Mutation Analysis of KCNQ1, KCNH2 and SCN5A Genes in Taiwanese Long QT Syndrome Patients
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SUMMARY
Long QT syndrome (LQTS) is a congenital arrhythmicogenic disorder that may lead to life-threatening ventricular arrhythmias, syncope, and sudden cardiac death (SCD). The affected patients have QTc-interval prolongation (> 440 ms in men and > 460 ms in women). The incidence has been estimated to be as high as 1 in 2,000 persons.1

To date, 15 genes have been linked to LQTS and more than 700 disease-causing mutations have been found. The total number of mutations is likely to increase.2 Three are major LQTS genes (KCNQ1, KCNH2 and SCN5A) and 10 are minor LQTS-susceptibility genes (AKAP9, CACNA1C, CALM1, CALM2, CAV3, KCNE1, KCNE2, KCNJ5, SCN4B, and SNTA1).3 Approximately 75% of patients with a clinically robust LQTS have mutations in one of the 3 major LQTS-causing genes (KCNQ1, 35%; KCNH2, 30%; and SCN5A, 10%). Less than 5% of LQTS cases have mutations in the 10 minor LQTS-susceptibility genes. These mutations can cause LQTS by altering cardiac repolarization currents.

KCNQ1 is located at chromosome 11p15.5, the gene encoding I\textsubscript{Ks} potassium channel \(\alpha\) subunit (K\textsubscript{vLQT1}, K\textsubscript{v7.1}). KCNH2 is located at chromosome 7q35-36, the gene encoding \(\alpha\) subunit (HERG, K\textsubscript{v11.1}). SCN5A is located at chromosome 3p21-p24, the gene encoding cardiac sodium channel \(\alpha\) subunit (Na\textsubscript{1.5}).4 These proteins are involved in the execution of the cardiac action potential (AP), directly or indirectly. LQTS is a result of a prolongation of the repolarization phase of the AP, caused by an increase in depolarizing inward currents (I\textsubscript{Na}) and/or a decrease in repolarizing outward currents (I\textsubscript{Ks} and I\textsubscript{Kr}). The delayed repolarization results in the appearance of early after-depolarizations, because of enhancement of the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger and the L-type Ca\textsuperscript{2+} channel.5 These may increase the risk of ventricular arrhythmias.

Key words: High resolution melting, Genetic, Cardiac disease, Sudden death

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Received for publication December 26, 2014. Revised and accepted January 26, 2015.
Released in advance online on J-STAGE June 26, 2015.
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tion scanning of genes for which large samples must be investigated.

In order to understand the spectrum of mutations causing LQTS in Taiwanese subjects, we screened 5 LQTS patients and the family members of 3 LQTS patients for mutations in the 3 most frequently implicated LQTS genes, KCNQ1, KCNH2 and SCN5A by HRM analysis and direct DNA sequencing.

**METHODS**

Sample preparation and DNA isolation: This study was approved by the Institutional Review Board of China Medical University Hospital (Taichung, Taiwan). Five patients were diagnosed recently with LQTS. The demographics and clinical features of the 5 patients with LQTS and the family members of 3 LQTS patients are given in Supplemental Table I. A characteristic ECG recording of torsades de pointes ventricular tachycardia was documented in one patient at an attack of syncope (Supplemental Figure 1). Genomic DNA samples were extracted from peripheral whole blood using Nucleospin Blood Kit (Macherey-Nagel, GmbH & Co. KG) according to the manufacturer’s protocol. Quantification of all DNA samples was made with a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Design of primers for HRM assay: We designed the primer sets on the KCNQ1, KCNH2, and SCN5A DNA sequences (GenBank accession number NM_000218, NM_000238 and NM_000335). In this study, all of the primers for HRM analysis were newly selected using Primer3 software. The coding exons of KCNQ1, KCNH2, and SCN5A were amplified using the intronic primers reported in Supplemental Tables II, III, and IV.

PCR amplification: Real-time PCR amplification and HRM analysis was performed with a LightCycler® 480 Real-Time PCR Systems (Roche Diagnostics, Penzberg, Germany) using a LightCycler® 480 High Resolution Melting Master kit (Roche Diagnostics) according to the manufacturer’s protocol.

PCR amplifications were carried out in a final volume of 10 μL containing 5 μL of Master mix (Taq polymerase, nucleotides and the High-Resolution Melting Dye), 0.25 μM of each primer, 30 ng DNA, and 2.5 mM MgCl₂. The PCR conditions were: pre-incubation step at 95°C for 10 minutes for activation of the polymerase, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension step at 72°C for 15 s. The melting program included 3 steps: denaturation at 95°C for 1 minutes, renaturation at 40°C for 1 minute, and subsequent melting that consisted of a continuous reading of fluorescence from 60°C to 90°C at the rate of 25 acquisitions per degree.

Gene scanning: HRM curve analysis was performed using LightCycler® 480 Gene Scanning Software, version 1.5. Gene scanning information comprises 3 steps: 1) normalization of melting curves, which involves equaling to 100% of the initial fluorescence and to 0% of the fluorescence remnant after DNA dissociation: 2) shifting of the temperature axis of the normalized melting curves to the point where the entire double-stranded DNA is completely denatured: and 3) the difference plot analyzes the differences in melting curve shape by subtracting the curves from wild-type and mutation DNA, therefore, differences in the plots help cluster the samples into groups.

**RESULTS**

HRM analysis and direct sequencing of LQTS-causing genes of KCNQ1: Mutational screening of KCNQ1 coding sequences by HRM analysis required the investigation of 16 coding exons. PCR optimization of 2 exons (1 and 16) did not obtain a sufficient amplicon quality for subsequent HRM analysis. Consequently, these 2 exons were analyzed by direct sequencing.

We identified one mutation and 3 polymorphisms of the KCNQ1 gene (Supplemental Table V). The direct sequencing confirmed the HRM results and found 3 genomic variants, c.435C > T (p.I145T) in exon 2 for the case 2 patient’s daughter (Supplemental Figure 2A), c.926C > T (p.T309I, pathogenic mutation) in exon 7 for case 2 (Supplemental Figure 2B), and c.1638G > A (p.S546S) in exon 13 for cases 1, 4, 5 and case 3 patient’s son (Supplemental Figure 2C). Direct sequencing of the two non-optimized exons (exons 1 and 16) detected a substitution c.1927G > A (p.G643S) in exon 16 for cases 2 and 4 (Supplemental Figure 2D).

HRM analysis and direct sequencing of LQTS-causing genes of KCNH2: Mutational screening of KCNH2 coding sequences by HRM analysis required the investigation of 15 coding exons. PCR optimization of 10 exons (1, 2, 4, 6, 7, 8, 9, 11, 12 and 13) did not obtain a sufficient amplicon quality for subsequent HRM analysis. Consequently, these 10 exons were analyzed by direct sequencing.

We identified one mutation and 3 polymorphisms of the KCNH2 gene after direct sequencing analysis for the non-HRM applicable exons (Supplemental Table VI), but no mutation or variant was detected in the HRM applicable exons. The genomic variants c.1467C > T (p.I489T) and c.1539C > T (p.F513F) are in exon 6 (Supplemental Figure 3A-B). These two alterations were observed concomitantly in 5 patients and there was a consistent pattern in mutation acquisition. Family 1 and the son of case 3 showed the heterozygous mutation and family 2, and cases 3 and 5 showed the homozygous mutation. The genomic variant c.1692A > G (p.L564L) is in exon 7 (Supplemental Figure 3C). All people showed the homozygous mutation. The genomic variant c.2229_2230delC (p.R744fs, pathogenic mutation) is in exon 9 (Supplemental Figure 3D). One case (case 1) revealed a 1-bp in-frame deletion involving nucleotides 2229 and 2230. Intriguingly, the c.2229_2230delC is similar to the c.2229_2230delC, which has been reported previously (rs8179013).

HRM analysis and direct sequencing of LQTS-causing genes of SCN5A: Mutational screening of SCN5A coding sequences by HRM analysis required the investigation of 27 coding exons. PCR optimization of 4 exons (1, 11, 16 and 27) did not obtain a sufficient amplicon quality for subsequent HRM analysis. Consequently, these 4 exons were analyzed by direct se-
quencing.

We identified one polymorphism of the SCN5A gene after HRM analysis and subsequent direct sequencing confirmation (Supplemental Table VII). This genomic variant (c.1141-3C > A) is located in intron 8 and family 3 has the mutation (Supplemental Figure 4A). The direct sequencing of the 4 non-optimized exons (exons 1, 11, 16 and 27) detected 4 substitutions. The genomic variant c.87A > G (p.A29A) is in exon 1 (Supplemental Figure 4B). Two families (1 and 3) and case 5 showed the heterozygous mutation and one family (2) and case 4 showed the homozygous mutation. The genomic variant c.1673A > G (p.H558R) is in exon 11 (Supplemental Figure 4C). One family (3) and the son of case 1 showed the heterozygous mutation. We also found heterozygous or homozygous mutation of c.1673A > G in non-LQTS cases. The genomic variant c.3183A > G (p.E1061E) is in exon 16 (Supplemental Figure 4D). All people showed the homozygous mutation. The genomic variant c.5454T > C (p.A1818A) is in exon 27 (Supplemental Figure 4E). Four people (cases 4 and 5 and the sons of case 1 and case 3) showed the heterozygous mutation and family 2 showed the homozygous mutation.

**DISCUSSION**

KCNQ1, KCNH2, and SCN5A mutations in LQTS have been previously reported in several studies. Numerous discrepancies in the KCNQ1 gene mutational status have been reported in several studies, and the results indicated different percentages ranging from 0% to 50%. One recent study demonstrated that the frequency of KCNQ1 mutations was 31% in a cohort of 70 cases in the Danish population. Doolan, et al found that the frequency of KCNQ1 mutation was 0% in 59 LQTS in an Australian population. Allegue, et al found that the KCNQ1 mutation was approximately 7% in 14 LQTS Spanish patients. Tester, et al demonstrated that the frequency of KCNQ1 mutation was approximately 10% in 49 LQTS patients in the United States. In a Taiwanese population, the frequency of KCNQ1 mutations was reported as being about 25% in 16 LQTS patients. In the present study, we showed that 20% of LQTS patients harbor KCNQ1 mutations, and our results were similar to those by Jimmy, et al who used direct sequencing technology. Similarly, the KCNQ2 and SCN5A mutation detection rate was extremely variable according to these studies. Future studies are required to collect more LQTS samples to confirm these data.

HRM analysis was successfully applied to scan for mutations in target genes. It has been applied to detect mutations of KCNQ1, KCNH2 and SCN5A. Millat, et al reported 2 exons (exon 1 and 16) of the KCNQ1 gene did not obtain a sufficient amplicon quality for subsequent HRM analysis and Farrugia, et al found 3 exons (exons 1, 13 and 16). In the present study, we showed that exons 1 and 16 were not suitable for HRM analysis and our results were similar to those of Millat, et al. We used the same instrument with the same software and same PCR kit as Farrugia, et al. One of the hypotheses that might explain the difference in the results for exon 13 between our study and that of Farrugia, et al is that the method is the use of different primer pairs (147 bp versus 244 bp) and different amplification protocols (PCR versus touchdown PCR). Millat, et al used the Rotor-Gene 6000 analyser from QIAGEN and a different PCR kit, including a LightCycler® 480 High Resolution Melting Master kit, LightCycler® 480 Probes Master kit plus SYTO-9 dye, SYBR® GreenER™ qPCR SuperMix, Hot Goldstar DNA polymerase kit, and a FailSafe™ PCR System.

HRM analysis provides a convenient way to detect mutations in a multi-exon gene without performing next-generation sequencing (NGS). Although HRM analysis has many advantages, it also has some limitations. It cannot detect mutations encompassing the whole gene or entire exon. In addition, amplicon length is generally recommended for HRM analysis. With decreasing product length, the melting temperature differences among the genotypes increase; therefore, allowing better differentiation between mutant and non-mutant samples. Amplicon length may also influence the sensitivity and specificity of genotyping. Heterozygotes are easily identified from their melting curves but homozygotes are more difficult to detect.

We have identified 2 mutations (p.T309I in KCNQ1 and p.R744fs in KCNH2) and 11 polymorphisms in 5 Taiwanese LQTS patients and the family members of 3 LQTS patients referred for three-gene screening. These mutations and polymorphisms have previously been described. Hsueh, et al found p.R744fs in KCNH2 first and they performed functional studies on this mutation. They reported that the addition of GEP to the C-terminal of p.R744fs changed the maturation process of the mutant protein but the channel was still assembly defective. In the present study, one patient had a KCNH2 p.R744fs mutation and another patient carried a KCNQ1 p.T309I mutation; both patients presented with recurrent syncope due to ventricular fibrillation and received implantable cardioverter defibrillator therapy. The genetic variant c.1673A > G (p.H558R) in exon 11 of SCN5A has been reported as a pathogenic mutation in some studies, although the study of Iwasa, et al suggested that the mutation was a non-pathogenic polymorphism. We found a heterozygous or homozygous change of the mutation in non-LQTS cases, and our results support the results of Iwasa, et al.

In conclusion, we have demonstrated the prevalence of KCNQ1, KCNH2, and SCN5A mutations and polymorphisms in a Taiwanese population. Although mutations in LQTS are common (75%) in Western populations, we identified 2 of 5 LQTS patients (40%) with disease-causing mutations in Taiwan.

**References**

6. Tester DJ, Will ML, Haglund CM, Ackerman MJ. Compendium

**SUPPLEMENTAL FILES**
Supplemental Table I, II, III, IV, V, VI, VII
Supplemental Figure 1, 2, 3, 4
Please find supplemental files: https://www.jstage.jst.co.jp/article/ihj/56/4/56_14-428/_article/supplement