. To screen coding regions of genes *SCN1B*, *KCND3*, and 10 exons of *ANK2* following methods were used: PCR, SSCP, and DNA sequencing. Five polymorphisms were found in screened candidate genes, 2 polymorphisms in *KCND3* and 3 in *SCN1B*. None of found polymorphisms has coding effect nor is located close to splice sites or has any similarity to known splicing enhancer motifs. Polymorphism G246T in *SCN1B* is a novel one. No mutation directly causing LQTS was found. Molecular mechanism of LQTS genesis in these patients remains unclear.

**Keywords:** *ANK2*, candidate genes, *KCND3*, long QT syndrome, *SCN1B*.

## Introduction

The long QT syndrome (LQTS) is a monogenic disorder characterized by prolongation of the QT interval on electrocardiogram and syncope or sudden death caused by polymorphic ventricular tachycardia (torsades de pointes).

In general, mutations in cardiac ion channel genes (*KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*) have been identified as a cause for LQTS (Splawski et al. 2000). Recently also mutation in cytoskeletal protein gene *ANK2* has been linked to LQTS in one French family (Mohler et al. 2003, 2004). About 50-60% of LQTS patients have an identifiable LQTS-causing mutation in one of the 5 most prevalent cardiac channel genes (Tester et al. 2005). In the other the disease mechanism remains unclear. The aim of this study is to test the hypothesis that other cardiac ion channel genes could be involved in LQTS pathophysiology: *SCN1B* gene encodes  $\beta 1$  subunit of cardiac  $\text{Na}^+$  channel and plays an important role in channel inactivation (Wallace et al. 2002). *KCND3* is a gene that encodes the  $\text{K}^+$  channel that underlies the potassium transient outward current  $I(\text{to})$  in the human ventricle (Dixon et al. 1996). Targeted mutational analysis of *ANK2* was also completed.

## Methods

### *Group of patients*

The group of patients was recruited from 25 unrelated individuals who were consecutively referred to the Department of Internal Medicine and Cardiology with suspicion on LQTS. They were examined clinically including bicycle ergometry. In all of them intermediate or high probability of LQTS was present based on diagnostic score (Schwartz et al. 1993). In this scoring system to various symptoms various point values are assigned, a value of 2-3 indicates intermediate probability and  $\geq 4$  points indicates high probability of LQTS diagnosis.

Informed consent was obtained from all the individuals and peripheral blood samples were taken. Mutational analysis of *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2* genes was performed

by methods published elsewhere (Splawski et al. 2000). In 11 patients the KCNQ1 gene mutations and in 2 patients the KCNH2 gene mutations were present. In the other 12 patients no mutation in the above mentioned LQTS related genes was found. These 12 individuals were subjects of this study - mutational analysis was extended to following genes: *SCN1B*, *KCND3* and exons 36 – 37 and 39 – 46 of *ANK2*. The brainspecific exon 38 was not analyzed in this study. Clinical characteristics of study subjects are summarized in Table 1.

#### *Genomic DNA Extraction and PCR*

Genomic DNA was extracted from samples of peripheral blood according to the standard protocol using DNA BloodSpin Kit and the standard chloroform/ethanol method. Eleven oligonucleotide primer pairs were used to amplify coding area of *KCND3*, as proposed by Postma et al. 2000, only the fifth segment of the first coding exon had different reverse primer (GGT CAT CCA GCT GCC CTC CAA CCT), which agrees with sequence of *KCND3* in NCBI database (accession number NT 019273). Ten primer pairs were disposed to amplify chosen exons of *ANK2* (from 36 to 46 except 38). These primers were taken from Mohler et al. 2003. For the PCR amplification of *SCN1B* gene coding and UTR region were suggested primers using Primer3 (Rozen and Skaletsky 2000), (Table 2.). Sequences of these primers agree with sequence of *SCN1B* in Ensembl database (© 2006 [WTSI](#) / [EBI](#), ENSG00000105711).

#### *SSCP analysis*

For the analysis of *KCND3* and *ANK2* 3 µL aliquots of the amplified sample were mixed with 5 µL of bromphenol blue loading dye, for analysis of *SCN1B* 1 µL aliquots of the amplified sample were mixed with 3 µL of bromphenol blue loading dye and 6 µL of destilated wather. Samples were subsequently denatured by heating at 94°C for 5 minutes, and placed into cold water to avoid renaturation. Then the samples were loaded on 9% (*KCND3*), 11% (*ANK2*) and 10% (*SCN1B*) non-denaturing PAA gel. Electrophoresis was performed at 200 V and 10°C for 3 hours

(*KCND3* and *ANK2*) and at 120 V and 18°C for 12 hours (all exons of *SCN1B* except exon 3).

Exon 3 was performed at 10°C.

### *DNA sequencing*

Genomic DNA obtained from 12 unrelated LQTS patients was screened for mutations in the coding regions of the genes *SCN1B*, *KCND3* and a part of the gene *ANK2* using SSCP analysis. If we have found three or four single strand bands in the SSCP patterns then we have done sequencing with forward and reverse sequencing primers. For purification of the amplified samples MinElute PCR Purification Kit (QIAGEN) was used. For cycle sequencing we used Big Dye Terminator Kit (Applied Biosystems) and for purification of samples after cycle sequencing DyeEx2.0 Spin Kit (Qiagen). Exons were sequenced by instrument ABI PRISM 310 (Applied Biosystems, USA).

## **Results**

The result of the mutation screening is listed in the Table 3. None of found SNPs led to amino acid changes, were located close to splice sites or had any similarity to known splicing enhancer motifs.

## **Discussion**

About 50-60% of LQTS patients have an identifiable LQTS-causing mutation in one of the five most prevalent cardiac channel genes. In the other the pathophysiology remains unknown. Possible mechanisms include involvement of other ion channels but also regulatory and other proteins. Recently in several new genes possible link to LQTS was found: *KCNJ2* mutations in Andersen-Tawil syndrome (Tawil et al. 1994, Plaster et al. 2001), *CACNA1c* mutation in Timothy syndrome (Splawski et al. 2005), *CAV3* and *SCN4B* mutations in some LQTS patients (Vatta et al.

2006, Medeiros-Domingo et al. 2007). These are either rare complex neurological disorders (prolonged QT interval being not the leading symptom) or there are only anecdotal cases. Thus it is still discussed if these diseases should be included among LQTS.

In this study we tested the hypothesis of other ion channels involvement. Three candidate genes were chosen for mutational analysis in 12 patients with clinical diagnosis of LQTS. *KCND3* encodes the K<sup>+</sup> channel that underlies the potassium transient outward current I<sub>to</sub> in the human ventricle (Dixon et al. 1996). I<sub>to</sub> is especially important during the early phase of repolarization, as it sets the plateau voltage of both action potential (Tseng 1999).

In contrast to other genes, *ANK2* does not encode an ion channel but encodes a structural protein called ankyrin B that is most likely implicated in ion channels anchoring to the cellular membrane. A clue to the basis of this variant emerged from a study by Chauhan et al. 2000, in which sodium channel activity in mice lacking ankyrin B – not an ion channel protein, but an adaptor protein that associated with cytoskeletal interactions – was altered. In this study, the ankyrin B spectrin-binding domain encoded by exons 36 and 37 of *ANK2* and the entire C-terminal domain encoded by exons 39-46 were screened. The brain-specific exon 38 and membrane-binding domain were not analyzed in this study.

*SCN1B* encodes the voltage-gated Na<sup>+</sup>-channel  $\beta$ 1 subunit (SCN1B). *SCN1B* is expressed in brain, skeletal muscle and heart. The  $\alpha$  subunit alone can display functional channel properties, but requires the  $\beta$  subunits to modulate Na<sup>+</sup>-channel inactivation. Mutations in Na<sup>+</sup>-channel genes are known to cause paroxysmal excitability phenomena in skeletal muscle (myotonia, periodic paralysis) and heart (long QT syndrome) (Wallace et al. 2002). In the mutation database (Stenson et al. 2003) only one mutation in the SCN1B gene has been published. It is the substitution mutation C387G (on protein level C121W). This mutation causes a disruption of disulfide bridge and may alter the secondary structure of the extracellular domain (Wallace et al. 2002).

In our study, two allelic variants were found in *KCND3*. All variants were single nucleotide polymorphisms (SNPs) in coding regions. None of the coding SNPs led to amino acid changes,

were located close to splice sites or had any similarity to known splicing enhancer motifs (Liu et al. 1998). All SNPs in *KCND3* were described in normal population too (Frank-Hansen et al. 2005). We assume that all the changes detected in the LQTS patient material were normal variants.

No allelic variant has been found in *ANK2*.

Three allelic variants (all SNPs) were found in *SCN1B* gene, two of them T9204C and A9248C were detected previously (Ensembl database, © 2006 [WTSI](#) / [EBI](#), ID:ENSG00000105711), G246T has not been published yet. By the help of ESEfinder Release 2.0 (Cartegni et al. 2003) we have found that G246T and T9204C substitution does not have any influence on DNA splicing. The substitution A9248C causes loss of one binding place for SC35 and SRp40 splicing proteins.

The fact that no mutations have been found, can have several reasons. The SSCP methodology can detect 88 and 90 % of mutations (Fodde and Losekoot 1994) and therefore we can not fully exclude the presence of mutations within analysed genes in these individuals. The SSCP method is sensitive for the detection of point mutations or small deletions, but it can possibly miss large deletions. The SSCP method is the most widely used screening method because of its relative simplicity and low costs. Compared to more advanced methods, such as TmHPLC (Temperature Modulated High-Performance Liquid Chromatography) the SSCP has relatively low sensitivity.

LQTS causing mutations could be also present in such regions of ion channel genes which are usually not examined. Alternatively, intronic mutations or mutation in the promoter and regulatory regions could be responsible for the disease symptoms. The possibility of other than ion channel genes being involved in the etiology of the long QT syndrome should not be refused, because of huge genetic heterogeneity of this disorder (see the above mentioned genes with recently discovered possible link to QT interval prolongation). This heterogeneity makes genetic testing much more difficult than in case if a single gene were responsible for the disease.

Molecular mechanism of LQTS genesis in our group of patients remains unclear.

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Table 1. Clinical characteristics of study subjects

Patient	Age	Sex	QTc (ms)	Syncope	Torsades de pointes	LQTS in family	Diagnostic score (points)
IB	64	F	0,48	0	0	+	4
MK	62	F	0,5	+	+	+	7
MI	42	F	0,48	+	0	+	5
LP	13	M	0,47	0	0	+	3
MZ	25	F	0,5	+	0	+	5
MK	25	M	0,47	+	0	+	4
PD	25	F	0,5	+	0	0	4
MD	58	F	0,54	+	+	0	6
JJ	46	M	0,47	0	0	+	3
AL	20	F	0,49	+	0	0	4
MS	16	F	0,48	+	+	0	5
ŠK	17	F	0,48	+	0	+	5

Sex: M – male, F – female, QTc – QT interval corrected to heart rate according to Bazett formula ( $QTc = QT/RR^{1/2}$ ), diagnostic scoring: 2-3 points – intermediate probability of LQTS,  $\geq 4$  points – high probability of LQTS

Table 2. Oligonucleotide primers for detection of mutations in <i>SCN1B</i>			
Exon	Size, bp	Forward Primer Sequence	Reverse Primer Sequence
1.1	250 bp	5'-GGTGCACCTAGCGGATGT-3'	5'-TATTAATAGCGGGGCGAGAG-3'
1.2	284 bp	5'-GCTCCCGGGGACATTCTA-3'	5'-AACTTCTGAAGCTGACTTGG-3'
2	186 bp	5'-CTGTCCCCACAGTGTCT-3'	5'-CGCACACCTTGACAAACT-3'
3	266 bp	5'-CTGGCTACCCCTAGATCCT-3'	5'-ACCCGACTCACCTTTGTC-3'
4	200 bp	5'-CTGGGCTACCCCTTAACC-3'	5'-ACCTCCCAGCCACCCTACT-3'
5	196 bp	5'-GGTCTGATGATGGGGTCACT-3'	5'-GCAAGAGAGGGGGAATTAGG-3'
6.1	267 bp	5'-CCTAATTCCCCCTCTCTTGC-3'	5'-AGCGGCAGTATTGCTTTACC-3'
6.2	289 bp	5'-CACTTTCGCCTCCTCCAG-3'	5'-CGGAGTGGGTCCCAGAAC-3'
6.3	275 bp	5'-GATGATGGGCTGGAGCAG-3'	5'-GGGGCTCACAATCGAAACTA-3'

Table 3. Single nucleotide polymorphisms in <i>SCN1B</i> , <i>KCND3</i> and <i>ANK2</i> genes			
<b>patient</b>	<b><i>SCN1B</i></b>	<b><i>KCND3</i></b>	<b><i>ANK2</i></b>
<b>I</b>	non	non	non
<b>II</b>	non	C264T/non	non
<b>III</b>	IVS+15 G>T/non = G246T/non	non	non
<b>IV</b>	IVS+15 G>T/non = G246T/non	C264T/non	non
<b>V</b>	non	non	non
<b>VI</b>	non	non	non
<b>VII</b>	non	C264T/C264T	non
<b>VIII</b>	non	non	non
<b>IX</b>	non	G669C/non	non
<b>X</b>	non	non	non
<b>XI</b>	T9204C/T9204C, A9248C/A9248C	non	non
<b>XII</b>	non	non	non