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Considerations when using next-generation sequencing for genetic diagnosis of long-QT syndrome in the clinical testing laboratory

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ABSTRACT

Background: Congenital long-QT syndrome (LQTS) is a potentially lethal cardiac electrophysiologic disorder characterized by QT interval prolongation and T-wave abnormalities. At least 13 LQTS-associated genes have been reported, but the high cost and low throughput of conventional Sanger sequencing has hampered the multi-gene-based LQTS diagnosis in clinical laboratories.
Methods: We developed an NGS (next-generation sequencing)-based targeted gene panel for 13 LQTS genes using the Ion PGM platform, and a cohort of 36 LQTS patients were studied for characterization of analytical performance specifications.
Results: This panel efficiently explored 212 of all 221 coding exons in 13 LQTS-associated genes. And for those genomic regions covered by the design of the NGS panel, the analytical sensitivity and analytical specificity for all potentially pathogenic variants were both 100% and showed 100% concordance with clinically validated Sanger sequencing results in five major LQTS genes (*KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2*).
Conclusion: This is the first description of an NGS panel targeting a multi-gene panel of 13 LQTS-associated genes. We developed and validated this robust, high-throughput NGS test and informatics pipeline for LQTS diagnosis suitable for the clinical testing laboratory.

1. Introduction

Congenital long-QT syndrome (LQTS) is a potentially lethal cardiac electrophysiologic disorder characterized by QT interval prolongation and T-wave abnormalities on surface electrocardiogram (ECG) and increased risk for syncope, seizures, and sudden cardiac death [1]. LQTS comprises two major hereditary variants [2], namely, autosomal dominantly inherited Romano–Ward syndrome and autosomal recessive Jervell and Lange Nielsen syndrome, in addition to other rare subtypes including Andersen syndrome and Timothy syndrome. The prevalence of LQTS is estimated to be 1/2000 in the general population [3], and the prevalence is of comparable magnitude among different ethnicities [4].

Since the sentinel discovery of the first LQTS-susceptibility locus on 11p15.5 [5] followed by the identification of key cardiac potassium channel genes (*KCNQ1* [6], *KCNH2* [7]) and a sodium channel gene (*SCN5A* [8]) as causative genes for LQTS1-3, at least 13 LQTS-associated genes have been reported to date [4]. The overall mutation detection rate of LQTS is about 70%, and the majority of identified mutations are found in the three major LQTS genes: 42–52% mutations in *KCNQ1* (LQT1), 32–45% mutations in *KCNH2* (LQT2), and 8–13% mutations in *SCN5A* (LQT3) [4]. All types of mutations are found in LQTS: approximately 70% are missense, 15% are frameshift, and in-frame deletions, nonsense, and splice site variants each account for 3%–6% of all mutations. Although some recurrent mutations have been reported, most affected families typically segregate a unique private mutation.

The genetic testing method currently most widely employed for LQTS diagnosis in clinical testing laboratories is Sanger sequencing for each coding exon of the major LQTS genes. However, the high cost and low throughput of consecutive, conventional Sanger sequencing in LQTS has hampered the comprehensive multi-gene-based LQTS diagnosis in clinical laboratories. Additionally, in patients with clinically definite LQTS, about 30% remain mutation negative even after a comprehensive assessment of the major LQTS genes.

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Next-generation sequencing (NGS), with its increased sensitivity and remarkable throughput, is rapidly entering the clinical testing space [9]. However, validation of NGS for use in a clinical testing laboratory requires rigorous establishment of analytical performance specifications. While guidelines regarding validation and standardization of NGS in clinical laboratory practice are being developed, these will continue to evolve as genotyping technologies and bioinformatics evolve, and as NGS clinical testing practices accumulate. In this study, we describe development of an NGS-based targeted gene panel for 13 LQTS genes using the Ion PGM platform, and we propose a feasible strategy to characterize the NGS performance specifications for clinical testing laboratories.

2. Material and methods

2.1. Patients

Clinical specimens from patients with LQTS requested for mutation analysis at Seoul St. Mary's Hospital, Seoul, Korea, from 2010 to 2013 were analyzed in this study. All patients underwent complete clinical evaluation, including ECG, transthoracic echocardiogram, 24-h ECG Holter recording, and exercise testing. Each individual showed prolongation of the corrected QT interval (QTc ≥ 460 ms) and/or documented torsade de pointes, ventricular fibrillation, cardiac arrest, or aborted sudden cardiac death. Written informed consent was obtained from the patients and/or their family members. This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board (IRB)/Ethics Committee of Seoul St. Mary's Hospital (IRB No·KC15TISI0769).

2.2. DNA extraction

Thirty-six peripheral blood samples from 36 probands with LQTS were provided under informed consent. Genomic DNA was isolated from peripheral blood leukocytes using the QIAmp DNA Mini Kit (Qiagen, Hamburg, Germany). DNA concentration was measured with a Qubit fluorometer (Life Technologies, San Francisco, CA, USA) in combination with the Qubit dsDNA HS assay kit according to the manufacturer's instructions, and DNA quality was confirmed by agarose gel electrophoresis.

2.3. Custom resequencing panel design

A custom panel targeting 13 known LQTS-associated genes, namely, *KCNQ1* (NM_000218.2), *KCNH2* (NM_000238.3), *SCN5A* (NM_198056.2), *ANK2* (NM_001148.4), *KCNE1* (NM_000219.3), *KCNE2* (NM_172201.1), *CAV3* (NM_033337.2), *SCN4B* (NM_174934.3), *AKAP9* (NM_005751.4), *SNTA1* (NM_003098.2), *KCNJ5* (NM_000890.3), *CACNA1C* (NM_000719.6), and *KCNJ2* (NM_000891.2) was designed using the Ion AmpliSeq Designer and the "white glove" design option, a program that facilitates additional customization to design amplicons for difficult regions. Two primer pools to amplify 436 amplicons, covering the entire coding regions of the 13 LQTS genes including 5 bp of 5′ and 3′ intronic sequence for each exon, were designed. Target design rates were 99%, 99.5%, 98.7%, 100%, 100%, 100%, 100%, 100%, 99.3%, 99.6%, 100%, 99.6%, and 100% for *KCNQ1*, *KCNH2*, *SCN5A*, *ANK2*, *KCNE1*, *KCNE2*, *CAV3*, *SCN4B*, *AKAP9*, *SNTA1*, *KCNJ5*, *CACNA1C*, and *KCNJ2*, respectively, totaling 52.53 kb (Supplementary Table 1).

2.4. Amplicon library preparation

The construction of the library involved the following steps: multiplex PCR, primer digestion and phosphorylation, ligation of platform-specific adapters, and purification. For multiplex PCR, 20 ng of DNA was amplified using the LQTS custom panel and the Ion AmpliSeq™ HiFi Master Mix (Ion Ampliseq Library kit 2.0). Amplicons were then digested, barcoded, and purified using the Ion Ampliseq Library kit 2.0 and Ion Xpress Barcode Adapters kit (Life Technologies) according to the manufacturer's instructions. The library was quantified using the Qubit fluorometer and the Qubit dsDNA HS assay kit (Life Technologes).

2.5. Sequencing

Eight pM of each library was pooled, multiplexed, and clonally amplified on Ion sphere particles (ISP) by emulsion PCR performed on the Ion One Touch 2 instrument with the Ion PGM template OT2 200 kit (Life Technologies) according to the manufacturer's instructions. Quality control was performed using the ISP Control kit (Life Technologies) to ensure that 10–30% template-positive ISP was generated in the emulsion PCR. Finally, the template ISPs were enriched, loaded on an Ion 318 chip, and sequenced on a PGM sequencer with the Ion PGM sequencing 200 kit v2 according to the manufacturer's instructions.

2.6. Data analysis

Sequence data were processed for primary and secondary analyses using standard Ion Torrent Suite Software running on the Torrent Server. Raw signal data were analyzed using Torrent Suite v 4.0.2. The pipeline included signal processing, base calling, quality score assignment, adapter trimming, PCR duplicate removal, read alignment, quality control of mapping quality, coverage analysis, and variant calling. Sequenced reads were aligned against the UCSC hg19 reference genome (Genome Reference Consortium GRCh37). The variant caller parameter setting was germline, low stringency. Following primary and secondary analyses, tertiary analysis was performed using Ion Reporter Software for variant annotation starting with a variant file in variant call format produced by the Torrent Variant Caller plugin.

2.7. Panel amplicon performance evaluation

The sequencing efficiency of each amplicon was evaluated using amplicon read ratios. These were calculated as the number of amplicon reads for each amplicon as a percentage of the total number of all amplicon reads [10]. And for analysis purposes, a quartile-grading scale of amplicon performance was used.

2.8. Sanger sequencing

All potentially pathogenic variants as well as low-coverage regions were verified by Sanger sequencing. PCR amplicons were bi-directionally sequenced using the Big Dye terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Chromatograms were analyzed with Sequencher software version 5.0 (Gene Codes, Ann Arbor, MI, USA). To classify variants, we followed the standards and guidelines of the American College of Medical Genetics and Genomics for the interpretation of sequence variants [11], and all variants were scored and classified into five pathogenicity groups (class 1: benign; class 2: likely benign; class 3: uncertain significance (VUS); class 4: likely pathogenic; class 5; pathogenic). To define novel variants, we searched locus-specific databases (LOVD) as well as public databases (HGMD, ExAC, 1000genomes, dbSNP) including an ethnic-specific Korean genome database containing 1244 alleles: Korean Reference Genome Database (KRGDB; http://152.99.75.168/KRGDB/). In silico analyses of missense variants were performed using Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant From Tolerant (SIFT; which predicts whether protein substitutions are tolerated, http://sift.jcvi.org/), MutationTaster2 (http://www.mutationtaster.org), and Align-GVGD (agvgd.iarc.fr) to assess if the substitutions were predicted to be potentially pathogenic. Also, for missense variants, evolutionary conservation of the involved amino acids was investigated using the resources at the Evola website (http://www.h-invitational.jp/hinv/ahg-