Targeted next-generation sequencing for research and diagnostics in congenital heart disease, and cleft lip and/or palate

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Abstract. Congenital heart disease (CHD), and cleft lip and palate (CLP) are currently the most common types of structural malformation in infants. Various methods have been used to identify the disease-associated genes. However, targeted next-generation sequencing (NGS) is not yet considered an option for routine use. Thus, the present study aimed to assess the safety and feasibility of using targeted NGS in patients with CHD concomitant with CLP. Between November 2015 and May 2017, a total of 17 patients with CHD concomitant with CLP, who were excluded from a diagnosis of trisomy syndrome, were selected at The Second Xiangya Hospital of Central South University (Changsha, China). Genomic DNA was extracted from peripheral blood samples of the patients. The copy number variants (CNVs) were determined by conducting a single nucleotide polymorphism (SNP) array with Illumina HumanOmni1-Quad Beadchip, while information on other gene mutations was obtained from targeted sequencing. The functions of gene mutations were then predicted using the PolyPhen-2, SIFT and Mutation Taster tools. Finally, Sanger sequencing was used to verify the mutations. The results identified no pathogenic mutations in CNVs analyzed by high-throughput SNP sequencing. Targeted NGS results demonstrated that 10 patients (58.8%) carried gene mutations, including 4 (23.5%) genetically diagnosed cases and 6 (35.3%) cases with unknown etiology. The 4 known diseases were Opitz G/BBB syndrome caused by *MID1* gene mutation, Loeys-Dietz syndrome caused by *TGFBR1* gene mutation, Ritscher-Schinzel/3C syndrome caused by *KIAA0196* gene mutation and CHARGE syndrome caused by *CHD7* gene mutation. The remaining 6 cases were not genetically diagnosed, although they carried candidate genes. In conclusion, the present study demonstrated that targeted NGS was an effective and accurate candidate gene detection method in patients with CHD concomitant with CLP.

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Abbreviations: CHD, congenital heart disease; CLP, cleft lip and palate; SNP, single nucleotide polymorphisms; CNVs, copy number variations; CL, cleft lip; CP, cleft palate; NGS, next-generation sequencing; PCR, polymerase chain reaction; LDS, Loeys-Dietz syndrome; RSS, Ritscher-Schinzel syndrome

Key words: targeted next-generation sequencing, copy number variants, single nucleotide polymorphism array, congenital heart disease, cleft lip, cleft palate

Introduction

Congenital heart disease (CHD), and cleft lip and palate (CLP) represent birth defects with the highest rates of incidence worldwide. Furthermore, the incidence rate of CHD in patients with CLP is 6.5-12.7%, which is notably higher in comparison with that of the general population (1,2). In addition to distorting the facial appearance, CLP can negatively affect normal infant activities, such as suckling and speaking (3). Clinically, CHD and CLP are commonly referred to as the main phenotypes, although specific syndromes are also described in certain patients, such as velocardiofacial syndrome, solitary median maxillary central incisor syndrome and Wolf-Hirschhorn syndrome, among others (4-6). Children with CLP typically require multiple surgical interventions and numerous sessions of speech therapy from infancy to early adulthood to achieve near-normal appearance and function (7). Genetic studies have suggested that deletion of the chromosome fragments and single gene mutation are both observed in these syndromes (4-6,8). However, the hereditary background of patients with such syndromes currently remains clear.

With the development of genetic sequencing technologies, numerous novel methods have been suggested as important techniques to identify disease-associated genes, including single nucleotide polymorphism (SNP) array, copy number variation (CNV) analysis, and targeted and whole exome sequencing (9-11). Traditionally, genetic testing in DNA-based diagnostic laboratories involves sequential Sanger sequencing of known disease genes. However, the diagnostic yield of next-generation sequencing (NGS) exceeds that of Sanger sequencing in genetic diseases, since multiple genes can be analyzed in a single experiment. Thus, the introduction of NGS has provided revolutionary opportunities for comprehensive genetic testing in research and diagnostics.

In the present study, the effectiveness and accuracy of using targeted NGS to determine candidate genes in patients with CHD concomitant with CLP were assessed.

Patients and methods

Patients. A total of 17 patients with CHD concomitant with CLP treated at The Second Xiangya Hospital of Central South University (Changsha, China) between November 2015 and May 2017 were enrolled into the present study (Fig. 1). The study group comprised of 14 male and 3 female patients aged 4-108 months (mean age, 42.8±32.9 months) with a mean body weight of 17.6±6.9 kg (Table I). The patient selection criteria in terms of CHD were as follows: i) Exhibiting typical clinical manifestations and symptoms of CHD on physical examination, including cyanosis and/or cardiac murmur; and ii) diagnosis of CHD by transthoracic echocardiography (12). In terms of CLP the inclusion criteria included the following: i) Typical clinical manifestations and symptoms on physical examination, including cleft lip (CL) and/or cleft palate (CP) (13,14); ii) stomatological diagnosis; and iii) amalgamation or non-merger of other malformations, or growth/mental retardation. The patient exclusion criteria were as follows: i) Patients without CHD and CLP; ii) cases diagnosed with trisomy 18 or 21 syndrome; and iii) refusal of participation by the patient's parents or guardians.

The study protocol was approved by the Review Board of The Second Xiangya Hospital of Central South University, and the relatives of study subjects provided informed consent for participation. All experiments were performed in accordance with relevant guidelines and regulations.

Blood sample collection and DNA extraction. Peripheral blood samples (600 μl) obtained from each patient were collected into 1.5 ml Eppendorf tubes (Eppendorf, Hamburg, Germany) containing protein kinase (20 μl) and cell lysate (200 μl). Tubes were agitated for 1 min, centrifuged for 10 sec at 4˚C at 9,295 x g, and subjected to genomic (g)DNA extraction using a DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocols using a QIAcube automated DNA extraction device (Qiagen, Inc.). The gDNA solution generated was stored at -80˚C. Subsequently, a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to determine the quantity and quality of the DNA samples, and 3 μg DNA from each sample was then used in subsequent assays (15-17).

SNP array analysis. Genomic DNA samples of the patients were used to conduct SNP array analysis at a final concentration of 50 ng/ml. The signal intensities of SNP probes were determined by employing an Illumina BeadScan genotyping system (Beadstation Scanner 500; Illumina, Inc., San Diego, CA, USA) with a HumanOmni1-Quad Beadchip (Illumina, Inc.), according to the manufacturer's protocol.

Targeted NGS. A targeted NGS gene panel for 455 genes that have been associated with CHD or CLP in previous studies (8,16,18,19) was employed (Table II). Targeted NGS, including library construction, capture and sequencing, was performed by Agilent Technologies, Inc. (Santa Clara, CA, USA). Enrichment of target regions and library preparation were performed using a SureSelectXT2 Custom kit (1-499 kb; Agilent Technologies, Inc.) according to the manufacturer's protocol. Library DNA concentrations were determined using an Agilent QPCR NGS Library Quantification kit (G4880A; Agilent Technologies, Inc.), with each sample at a final concentration of 10 nmol/l. Subsequently, samples were ordered with a HiSeq2000 sequencing system using TruSeq chemistry and protocols (version 3; Illumina, Inc.) (20).

Data analysis and filtering. The Ensembl database (release 95; https://www.ensembl.org/) was used for variant annotation. Filtering was performed with ANNOVAR Documentation (http://annovar.openbioinformatics.org/), using the following SNP databases for filtering: dbSNP (build 138; https://www.ncbi.nlm.nih.gov/snp), Exome Variant Server (release ESP6500SI-V2; http://evs.gs.washington.edu/EVS/), 1000 Genomes Project (released May 2012; http://www.internationalgenome.org/home) and HapMap CHB (release 28; http://hapmap.ncbi.nlm.nih.gov/). In order to predict the possible impact of variants, the following tools were used: SIFT (version 6.2.1, https://sift.bii.a-star.edu.sg/), Polyphen-2 (version 2.2.2; http://genetics.bwh.harvard.edu/pph2/), Mutation-Taster (version 2; http://www.mutationtaster.org/) and Human Splicing Finder (version 3.1, http://www.umd.be/HSF3/). The filtering strategies used are displayed in Fig. 2.

Variant validation. Variants warranting further investigation included novel variants, which were predicted to be 'likely pathogenic' or 'pathogenic' according to PolyPhen-2, Mutation-Taster and SIFT predictions, or were indicated to be 'likely pathogenic' and possessed minor allele frequencies of <0.1%, as predicted by ExAC browser (version 0.3.1; http://exac.broadinstitute.org/). Variants and samples from the parents of certain patients were assessed by Sanger sequencing. To confirm the disease-associated genes, the relevant literature was surveyed on PubMed (https://www.ncbi.nlm.nih.gov/pubmed); example literature searches included: *MID1*, Opitz G/BBB syndrome, 2007.1.1-2018.10.31, English; *TGFBR1*, Loeys-Dietz syndrome, 2007.1.1-2018.10.31, English; *KIAA0196*, Ritscher-Schinzel syndrome, 2007.1.1-2018.10.31, English; *CHD7*, CHARGE syndrome, 2007.1.1-2018.10.31, English.

Polymerase chain reaction (PCR). Entire exon and exon-intron junctions of genes were amplified by PCR. Genomic DNA (0.5 μl) obtained from peripheral blood samples of patients was added to 11 μl double-distilled water, 0.5 μl forward primer, 0.5 μl reverse primer and 2x PCR Master Mix (12.5 μl; Nanjing Saihongrui Biotechnology Co., Ltd., Nanjing, China) containing 2X Taq DNA Polymerase. qPCR was conducted as follows: Initial denaturation at 94˚C for 5 min; 35 cycles of denaturation at 94˚C for 30 sec, annealing at 50˚C for 30 sec and extension at 72˚C degrees for 30 sec; and final extension at 72˚C degrees for 10 min. Sequences of the PCR products were determined using an ABI 3100 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The primer sequences are listed in Table III.

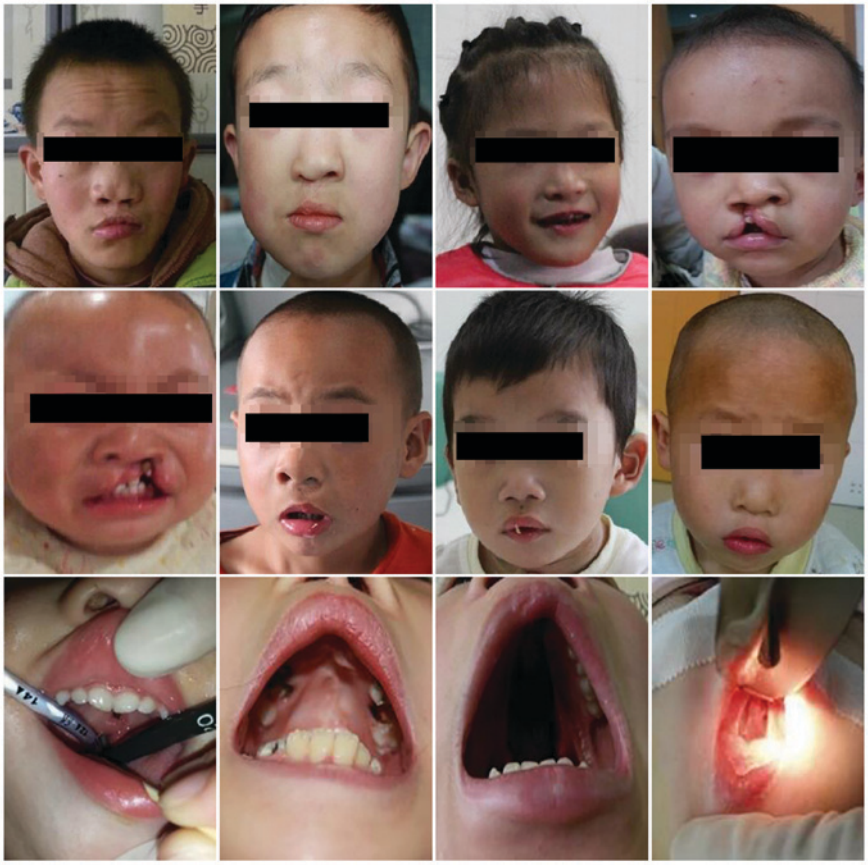

Figure 1. Characteristic examples of left lip and/or palate phenotype in patients.

Results

Screening outcomes. No pathogenic mutations were identified in CNVs analyzed by high-throughput SNP sequencing. The targeted NGS results demonstrated that 10 out of the 17 patients (58.8%) carried gene mutations, including 4 cases (23.5%) that were genetically diagnosed and 6 cases (35.3%) with unknown etiology; the remaining 7 patients did not carry known mutations. The diseases involved in the 4 known cases and the associated genes were as follows: Opitz G/BBB syndrome caused by midline-1 (*MID1*) gene mutation; Loeys-Dietz syndrome (LDS) caused by transforming growth factor-β receptor type I (*TGFBR1*) gene mutation; Ritscher-Schinzel syndrome (RSS; also known as 3C syndrome) caused by *KIAA0196* gene mutation; and CHARGE syndrome caused by chromodomain-helicase-DNA-binding protein 7 (*CHD7*) gene mutation.

Prediction of gene function. Characteristics of the named gene mutations were predicted through the PolyPhen-2, SIFT and Mutation Taster programs (Table IV). Among the genetically diagnosed cases, the *MID1* (c.G1477C, p.A723V) and *TGFBR1* (c.T1400A, p.M467K) gene mutations were predicted to be 'pathogenic', 'likely pathogenic' and 'pathogenic' by SIFT, PolyPhen-2 and Mutation Taster, respectively. The *CHD7* gene mutation (c.C4894T, p.R1632C) was predicted to be 'pathogenic' by both SIFT and Mutation Taster, while the PolyPhen-2 program predicted this mutation to be 'benign'. The *KIAA0196* gene mutation (c.A2533G, p.T845A) was predicted to be 'likely

Table I. Patient characteristics.

Characteristic	Value
Total no.	17
Males/females	14/3
Mean age (months)	42.8±32.9
Mean weight (kg)	17.6±6.9
Cardiac phenotype	
ASD	1
VSD	7
DORV	1
ASD + VSD	3
ASD + PLSVC	1
TOF + PLSVC	1
VSD + PFO	1
TOF + ASD	1
PDA + PFO	1
Maxillofacial phenotype	
CL	5
CP	9
CLP	3

VSD, ventricular septal defect; ASD, atrial septal defect; TOF, tetralogy of Fallot; PLSVC, persistent left superior vena cava; PDA, patent ductus arteriosus; PFO, patent foramen ovale; DORV, double outlet right ventricle; CLP, cleft lip and cleft palate; CP, cleft palate; CL, cleft lip.

Table II. Target regions.

Regions 1	*Regions 2*	*Regions 3*	*Regions 4*	*Regions 5*	*Regions 6*	*Regions 7*	*Regions 8*	*Regions 9*	*Regions 10*
ABCC9	*ACE*	*ACP6*	*ACTA2*	*ACTB*	*ACTC1*	*ACTN2*	*ACVR1*	*ACVR2B*	*ADRB1*
ADRB2	*ADRB3*	*AGL*	*AGT*	*AGTR1*	*AHSA2*	*ANKRD1*	*APOBEC2*	*ARL13B*	*ASXL2*
ATE1	*ATP1A2*	*ATP4A*	*ATP4B*	*BAT1*	*BBIP1*	*BBS1*	*BBS10*	*BBS12*	*BBS2*
BBS4	*BBS5*	*BBS7*	*BBS9*	*BCL11A*	*BCL6*	*BCL9*	*BCOR*	*BICC1*	*BMP7*
BMPR1A	*BMPR1B*	*BMPR2*	*BUB1B*	*C1ORF106*	*CACNA1C*	*CACNA2D1*	*CALM1*	*CALM2*	*CALR3*
CASQ2	*CAV3*	*CCDC39*	*CCDC40*	*CCT4*	*CDH1*	*CDH2*	*CDKN1C*	*CER1*	*CFC1*
CHD1L	*CHD7*	*LDB3*	*FH19*	*APPB1*	*FIBP*	*KMT2D*	*GPD1*	*TTC8*	*NGF*
HMGCL	*STIL*	*MKKS*	*RPSA*	*ARL6*	*NELFA*	*GRID2*	*KCNH2*	*TRIM32*	*CHRAC1*
CHRD	*CHRNG*	*PQBP1*	*CITED2*	*CLDN7*	*CLUL1*	*CNTF*	*COL11A1*	*COL11A2*	*COL2A1*
COL3A1	*CREBBP*	*CRELD1*	*CRHBP*	*CRX*	*CRYAB*	*CSRP1*	*CSRP3*	*CTLA4*	*CTNNA3*
CUL3	*CYP11B2*	*DAND5*	*DAPK3*	*DES*	*DHCR24*	*DHCR7*	*DHODH*	*DLL1*	*DMRT2*
DNAI1	*DNAI2*	*DOLK*	*DOT1L*	*DPP6*	*DPPA4*	*DSC2*	*DSG2*	*DSP*	*DST*
DTNA	*DVL1*	*DVL2*	*DZIP1*	*EDNRA*	*EDNRB*	*EED*	*EFNB1*	*EHMT1*	*ELN*
EMD	*EP300*	*ESCO2*	*EVC*	*EVC2*	*EYA4*	*EZH1*	*EZH2*	*FBN1*	*FBN2*
FGB	*FKTN*	*FLNA*	*FLNB*	*FMO5*	*FOXA2*	*FOXC1*	*FOXC2*	*FOXH1*	*FOXJ1*
FOXL2	*FTO*	*FXN*	*GAA*	*GADL1*	*GALNT11*	*GATA4*	*GATA5*	*GATA6*	*GATAD1*
GDF1	*GJA1*	*GJA5*	*GJA8*	*GJA9*	*GLA*	*GLI2*	*GLI3*	*GPC3*	*GPD1L*
GPR161	*GPRC6A*	*GSK3B*	*HAND1*	*HAND2*	*HCN4*	*HES1*	*HES4*	*HEY2*	*HFE*
HOXA1	*HUWE1*	*HYLS1*	*ID2*	*IDUA*	*IER2*	*IFNG*	*IFT122*	*IFT172*	*IFT20*
IFT57	*IFT88*	*IGFBP4*	*IGFBP5*	*IHH*	*IL10*	*IPPK*	*ISL1*	*JAG1*	*JARID2*
JAZF1	*JPH2*	*JUP*	*KCND2*	*KCND3*	*KCNE1*	*KCNE1L*	*KCNE2*	*KCNE3*	*KCNE4*
KCNH2	*KCNJ11*	*KCNJ2*	*KCNJ5*	*KCNJ8*	*KCNMB1*	*KCNQ1*	*KDM5A*	*KDM5B*	*KDM6A*
KIAA0196	*KIAA1841*	*KIF3A*	*KIF3B*	*KIF3C*	*KIFAP3*	*KLF13*	*KRAS*	*LAMA4*	*LAMP2*
LBR	*LDB3*	*LEFTY1*	*LEFTY2*	*LEMD3*	*LIPC*	*LLPH*	*LMNA*	*LPIN1*	*LRRC50*
LRRC6	*MARK2*	*MAX*	*MED13L*	*MED20*	*MEF2A*	*MEF2C*	*METT10D*	*MGAT1*	*MGP*
MICA	*MICB*	*MID1*	*MKKS*	*MKRN2*	*MKS1*	*MNDA*	*MSX2*	*MYBPC3*	*MYH10*
MYH11	*MYH6*	*MYH7*	*MYL2*	*MYL3*	*MYLK2*	*MYOZ2*	*MYPN*	*NAA15*	*NCOR2*
NEBL	*NEK2*	*NEXN*	*NF1*	*NFATC1*	*NFATC3*	*NFATC4*	*NFKBIL1*	*NIPBL*	*NKD1*
NKX2-5	*NKX2-6*	*NKX3-2*	*NODAL*	*NOS3*	*NOTCH1*	*NOTCH2*	*NOTCH2NL*	*NOTCH3*	*NOTCH4*
NOTO	*NPHP3*	*NPPA*	*NPPB*	*NSD1*	*NUB1*	*NUMBL*	*NUP188*	*OBSCN*	*OFD1*
OSR1	*PAFAH1B1*	*PAPOLG*	*PCMTD2*	*PCSK5*	*PDLIM3*	*PEX1*	*PEX13*	*PHF8*	*PHYHD1*
PIFO	*PITX2*	*PKD1L1*	*PKD2*	*PKP2*	*PLA2G7*	*PLAGL1*	*PLN*	*PPM1K*	*PPP3CA*
PQBP1	*PRC1*	*PRDM1*	*PRKAB2*	*PRKAG2*	*PROX1*	*PSEN1*	*PSEN2*	*PTCH1*	*PTCH2*
PTPLA	*PTPN11*	*PTPN22*	*PTPRC*	*RAB10*	*RAB23*	*RAF1*	*RAI1*	*RAI2*	*RANGRF*
RAPGEF5	*RBM20*	*REL*	*RFX2*	*RFX3*	*RIT1*	*RNF20*	*ROCK2*	*ROR2*	*RPGRIP1L*

Table II. Continued.

Regions 1	*Regions 2*	*Regions 3*	*Regions 4*	*Regions 5*	*Regions 6*	*Regions 7*	*Regions 8*	*Regions 9*	*Regions 10*
RUNX2	S100Z	SALL1	SALL2	SALL4	SATB2	SCN1B	SCN3B	SCN4B	SCN5A
SDC2	*SDHA*	*SEL1L3*	*SEMA3E*	*SESN1*	*SETBP1*	*SGCA*	*SGCB*	*SGCD*	*SGCE*
SGCG	*SHH*	*SHOC2*	*SIX3*	*SLC26A2*	*SLC2A10*	*SLMAP*	*SMAD2*	*SMAD5*	*SMARCD3*
SMO	*SMYD1*	*SMYD2*	*SNAI1*	*SNTA1*	*SOD2*	*SOS1*	*SOX17*	*SOX9*	*SRF*
STIL	*SUFU*	*SUPT3H*	*SUPT5H*	*SUV420H1*	*TAZ*	*TBX1*	*TBX20*	*TBX3*	*TBX5*
TCAP	*TCF21*	*TCOF1*	*TDGF1*	*TFAP2A*	*TFAP2B*	*TGFB1*	*TGFBR1*	*TGFBR2*	*TGIF1*
TLL1	*TMBIM4*	*TMEM195*	*TMEM43*	*TMPO*	*TNF*	*TNFRSF21*	*TNNC1*	*TNNI3*	*TNNT2*
TP63	*TPM1*	*TRDN*	*TRPM4*	*TSC1*	*TSEN15*	*TTC21B*	*TTC30A*	*TTR*	*TWIST1*
TXNDC3	*UBE2B*	*UBR1*	*UMODL1*	*USF1*	*USP34*	*USP44*	*VANGL2*	*VCL*	*VEGFA*
VEGFC	*VIT*	*WDR5*	*WHSC1*	*WNT3A*	*XPO1*	*ZEB2*	*ZFPM1*	*ZIC3*	*ZNF480*
ZNF528	*ZNF534*	*ZNF610*	*ZNF638*	*ZNHIT3*					

Table III. Primer sequences.

Gene	Primers (5'-3')
MID1	F: CACTTTGTGATAGGAGGCATGA
	R: ACACATTTCCAGCTACTCCATAG
TGFBR1	F: CACCAGTACCCTATTGATGGAAA
	R: AGTTCAGGCAAAGCTGTAGAA
KIAA0196	F: CATATTTGGTGCCGCATGTC
	R: AGAAGCCTGTCTAAGCCATTTA
CHD7	F: AATGTTTCAGGGTGGAGA
	R: CAGGACCTTGTTACAGTGAT
NPHP3	F: CACAATGCAGTATAGCACACAAA
	R: CTTATCTGTTCCAGCCACACT
PCSK5	F: GTGCCTTGTTAATCCCTTTACAC
	R: CAGGCTGCTCTTGCTCTT
FBN2	F: TAATGAGTGTGTCGCCCTTC
	R: TGGCACTTAGTATGTTTCCAGAG
DLL1	F: CTGTCTTTGGTTTGTCTGGTTTC
	R: AAGTCGTTCACGCCATCC
NOTCH3	F: TCCTAAACTCACCCTGTCCT
	R: GCACAGTCGTAAGTGAGGTC
COL3A1	F: AAGCAGCATCACTGTCATCTAA
	R: AGAACTGCCCATTTGTGGT
CHD1L	F: GAACTCGCTCTGAGGTTCAA
	R: AACTTCTAAGGTCACAGGTTAGG
FMO5	F: GCTGAGTAAAGAGAACACTTGGA
	R: TGGCTGTTTGGCTAATCTCTAC
LLPH	F: GGATGAACCAAAGGCAAAGAAA
	R: GAGGAGTTATGCTGGGTTTGA
PPARGC1A	F: GGATGAACCAAAGGCAAAGAAA
	R: GAGGAGTTATGCTGGGTTTGA
PTPN22	F: GGACATAGAGCTGAATTTGCTTC
	R: CAAAGACAAGCTCTCTATAAGGTAGA
TTC30A	F: GGAACTCTTTATTGTGCCAAAGG
	R: CTGGACTCATCTGTGACTGTATTC
OBSCN	F: CCACGCTGGACTCCATTAG
	R: GCACAGATGGGTGGATGAA
DAND5	F: GTCATTGCTCCTCTCTCTACATC
	R: ACGTCTTTCTTGGTCCATCTC
STIM2	F: CTAGAGCTTGTGCATGGGAA
	R: GACTTTGCTCTGCAGTTTGTAAG

F, forward; R, reverse; *MID1*, midline-1; *TGFBR1*, transforming growth factor-β receptor type I; *CHD7*, chromodomain-helicase-DNA-binding protein 7; *NPHP-3*, nephrocystin-3; *PCSK5*, proprotein convertase subtilisin/kexin type 5; *FBN2*, fibrillin 2; *DLL1*, delta-like protein 1; *NOTCH3*, Notch 3; *COL3A1*, collagen type III α1 chain; *CHD1L*, chromodomain helicase DNA binding protein 1 like; *FMO5*, flavin containing monooxygenase 5; *LLPH*, LLP homolog, long-term synaptic facilitation factor; *PPARGC1A*, peroxisome proliferator activated receptor gamma coactivator 1 alpha; *PTPN22*, protein tyrosine phosphatase non-receptor type 22; *TTC30A*, tetratricopeptide repeat domain 30A; *OBSCN*, obscurin; *DAND5*, DAN domain BMP antagonist family member 5; *STIM2*, stromal interaction molecule 2.

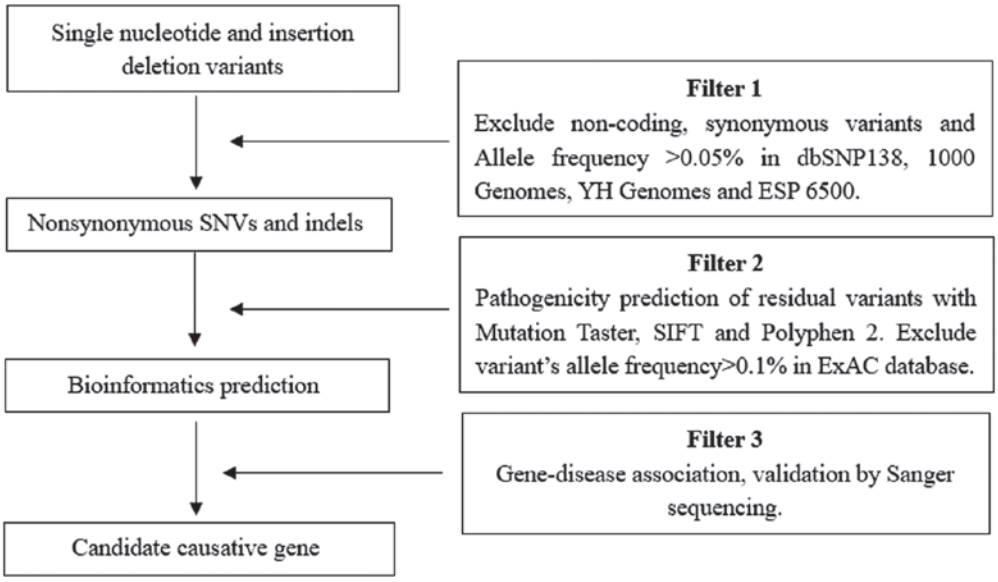

Figure 2. Flow chart representing the filtering strategies employed in the present study.

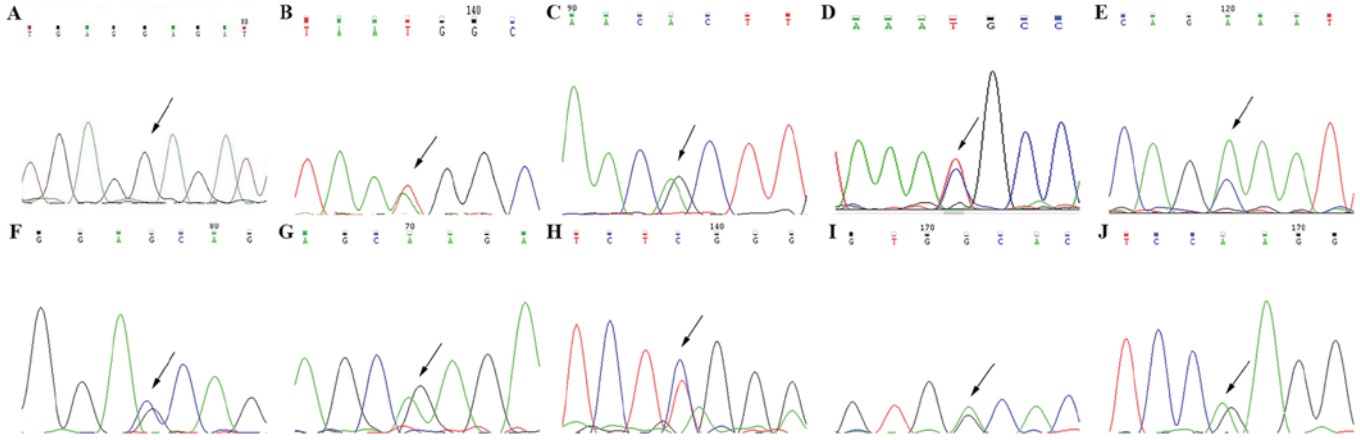

Figure 3. Results of Sanger sequencing. Results for patients 1 to 10 are presented in graphs A-J, respectively. The first four patients were diagnosed as follows: Patient 1, Opitz G/BBB syndrome; patient 2, Loeys-Dietz syndrome; patient 3, Ritscher-Schinzel syndrome; and patient 4, CHARGE syndrome.

pathogenic' by PolyPhen-2 and 'pathogenic' by Mutation Taster, while the SIFT program predicted this mutation to have 'uncertain significance'. Potential pathogenic genes were identified in the remaining 6 cases; however, literature searches did not reveal previously reported associations between the genes and diseases of interest. Therefore, the cases cannot be diagnosed based upon the identified mutations.

Sanger sequencing. Finally, Sanger sequencing verified each mutation (Fig. 3), which was followed by review of the literature in the context of each mutation to obtain the genetic diagnosis (8,21-23). The parents of certain patients were also studied through the use of Sanger sequencing, but the identified gene mutations were not detected in any of the parents (data not shown).

Discussion

CHD and CLP are characterized by anomalous anatomical structures, caused by abnormal development of the heart and large blood vessels in CHD (24), or abnormal fusion of the lip and palate during embryonic development in CLP (25). These diseases can severely affect neonatal health, thus representing a burden to families and the society (16). With the rapid development of genetic sequencing technology, a number of methods are considered to be important in identifying disease-associated genes, including Sanger sequencing, SNP array, CNV analysis, and targeted and whole exome sequencing.

In the current study, no pathogenic mutations were identified in CNVs analyzed by high-throughput SNP sequencing. Certain gene mutations were successfully identified in 10 patients (58.8%) via targeted NGS. According to the clinical phenotype of the patient and the mutation site of the candidate pathogenetic gene, 4 of these patients were diagnosed with a known genetic syndrome. To the best of our knowledge, it appears that the present study identified for the first time a mutation (c.G1477C, p.A723V) in the *MID1* gene as a possible cause of ventricular septal defect and CL in an

Table IV. Results of targeted sequencing.

Patient no.	Age (months)	Sex	CHD	CLP	Gene	Chromosome	Prediction tool			CCDS	Amino acid
							SIFT	PolyPhen-2	Mutation taster		
1	108	Male	VSD	CL	*MID1*	Xp22.2	Pathogenic	Likely pathogenic	Pathogenic	G1477C	A723V
2	48	Male	TOF, PLSVC	CLP	*TGFBR1*	9q22.3	Pathogenic	Likely pathogenic	Pathogenic	T1400A	M467K
3	9	Male	PDA, PFO	CL	*KIAA0196*	8q24.13	Uncertain significance	Likely pathogenic	Pathogenic	A2533G	T845A
4	60	Male	VSD, ASD	CL	*CHD7*	8q12.2	Pathogenic	Benign	Pathogenic	C4894T	R1632C
5	4	Female	VSD, ASD	CP	*NPHP3*	3q22.1	Uncertain significance	Benign	Pathogenic	C1228A	Q410K
6	36	Male	VSD	CL	*PCSK5*	1q21.1	Pathogenic	Likely pathogenic	Pathogenic	C5041G	P1681A
7	24	Male	DORV, VSD	CP	*FBN2*	5q23.3	Uncertain significance	Likely pathogenic	Pathogenic	G6154A	E2052K
8	72	Male	ASD, PLSVC	CP	*DLL1*	6q27	Pathogenic	Likely pathogenic	Pathogenic	C1307T	S436L
9	84	Female	VSD	CP	*NOTCH3*	19p13.12	Pathogenic	Likely pathogenic	Pathogenic	G515A	G172D
10	30	Male	ASD	CP	*COL3A1*	2q32.2	Uncertain significance	Likely pathogenic	Pathogenic	G1472A	R491Q

CHD, congenital heart disease; VSD, ventricular septal defect; ASD, atrial septal defect; TOF, tetralogy of Fallot; PLSVC, persistent left superior vena cava; PDA, patent ductus arteriosus; PFO, patent foramen ovale; DORV, double outlet right ventricle; CLP, cleft lip and palate; CP, cleft palate; CL, cleft lip; MID1, midline-1; TGFBR1, transforming growth factor-β receptor type I; CHD7, chromodomain-helicase-DNA-binding protein 7; NPHP-3, nephrocystin-3; PCSK5, proprotein convertase subtilisin/kexin type 5; FBN2, fibrillin 2; DLL1, delta-like protein 1; NOTCH3, Notch 3; COL3A1, collagen type III α1 chain; CCDS, consensus coding sequence.

Opitz G/BBB syndrome patient. The *MID1* gene is located on the short arm of the X chromosome, is approximately 300 kb, and includes 9 coding exons and multiple non-coding exons. In early embryonic development, the *MID1* gene is highly expressed in the heart, facial region and central nervous system (26,27). In total, >90 different mutations of the *MID1* gene have been reported in the literature, and point mutations in this gene have been suggested to cause Opitz G/BBB syndrome (18,21,28-31). The MID1 protein encoded by the *MID1* gene is a ubiquitin ligase that interacts with the α4 protein, which is linked to the protein phosphatase PP2A and forms the complex *MID1*-α4-PP2A (27,32). This complex is closely associated with the development of the ventral midline; therefore, this is also the main reason for the abnormal development of the ventral midline structure caused by *MID1* gene mutation (33).

LDS is characterized by vascular abnormalities (cerebral, thoracic, and abdominal arterial aneurysms and/or dissections), skeletal manifestations, craniofacial features (such as CP) and cutaneous findings (34). Approximately a third of LDS cases are caused by *TGFBR1* mutation, while two thirds are caused by *TGFBR2* mutation; the mutation site is mostly located in the serine-hydroxybutyrate enzyme activation coding region, located in the intracellular portion of the TGF-β receptor (35). The TGF-β type I receptor is necessary for the fusion of the upper lip and soft palate (36,37), and the *TGFBR1* gene serves a major role in the development of the heart (22). In the present study, the *TGFBR1* gene mutation was predicted to be 'pathogenic', 'likely pathogenic' and 'pathogenic' by the SIFT, PolyPhen-2 and Mutation Taster programs, respectively. It was also identified for the first time that mutation (c.T1400A, p.M467K) in the *TGFBR1* gene was a possible cause of tetralogy of Fallot and CL in the LDS patient.

RSS is a clinically heterogeneous disorder characterized by distinctive craniofacial features (including CP) in addition to cerebellar and cardiac anomalies (8). To date, two articles (8,38) have been reported on cases of RSS caused by *KIAA0196* gene mutation. Mutation (c.A2533G, p.T845A) in *KIAA0196* gene was investigated in the present study, which is a novel candidate gene involved in heart development. The *KIAA0196* gene is situated at 8q24.13 of chromosome 8, and the encoded protein of this gene is known as strumpellin, which is comprised of 1,159 amino acids and is highly conserved (8). This protein is ubiquitously expressed in multiple systems and is highly expressed in skeletal muscle. *KIAA0196* mutations have been reported to cause hereditary spastic paraplegia (39), while a complex overlapping phenotype, particularly with CHD, has been rarely reported. In 2013, Elliott *et al* (8) detected *KIAA0196* gene mutations in 8 patients with RSS/3C syndrome. The expression of strumpellin protein was also reduced by 60%, and the patients exhibited abnormal phenotype of heart development defects. In addition, previous studies have indicated that there are genes that cause cardiac abnormalities in the 8q24 interval, and suggested that the *KIAA0196* gene is incorporated into the interval (8,40). These studies also suggested that the *KIAA0196* gene may serve a role in the pathogenesis of cardiac developmental disorders.

CHARGE syndrome is a congenital condition comprising of choroid disease, heart disease, atresia choanae, retarded growth and development, genital hypoplasia, and ear anomalies and/or deafness; facial palsy, micrognathia, CP and swallowing difficulties are also common (41). In the present study, the *CHD7* gene mutation (c.C4894T, p.R1632C) was predicted to be 'pathogenic' by both SIFT and Mutation Taster, while the PolyPhen-2 program predicted this mutation to be 'benign'. To date, ~193 mutations of the *CHD7* gene have been reported to lead to CHARGE syndrome (23,42). The *CHD7* gene encodes the CHD7 protein, which serves a role in chromatin remodeling, cell cycle regulation, apoptosis regulation, transcriptional regulation and embryonic stem cell diversity (43). Studies have demonstrated that the *CHD7* gene is expressed in a number of fetal tissues, including the fetal eye, ear, brain cells, olfactory bulb and heart tube, among others (19,43). The majority of mutations lead to the production of non-functional CHD7 protein, which may disrupt chromatin remodeling and gene expression. Regulation changes in *CHD7* gene expression during embryonic development may lead to symptoms and signs of CHARGE syndrome (19).

The remaining 6 cases were not genetically diagnosed, although candidate genes were identified, including nephrocystin-3 (*NPHP-3*), proprotein convertase subtilisin/kexin type 5 (*PCSK5*), fibrillin 2 (*FBN2*), delta-like protein 1 (*DLL1*), Notch 3 (*NOTCH3*) and collagen type III α1 chain (*COL3A1*). The *NPHP3* gene mutation (c.C1228A, p.Q410K) may impact the development of cilia tissue, while it has been reported that primary ciliary dyskinesia is associated with the development of CHD (44). In addition, the *PCSK5* gene mutation (c.C5041G, p.P1681A) was predicted to be 'pathogenic', 'likely pathogenic' and 'pathogenic' by the SIFT, PolyPhen-2 and Mutation Taster tools, respectively. In mice, the *PCSK5* gene causes VACTERL syndrome, which comprises of deformity in vertebral, anorectal, cardiac, tracheoesophageal, renal, limb and other systems (45). The *FBN2* gene mutation (c.G6154A, p.E2052K) may cause congenital contractural arachnodactyly. The cardiovascular phenotype of this syndrome is milder and less common in comparison with that of Marfan syndrome, and the ascending aorta is also slightly dilated, which may be combined with other intracardiac malformations (46). Furthermore, the SIFT, PolyPhen-2 and Mutation Taster programs respectively predicted the *DLL1* gene mutation (c.C1307T, p.S436L) to be 'pathogenic', 'likely pathogenic' and 'pathogenic'. The protein encoded by the *DLL1* gene is a ligand in the Notch signaling pathway, which mainly regulates the apoptosis of hematopoietic cells and signals between cells. The Notch signaling pathway serves an important role in embryonic differentiation and in homeostasis in adults, as well as in the development of various systems (47). Additionally, the protein encoded by the *NOTCH3* gene, which was found to be affected by mutation (c. G515A, p. G172D) in the present study, is among the key proteins in the Notch signaling pathway. The disease caused by the mutation is an autosomal dominant arteriopathy associated with subcortical infarcts and leukoencephalopathy (48). Although CHD or CLP caused by mutations in the *NOTCH3* gene has not been reported to date, the role of the Notch signaling pathway in the growth and development of various systems is widely recognized (47). The *COL3A1* gene mutation (c.G1472A, p.R491Q) was predicted to be 'likely pathogenic' and 'pathogenic' by PolyPhen-2 and Mutation Taster, respectively, whereas the SIFT program

predicted this mutation to be of 'uncertain significance'. This gene mutation may cause Ehlers-Danlos syndrome and aortic aneurysm. According to review of the literature, it was noted that all of the mutation sites determined in the current study are novel mutation sites that have not been previously reported to the best of our knowledge. Samples obtained from the parents of certain patients were also examined by Sanger sequencing, however, these parents did not carry the identified genes mutations. The results should be further verified in an animal model, such as zebra fish or mouse.

When considering all forms of genetic sequencing technology, the advantages of targeted NGS are evident. Firstly, the amount of tedious and repetitive work for researchers is reduced, owing to the fact that this method can rapidly analyze large quantities of genetic information. NGS enables thousands of genes to be analyzed simultaneously, or a smaller subset of genes (a 'mini-genome' or disease-specific panel) to be examined in a single assay. However, the limitation of sample size and the possibility of leak detection of base point mutation exist in SNP array technology. Secondly, targeted NGS not only allows focusing on specific genes associated with pathological expression, but can also improve the coverage and expressive quality of exons due to its efficient enrichment. Large-scale parallel sequencing of a specifically selected part of the genome (for example, the exome or a specific set of genes relevant to a disease phenotype) leads to a higher sequencing coverage as compared with that of whole-genome sequencing (49). Furthermore, for a specific phenotype or disease, targeted sequencing has lower cost and is more rapid than whole exome sequencing, since this technology reduces the genetic discovery that may be irrelevant to the disease (49). In addition, targeted NGS may be more clinically useful in comparison with other sequencing techniques, owing to faster turnaround time (reduced sequencing volume and associated data analysis), higher and more reliable coverage, and the ability to avoid incidental findings. However, this method is associated with certain disadvantages including the limitation of requiring known virulence genes and its lack of suitability for a single sample.

Notably, the implementation of NGS in clinical practice has altered the way genetic counsellors and other clinicians approach genetic testing. Molecular diagnostics may now be performed at an early stage of disease, often enabling a broader set of therapeutic options and a lengthened window of opportunity to ameliorate disease progression (50). The identification of underlying genetic defects can also improve diagnosis of the disease prior to genetic counselling and enable prenatal testing.

In conclusion, using targeted NGS technology, the present study determined 10 individual mutations (58.8%) in candidate disease genes, which are possible causes of CHD and CLP in patients. The targeted NGS was demonstrated to be an effective and accurate method for providing a specific diagnosis of CHD and CLP, despite the presence of diverse phenotypes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HB and TZ conceived and designed the study, and drafted the manuscript. HB and LL collected the data. HB, TZ and SH were involved in data cleaning and verification. HB, TZ and ZT analyzed the data. All authors were involved in the final draft of the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Review Board at The Second Xiangya Hospital of Central South University (China), and the relatives of study subjects provided informed consent. All experiments were performed in accordance with relevant guidelines and regulations.

Patient consent for publication

Written informed consent was obtained from the their parent, guardian or next of kin for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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