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Denaturing high-performance liquid chromatography screening of the long QT syndrome-related cardiac sodium and potassium channel genes and identification of novel mutations and single nucleotide polymorphisms

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Abstract Mutations in cardiac potassium and sodium channel genes are responsible for several hereditary cardiac arrhythmia syndromes. We established a denaturing high-performance liquid chromatography (DHPLC) protocol for rapid mutation screening of these genes, and reported mutations and variations identified by this method. We included 28 patients with Brugada syndrome, 4 with congenital long QT syndrome (LQTS),

11 with drug-induced LQTS, 4 with idiopathic ventricular fibrillation, and 50 normal volunteers. Polymerase chain reactions were performed to amplify the entire coding region of these genes. DHPLC was used to screen for heteroduplexes then DNA sequencing was performed. With this method, we identified the mutation(s) in all four patients with congenital LQTS (*KCNQ1* A341V, *KCNH2* N633D, *KCNH2* 2768Cdel and

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KCNE1 K70 N Y81C double mutations). We also identified the *SCN5A* A551T mutation in 1 of the 28 patients with Brugada syndrome. All the above-mentioned mutations were novel except *KCNQ1* A341V. No mutations were identified in patients with drug-induced LQTS or idiopathic ventricular fibrillation. In total, 25 single nucleotide polymorphisms were identified, 10 of which were novel. In conclusion, DHPLC is a sensitive and rapid method for detection of cardiac sodium and potassium channel gene mutations.

Keywords Na-channel · K-channel · Long QT syndrome · Sudden death

Introduction

Congenital long QT syndrome (LQTS) is a hereditary arrhythmogenic disorder characterized by prolonged cardiac repolarization and potentially lethal ventricular arrhythmias (*torsade des pointes*). Although some patients may remain asymptomatic, others may suffer from attacks of ventricular arrhythmias causing syncope, seizure or even sudden cardiac death. Past genetic studies have demonstrated genetic heterogeneity in congenital LQTS, and at least six loci have been identified (Priori 2004). The first identified locus (LQT1) was mapped to 11p15.5 and the gene responsible is *KCNQ1*. Later studies identified other responsible genes, including *KCNH2* (LQT2), *SCN5A* (LQT3), *ANK2* (LQT4), *KCNE1* (LQT5) and *KCNE2* (LQT6). Most of these genes encode cardiac ion channels with the exception of *ANK2*, which encodes an anchoring protein for membrane-associated proteins. *KCNQ1* assembles with *KCNE1* to form the cardiac I_{Ks} channel. *KCNH2* and *KCNE2* are the α and β subunits of I_{Kr} , respectively. *SCN5A* encodes the pore-forming subunit of the cardiac sodium channel. Mutations in these cardiac ion channels cause alterations in channel function leading to prolonged cardiac repolarization and clinical LQTS.

Mutations in different long QT genes can all cause LQTS, but with different triggers for attacks and with differing prognosis (Priori 2004). Mutations in different genes may require different treatment strategies although they all have QT prolongation. Furthermore, mutations in these long QT-related genes can result in diseases other than LQTS. *KCNQ1* is also responsible for familial atrial fibrillation, while *SCN5A* mutations are responsible for Brugada syndrome, congenital cardiac conduction defect and sudden infant death syndrome (Priori 2004). Mutations in these genes might also be responsible for drug-induced LQTS (Paulussen et al. 2004).

Detection of mutation in these genes is therefore important for genetic diagnosis and screening of these "cardiac channelopathies." However, polymerase chain reaction (PCR) and DNA sequencing strategies to detect mutations are time- and effort-consuming. For instance,

the *SCN5A* gene has 28 exons and the coding region comprises more than 6,000 bp. *KCNQ1* and *KCNH2* have 16 and 15 exons, respectively. Therefore, a reliable mutation screening method would greatly enhance the efficacy of identifying mutations. Single strand conformation polymorphism analysis is a common method for mutation screening (Orita et al. 1989). However, it has a maximal sensitivity of around 80%. In the present study, we set up an economic denaturing high-performance liquid chromatography (DHPLC) technique to screen for variations in these genes. Here, we report a detailed protocol as well as the mutations and polymorphisms identified using this method.

Materials and methods

Human subjects

The human subjects included (1) 28 patients with Brugada syndrome, (2) 4 patients with congenital LQTS, (3) 11 patients with drug-induced LQTS, (4) 4 patients with idiopathic ventricular fibrillation of non-Brugada type, and (5) 50 volunteer individuals with normal ECG and no clinical cardiac arrhythmias. The investigation conforms to the principles outlined in the Declaration of Helsinki. This study was approved by the Institutional Reviewing Board and informed consent was obtained from all subjects. Peripheral blood samples were collected and genomic DNA extracted using a modified proteinase K method (Qiagen, Hilden, Germany).

PCR amplification

PCR was performed in thin-walled PCR tubes in a total volume of 25 μ l containing 100 ng genomic DNA, 0.12 μ M of each primer, 100 μ M dNTPs, 0.5 units of AmpliTaq Gold (PE Applied Biosystems, Foster City, CA) and 2.5 μ l GeneAmp 10 \times buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), in 2 mM $MgCl_2$ as provided by the manufacturer. Amplification was performed in a multiblock system thermocycler (ThermoHybaid, Ashford, UK). PCR amplifications were performed with an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and an elongation step at 72°C for 1 min unless otherwise specified in Electronic Supplementary Material (S1). The final extension step was 72°C for 10 min. The primer sequences for the amplifications are listed in S1 together with the optimal conditions. The primers were designed to cover the entire coding region of the genes based on information available in the literature (Syrris et al. 2001) as well as sequence data in GenBank (accession numbers: AJ006345 for *KCNQ1*, AC006343 for *KCNH2*, AC137587 for *SCN5A*, AP001720 for *KCNE1* and AF302095 for *KCNE2*). The primers were chosen to

match the requirement for subsequent DHPLC analysis as judged by a computer program from Transgenomic (San Jose, CA). Multiple primer pairs were used for large exons.

DHPLC analysis

The DHPLC system used for analyzing heteroduplexes was a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic). DHPLC was carried out on automated HPLC instrumentation equipped with a DNASep column (Transgenomic). DHPLC-grade acetonitrile (9017-03, JT Baker, Phillipsburg, NJ) and triethylammonium acetate (TEAA, Transgenomic, Crewe, UK) were used to constitute the mobile phase. The mobile phases comprised 0.05% acetonitrile in 0.1 M TEAA (eluent A) and 25% acetonitrile in 0.1 M TEAA (eluent B). For heteroduplex detection, crude PCR products, subjected to an additional 3 min 95°C denaturing step followed by gradual reannealing from 95 to 65°C over a period of 30 min prior to analysis, were eluted at a flow rate of 0.9 ml/min. The start- and end-points of the gradient by mixing eluents A and B, and the temperature required for successful resolution of heteroduplex molecules, was adjusted by using an algorithm provided with WAVEmaker system control software version 4.1.42 (Transgenomic). PCR product (8 µl) was injected for analysis for each run. Individual analytical gradient conditions for DHPLC runs are described in Electronic Supplementary Material (S2) and are expressed as a percentage of eluent B. The flow rate was 0.9 ml/min, and the UV detector was set to 260 nm. Heterozygous profiles were identified by visual inspection of the chromatograms on the basis of the appearance of additional, earlier eluting, peaks. Corresponding homozygous profiles show only one peak.

For amplicons of some exons, two melting domains were predicted according to the base sequence. Two DHPLC conditions were applied to these amplicons for higher sensitivity of detection (S2).

DNA sequencing

Amplicons were purified by solid-phase extraction and were bidirectionally sequenced with the PE Biosystems *Taq* DyeDeoxy terminator cycle sequencing kit (PE Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing reactions were separated on a PE Biosystems 373A/3100 sequencer. Sequencing reactions were performed for the amplicons whose DHPLC elution pattern revealed the presence of heteroduplex. If a mutation in *SCN5A* was not identified for patients with Brugada syndrome, DNA sequencing reactions were performed irrespective of the *SCN5A* DHPLC patterns to verify the accuracy of the DHPLC results. Sequencing results were compared with the sequence in GenBank (accession numbers as above). The adenosine of the start codon (ATG) was numbered as the first nucleotide when expressing genetic variations.

Results

Using the DHPLC method, we successfully identified 28 heteroduplex patterns in the PCR products. Figure 1 illustrates these heteroduplex patterns and their corresponding homoduplex patterns. Further DNA sequencing reactions confirmed the presence of heterozygous variations in these amplicons. We define "mutation" as base alterations that were not found in volunteer samples, and "single nucleotide polymorphisms (SNPs)" as base alterations that were found in

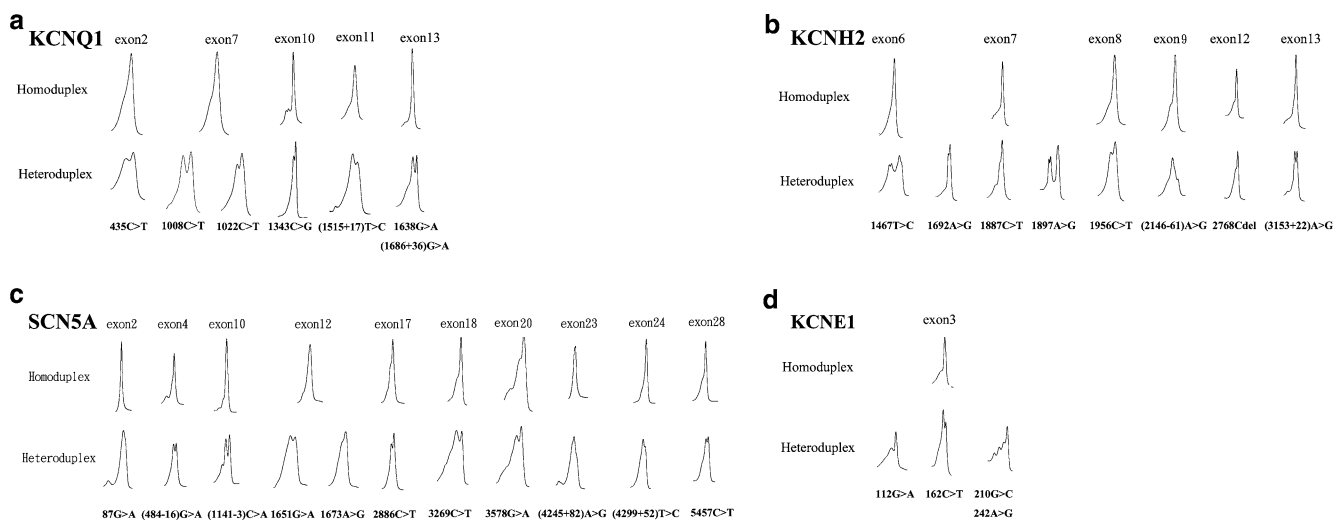


Fig. 1a–d Denaturing high-performance liquid chromatography (DHPLC) patterns of heteroduplex PCR products of *KCNQ1* (a), *KCNH2* (b), *SCN5A* (c) and *KCNE1* (d) genes. For homoduplexes,

the elution pattern revealed a single peak. On the other hand, heteroduplexes revealed additional elution peaks due to a difference in melting temperature

Table 1 Mutations identified in study patients. *AA* Amino acid change, *BS* Brugada syndrome, *CLQTS* congenital long QT syndrome

Gene and location	Base change	AA	Disease	First reported
<i>KCNQ1</i> exon7	1022C>T	A341V	CLQTS	Wang et al. (1995)
<i>KCNH2</i> exon7	1897A>G	N633D	CLQTS	This report
<i>KCNH2</i> exon12	2768Cdel	Frame shift	CLQTS	This report
<i>SCN5A</i> exon12	1651G>A	A551T	BS	This report
<i>KCNE1</i> exon3	210G>C	K70N	CLQTS ^a	This report
<i>KCNE1</i> exon3	242A>G	Y81C	CLQTS ^a	This report

^aThe same patient with double mutation

volunteer samples. We identified six mutations (Table 1) and 25 SNPs (Table 2) in these genes. Because there were two tightly-linked SNPs in three amplicons, only 28 heteroduplex patterns were identified. Of these, ten of the SNPs and five of the mutations were novel.

Mutations

In total, six mutations were found in the present study (Table 1). There were four patients with congenital LQTS. The mutation responsible was identified in all four patients (*KCNQ1* in 1, *KCNH2* in 2 and *KCNE1* in 1). The patient with the *KCNQ1* A341V mutation presented with an attack during swimming, which is characteristic for LQT1 disease. The patient with the *KCNH2* N633D mutation had clusters of attacks during pregnancy. The other patient with a *KCNH2* mutation had a deletion of the 2,768th base, resulting in a frame shift. The patient with *KCNE1* disease had double mutations in this gene (K70N and Y81C). Subsequent TA cloning and

sequencing confirmed that the two mutations are located on different chromosomes. The clinical attacks were sympathetic dependent, and responded well to better adrenergic-blocking agents. There was no hearing impairment. In the 11 patients with drug-induced LQTS, no mutations were detected in any of the genes.

The present study included 28 patients with Brugada syndrome. We identified a unique heteroduplex pattern in exon 12 of *SCN5A*, which was not found in normal individuals. Further DNA sequencing revealed the presence of a mutation. The 1651G>A mutation resulted in an amino acid change from alanine to threonine at position 551 (A551T). The patient with the 1651G>A mutation had his first syncopal attack at 46 years of age. His resting ECG showed persistent co-ved-type Brugada ECG in the right precordial leads. This patient's son also carried the same mutation while his daughter did not; neither had ECG or clinical manifestation of Brugada syndrome. For the other 27 patients with Brugada syndrome, no mutation was identified in *SCN5A* with DHPLC. Direct DNA

Table 2 Single nucleotide polymorphisms and their incidences in the normal population and in patients with drug-induced long QT syndrome. *AA* Amino acid change, *DLQTS* incidence in drug-induced long QT syndrome, *Normal* incidence in normal population

Gene and location	Base change	AA	Normal (%)	DLQTS (%)	First reported
<i>KCNQ1</i> exon2	435C>T	–	22	18	Itoh et al. (1998)
<i>KCNQ1</i> exon7	1008C>T	–	2	9	This report
<i>KCNQ1</i> exon10	1343C>G	P448R	28	0	Splawski et al. (2000)
<i>KCNQ1</i> IVS11	(1515+17)T>C	–	6	0	This report
<i>KCNQ1</i> exon13	1638G>A	–	46	36	Lee et al. (1997)
<i>KCNQ1</i> IVS13	(1686+36)G>A	–	46	36	Jongbloed et al. (2002)
<i>KCNH2</i> exon6	1467T>C	–	48	27	Akimoto et al. (1998)
<i>KCNH2</i> exon6	1539C>T	–	48	27	Akimoto et al. (1998)
<i>KCNH2</i> exon7	1692A>G	–	18	18	Akimoto et al. (1998)
<i>KCNH2</i> exon7	1887C>T	–	2	0	This report
<i>KCNH2</i> exon8	1956C>T	–	16	9	Larsen et al. (1999)
<i>KCNH2</i> IVS8	(2146-61)A>G	–	14	9	This report
<i>KCNH2</i> IVS13	(3153+22)A>G	–	38	27	Larsen et al. (2001)
<i>SCN5A</i> exon2	87G>A	–	42	18	Takahata et al. (2003)
<i>SCN5A</i> IVS4	(484-16)G>T	–	2	0	This report
<i>SCN5A</i> IVS9	(1141-3)C>A	–	14	0	This report
<i>SCN5A</i> exon12	1673A>G	H558R	18	0	Iwasa et al. (2000)
<i>SCN5A</i> exon17	2886C>T	–	2	0	This report
<i>SCN5A</i> exon18	3269C>T	P1090L	4	0	Iwasa et al. (2000)
<i>SCN5A</i> exon20	3578G>A	R1193Q	2	0	Takahata et al. (2003)
<i>SCN5A</i> IVS23	(4245+82)A>G	–	24	0	This report
<i>SCN5A</i> IVS24	(4299+52)T>C	–	32	18	This report
<i>SCN5A</i> exon28	5457C>T	–	48	54	Wang et al. (1995)
<i>KCNE1</i> exon3	112G>A	G38S	30	9	Lai et al. (1994)
<i>KCNE1</i> exon3	162C>T	–	2	0	This report

sequencing was performed for all PCR products of *SCN5A* irrespective of their DHPLC patterns. This confirmed that there was no mutation in the *SCN5A* gene. For idiopathic ventricular fibrillation other than Brugada or long QT type, no mutations in LQTS-related channel genes were identified.

SNPs identified in normal individuals and in drug-induced LQTS

We screened for base variation in 50 subjects (100 alleles) with no ECG abnormalities or clinical arrhythmias. We identified 22 heteroduplex DHPLC elution patterns. Further DNA sequencing identified 25 SNPs, 10 of which were novel. Table 2 shows the base variations as well as their incidence of heterozygosity. All the variations are SNPs and ten of them have never been reported before. Among the 25 SNPs, 8 were intronic SNPs, 12 were synonymous SNPs in the coding region, and 5 were non-synonymous SNPs with amino acid changes. In the 11 patients with drug-induced LQTS, 14 SNPs were identified. However, all these SNPs were also observed in normal individuals (Table 2).

Discussion

Cardiac channelopathies have been recognized as an important cause of various cardiac arrhythmias; most of these arrhythmias are potentially lethal. Molecular genetic diagnosis contributes significantly to the identification of subjects at risk and to further management of patients with these diseases. DHPLC has been applied to detection of mutation in several genes with high accuracy (Xiao and Oefner 2001). However, its application to cardiac channelopathy genes has rarely been reported, with only one report on DHPLC screening of LQTS-related cardiac potassium channel genes (Jongbloed et al. 2002). DHPLC screening of the entire *SCN5A* gene has not previously been reported. In the present study, we set up a protocol for heteroduplex screening using DHPLC. With this method, we identified six disease-causing mutations and 25 SNPs. We demonstrated DHPLC to be a powerful tool in screening for variations in LQTS-related cardiac potassium and sodium channel genes. This will greatly enhance the potential for molecular diagnosis of cardiac channelopathies. The novel mutations and SNPs reported here also add to the compendium of the *SCN5A* variation database.

Patients with Brugada syndrome

Brugada syndrome is an autosomal dominant hereditary sudden cardiac death syndrome. Molecular genetic studies have demonstrated that a mutation in the *SCN5A* gene is responsible for this disease in 15–20% of patients (Priori et al. 2000). To date, more than 30 distinct mutations have been reported, most of these in

Western populations. In the present study, we identified a novel *SCN5A* mutation in Brugada syndrome, and found the incidence of *SCN5A* mutation in patients with Brugada syndrome to be 3.6% (1 in 28). Chen et al. (2004) reported the incidence of *SCN5A* mutation as 0% in 48 unrelated Chinese patients with Brugada syndrome. On the other hand, Mok et al. (2004) reported an incidence of 14% in 36 Chinese patients with Brugada syndrome. This discrepancy could be resolved by more genetic studies in Chinese patients. In another report by Schulze-Bahr et al. (2003), the incidence of *SCN5A* mutation differed significantly between patients with familial or sporadic Brugada syndrome; the incidence in familial Brugada syndrome was 38%, while the incidence in sporadic cases was 0%. All 28 patients in the present study had sporadic Brugada syndrome. The low incidence of mutation (1/28) of the *SCN5A* gene in our patients with Brugada syndrome indicates that all our patients represent sporadic cases.

SNPs in the general population

Twenty-five SNPs were identified in normal individuals. Of these, 15 have been reported previously (Akimoto et al. 1998; Itoh et al. 1998; Iwasa et al. 2000; Lai et al. 1994; Larsen et al. 1999, 2001; Lee et al. 1997; Splawski et al. 2000; Takahata et al. 2003) and 10 were novel. When compared to previous reports, we found that there were significant inter-ethnic differences. For instance, *SCN5A* 1673A > G (H558R) is a frequently reported SNP. This SNP results in a decrease of sodium current when expressed in vitro (Makielski et al. 2003). It has also been reported that this SNP interacts with other variations of the *SCN5A* gene, altering susceptibility to cardiac arrhythmias (Viswanathan et al. 2003; Ye et al. 2003). The frequency of this allele in Western studies ranged from 20 to 30%. In the present study, the incidence (18%) was slightly lower compared to previous reports. Splawski et al. (2002) reported S1102L as another common SNP of the *SCN5A* gene. This *SCN5A* variant was identified in 13.2% of African Americans. Functional studies revealed a change in opening kinetics, and this variant form is associated with cardiac arrhythmia death in African Americans. However, this variation was not found in the Chinese population in the present study. On the other hand, a frequent SNP, 87G > A, reported in Japanese and Chinese populations (Mok et al. 2004; Takahata et al. 2003) has not been seen in Western populations. This SNP might be specific for the Asian population. The SNP (1113-3)C > A, an SNP in intron 9 within a donor-recipient mRNA splice site, has not been reported in other ethnic groups. It is unclear whether the change from CAG into AAG near exon 10 affects the splicing process.

Patients with congenital LQTS

Patients with LQTS have prolonged cardiac repolarization and *torsade des pointes* in common but they differ in

the triggering factors for attacks (Priori 2004). They also differ in prognosis and frequency of attack as well as response to beta-blocker treatment. Therefore, genetic diagnosis is essential in designing gene-specific treatment for patients with LQTS. Another issue in clinical management of LQT families is the wide range of QTc intervals among subjects carrying mutations. Some carriers of the mutation may have apparently normal QT intervals although they are actually at risk of developing lethal ventricular arrhythmias. Genetic diagnosis can therefore be applied to identify these patients and protect them from ignorance of their situation.

We included four patients with congenital LQTS in the present study. Mutations were detected in all four patients by DHPLC screening (*KCNQ1* A341V, *KCNH2* N633D, *KCNH2* 2768Cdel and *KCNE1* K70 N plus Y81C double mutations). The *KCNQ1* A341V mutation has been reported to be responsible for congenital LQTS in many different ethnic groups. The *KCNH2* N633D mutation was novel. However, a mutation at the same amino acid has been reported (N633S) (Satler et al. 1998). This amino acid is highly conserved among many species. For *KCNH2* 2768Cdel, a frame shift occurs and it is very unlikely that the truncated protein has normal function. For the *KCNE1* double mutation, both amino acids are highly conserved. Site-directed mutagenesis has revealed the functional importance of amino acid Y81 (Abitbol et al. 1999). For the above reasons, these base variations are considered disease-causing mutations. Further functional studies on these mutations will be worthwhile and will help elucidate their pathophysiological effects.

Patients with drug-induced LQTS

While congenital long QT is a rare disease, drug-induced LQTS is a more prevalent disorder. Genetic background may play an important role in the pathogenesis of drug-induced LQTS and this kind of adverse drug reaction can sometimes be idiosyncratic. It has been reported that drug-induced LQTS is a “*forme fruste*” of congenital LQTS. Mutations with minor functional changes are compatible with normal life and *torsades des pointes* happen only upon use of offending agents. Past research targeted at identifying variations in all LQT genes has demonstrated that 5–15% of patients have minor mutations in these long QT genes (Paulussen et al. 2004). In a large-scale screening study of 95 patients, Yang et al. (2002) reported that the incidence of *SCN5A* SNPs was not significantly different between patients and a control group. Although they also identified three novel variations of *SCN5A* gene in these patients, a causal relationship was not confirmed because functional expression of the mutated channels revealed no alterations in I_{Na} . In the present study, we also identified 14 SNPs in patients with drug-induced LQTS. Among the 14 SNPs, 13 resulted in no amino acid change. The only

non-synonymous SNP was LQT5 G38S. Functional expression studies have demonstrated that there were no changes in the electrophysiological properties of this allele (Lai et al. 2000). Furthermore, all the SNPs were present in normal individuals. Therefore, our results suggest that common SNPs in LQT genes are not associated with drug-induced LQTS.

Conclusions

The DHPLC technique was applied to screen LQTS-related cardiac potassium and sodium channel genes for genetic variations. We established a detailed protocol and demonstrated the accuracy of this method. The DHPLC method is simple, rapid, economic, and highly sensitive. Using this method, six mutations and 25 SNPs were identified.

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