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A novel variant in *TBX20* (p.D176N) identified by whole-exome sequencing in combination with a congenital heart disease related gene filter is associated with familial atrial septal defect*#

Ji-jia LIU[1], Liang-liang FAN[2], Jin-lan CHEN[1], Zhi-ping TAN[3], Yi-feng YANG[†‡1]

([1]*Department of Cardiothoracic Surgery, the Second Xiangya Hospital, Central South University, Changsha 410011, China*)

([2]*Department of Cell Biology, School of Life Sciences, Central South University, Changsha 410013, China*)

([3]*Center of Clinical Gene Diagnosis and Therapy, the State Key Laboratory of Medical Genetics, the Second Xiangya Hospital, Central South University, Changsha 410011, China*)

[†]E-mail: yangyifengcsuxy@gmail.com

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Abstract: Congenital heart disease (CHD) is the leading cause of birth defects, and its etiology is not completely understood. Atrial septal defect (ASD) is one of the most common defects of CHD. Previous studies have demonstrated that mutations in the transcription factor T-box 20 (TBX20) contribute to congenital ASD. Whole-exome sequencing in combination with a CHD-related gene filter was used to detect a family of three generations with ASD. A novel *TBX20* mutation, c.526G>A (p.D176N), was identified and co-segregated in all affected members in this family. This mutation was predicted to be deleterious by bioinformatics programs (SIFT, Polyphen2, and MutationTaster). This mutation was also not presented in the current Single Nucleotide Polymorphism Database (dbSNP) or National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP). In conclusion, our finding expands the spectrum of *TBX20* mutations and provides additional support that *TBX20* plays important roles in cardiac development. Our study also provided a new and cost-effective analysis strategy for the genetic study in small CHD pedigree.

Key words: Congenital heart disease (CHD), Atrial septal defect (ASD), Whole-exome sequencing, CHD-related gene filter, *TBX20*

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1 Introduction

Congenital heart disease (CHD) is the most common birth defect and the leading non-infectious cause of death in the newborn, affecting 19–75 per 1000 live births. Since CHD could cause prenatal lethality, the actual incidence may be much higher (Pierpont *et al.*, 2007; Bruneau, 2008; Richards and Garg, 2010). Atrial septal defect (ASD; OMIM 612794) is one of the most common forms of CHD and occurs in both isolation and other complex cardiac malformations.

Genetically, CHD is a very heterogeneous disease. To date, the amount of genes related to CHD including ASD has been identified (Andersen *et al.*, 2013): (1) transcription factors and co-factors, e.g., *GATA4* (OMIM 600576), *NKX2-5* (OMIM 600584), *TBX5* (OMIM 601620), and *TBX20* (OMIM 606061); (2) ligands-receptors, e.g., *CRELD1* (OMIM 607170);

‡ Corresponding author

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(3) structure protein of sarcomere, e.g., *MYH6* (OMIM 160710), *MYH7* (OMIM 160760), and *ACTC1* (OMIM 102540) (Posch *et al.*, 2010b; Wessels and Willems, 2010; Ware and Jefferies, 2012; Andersen *et al.*, 2013; Fahed *et al.*, 2013).

T-box 20 (TBX20) is a member of the T-box family that encodes the transcription factor TBX20. TBX20 carries strong transcriptional activation and repression domains, and physically or genetically interacts with several cardiac development transcription factors, including NKX2-5, GATA4, GATA5, and TBX5 regulating various aspects of embryonic heart development. In the developing mouse embryos, *tbx20* is expressed in cardiac progenitor cells, as well as in the developing myocardium and endothelial cells associated with endocardial cushions, the precursor structures for the cardiac valves and the atrioventricular septum, which implies that *tbx20* is essential for proper heart development. Loss function of *tbx20* in the mouse has been found in connection with various forms of congenital heart defects, including defects in septation, valvulogenesis, cardiomyopathy, and arrhythmia (Stennard *et al.*, 2003; Stennard *et al.*, 2005; Kirk *et al.*, 2007; Liu *et al.*, 2008; Posch *et al.*, 2010a; Sotoodehnia *et al.*, 2010; Shen *et al.*, 2011; Zhang *et al.*, 2011; Qiao *et al.*, 2012).

In our study, by using whole-exome sequencing in combination with a CHD-related gene filter, all non-coding and synonymous variants, as well as variants present in the Single Nucleotide Polymorphism Database (dbSNP), 1000 Genomes, HapMap, YH, and Exome Sequencing Project (ESP) databases and variants which are not in 455 CHD-related genes (Data S1) were excluded initially. According to prediction by three bioinformatics programs (SIFT, Polyphen2, and MutationTaster) and co-segregation analysis, we identified a novel mutation (c.526G>A/p.D176N) in exon3 of *TBX20* in all affected members in three generations of a family with ASD. To the best of our knowledge, this mutation has not been reported in previous studies.

2 Materials and methods

2.1 Subjects

A family from Hunan Province in central-south China with seven members across three generations participated in this study. Three patients were diagnosed as having ASD (I1, II2, and III1) (Table 1; Fig. 1a). All patients were diagnosed by transthoracic echocardiograms in the Department of Cardiothoracic Surgery of the Second Xiangya Hospital, China. All family members were provided informed consent for collection, storage, and use of DNA for the purpose of research. A proband (III1 in Fig. 1a) consented specifically for whole-exome sequencing. This study protocol was approved by the Review Board of the Second Xiangya Hospital of the Central South University, China.

2.2 Methods

2.2.1 DNA extraction

Genomic DNA (gDNA) was extracted from peripheral blood lymphocytes of the participants. gDNA was prepared using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) on the QIAcube automated DNA extraction robot (Qiagen, Hilden, Germany) as previously described (Tan *et al.*, 2012).

2.2.2 Targeted capture and massive parallel sequencing

Exome capture and high-throughput sequencing (HTS) were performed in the State Key Laboratory of Medical Genetics of China in collaboration with Beijing Genomic Institute (BGI Shenzhen, China) (Wang *et al.*, 2010). gDNA (5 μg) from the proband (III1) in this family was captured with the NimbleGen SeqCap EZ library exome capture reagent (Roche Inc., Madison, USA) and sequenced (Illumina HiSeq2000, 90 base paired-end reads; Illomina Inc., USA). Briefly, gDNA was randomly fragmented by a Covaris S2 instrument (Covaris Inc., USA). Then, the 250–300 bp fragments of DNA were subjected to three enzymatic steps: end repair, A-tailing, and adapter ligation. Once the DNA libraries were indexed, they were amplified by ligation-mediated polymerase chain reaction (PCR). Extracted DNA was purified and hybridized to the NimbleGen Seqcap EZ Library. Each captured library was then loaded onto the Illumina HiSeq2000 platform. Illumina base calling software V1.7 was employed to analyze the raw image files with default parameters.

Table 1 Summary of the family with atrial septal defect (ASD)

Family	Age	CHD	Echocardiography				TBX20	
			ASD size (mm)	RA (mm)	RV (mm)	LVEF (%)	DNA	Protein
III1 (proband)	7 months	ASD	15	29	26	69	526G>A	D176N
I1	59 years	ASD	2	35	34	60	526G>A	D176N
I2	61 years	No		33	30	62		
II1	31 years	No		32	30	65		
II2	28 years	ASD	12	43	41	63	526G>A	D176N
II3	25 years	No		32	31	69		
III2	3 years	No		24	22	72		

CHD: congenital heart disease; RA: right atrium; RV: right ventricle; LVEF: left ventricular ejection fraction

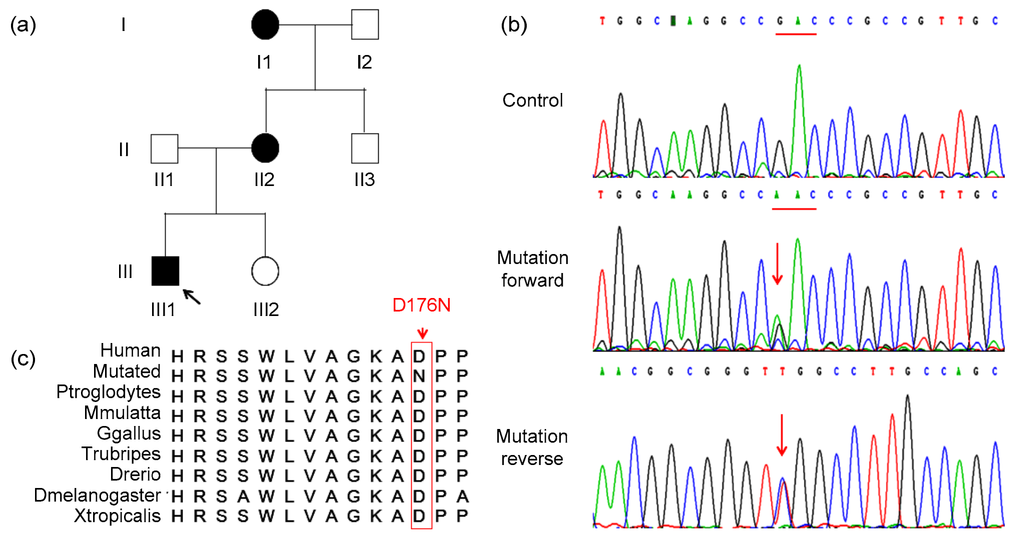

Fig. 1 Sequencing and analysis of *TBX20* mutation (p.D176N) in the family with ASD

(a) Pedigree of the family affected with ASD. Family members are identified by generations and numbers. Squares: male members; circles: female members; black symbols: affected members; white symbols: unaffected members; arrow: proband. (b) Sequencing results of the *TBX20* mutation. Sequence chromatogram indicates a G to A transition of nucleotide 526. (c) Alignment of multiple TBX20 protein sequences across species. The D176 affected amino acid locates in the highly conserved amino acid region in different mammals

2.2.3 Read, mapping and variant detection

Single-nucleotide polymorphism (SNP) analysis was performed as previously described (Gao *et al.*, 2013): (1) reads were aligned to the NCBI human reference genome (gh19/NCBI 37.1) with SOAPaligner method V2.21; (2) for paired-end reads with duplicated start and end sites, only one copy with the highest quality was retained and the reads with adapters were removed; (3) SOAPsnp V1.05 was used to assemble the consensus sequence and call genotypes; (4) small insertions and deletions (INDELs) detection was used with the Unified Genotyper tool from GATK V1.0.4705.

2.2.4 Filtering and annotation

Five major steps were taken to prioritize all the high-quality variants among CHD-related genes (Gao *et al.*, 2013): (1) variants within intergenic, intronic, and untranslated regions (UTRs) and synonymous mutations were excluded from later analysis; (2) variants in dbSNP132 (http://www.ncbi.nih.gov/projects/SNP/), the 1000 Genomes project (1000G, http://www.1000genomes.org), and HapMap project (ftp://ftp.ncbi.nlm.nih.gov/hapmap) were excluded; (3) variants in YH database (http://yh.Genomics.org.cn/) and National Heart, Lung, and Blood Institute (NHLBI) ESP database (http://evs.gs.washington.

edu/EVS/) were further excluded; (4) variants not in 455 CHD-related genes (Data S1) were excluded (Wilde and Behr, 2013; Zaidi *et al.*, 2013); (5) SIFT (http://sift.bii.astar.edu.sg/), Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), and MutationTaster (http://www.mutationtaster.org) were used to predict the possible impact of variants.

2.2.5 Mutation validation and co-segregation analysis

Sanger sequencing was used to validate the candidate variants found in the whole-exome sequencing. Segregation analysis was performed in all family members. Primer pairs used to amplify fragments encompassing individual variants were designed by an online tool PrimerQuest (Integrated DNA Technologies, Inc.; http://www.idtdna.com/Primerquest/Home/Index) and the sequences of PCR primers will be provided upon request.

3 Results

3.1 Patient characteristics and phenotype information

A Chinese family with isolated secundum ASD was first identified after the proband (III1) was referred for evaluation of a murmur at 7 months old. The echocardiography described a dilated right atrium, dilated right ventricle, and a secundum ASD measuring 15 mm in dimension. Meanwhile, the family history revealed that there were an additional two related living individuals diagnosed as having ASD. Both the proband (III1) and II2 underwent successful surgical repairs. I1 without any treatment was diagnosed with secundum ASD by echocardiography (Fig. 1a; Table 1). No other malformations were observed in the three affected members, which indicated that this family CHD is an isolated or non-syndromic CHD with an autosomal dominant pattern.

3.2 Exome sequencing and co-segregation analysis

To detect the causative genetic alteration in this family, whole-exome sequencing in combination with a CHD-related gene filter was performed on the proband (III1). The result demonstrated a set of 19 single nucleotide variants in 16 CHD candidate genes after filtering (Table 2). Co-segregation analysis of six causative variants (*OBSCN*, *USF1*, *TBX20*, *LDB3*, *MYH6*, and *IFT20*) (Table 2), which were predicted by three programs (SIFT, MutationTaster, and Polyphen2) showed that only *TBX20* gene mutation segregated in all affected family members (Figs. 1a and 1b; Table 1). Unaffected family members who were assessed did not carry the mutation. The missense mutation (c.526G>A) results in a substitution of aspartic acid by asparagine in the TBX20 protein (p.D176N). This newly identified c.526G>A mutation was not found in our 200 control cohorts (Tan *et al.*, 2012). This mutation was also not presented in the current dbSNP and NHLBI ESP.

3.3 Variant analysis

The aspartic acid residue at position 176 in TBX20 protein is highly evolutionarily conserved in diverse species including chimp, monkey, chicken, pufferfish, zebrafish, melanogaster, and frog (Fig. 1c). Three programs for analyzing protein functions, Polyphen2, SIFT, and MutationTaster, predicted that the p.D176N variants are probably damaging (0.985), damaging (0.004), and disease causing (23), respectively.

4 Discussion

Due to the complexity of CHD attributed by both genetic and nongenetic effectors, the etiology of CHD is still not completely understood. To date, approximately 500 genes have been revealed to be related to cardiac development defects in mice when mutated, and 55 human genes have been identified associated with CHDs (Andersen *et al.*, 2013; Fahed *et al.*, 2013; Wilde and Behr, 2013; Zaidi *et al.*, 2013). Complex or rare Mendelian disorders in small CHDs pedigree make the discovery of novel genes difficult or impossible using the traditional approach (Rabbani *et al.*, 2012).

However, next-generation sequencing technologies such as the whole-exome sequencing approach are improving as rapid, high-throughput, and cost-effective approaches to fulfill medical sciences and research demands (Ng *et al.*, 2009; Metzker, 2010; Ku *et al.*, 2011). In our study, the pedigree is really small and it is difficult to discover a new causative gene. Therefore, we initially hypothesized that the

Table 2 Variants identified by whole-exome sequencing in combination with CHD candidate gene filter

Gene	Chr	Base position	RB	AB	Mutation	Amino acid alteration	Sorting intolerant from tolerant	Polyphen2	MutationTaster
NOTCH2NL	chr1	145273345	T	C	Missense	S>P	rs10910779		
NOTCH2	chr1	120539661	C	T	Missense	R>Q	rs146498360		
OBSCN	chr1	228562288	G	A	Missense	G>R	Damaging (0.004)	PD (0.997)	DC (125)
USF1	chr1	161011931	T	G	Missense	Y>C	Tolerated (0.199)	PD (0.560)	DC (194)
ZNF638	chr2	71576412	A	G	Missense	I>V	rs12612365		
ZNF638	chr2	71650308	G	A	Missense	A>T	Damaging (0.024)	Benign (0.094)	Polymorphism (58)
VEGFC	chr4	177605086	C	T	Missense	M>I	Tolerated (0.103)	Benign (0.000)	Polymorphism (10)
DST	chr6	56472194	C	T	Missense	C>Y	rs185733722		
TBX20	chr7	35288308	C	T	Missense	D>N	Damaging (0.004)	PD (0.985)	DC (23)
LRRC6	chr8	133634908	G	T	Missense	P>H	rs76147813		
LDB3	chr10	88469751	C	T	Missense	A>V	Tolerated (0.291)	PD (0.745)	DC (64)
PTPN11	chr12	112892433	T	G	Nonsense	Y>*	rs76982592		
PTPN11	chr12	112892407	T	G	Missense	S>A	rs79068130		
MYH6	chr14	23855762	A	T	Missense	I>N	Damaging (0.000)	Benign (0.248)	DC (194)
MYH6	chr14	23871682	C	T	Missense	G>S	rs148962966		
IFT20	chr17	26658963	T	G	Missense	N>H	Damaging (0.015)		DC (68)
DSC2	chr18	28651796	G	T	Missense	R>S	Tolerated (0.382)	Benign (0.095)	Polymorphism (110)
DOT1L	chr19	2211146	T	C	Missense	V>A	Damaging (0.014)	Benign (0.001)	Polymorphism (64)
EP300	chr22	41527628	A	G	Missense	S>G	rs146242251		

Chr: chromosome; RB: reference sequence base; AB: alternative base identified; PD: probably damaging; DC: disease causing. Variants were share by two family members (III1 and II1) after filtering. Each row represents a single variant. Shaded rows represent the five variants that were validated independently and screened for in affected family members. Only *TBX20* (in box) was segregated with disease in this family

causative gene is in the list of related genes for CHD (Data S1) after analysis of whole-exome sequencing data. According to prediction by three bioinformatics programs (SIFT, Polyphen 2, and MutationTaster), six candidate causative genes were highly suspicious (*OBSCN*, *USF1*, *TBX20*, *LDB3*, *MYH6*, and *IFT20*; Table 2). Co-segregation analysis demonstrated that only *TBX20* gene mutation (c.526G>A/p.D176N) was segregated in all affected family members. If the variant is not in the 455 CHD-related genes, much more work needs to be done, such as whole-exome sequencing on all other family members. If so, it is inevitable that the cost and workload will increase. Therefore, our research provided a new and cost-effective strategy for genetic study in small CHD pedigree (Fig. 2).

TBX20 plays a critical role in embryonic development and organogenesis, including cell type specification, tissue patterning, and morphogenesis (Smith, 1999; Packham and Brook, 2003; Showell *et al*., 2004). Inherited *TBX20* mutations (I152M, Q195*) in patients with ASD were first identified using the first generation sequencing technology (Kirk *et al*., 2007). The author reported missense (I152M) and nonsense (Q195*) mutations in two families with isolated ASD or/and other cardiac structure anomaly. Subsequently, other studies identified other *TBX20* mutations via the first generation sequencing technology. Liu *et al.* (2008) found a number of variants among Chinese patients with ASD with or without other congenital heart defects, including tetralogy of Fallot (TOF), total anomalous pulmonary venous connection (TAPVC). Qian *et al.* (2009) reported that two different variants of *TBX20* were found in four children with ASD with or without other CHD. Posch *et al.* (2010a) identified a *TBX20* missense variant in a patient with ASD with additional TOF and cardiac valve defect (Table 3).

In this study, the whole-exome sequencing in combination with a CHD-related gene filter was performed to investigate a family with ASD. A novel mutation c.526G>A in *TBX20* causing a missense

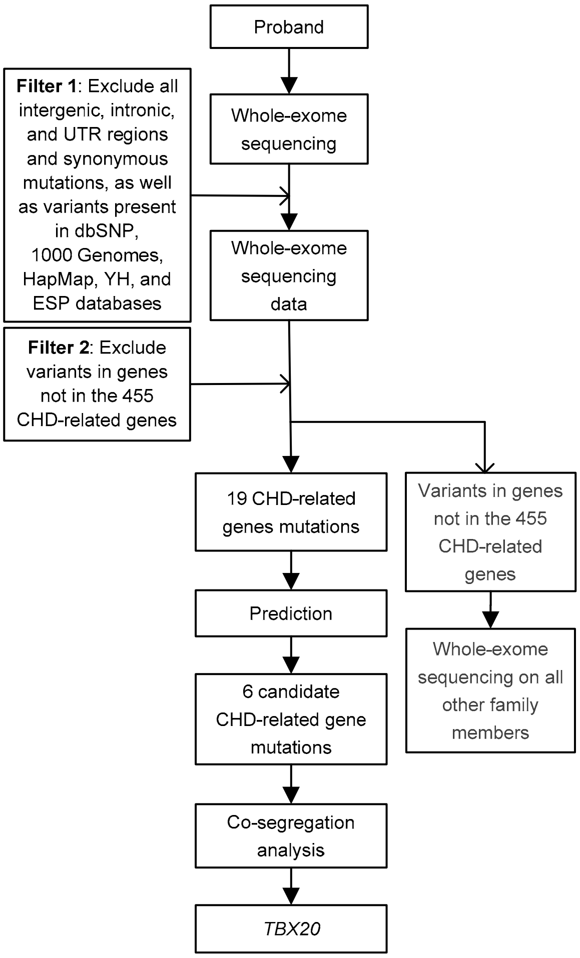

Fig. 2 Analysis strategy for a novel causative mutation in small CHD pedigree

Table 3 Summary of identified ASD-related *TBX20* gene mutations

Reference	Nucleotide change	AA change	Cardiac defect
Kirk *et al.* (2007)	456C>G	I152M	ASD,VSD, PFO
	583C>T	Q195*	ASD, CoA, MVP, MR, DCM
Liu *et al.* (2008)	187G>A	A63T	ASD
	361A>T	I121F	TAPVC, ASD
Qian *et al.* (2009)	597C>G	H186D	ASD, MR, TOF, cleft mitral valve
	601T>C	L197P	ASD, TOF
Posch *et al.* (2010)	374C>G	I121M	ASD, TOF, cardiac valve defect

AA: amino acid; ASD: atrial septal defect; CoA: coarctation of aorta; DCM: dilated cardiomyopathy; MR: mitral regurgitation; MVP: mitral valve prolapse; PFO: patent oval foramen; TAPVC: total anomalous pulmonary venous connection; TOF: tetralogy of Fallot; VSD: ventricular septal defect

change (p.D176N) that affected a highly conserved residue in an evolutionarily conserved protein was identified. The p.D176N was not found in the public databases and our 200 control cohorts. Meanwhile, Polyphen2, SIFT, and MutationTaster predicted that p.D176N will be deleterious in its effect. Co-segregated analysis showed that p.D176N segregates with disease in this family. These findings demonstrated that this variant should not be excluded from further study.

This identified missense change (p.D176N) is in the T-box DNA binding domain of TBX20 (109–288 AA). TBX20 associated directly with other cardiac transcription factors, namely, the homeodomain factor NKX2-5 and zinc finger factor GATA4 and GATA5 (Stennard *et al.*, 2003). Modification of amino acid from aspartic acid to asparagine may not prevent binding to its target DNA site, but there are other possibilities, such as an influenced rate of scanning of DNA or co-factors for interaction, or abnormal structure stability when bound to co-factors (Posch *et al.*, 2010a). Previous studies have demonstrated that identified ASD-related *TBX20* mutations are all in the T-box DNA binding domain (109–288 AA) except p.A63T (Fig. 3; Table 3) (Kirk *et al.*, 2007; Liu *et al.*, 2008; Qian *et al.*, 2009; Posch *et al.*, 2010a). Therefore, although *in vitro* assays were not performed in our study, we still believed that the mutation (p.D176N) in this study plays a critical role in CHDs. In our further analysis, the functional test will be performed.

In summary, we reported a novel *TBX20* mutation (p.D176N) in a three-generation family with three ASD patients. The present identification of a novel mutation not only further supports the important role of cardiac transcription factor TBX20 in congenital ASD, but also expands the spectrum of *TBX20* mutations and will contribute to the genetic diagnosis and counseling of families with CHD. Meanwhile, our study provided a new and cost-effective analysis strategy for the genetic study in small CHD pedigree.

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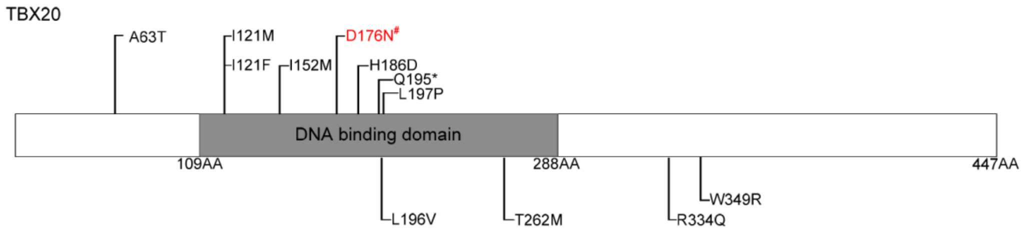

Fig. 3 Schematic representation of TBX20 protein structure with exonic germline mutations related to non-syndromic CHD indicated

All mutations related to ASD are represented on the top. Mutations found in patients with CHD other than ASD are shown below the structural domain. # indicate the novel mutation in our study

Compliance with ethics guidelines

Ji-jia LIU, Liang-liang FAN, Jin-lan CHEN, Zhi-ping TAN, and Yi-feng YANG declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for which identifying information is included in this article.

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List of electronic supplementary materials

Data S1 Four hundred and fifty-five candidate genes for congenital heart disease (CHD)

中文概要：

本文题目：全外显子测序结合先天性心脏病相关基因过滤在一家族性房间隔缺损家系中检测出新的致病突变 ***TBX20*****（D176N）**

A novel variant in *TBX20* (p.D176N) identified by whole-exome sequencing in combination with a congenital heart disease related gene filter is associated with familial atrial septal defect

研究目的：寻求该房间隔缺损家系遗传致病原因。

创新要点：1．鉴定出一个全新的家族性房间隔缺损相关性 *TBX20* 突变；2．首次使用全外显子测序结合先天性心脏病相关基因过滤的方法来研究小家系遗传致病因素；3．*TBX20* 的 T-box DNA 结合域的突变与先天性心脏病有关。

研究方法：对一个临床发现的房间隔缺损家系（图 1a）的先证者进行全外显子测序，运用公共数据库过滤后，使用先天性心脏病相关基因再次过滤，得到了 19 个候选基因；然后，运用 SIFT、Polyphen-2 和 MutationTaster 等软件预测，排除了 13 个多态性位点（表 2）；最后，运用共分离检测（聚合酶链式反应产物直接测序），找到该家系致病的遗传因素，即 *TBX20* 基因发生了错义突变（D176N）（图 2），该突变位点在 ESP 和 dbSNP 数据库中也未曾发现，且该位点在多种生物中高度保守（图 1c）。

重要结论：1. 本研究发现的 *TBX20* 突变（D176N）是该房间隔缺损家系致病的原因，同时该突变位点为世界上首次报道；2. 全外显子测序结合先天性心脏病相关基因过滤是一个分析小家系遗传致病因素的有效又经济的方法。

关键词组：先天性心脏病；房间隔缺损；全外显子测序；先天性心脏病相关基因；*TBX20*