Clinical application of targeted next-generation sequencing in fetuses with congenital heart defect

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KEYWORDS: congenital heart defects; pathogenic variant; prenatal diagnosis; targeted next-generation sequencing

ABSTRACT

Objectives To assess the value of targeted next-generation sequencing (NGS) in prenatal diagnosis of congenital heart defects (CHD) and to investigate the genetic etiology of prenatal CHD.

Methods Forty-four fetuses with CHD, normal molecular karyotype and negative chromosomal microarray results underwent targeted NGS. Fetal genomic DNA was extracted directly from amniotic fluid cells in each prenatal case. A customized targeted-NGS panel of 77 CHD-associated genes was designed to detect variants in the coding regions and the splicing sites of these genes.

Results The detection rates of targeted NGS for pathogenic and likely pathogenic variations were 13.6% (6/44) and 2.3% (1/44), respectively. The turnaround time of the test was 3 weeks. The six pathogenic variations were identified on the genes CHD7 (CHARGE syndrome), CITED2 (tetralogy of Fallot, ventricular septal defect and atrial septal defect), ZFPM2 (tetralogy of Fallot), MYH6 (atrial septal defect, familial isolated dilated cardiomyopathy) and, in two cases, KMT2D (Kabuki syndrome). The likely pathogenic variation was detected on JAG1, which is associated with tetralogy of Fallot and Alagille syndrome. Sanger sequencing in the fetuses and their parents indicated that all seven mutations were de novo. Variations of uncertain significance were detected in 79.5% of cases.

Conclusions Targeted NGS in fetuses with isolated and non-isolated CHD achieved a high diagnostic yield in our cohort, with an acceptable turnaround time for the prenatal setting. Our results have important implications for clinical management and genetic counseling. Copyright © 2018 ISUOG. Published by John Wiley & Sons Ltd.

INTRODUCTION

Congenital heart defects (CHD) are the most common forms of birth defects, with an estimated incidence of 4–8 per 1000 live births, and account for 30–50% of cases of infant mortality1,2. It has been hypothesized that the etiology of CHD is multifactorial inheritance resulting from interaction of genetic and environmental factors3. However, knowledge is limited regarding the genetic basis of CHD, despite the publication of several studies in this area in past decades.

The genetic etiology of CHD is attributed to chromosomal anomalies and single-gene disorders4. Chromosomal anomalies identified through traditional karyotype analysis represent the underlying cause in 3–18% of patients affected by CHD5, and the reported prevalence is higher when using techniques such as fluorescence in-situ hybridization and chromosomal microarray analysis (CMA)6. Besides chromosomal anomalies, single-gene disorders, in which gene mutations are associated with syndromic or non-syndromic cardiac defects, also contribute
to CHD. Approximately 3–5% of CHD are associated with Mendelian syndromes caused by single-gene dysfunction. Cardiac defects can be found in 90–97% of patients with Alagille syndrome, 75–85% of patients with CHARGE syndrome and 75% of patients with Holt–Oram syndrome. Several gene mutations have been associated with non-syndromic cardiac defects, although the exact proportion of CHD cases attributed to this cause remains unknown.

In the past decade, G-banding karyotyping and CMA have become the major methods used to identify chromosomal anomalies. However, next-generation sequencing (NGS) and whole-exome sequencing enable rapid analysis of a large number of gene sequences and facilitate discovery of novel causative genes in many genetic diseases. Compared with exome sequencing, gene-panel-based NGS captures selected regions of genes that are known to be associated with the disease of interest, which can be achieved with lower cost, shorter turnaround time and easier variant interpretation. Targeted NGS has been used to investigate the genetic etiology of CHD in the postnatal setting; however, no data are available on its role in prenatal diagnosis of CHD.

In this study, a gene panel targeting 77 CHD-associated genes was customized for NGS and used for genetic analysis of a prenatal series of CHD fetuses without chromosomal abnormalities, in order to evaluate this technique as a prenatal diagnostic tool for fetal CHD.

METHODS

Between January 2013 and October 2014, 1126 pregnancies were diagnosed with CHD during routine prenatal ultrasound screening of 41,142 women at the Affiliated Obstetrics and Gynecology Hospital of Nanjing Medical University, a tertiary referral center for prenatal genetic counseling and prenatal diagnosis located in eastern China. Of these, CHD fetuses with normal karyotype and negative CMA results were eligible for targeted-NGS testing. Eligible fetuses whose parents provided informed consent for targeted NGS were included in the study. Since this was a research-based study, results of only pathogenic variants were provided to the parents during the pregnancy. The study was approved by the research ethics board of the Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University.

Extraction of genomic DNA

After obtaining informed consent, 20 mL samples of clear amniotic fluid were obtained and genomic DNA was extracted from the samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Quality and quantity control of genomic DNA was performed using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoresis. A total of 0.25 and 1 μg of genomic DNA was used for CMA analysis and targeted NGS, respectively.

Targeted NGS

Based on a literature review (Appendix S1) and CHD-Wiki (http://www.bioinformatics.org/wiki/CHDWiki), we identified 77 genes (Table 1) for which potentially pathogenic mutations associated with isolated or syndromic CHD has been reported previously. These CHD-related syndromes include cardiofaciocutaneous syndrome, Holt–Oram syndrome, Noonan syndrome, CHARGE syndrome, Ellis–van Creveld syndrome, Rubinstein–Taybi syndrome, Alagille syndrome, Char syndrome, Okihiro syndrome, Axenfeld–Rieger syndrome and Kabuki syndrome.

Customized NimbleGen SeqCap EZ probes were used to capture target regions in these genes. Target regions included all the coding DNA sequence and 10 bp of the flanking intronic sequence on either side of the genes. The total size of the target genomic regions was 1.5 Mb in length. A total of 1 μg genomic DNA was fragmented into the optimum size of 200 (100–300) bp.

### Table 1

Panel of 77 genes associated with congenital heart defects (CHD) used for targeted next-generation sequencing in 44 fetuses with CHD

<table>
<thead>
<tr>
<th>Genes associated with syndromic CHD (n = 43)</th>
<th>Genes associated with non-syndromic CHD (n = 20)</th>
<th>Genes associated with both syndromic and non-syndromic CHD (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRX</td>
<td>BCOR</td>
<td>BRAF</td>
</tr>
<tr>
<td>EHMT1</td>
<td>EVC</td>
<td>EVC2</td>
</tr>
<tr>
<td>HRAS</td>
<td>EVC</td>
<td>KMT2D</td>
</tr>
<tr>
<td>MID1</td>
<td>NFI</td>
<td>NIPBL</td>
</tr>
<tr>
<td>RAII</td>
<td>RBM10</td>
<td>TGFB2</td>
</tr>
<tr>
<td>SHOC2</td>
<td>SLC2A10</td>
<td>SOS1</td>
</tr>
<tr>
<td>ZEB2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ACTC1</th>
<th>ACVR1</th>
<th>ALDH1A2</th>
<th>ANKR1D1</th>
<th>CITED2</th>
<th>CRELD1</th>
<th>FOXH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF1</td>
<td>LEFTY2</td>
<td>MEDI3L</td>
<td>MYH11</td>
<td>MYH6</td>
<td>MYH7</td>
<td>NKX2.5</td>
</tr>
<tr>
<td>NKX2-6</td>
<td>NODAL</td>
<td>TAB2</td>
<td>TBX20</td>
<td>ZFPM2</td>
<td>ZIC3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ACVR1</th>
<th>CFC1</th>
<th>ELN</th>
<th>FBN1</th>
<th>FLNA</th>
<th>GATA4</th>
<th>GATA6</th>
</tr>
</thead>
<tbody>
<tr>
<td>GJA1</td>
<td>JAG1</td>
<td>NOTCH1</td>
<td>PDGFRA</td>
<td>RAI1</td>
<td>TBX1</td>
<td>TFAP2B</td>
</tr>
</tbody>
</table>
bp by sonication. Target regions were then captured by the customer-designed probes, enriched by PCR and sequenced on an Illumina Hiseq2500 (PE100) (Illumina, San Diego, CA, USA).

Data analysis and filtering

Preliminary data analysis (including read alignment, variant calling and annotation) was carried out by Beijing Genomics Institute (Shenzhen, China). After quality control of raw reads, sequencing data were aligned to the human reference genome (UCSC hg19 Feb.2009, http://genome.ucsc.edu/) using BWA 0.6.2-r12616. Sequence variations (single-nucleotide variants (SNVs) and short insertions and deletions (indels)) were called by GATK17 and annotated by a self-developed pipeline. The minor allele frequency (MAF) of variations was obtained from four databases: the Single Nucleotide Polymorphism database 137 (dbSNP137, http://www.ncbi.nlm.nih.gov/SNP/), the 1000 Genomes Project (http://www.internationalgenome.org/), the National Heart, Lung and Blood Institute Exome Sequencing Project (NHlBI-ESP) 6500 (http://evs.gs.washington.edu/EVS/) and the Human Genetic Variation Database of Beijing Genomics Institute (HGVD, http://www.hgvd.genome.med.kyoto-u.ac.jp/). The effect of variants on encoding proteins or splice sites was predicted by in-silico programs including SIFT (http://sift.jcvi.org/), Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) and Condel (http://hg.upb.evl.edu/condel).

Validation of variants by PCR and Sanger sequencing

Genomic DNA sequence flanking variants were extracted from hg19 for further identification. For amplification of sites, validation primers were designed using Primer Premier 5 software (Premier Biosoft International) with standard parameters. Primers and PCR conditions are available on request. Putative fragments were amplified by PCR using these primers, and PCR products were purified and sequenced on an ABI 3730xl (Thermo Fisher Scientific) DNA analyzer.

Variation analysis

A preliminary filter process was performed to filter out common variations and synonymous variations. Variants with MAF ≤ 0.01, those not reported in the four large population databases, and non-synonymous/splicing/frameshift/cds-ins/cds-loss variants were prioritized for further analysis. All of the above selected variants were then classified as pathogenic, likely pathogenic, of uncertain significance, likely benign or benign according to the American College of Medical Genetics and Genomics (ACMG) guidelines18.

RESULTS

Between January 2013 and October 2014, 1126 pregnancies were diagnosed with congenital heart abnormalities in our department. Following genetic counseling, 136 women opted for amniocentesis in order to obtain further genetic diagnosis through karyotyping and/or CMA. Of these, 102 samples were associated with a normal karyotype and negative CMA result. After excluding four cases with inadequate DNA samples, informed consent for targeted NGS was sought from the parents of the other 98 fetuses and was obtained in 44 cases. Enrollment and screening results of the study participants are shown in Figure 1. Mean gestational age was 25.2 (range, 24–27) weeks. The prevalence of different types of CHD in the study population is shown in Table 2.

G-banding karyotyping and SNP-array analysis

Of the 136 prenatal CHD cases that underwent amniocentesis in our center, 18 had an abnormal karyotype. The remaining 118 cases underwent SNP-array analysis and pathogenic copy number variations (CNVs) were detected in 16 (13.6%) (Figure 1). Forty-four fetuses with negative SNP-array results were included in this study. Among these, 33 had isolated cardiac defects and 11 had cardiac abnormalities combined with other defects (Table 2).
Table 2 Prevalence of congenital heart defect in 44 fetuses with normal karyotype and negative chromosomal microarray results, which underwent targeted next-generation sequencing

<table>
<thead>
<tr>
<th>Cardiac defect</th>
<th>Patients (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated cardiac defects</td>
<td>33</td>
</tr>
<tr>
<td>Tetralogy of Fallot</td>
<td>8</td>
</tr>
<tr>
<td>Septal defect (VSD/ASD)</td>
<td>6</td>
</tr>
<tr>
<td>Aortic arch abnormality</td>
<td></td>
</tr>
<tr>
<td>Persistent left superior vena cava</td>
<td></td>
</tr>
<tr>
<td>Multiple heart abnormalities</td>
<td>15</td>
</tr>
<tr>
<td>Cardiac defect associated with:</td>
<td>11</td>
</tr>
<tr>
<td>Renal dysplasia</td>
<td>3</td>
</tr>
<tr>
<td>Skeletal dysplasia</td>
<td>3</td>
</tr>
<tr>
<td>Craniofacial dysplasia</td>
<td>1</td>
</tr>
<tr>
<td>Cranioencephalic dysplasia</td>
<td>2</td>
</tr>
<tr>
<td>Digestive tract anomaly</td>
<td>2</td>
</tr>
</tbody>
</table>

ASD, atrial septal defect; VSD, ventricular septal defect.

Targeted NGS

For each sample analyzed by targeted NGS, a minimum of 224 (maximum, 542) Mb of data was mapped to target regions with a mean depth of 221.95 ×. The coverage of target regions was 98.90% and the coverage of targeted regions covered with a depth of 20 × was 97.65% (Figure S1).

Interpretation of variations

In the 44 fetuses that underwent targeted NGS, a total of 255 rare variations located in the target regions were identified using our panel analysis, including six pathogenic variations, 243 variations of uncertain significance (VUS) (in 35 fetuses; data not shown), and five likely benign variations (data not shown). Thus, the detection rates of pathogenic, likely pathogenic, of uncertain significance and likely benign variations were 13.6% (6/44), 2.3% (1/44), 79.5% (35/44) and 4.5% (2/44), respectively.

Identification of mutation

To confirm the variants identified by targeted NGS, Sanger sequencing was performed in the fetuses and their parents. All seven variants were confirmed in the fetuses (Figure 2), but were not detected in their parents, suggesting that the mutations were de novo (data not shown). The parents elected to terminate the pregnancy in four of the six cases with congenital heart defects (CHD) detected on ultrasound, and with normal karyotypes and negative chromosome microarray results.
Targeted NGS in fetuses with CHD

Figure 2 Mutation sites (arrows) in genes associated with congenital heart defects (CHD), identified by targeted next-generation sequencing and validated by Sanger sequencing in seven fetuses with CHD and normal karyotype.

parents chose to continue the pregnancy despite obtaining the pathogenic variant results.

DISCUSSION

In this study, we performed a genetic analysis on 44 fetuses with CHD using a customized gene panel of 77 CHD-associated genes, and evaluated this technique in prenatal diagnosis of CHD. In the cohort of 44 fetuses with normal karyotype and negative CMA results, targeted NGS identified six pathogenic and one likely pathogenic variations, yielding a detection rate of 13.6% (6/44) for CHD-related variants. When combined with the results of karyotyping and CMA in the original cohort, the total detection rate of genetic abnormalities was 29.4% (40/136). To the best of our knowledge, this is the first study to perform targeted NGS on prenatal CHD cases. Our results indicate that this strategy is a useful and effective tool for prenatal genetic diagnosis.

The three pathogenic variants affecting CHD7, CITED2 and ZFPM2 that were detected in our cohort have been well-described in relation to CHD. One case with tetralogy of Fallot (TOF) had a frameshift deletion in CHD7, which is responsible for CHARGE syndrome19. The CITED2 variant detected in another case with ventricular septal defect (VSD) and atrial septal defect (ASD) is known to cause cardiac septal defects20. A missense variant in ZFPM2 was detected in a fetus with extracorporeal heart, VSD and polycystic kidney; mutations in this gene have been associated with a range of anomalies including diaphragmatic hernia, double outlet right ventricle and TOF21-23.

We also found three novel pathogenic mutations, one in MYH6 and two in KMT2D, and one novel likely pathogenic mutation in JAG1. One fetus with single atrium, single ventricle and aortic atresia had a missense mutation in MYH6, which has been reported to cause ASD/VSD15,24-26, tricuspid atresia, transposition of the great arteries, persistence of foramen ovale and aortic stenosis27, and hypoplastic left heart syndrome28. Nonsense and frameshift mutations in KMT2D were detected in two fetuses accompanied by right renal agenesis. Missense and nonsense mutations in KMT2D are known to cause Kabuki syndrome combined with CHD24,29, and frameshift mutations in KMT2D have been implicated previously in severe CHD30,31. A missense mutation in JAG1, which is known to cause TOF, ASD and persistence of foramen ovale32, was found in another case with cardiac and skeletal dysplasia. The JAG1 mutation was classified as a likely pathogenic variation as the function of this site needs further study.

The value of targeted NGS in genetic diagnosis of familial CHD has been discussed in two recently published studies. Blue et al.15 sequenced 57 CHD-related genes in probands from 16 families with a history of CHD,
and identified five (31%, 5/16) potential disease-causing variations. Jia et al.14 assessed the diagnostic value of targeted sequencing of 57 CHD-implicated genes in 36 patients from 13 well-selected families with non-syndromic CHD and identified potential causative variations in six (6/13, 46%) families. The detection rate in the studies by Blue et al. and Jia et al. was higher than that in our study, which could be attributed to the following reasons: (1) the CHD types assessed in these studies included double-inlet left ventricle, patent ductus arteriosus, mitral stenosis and major aortopulmonary collaterals, whereas CHD types in our study involved a single atrium, single ventricle, aortic atresia, extracorporeal heart and visceral inversion; (2) the studies by Blue et al. and Jia et al. involved familial CHD cases that were prone to association with genetic causes other than environmental factors, whereas all cases in our study were sporadic; (3) the variants identified in our study were interpreted according to the ACMG guidelines16, unlike the interpretation standards used in the other studies; and (4) in contrast to our prenatal cohort, the studies of Blue et al. and Jia et al. included postnatal patients, in whom the phenotype is much more obvious than in fetuses, contributing to an increased diagnostic rate. Nevertheless, our prenatal study provides an interesting supplement for investigating the value of targeted NGS in prenatal diagnosis of CHD.

Targeted NGS has several advantages that make it an effective method for diagnosing patients with sporadic (or familial) CHD. First, in diseases with genetic and clinical heterogeneity, targeted NGS can be used to analyze the underlying genetic reason for the disease with a 3-week turnaround time and low cost compared with whole-exome or whole-genome sequencing. Second, since clinical features of syndromes may present at different stages, it is difficult to ascertain whether a fetus is affected by a syndromic or isolated cardiac defect, and, in these cases, early genetic diagnosis can provide valuable information on disease prognosis and genetic counseling. In our study, three of the six pathogenic variants identified by targeted NGS were related to syndromes with phenotypes not detectable prenatally. In families with genetic reproductive risks and family members with a high risk of cardiac defects, targeted NGS can be of great value in genetic counseling, including preconception counseling, prenatal diagnosis and future family planning.

One limitation of the targeted NGS technique is that it focuses exclusively on variations in exons and exon–intron boundaries of the target genes, which are known to harbor the majority of monogenic mutations, thus overlooking variants that disrupt regulatory elements located in deep introns, untranslated regions or intergenic regions. Exon sequencing or whole-genome sequencing would be required to identify further novel pathogenic genes and non-coding region variants in the remaining fetuses with CHD. In this study, the VOUS rate was as high as 79.5%, which could be attributed to the small sample size of the study and the fact that, despite all candidate genes in the panel being associated with CHD, not all variants have been studied in detail. The high rate of VOUS is a limitation of this approach, but these results should help enrich the pool of variants of CHD-associated genes. As research findings increase, a greater number of rare variants will be characterized and the VOUS rate will decline in the future.

In conclusion, targeted NGS can be a useful method in prenatal genetic diagnosis of CHD. A definite genetic diagnosis of the disease would allow a more comprehensive genetic prognosis. The method is also appropriate for individuals with a high risk of cardiac defects and families with genetic reproductive risk of cardiac diseases.

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