Detection and validation of six pathogenic variants in Chinese inherited heart disease patients using clinical whole-exome sequencing

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Abstract

The targeted next-generation sequencing (NGS) was employed in detecting the pathogenic mutations in inherited heart disease patients in the present study. Two main methods, the NGS and the classic Sanger sequencing, were used in this study. And, the whole-exome sequencing (WES) was specifically used in this study.

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Abstract

The inherited heart diseases related genes are involving various mutation types and regions, and targeted next-generation sequencing (NGS) was employed in detecting the pathogenic mutations in inherited heart disease patients in the present study. Five literature-annotated disease mutations (*PKP2* gene, c.148_151delACAG, NM_001005242;*ABCC9* gene, c.3589C>T, NM_005691; *PKP2* gene, c.1237C>T, NM_001005242; *PKP2* gene, c.2490-6T>C, NM_001005242; *TNNT2* gene, c.650_652delAGA, NM_000364) and one novel mutation (*FBN1* gene, c.8286_8289delCATC, NM_000138) were found in our patients. Two main methods, the NGS and the classic Sanger sequencing, were used in this study. The whole-exome sequencing (WES) was specifically used in our study. Combined applications of the NGS platform and bioinformatics are proved to be effective methods for inherited heart disease diagnosis.

Key words: Inherited heart disease; next-generation sequencing; whole-exome sequencing

Key Clinical Message

The whole-exome sequencing (WES) and combined applications of the NGS platform and bioinformatics are proved to be effective methods for inherited heart disease diagnosis.

Introduction

The inherited heart diseases include arrhythmia syndromes and cardiomyopathies, and they also are the main cause of heart failure and sudden cardiac death. The autosomal dominant inheritance pattern is showed in most inherited heart diseases and genetic testing of an affected patient is a clinical recommendation (*Singer et al., 2021*). Inherited arrhythmia syndromes include several different diseases: Brugada syndrome (BrS), catecholaminergic polymorphic ventricular tachycardia (CPVT), idiopathic ventricular fibrillation (IVF), long QT syndrome (LQTS), progressive cardiac conduction system disease (PCCD), and short QT syndrome (SQTS), and the heart of patients with these diseases is typically structurally normal. Patients with inherited arrhythmia syndromes are probably at increased risk for sudden cardiac death (SCD) and an autopsy for the sudden cardiac death patients is typically negative (*Olde et al., 2016* ; *Gray et al., 2016*). Inherited cardiomyopathies are a group of heart muscle diseases that are characterized by heterogeneous phenotypes, encompassing arrhythmogenic right ventricular cardiomyopathy (ARVC), dilated cardiomyopathy (DCM), and hypertrophic cardiomyopathy (HCM). The genes pathogenically involved in these diseases are diverse and overlap among the phenotypes, and these diseases are associated with the mutations in a large number of genes (*Forleo et al., 2017*).

Next-generation sequencing (NGS) is getting increasingly important and useful in DNA sequencing and causative mutations detection, with the rapid clinical genetic diagnosis development (*Xie et al., 2012*). The inherited heart diseases related genes are involving various mutation types and regions, and therefore NGS was employed in detecting the pathogenic mutations in inherited heart disease patients in the present study by providing comprehensive mutations detection from small indel to large deletions and duplications. Whole-exome sequencing (WES) is a diagnostic approach for the identifying molecular defects in patients with suspected genetic disorders (*Yang et al., 2013*). In this study, we employed WES, containing more than 22,000 functional genes, to detect the clinically pathogenic variants from exomes in inherited heart disease patients. We performed molecular screening in 9 unrelated patients to investigate the genetic overlap between phenotypes and to identify gene-phenotype associations using the Illumina NovaSeq 6000 platform.

Material and methods

Ethics statement

Written informed consent was obtained from the patients participating in the study before collecting 2ml of their peripheral blood for the experiment. The patients were recruited in this study of clinical and molecular diagnosis of inherited heart disease patients related genes approved by the Institutional Ethics Committees at Third Xiangya Hospital of Central South University. Genomic DNA was extracted from peripheral blood samples following the manufacturer's standard procedure using the HiPure Blood DNA Midi Kit III (Magen, Guangzhou, China).

Whole-exome sequencing

Whole-exome sequencing and analysis protocols developed by Agilent Technologies (2017), Inc. (Santa Clara, USA) were adapted for the clinical test of whole-exome sequencing. Briefly, genomic DNA samples were fragmented with the use of M220 Focused-ultrasonicator (Covaris, Massachusett, USA). The fragment was ranging from 150 bp to 250 bp. The ends were repaired with Hieff NGS® UltimaTM Endprep Mix Kit (Yeasen, Shanghai, China), ligated to Illumina multiplexing paired end adapters with Hieff NGS® UltimaTM DNA Ligation Module (Yeasen, Shanghai, China), purified by AMPure XP Beads (Beckman Coulter, Illinois, USA), and amplified by means of a PCR assay with the use of primers with sequencing barcodes (indexes) by 2×Super Canace® II High-Fidelity Mix for Library Amplific ation Kit (Yeasen, Shanghai, China). Library enrichment for WES was conducted by Agilent SureSelect Human All Exon v6 (Santa Clara, CA, USA). Enriched samples were sequenced using an NovaSeq 6000 platform (San Diego, CA, USA). Mean coverage of the sequences was 95.6×, and on average 97.4% of base pairs with >10× coverage were successfully detected. Sequencing data were aligned to the hg38 reference genome.

Variant calling and filtering

Variant calling was performed using the Genome Analysis Toolkit (GATK) HaplotypeCaller function (version 4.1.2.0). Variant filtering was performed using GATK VariantFiltration function (version 4.1.2.0) (*Van der Auwera et al., 2013*). Sequencing data were aligned to reference genome (version hg38) using BWA (*Jo & Koh, 2015*). In order to assess the potential functional impacts of variants, five bioinformatics algorithms were utilized in our study: PolyPhen-2 (PP2), Sorting Tolerant From Intolerant (SIFT), Meta-support vector machine (Meta-SVM), megalencephaly-capillary malformation (MCAP), and Mutation Taster.

Candidate mutation confirmed by Sanger sequencing

The potential mutated bases and flanking sequence of genes were amplified by polymerase chain reaction (PCR) and sequenced by Sanger sequencing.

(R42: forward primer 5'-TACACACCAAAAATTCTGCATAGC

and reverse primer 5'-CCAGCTGAGTACGGCTACATC;

R44: forward primer 5'-CATCAGTTTGTCACTGTGTTGAGT

and reverse primer 5'-TTTGGGCTATTTGTCTTGTCATTA;

R46: forward primer 5'-GAGTCTAAGCCAGCAGGTAACAAT

and reverse primer 5'-TAGCCGGGGTATATTCTACAAGAG;

R47: forward primer 5'-TCACCCAGTTCACCACTGAG

and reverse primer 5'-ATGCAGCATAAGGCAGAAAATTG;

R51: forward primer 5'-TTGGGCTGGGTAGTAGAAAAATAG

and reverse primer 5'-GCACTGTGCTAGTGGTTTAAAAAG;

R52: forward primer 5'-GCAGGAGAGCATCTAGTTCAATC

and reverse primer 5'-CAGGAAGAAGAGCATAAGAACCTG).

All nucleotide positions were determined according to the standard gene reference sequence.

Results

Clinical features of the patients

The clinical manifestations of all patients are shown in Table 1. A total of 6 patients (4 men, 2 women; mean age: 39.8 +- 13.3 years; range: 16 to 52 years) were included in this study.

Variant assessment

Totally 6 rare heterozygous variants in 4 genes were detected in our study (Table 1).

There is a heterozygous mutation in *ABCC9* c.3589C>T (p.Arg1197Cys) gene located on the 12: 21981972 chromosome in patient R44 (Figure 2A). In East Asian the frequency of heterozygote was 0.13%, which is more than caucasian (<0.1%). The sequence at this locus is moderate conservative and computer-aided analysis predicts that this variation is more likely to affect function. According to American College of Medical Genetics and Genomics (ACMG) standard, this variation should be categorized into "uncertain significance". Both Brugada (BrS) syndrome and short QT syndrome (SQTS) are *ABCC9* gene related diseases and autosomal dominant inheritance. The patient R47 was affected by the heterozygous frameshift mutation c.8286_8289del (p.Ile2763Leufs*15) in *FBN1* gene located on the 15:48703514 chromosome (Figure 2B). According to ACMG standard, this variation should be categorized into "pathogenic". This is a novel mutation in*FBN1* gene. All of patient R46, R42, and R51 have *PKP2* gene mutations. Patient R42 was affected by the heterozygous frameshift mutation c.148_151del (p.Thr50Serfs*61) in *PKP2* gene located on the 12:33049515 chromosome (Figure 2C). According to ACMG standard, this variation should be categorized into "pathogenic". Patient R46 was affected by the heterozygous nonsense mutation c.1237C>T (p.Arg413Ter) in *PKP2* gene located on the 12:33003841 chromosome (Figure 2C). According to ACMG standard, this variation should be categorized into "pathogenic". Patient R51 was affected by the heterozygous nonsense mutation c.2490-6T>C in *PKP2*gene located on the 12:32945671 chromosome (Figure 2C) and this mutation leads to a splicing abnormality, thereby causing exon 13 extension. According to ACMG standard, this variation should be categorized into "likely pathogenic". *PKP2* is a susceptibility gene for familial arrhythmogenic right ventricular dysplasia type 9 (ARVD9), which is an autosomal dominant disease. Patient R52 was affected by missing codon 210 (lysine) due to the heterozygous mutation c.650_652delAGA (p.Lys217del) in *TNNT2* gene located on the 1:201331099 chromosome (Figure 2D). According to ACMG standard, this variation should be categorized into "pathogenic". *TNNT2* is a susceptibility gene for dilated cardiomyopathy type 1D, which is an autosomal dominant disease.

Sanger sequencing

Sanger sequencing was performed to analyze the mutations of inherited heart diseases related genes in order to verify the accuracy of the potential mutations identified by NGS. The results showed complete consistency between the NGS and Sanger sequencing, indicating that NGS used in this study has high accuracy (Figure 1).

Discussion

As an emerging technology, NGS is powerful and comprehensive in exploring genetic mutations associated with a variety of human diseases (*Forleo et al., 2017*). In our study, WES is employed to identify genetic variations related to inherited arrhythmia syndromes and cardiomyopathies in patients. Our study aimed to find out novel mutations related to inherited heart diseases for better laboratory diagnosis and clinical management in future.

ABCC9 gene

In our study, the clinical diagnosis of patient R44 with mutation c.3589C>T in *ABCC9* gene is Brugada (BrS) syndrome and short QT syndrome (SQTS). The variation, c.3589C>T, has been reported in Hu et al. (*Hu et al., 2014*), which is the first to identify *ABCC9* as a susceptibility gene for early repolarization syndrome (ERS) and BrS. BrS is an inherited cardiac disease leading to ventricular fibrillation and an increased risk of sudden cardiac death (SCD) in structurally normal hearts (*Sarquella-Brugada et al., 2016* ; *Sieira et al., 2016*). Genetic characterization of the Brs syndrome might not only be able to better establish the diagnostic ECG pattern (ST elevation, sinus node dysfunction, or conduction abnormalities) but also to clarify the overlap syndromes (*Sieira et al., 2017*). SQTS is a rare, life-threatening, inherited heart disease characterized by ventricular tachyarrhythmias leading to syncope and sudden cardiac death (*Bjerregaard et al., 2018* ; *El-Battrawy et al., 2018* ;*Campuzano et al., 2018*). Genetic testing is essential

in diagnosing the disease (*Bjerregaard et al., 2018*). Clinical manifestations associated with SQTS may range from asymptomatic, palpitations, syncope, dizziness, atrial fibrillation, ventricular arrhythmias, and SCD (*El-Battrawy et al., 2018* ; *Campuzano et al., 2018*). In a published study (*Bienengraeber et al., 2004*), researchers identified two mutations in *ABCC9* , encoding the regulatory SUR2A subunit of the cardiac K_{ATP}channel, by scanning of genomic DNA from individuals with heart failure and rhythm disturbances due to idiopathic dilated cardiomyopathy.

FBN1 gene

The clinical diagnosis of patient R47 with mutation c.8286_8289delCATC in *FBN1* gene is Marfan syndrome. c.8286_8289delCATC is a novel mutation, which is the first report in *FBN1* gene. *FBN1* is a susceptibility gene for Marfan syndrome, which is an autosomal dominant disease. The downstream truncation variation of this frameshift mutation in FBN1 gene in the individual has been reported as disease-causing mutation in many literatures (*Jensen et al., 2014* ; *Baudhuin et al., 2014* ; *Aalberts et al., 2014*). Mutations in the *FBN1* gene, encoding fibrillin-1, lead to a series of severe cardiovascular inherited complications and connective tissue disorders, such as Marfan syndrome (MFS), whose major features include tall stature and arachnodactyly, ectopia lentis, and thoracic aortic aneurysm and dissection (*Aalberts et al., 2014* ; *Zeng et al., 2018* ; *Faivre et al., 2007* ; *Sakai et al., 2016*). Since more than one thousand individual mutations in *FBN1* are associated with Marfan syndrome and mutations in specific regions of*FBN1* can cause the opposite features, it is difficult to identify genotype-phenotype correlations (*Sakai et al., 2016*). Dr. Baudhuin and her co-workers observed that a higher frequency of truncating or splicing *FBN1* variants in Ghent criteria-positive patients, and found that missense mutations are the most common type of*FBN1* mutation, by investigating *FBN1* genotype-phenotype correlations with aortic dissection and prophylactic aortic surgery in patients with Marfan syndrome. They also concluded that *FBN1*mutations have been shown to occur with limited genotype-phenotype correlations across the gene in their study (*Baudhuin et al., 2014*). In Aalberts et al., the data show that in those patients without an *FBN1* mutation, the prevalence of left ventricle (LV) dilatation was significantly higher than in those patients with such a mutation (*Aalberts et al., 2014*).

PKP2 gene

In 2004, for the first time, Gerull and colleagues reported the link between heterozygous mutations in the *PKP2* gene, coding for Plakophilin-2 (PKP2), which is one of the structural components of the cardiac desmosome, part of the Armadillo family of proteins, and known for its role in cell-cell adhesion, and they found that there is a connection between *PKP2* expression and the cardiac transcriptional program (*Cerrone et al., 2019* ; *Cerrone et al., 2017*). In 2016, a total homozygous *PKP2* gene deletion observed in two siblings with severe left ventricular noncompaction cardiomyopathy (LVNC) was identified for the first time, and this homozygous *PKP2* gene deletion leads to rapid and lethal cardiac failure in the patient (*Ramond et al., 2017*). According to a study in ARVC patients, frameshift and nonsense mutations account for 35% (79 out of 224) of *PKP2* genetic variations identified (*Li Mura et al., 2013*). *PKP2* is necessary in maintaining transcription of genes that control intracellular calcium cycling. Lack of PKP2 leads to disruption of intracellular calcium homeostasis and isoproterenol-induced arrhythmias, and may cause life-threatening arrhythmias even in the absence of structural disease (*Cerrone et al., 2017*). In Sonoda et al.'s cohort of 71 clinically diagnosed ARVC patients, one male patient (1.4%), who carries copy number variation (CNV), as the first case of ARVC in Asia, was identified with an extensive *PKP2* deletion. The deletion range extended 3' of*PKP2* , which is novel in ARVC patients (*Sonoda et al., 2017*). The same mutation, c.148_151delACAG, in PKP2 gene in R42 has been reported in clinical cases (*Tisma-Dupanovic et al., 2013* ;*Philips et al., 2014*). The patient was a presumed pathogenic mutation, which is heterozygous, in exon 1 of the PKP2 gene (*Tisma-Dupanovic et al., 2013*). Among the 42 arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) patients in Philips et al., the vast majority (76%) had a pathogenic mutation affecting the PKP2 gene (*Philips et al., 2014*). The same mutation, c.1237C>T, in *PKP2* gene in patient R46, who was diagnosed as a ARVD9 patient, has been reported in multiple clinical cases (*Campuzano et al., 2014* ; *Alcalde et al., 2014* ;*Fressart et al., 2010*). *PKP2* associated with the sudden cardiac death (SCD)-disease manifesting earlier onset or

more severe presentation of cardiomyopathies (*Campuzano et al., 2014*). Genetic analysis revealed that the truncating *PKP2* mutation is the most frequent arrhythmogenic right ventricular cardiomyopathy (ARVC) related genetic variation (*Alcalde et al., 2014* ; *Fressart et al., 2010*). The truncated proteins are considered responsible for the pathological phenotype due to their worse functional severity (*Alcalde et al., 2014*).

In our study, we found patient R51 was affected by the heterozygous nonsense mutation c.2490-6T>C in *PKP2* gene located on the 12:32945671 chromosome and this mutation leads to a splicing abnormality, thereby causing exon 13 extension. The mutation, c.2490-6T>C, in *PKP2* has been reported in Chen et al. The mutation in *PKP2* is the most prevalent causation leading to arrhythmogenic cardiomyopathy (ACM) and ventricular arrhythmic events are liable to be developed in the *PKP2* mutations carrier (*Chen et al., 2019*). The patient R51 is also a carrier of three heterozygous mutations: (1) a mutation c.1166-51G>A in*TRDN* gene located on the 6:123702586 chromosome. This variation should be categorized into "uncertain significance" and is related to catecholamin-sensitive pleomorphic ventricular tachycardia type 5 with or without myasthenia, which is autosomal recessive (https://www.omim.org/). (2) a mutation c.80756C>T (p.Pro26919Leu) in *TTN* gene located on the 2:179430103 chromosome. This variation should be categorized into "uncertain significance" and is related to familial hypertrophic cardiomyopathy type 9 (autosomal dominant), hereditary myopathy with early respiratory failure (autosomal dominant), early onset myopathy with lethal cardiomyopathy (autosomal recessive), dilated cardiomyopathy type 1G (-), delayed tibialis muscle atrophy (autosomal dominant), and limb band muscular dystrophy type 2J (autosomal recessive) (https://www.omim.org/). (3) a mutation c.64508G>T (p.Gly21503Val) in *TTN* gene located on the 2:179449963 chromosome. This variation should be categorized into "uncertain significance" and is related to familial hypertrophic cardiomyopathy type 9 (autosomal dominant), hereditary myopathy with early respiratory failure (autosomal dominant), early onset myopathy with lethal cardiomyopathy (autosomal recessive), dilated cardiomyopathy type 1G (-), delayed tibialis muscle atrophy (autosomal dominant), and limb band muscular dystrophy type 2J (autosomal recessive) (https://www.omim.org/). But in our case, the patient R51 has no clinical symptom related to above mentioned three gene mutations.

TNNT2 gene

The mutation c.650_652delAGA in *TNNT2* gene has been reported in several patients with dilated cardiomyopathy. The *TNNT2* mutations is thought to cause a fully penetrant and severe disease *(Otten et al., 2010* ; *Mogensen et al., 2004*). In 17 *TNNT2* mutations found in these patients, six (35%) were the specific *TNNT2*p.K217del mutation. Since an early age of disease manifestation was observed, the severe phenotype associated with the *TNNT2*p.K217del mutation can be confirmed (*Otten et al., 2010*). Besides, the experimental results suggest that this mutation alters*TNNT2* activity. In Robinson et al., the deletion of codon 210 in cardiac troponin T has been reported to be a cause of inherited dilated cardiomyopathy (*Robinson et al., 2002*).

Conclusion

In this work, we found that one novel and five recorded mutations in four genes related to inherited heart diseases. The targeted next-generation sequencing was applied in this study to detect the candidate mutations. NGS has been proved to have considerable potential for both clinic and research use. The exon capture followed by NovaSeq 6000 sequencing was specifically used in our study. Combined applications of this platform and bioinformatics are very promising and effective methods for molecular screening of patients with inherited heard diseases, but it still remains that deficiencies on accuracy and stability need to be further investigated.

Conflict of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Authors Contribution

Study conduct: LC, FY, YB, ZZ, and HZ. Data collection: LC, SX, YB, YZ, and QW. Data analysis: YZ

and QW. Data interpretation: LC, YZ, and QW. Drafting manuscript: SX. Revising manuscript content: SX and LC. Approving final version of the manuscript: LC and HZ.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legendsFigure 1. Sanger sequencing analysis of six mutations in patients with inherited heart diseases. A. The heterozygous mutation (c.148_151delACAG) was identified in patient R42. B. The heterozygous mutation (c.3589C>T) was identified in patient R44. C. The heterozygous mutation (c.1237C>T) was identified in patient R46. D. The heterozygous mutation (c.8286_8289delCATC) was identified in patient R47. E. The heterozygous mutation (c.2490-6T>C) was identified in patient R51. F. The heterozygous mutation (c.650_652delAGA) was identified in patient R52.**Figure 2**. **(A)** There is a heterozygous mutation c.3589C>T (p.Arg1197Cys) in *ABCC9* gene located on the 12: 21981972 chromosome in patient R44.**(B)** There is a heterozygous mutation c.8286_8289del (p.Ile2763Leufs*15) in *FBN1* gene located on the 15:48703514 chromosome in patient R47.**(C)** There is a heterozygous mutation c.148_151del (p.Thr50Serfs*61) in *PKP2* gene located on the 12:33049515 chromosome in patient R42, a heterozygous mutation c.1237C>T (p.Arg413Ter) in *PKP2* gene located on the 12:33003841 chromosome in patient R46, and a heterozygous mutation c.2490-6T>C in *PKP2* gene located on the 12:32945671 chromosome in patient R51.**(D)** There is a heterozygous mutation c.650_652delAGA (p.Lys217del) in *TNNT2* gene located on the 1:201331099 chromosome in patient 52.**Table 1**. Genetic and clinical profile of each inherited heart disease patient carrying potentially pathogenic variants.