



Whole-exome sequencing revealed novel genetic alterations in patients with tetralogy of Fallot

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Background: The most prevalent cyanotic congenital heart disease (CHD) phenotype is tetralogy of Fallot (TOF). Rare genetic variations have been identified as significant risk factors for CHD. Thus, this research sought to identify the pathogenic variations and molecular etiologies of TOF.

Methods: This study employed whole-exome sequencing (WES) and Sanger sequencing to identify pathogenic variations in DNA samples from patients with TOF. The pathogenicity of the variations was predicted using an in-silico approach.

Results: We enrolled 17 patients with TOF in this study. Among these patients, 14 had mutations in TOF-related genes, including *G7B2*, *TBX15*, *CTNS*, *SPINK1*, *GATA6*, *PRIMOL*, *GDF15*, *SLC17A9*, *AIFM1*, *FOXC2*, *KLF13*, *ABCA4*, *CPA6*, *FKBP10*, *ASPA*, *SBF1*, *HBA2*, *IGLL1*, *GNE*, and *KLHL10*. We also gathered WES data from three participants without TOF, who comprised the control group, but no variations were found in the indicated genes. Further analysis showed that the patients with *FKBP10* and *GNE* variants had more serious clinical symptoms. Sanger sequencing confirmed that the two variants were heterozygous in TOF patients.

Conclusions: We identified several genetic variants associated with TOF and confirmed that *FKBP10* and *GNE* variants were associated with TOF severity. The findings of this study help researchers and clinicians on genetic counseling with the verification of the potential of WES in detecting TOF and help implement early interventions for patients with TOF.

Keywords: Tetralogy of Fallot (TOF); whole-exome sequencing (WES); *FKBP10*; *GNE*

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Introduction

Congenital heart disease (CHD) is the most prevalent birth abnormality, and affects around eight out of every 1,000 infants born (1-3). Tetralogy of Fallot (TOF) is the most prevalent and complicated cyanotic CHD, and affects around one out of every 2,500 newborns (4,5). TOF is a condition that affects the heart's outflow tract and is defined by four specific structural abnormalities identified postnatally: a ventricular septal defect, an anterocephalad deviation

of the outflow septum with aortic override, a variable obstruction of the right ventricular outflow tract (RVOT) (pulmonary stenosis), and right ventricle hypertrophy (6-9). With the help of surgical procedures performed throughout childhood, 85–90% of TOF patients now live until the age of at least 30 years (10). However, due to the severe morbidity associated with the scar tissue following surgery and pulmonary regurgitation in adulthood, only 25% of TOF patients have an event-free life beyond the age of 40 years (11,12).

The etiology of TOF is unknown, and no single gene is

responsible for the phenotypic characteristics of the disease (13,14). Research on syndromic TOF sufferers has provided crucial insights into some causal genes (15). Notably, 20% of the cases have been linked to a known syndrome or chromosomal abnormality (16). About 15% of TOF patients have 22q11.2 deletion syndrome for which T-box transcription factor is the primary cause (17,18). About 80% of TOF cases are non-syndromic, but the etiology of non-syndromic TOF is not known, mostly because the inheritance is non-Mendelian (19–21). As a result, a polygenic genetic architecture has been theorized and genome-wide techniques have been used to gain insights into the complicated genetic abnormalities responsible for TOF and other CHDs (20–23).

Research has shown that whole-exome sequencing (WES) may reveal novel CHD candidate genes (24). The aim of this study was to find TOF-related pathogenic genes through WES technology performed on the genes of 17 TOF patients, providing a basis for studying TOF pathogenesis. We present this article in accordance with the MDAR reporting checklist (available at <https://tp.amegroups.com/article/view/10.21037/tp-23-449/rc>).

Methods

Serum sample collection

A total of 17 patients with TOF who underwent cardiac surgery (7 males and 10 females) at Guizhou Provincial

People's Hospital between March 2018 and July 2021 were included in this study. TOF was confirmed by echocardiography, clinical symptoms, signs, and intraoperative findings. Inclusion criteria were confirmed diagnosis of TOF (via review of the medical chart and echocardiograms). Exclusion criteria were patients with TOF with pulmonary atresia, absent of pulmonary valve, or placement of right ventricle to pulmonary artery conduit in case of large coronary artery branch crossing RVOT. The patients' specific features are detailed in *Table 1*. The study protocol was approved by the Ethics Committee of Guizhou Provincial People's Hospital (No. 2018040) and was performed in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from the enrolled participants or their legal guardians.

DNA sample preparation

Genomic DNA was extracted using a QIAamp DNA Blood Kit (TransGen Biotech, China) from peripheral blood samples taken from the patients.

WES and data analysis

The Agilent SureSelect Human Exon V5 kit (Agilent Technologies) was used to extract and collect genomic DNA from the blood to generate the library for WES. The library preparations were sequenced on the Illumina HiSeq 2000 platform, supplied by Illumina Inc. (San Diego, CA, USA) and 100-bp paired-end reads were yielded.

Validation by Sanger sequencing

The primers for the standard polymerase chain reaction assays were designed using Primer 3 software (<https://bioinfo.ut.ee/primer3-0.4.0/>). Sanger sequencing was used to analyze the amplified DNA fragments directly. The DNASTAR program (<https://dnastar.com/>) was used to assess the sequencing data by comparing the findings to the reference sequences. The whole sequenced data were trimmed for low-quality sequences and aligned to UCSC human reference genome (GRCh38/HG38) using Burrows-Wheeler Alignment (BWA). The Genome Analysis Toolkit (GATK) and VarScan were used to detect single nucleotide polymorphisms (SNPs) and small insertions/deletions. ANNOVAR was used to annotate the variants with several databases including dbSNP, gnomAD, 1000 Genomes Project, and ExAC. Finally, four online

Highlight box

Key findings

- We performed whole-exome sequencing (WES) in a large cohort of tetralogy of Fallot (TOF) patients and identified very rare deleterious variations in several genes.

What is known and what is new?

- TOF is a complex congenital heart disease with clinical and genetic heterogeneity.
- We enrolled 17 patients with TOF in this study. Among these patients, 14 had mutations in TOF-related genes, including *G7B2*, *TBX15*, *CTNS*, *SPINK1*, *GATA6*, *PRIMOL*, *GDF15*, *SLC17A9*, *AIFM1*, *FOXC2*, *KLF13*, *ABCA4*, *CPA6*, *FKBP10*, *ASPA*, *SBF1*, *HBA2*, *IGLL1*, *GNE*, and *KLHL10*.

What is the implication, and what should change now?

- Our findings may help clinicians determine the potential of WES in detecting TOF and implement early interventions for patients with TOF.

Table 1 The clinical characteristics of the patients with TOF

Case ID	Age (years)	Sex	Echocardiography			Acropachia	Cyanosis
			RV diastolic diameter (mm)	RVAW (thickened) (mm)	RVOT (mm)		
TOF-1	3	Female	18.4	7.0	6.1	No	Yes
TOF-2	5	Female	11.5	8.6	4.0	No	No
TOF-3	3	Male	14.5	4.6	5.2	No	Yes
TOF-4	2	Female	10.7	5.8	5.0	No	Yes
TOF-5	22	Female	24.2	6.4	7.0	Yes	Yes
TOF-6	2	Female	13.2	7.0	5.2	No	No
TOF-7	29	Female	22.6	12.6	6.0	No	Yes
TOF-8	1	Female	8.0	6.5	7.2	No	Yes
TOF-9	1	Male	14.4	6.0	6.8	Yes	Yes
TOF-10	3	Male	15.9	7.4	–	Yes	Yes
TOF-11	56	Male	28.1	8.9	12.0	Yes	Yes
TOF-12	25	Female	22.9	8.0	4.0	No	Yes
TOF-13	2	Male	13.2	5.7	5.5	No	Yes
TOF-14	30	Female	22.9	10.0	12.5	Yes	Yes
TOF-15	12	Female	20.2	9.2	5.0	Yes	Yes
TOF-16	1	Male	13.0	7.1	3.5	No	Yes
TOF-17	–	Male	–	–	–	–	–

TOF, tetralogy of Fallot; RV, right ventricle; RVAW, right ventricular anterior wall; RVOT, right ventricular outflow tract.

mutation pathogenicity prediction and analysis softwares including PolyPhen, SIFT, MutationTaster, and FATHMM were used to predict the influence of polymorphic variation on coding proteins and conservation, so as to conduct mutation pathogenicity analysis. The primer sequences were as follows: forward primer (FKBP10-F), 5'-GGTCTTTCACGTCCTCCTGA-3', reverse primer (FKBP10-R), 5'-GAATCGAAGAGGGTGCCGT-3'; forward primer (GNE-F), 5'-TGGAGACAGGTTTGATGCCC-3', reverse primer (GNE-R), 5'-TGTCATAGGAAGGGCAGCCT-3'.

Statistical analysis

SPSS 20.0 statistical software was used to analyze the data. The number of mutations, disease-related mutations and sequencing depth of patients were listed and analyzed.

Results

Clinical features of patients with TOF

A total of 17 TOF patients were included in this study and the clinical features of the TOF patients are listed in *Table 1*.

Analysis of the WES findings in patients with TOF

WES was used to examine the genetic etiology of TOF in the 17 patients. Only variants with a minor allele frequency of <1% in the Single Nucleotide Polymorphism Database (dbSNP), 1000G (1000 Genomes Project, <http://www.internationalgenome.org/>), ESP6500 (Exome Variant Server, <http://evs.gs.washington.edu/EVS/>), and gnomAD databases (Genome Aggregation database, <https://gnomad.broadinstitute.org/>) were retained. The retained variations were filtered based on the TOF candidate genes that

Table 2 *In-silico* analysis of the variants found by whole-exome sequencing

Case ID	Gene	Variations (mRNA)	Variations (amino acid)	Pathogenicity evidence	gnomAD
TOF-1	<i>GJB2</i>	NM_004004.6:c.299_300del	p.His100fs	P	f=4.1e-5
	<i>TBX15</i>	NM_001330677.2:c.980G>A	p.Arg327His	VUS	f=5e-4
TOF-2	<i>CTNS</i>	NM_004937.3:c.611_613ACG	p.Asp205del	LP	f=8.2e-6
TOF-3	<i>SPINK1</i>	NM_001354966.1:c.194+2T>C	splicing	P	f=2.8e-4
TOF-4	<i>GATA6</i>	NM_005257.5:c.151G>A	p.E51K	P	f=1.7e-4
	<i>VSD</i>	NM_001300767.2:c.-123T>G	NA	P	f=4e-4
	<i>GDF15</i>	NM_004864.3:c.651G>C	p.Ala218Pro	VUS	f= NA
TOF-5	<i>SLC17A9</i>	NM_001302643.2:c.-56C>T	p.Arg9Cys	P	f= NA
TOF-8	<i>AIFM1</i>	NM_001130846.3:c.13C>T	p.Leu5Phe	P	f=2.3e-4
	<i>FOXC2</i>	NM_005251.2:c.1061T>A	p.Leu354Gln	LP	f= NA
TOF-9	<i>KLF13</i>	NM_001302461.2:c.319T>A	p.Ser107Thr	VUS	f= NA
TOF-11	<i>PRIMOL1</i>	NM_001300767.2:c.-123T>G	NA	P	f=4e-4
	<i>ABCA4</i>	NM_000350.3:c.1531C>T	p.Arg511Cys	LP	f=2.1e-4
	<i>KLF13</i>	NM_001302461.2:c.319T>A	p.Ser107Thr	VUS	f= NA
TOF-12	<i>CPA6</i>	NM_020361.5:c.544C>T	p.Arg182Ter	LP	f=2.5e-5
TOF-13	<i>FKBP10</i>	NM_021939.3:c.831dupC	p.Gly278fs	P	f=1.9e-4
TOF-14	<i>ASPA</i>	NM_000049.3:c.79G>A	p.Gly27Arg	P	f=1.6e-5
	<i>SBF1</i>	NM_001365819.1:c.4693A>G	p.Thr1565Ala	LP	f=3.2e-4
TOF-15	<i>HBA2</i>	NM_000517.6:c.427T>C	p.Ter143Gln	P	f=4.9e-5
	<i>IGLL1</i>	NM_001369906.1:c.428C>T	p.Pro143Leu	LP	f=2e-4
TOF-16	<i>GNE</i>	NM_005476.7:c.527A>T	p.Asp176Val	LP	f=4.1e-5
	<i>KLF13</i>	NM_001302461.2:c.310G>C	p.Glu104Gln	VUS	f= NA
TOF-17	<i>KLHL10</i>	NM_001329596.2:c.673G>A	p.Ala225Thr	P	f=4.2e-4
	<i>IGLL1</i>	NM_001369906.1:c.428C>T	p.Pro143Leu	LP	f=2e-4

TOF, tetralogy of Fallot; P, pathogenic; VUS, variants of uncertain significance; LP, likely pathogenic; NA, not available.

had been chosen. In total, 21 variations in 20 genes were discovered in 14 individuals, including 21 heterozygous variants in *GJB2* (Gap Junction Protein Beta 2), *TBX15* (T-Box Transcription Factor 15), *CTNS* (Cystinosin, Lysosomal Cystine Transporter), *SPINK1* (Serine Peptidase Inhibitor Kazal Type 1), *GATA6* (GATA Binding Protein 6), *PRIMOL* (Homo sapiens primase and DNA directed polymerase), *GDF15* (Growth Differentiation Factor 15), *SLC17A9* (Solute Carrier Family 17 Member 9), *AIFM1* (Apoptosis Inducing Factor Mitochondria Associated 1), *FOXC2* (Forkhead Box C2), *KLF13* (KLF Transcription Factor 13), *ABCA4* (ATP Binding Cassette Subfamily A

Member 4), *CPA6* (Carboxypeptidase A6), *FKBP10* (FKBP Prolyl Isomerase 10), *ASPA* (aspartoacylase), *SBF1* (SET Binding Factor 1), *HBA2* (Hemoglobin Subunit Alpha 2), *IGLL1* (Immunoglobulin Lambda Like Polypeptide 1), *GNE* (Glucosamine (UDP-N-Acetyl)-2-Epimerase/N-Acetylmannosamine Kinase), and *KLHL10* (Kelch Like Family Member 10) (Table 2). None of the above genes were discovered in the WES data of the three participants in the control group.

In addition, we analyzed the clinical symptoms of the TOF patients with gene variations and found that patients with *FKBP10* and *GEN* variants had more serious

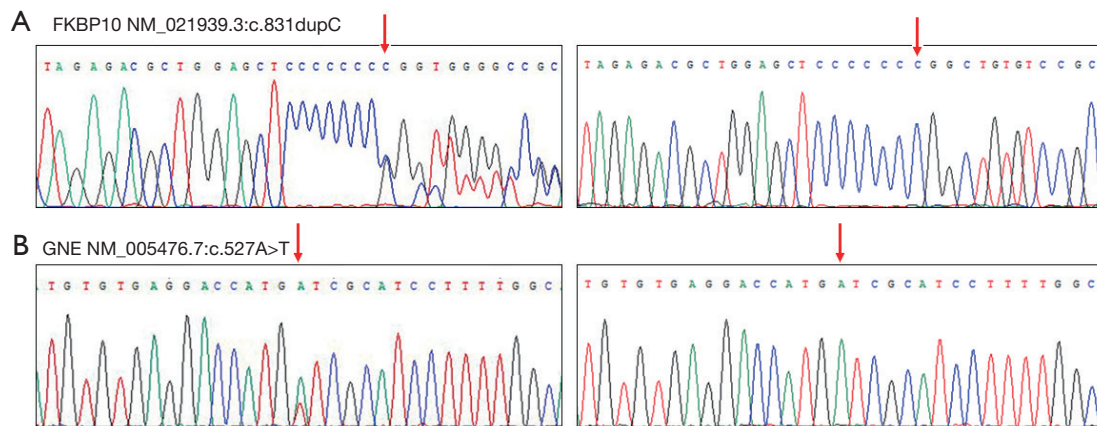


Figure 1 Confirmatory analysis of our whole-exome sequencing data by Sanger sequencing. (A) *FKBP10* and (B) *GNE*. *FKBP10*, FKBP Prolyl Isomerase 10; *GNE*, Glucosamine (UDP-N-Acetyl)-2-Epimerase/N-Acetylmannosamine Kinase.

clinical symptoms (thickening and obvious stenosis of RVOT, pulmonary valve thickening and stenosis). Sanger sequencing was used to validate the variants. Heterozygous variants c.831dupC in *FKBP10* (Figure 1A) and c.527A>T in *GEN* (Figure 1B) were identified in the TOF patients.

Analyses of the variations discovered in the TOF patients

An in-silico analysis was conducted to analyze the 21 different variations. First, we examined the frequency with which the variations appeared. The gnomAD database (Genome Aggregation database, <https://gnomad.broadinstitute.org/>) reported that all of these variants were unusual, and six of these variants were not found in the 1000G databases (1000 Genomes Project, <http://www.internationalgenome.org/>). Thus, the frequency of these mutations in the study was very low, which matched the phenomenon of the low incidence of TOF in the population (Table 2). We grouped variants into the following categories according to guidelines from the American College of Medical Genetics and Genomics (ACMG) and Association of Molecular Pathology (AMP): pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB) and benign (B) (25).

Discussion

The first clinical application of WES indicated that the success rate of mutation detection in individuals with a genetic illness was roughly 25% (26). A recent WES study has identified variants in *NOTCH1* and *FLT4*, the most commonly observed genes, in 7% of TOF cases (27). Jin *et al.* found dominant *FLT4* mutations accounting for

2.3% of TOF cases (28). Some of the mutations in our top gene candidates were de novo, while others were found in patients who appeared to be asymptomatic, which suggested that penetrance was only partially achieved. WES was conducted to analyze the DNA samples of 17 TOF patients, and 21 mutations related to TOF were found in 14 of the included patients. Our findings support the idea that genetic variations in several genes have a role in TOF etiology and that sporadic cases of unknown origin result from a common genetic variation in one of these genes.

FK506 binding protein 10 is encoded by the *FKBP10* gene, which is found on chromosome 17q21.2 and belongs to the immunophilins family of proteins with Peptidylprolyl cis/trans isomerase (PPIase) activity, which aids in the appropriate folding of type I collagen before the triple helices are assembled (29). Alanay *et al.* in 2010 reported that a mutation of the *FKBP10* gene was involved in osteogenesis imperfecta (OI) type XI (30). A total of 191 somatic *FKBP10* point mutations that are not synonymous have been documented in the COSMIC database (<http://cancer.sanger.ac.uk/cosmic>). In the Yupik Inuit community, Barnes *et al.* discovered the biallelic c.877 879delTAC (p.Tyr293del) mutation in *FKBP10*, which causes Kuskokwim syndrome with subtle skeletal symptoms, such as congenital contractures and osteopenia without fractures (31). In this study, we identified a novel mutation, c.831dupC, in a patient with TOF. Moreover, a mutation in *GNE* (UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase) was identified. The *GNE* c.527A>T (p.Asp176Val) variant is a well-described pathogenic variant (32,33). Previous research has shown that 20% of *GNE* myopathy mice (30 weeks of age)

develop endomysial fibrosis, amyloid deposition, and ringed vacuoles in cardiomyocytes (34).

Owing to the complexity of TOF, for personalized diagnosis and treatment for TOF patients, WES may be necessary to help identify the pathological mutations. The use of WES may not only reveal the genetic pathophysiology of individuals with unexplained TOF, but may also have extensive potential on the application in the screening and early identification of patients with the disorder. The poor treatment results and the uncertain etiology of TOF cause patients to suffer both psychologically and physically. More effective treatment options will be able to be designed and patient compliance will probably increase if the genetic causes of the disease can be identified early in the course of illness development. In addition, future causality studies are warranted to translate the observations into preclinical validation. This study has some limitations, and the sample size is small. In future, the number of samples should be further increased and an in-depth research will be conducted.

Conclusions

In conclusion, we identified several genetic variants that were associated with TOF and confirmed that variants of *FKBP10* and *GNE* were associated with TOF severity, which help determine the potential of WES in detecting TOF and implement early interventions for patients with TOF.

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Footnote

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Data Sharing Statement: Available at <https://tp.amegroups.com/article/view/10.21037/tp-23-449/dss>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tp.amegroups.com/article/view/10.21037/tp-23-449/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study protocol was approved by the Ethics Committee of Guizhou Provincial People's Hospital (No. 2018040) and was performed in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from the enrolled participants or their legal guardians.

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