

## Original Article

# Single nucleotide polymorphism of NKX2-5 gene with sporadic congenital heart disease in Chinese Bai population

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**Abstract:** Background: Congenital heart disease (CHD) is the most common birth abnormality, especially for sporadic CHD. However, the etiology of sporadic CHD is largely unknown. NKX2-5, the earliest sign of cardiac progenitor cell differentiation, plays a key role in cardiac morphogenesis, and the mutation of this gene can cause sporadic CHD. Purpose: To investigate the association of genetic variations of NKX2-5 with sporadic CHD in Chinese Bai people. Methods: The whole 2 coding exons and flanking intron sequences of NKX2-5 gene were screened using DNA sequencing in 70 Chinese Bai patients with sporadic CHD and 136 healthy controls. Results: A novel heterozygous DNA sequence variant (DSV), 1433A>G, was identified in one tetralogy of Fallot (TOF) patient and one persistent left superior vena cava (PLSVC) patient, but none in controls. The frequency of single nucleotide polymorphism (SNP) rs2277923 in CHD group was significantly higher than that in control group. The allele and genotype were associated with the occurrence of CHD. Conclusion: The novel DSV (1433A>G) may be relevant with TOF and PLSVC, and the SNP rs2277923 of NKX2-5 gene contributes to the risk of sporadic CHD in Chinese Bai people.

**Keywords:** Congenital heart disease, NKX2-5, single nucleotide polymorphism, Chinese Bai people

## Introduction

Congenital heart disease (CHD) is the main cause of death in infants, which affects 0.4%-1% of live births worldwide [1]. CHD can be classified as syndromic CHD and non-syndromic CHD, based on whether the heart is associated with external cardiac abnormalities. In non-syndromic CHD, there is only a heart defect, without external cardiac abnormality. It can be divided into two subtypes, including familial CHD and sporadic CHD. The total number of patients with sporadic CHD approximately accounts for 80% of CHD patients [2-4].

CHD is considered to be caused by genetic and environmental factors. Recent reports [5, 6] have stated that, an increasing number of genes, including cardiac transcription factor GATA4, NKX2-5 and TBX5, are associated with non-syndromic CHD. NKX2-5 is a homeobox transcription factor. It is the first known marker

to be a genetic cause of CHD, and is initially validated in pedigrees with autosomal dominant inheritance of cardiac septal defects [7]. The dosage-sensitive interdependence between cardiac transcription factors and chromatin remodeling complex has been observed in the heart development [8]. NKX2-5 gene knockout at mid-embryonic stage can cause premature death and defective cardiac morphogenesis [9]. Approximately 38 missense and nonsense mutations in NKX2-5 gene have been found, and these mutations mainly exist in Caucasian population (only 4 mutations found in Chinese patients). A total of 7 mutations (rs17052019, rs2277923, rs3729938, rs3729753, rs3729754, rs703752 and rs11552707) have been highly proved to be involved in congenital heart malformation [10].

The epidemiological investigation shows that, the incidence of CHD has significant difference among different nationalities in Yunnan prov-

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**Table 1.** Distribution of congenital heart disease in patients

Sort	Gender		Total (%)
	Male	Female	
VSD	5	8	13 (18.6)
ASD	8	18	26 (37.1)
PFO	3	3	6 (8.6)
TOF	2	3	5 (7.1)
PDA	0	4	4 (5.7)
VSD+ASD	2	1	3 (4.3)
VSD+PFO	1	1	2 (2.6)
ASD+PDA	0	1	1 (1.4)
ASD+PFO	1	0	1 (1.4)
RVOTS	0	1	1 (1.4)
TECD	1	0	1 (1.4)
TI	1	0	1 (1.4)
BAV	2	0	2 (2.6)
HCM	1	0	1 (1.4)
PLSVC	1	0	1 (1.4)
Else	1	1	2 (2.6)
Total	29	41	70 (100)

PFO, patent foramen ovale; TOF, tetralogy of Fallot; PDA, patent ductus arteriosus; RVOTS, right ventricular outflow tract septum; TECD, complete type endocardial cushion defect; TI, tricuspid insufficiency; BAV, bicuspid aortic valve; HCM, hypertrophic cardiomyopathy; PLSVC, persistent left superior vena cava.

ince of China. The incidence of CHD in Bai minority accounts for 8.12‰ of Yunnan people, with Dai of 5.39‰ and Han of 3.11‰ [11]. The Bai minority has a long history and culture in the Southwest border area of China, which mainly distributes in Dali Bai Minority Autonomous District, Yunnan, China. Historically, The Bai nationality is a mainstay group of the culture of Tibetan and Myanmar, which assimilate large numbers of people from different nationalities. Bai people descend from the ancient Baiman population within Kunming ethnic group (a branch of ancient Qiang nationality) living in Erhai lake area, and it also assimilates an array of Han people. Meanwhile, partial gene flow influence deriving from the central Asian region has existed in Bai people. This study investigated the distribution and frequency of above 7 mutations in NKX2-5 gene in Chinese Bai people with sporadic CHD. The objective is to clarify the correlation between the single nucleotide polymorphisms (SNPs) of NKX2-5 gene and sporadic CHD.

## Subjects and methods

### Subjects

A cohort of 70 CHD patients (29 male and 41 female; average age of  $14.0 \pm 7.3$  years), chosen based on ultrasound cardiogram imaging evidence of CHD, were recruited from Department of Cardiac Surgery, Yan'an Affiliated Hospital of Kunming Medical University (Kunming, China). The variety of CHD was listed in **Table 1**. CHD in all patients was confirmed by surgery. The 136 controls (57 male and 79 female; average age of  $13.5 \pm 3.4$  years) were selected from Department of Physical Examination, Yan'an Affiliated Hospital of Kunming Medical University (Kunming, China). Written informed consents were signed in all subjects. This study was approved by the Ethics Committee of Kunming Medical University. CHD patients and controls were all unrelated, and were the Chinese Bai people who settled in Dali Bai Minority Autonomous District in Yunnan, China. They had no migration history or history of marriage with other nationalities within three generations.

### DNA extraction and genotyping

2 mL of peripheral blood was collected from 70 patients and 136 healthy controls. The DNA was extracted using Axyprep Blood Genomic DNA Miniprep kit (Axygen Scientific Inc., CA, USA). 7 SNPs (rs17052019, rs2277923, rs3729938, rs3729753, rs3729754, rs703752 and rs11552707) were selected based on SNP database in NCBI and HapMap to examine the association between NKX2-5 gene and CHD. These SPNs were listed in **Table 2**. 3 primers (N1-N3) were designed by Prime 5.0 to amplify 7 SNPs in exon and 3'non-coding regions of NKX2-5 gene. N1 was used amplify rs17052-019 and rs2277923. N2 was used amplify rs3729938, rs3729753 and rs3729754. N3 was used amplify rs703752 and rs11552707. Primers and their characteristics are listed in **Table 3**.

PCR was performed to amplify 7 SNPs using the following systems: 2  $\mu$ L DNA sample, 0.15  $\mu$ L Taq polymerase (5 U/ $\mu$ L), 1  $\mu$ L forward and reverse primers (5  $\mu$ M for each), 2  $\mu$ L dNTP mixture, 2.5  $\mu$ L 10  $\times$  PCR buffer (Mg<sup>2+</sup> Plus), and 17.35  $\mu$ L ddH<sub>2</sub>O to total volume of 25  $\mu$ L. The 10  $\times$  PCR buffer (Mg<sup>2+</sup> Plus) contained 100 mM

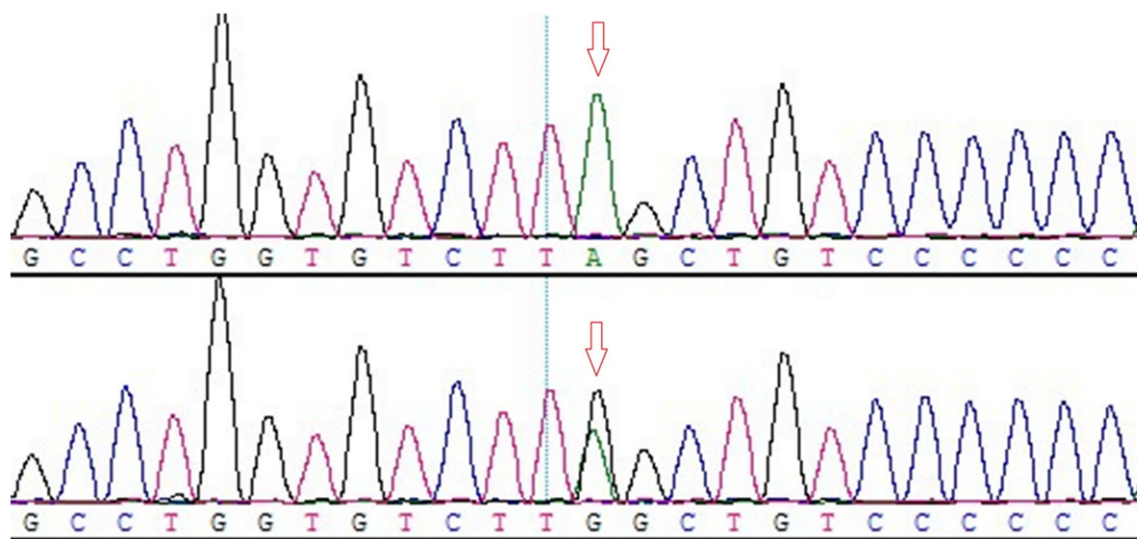
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**Table 2.** Information of 7 SNPs in NKX2-5

Chromosome position	mRNA position	dbSNP rs# cluster ID	Function	dbSNP allele	Protein residue	Amino acid position	Region
172662040	276	rs17052019	Missense/contig reference	C/A	Ala[A]/Asp[D]	16	Exon1
172662024	292	rs2277923	Synonymous/contig reference	G/A	Glu[E]/Glu[E]	21	Exon1
172660011	765	rs3729938	Missense/contig reference	G/C	Cys[C]/Ser[S]	179	Exon2
172659941	835	rs3729753	Synonymous/contig reference	C/G	Leu[L]/Leu[L]	202	Exon2
172659915	861	rs3729754	Missense/contig reference	T/C	Leu[L]/Pro[P]	211	Exon2
172659511	1265	rs703752	3'UTR	A/G			3'UTR
172659237	1539	rs11552707	3'UTR	G/T			3'UTR

**Table 3.** Sites of NKX2-5-specific primers

Primer	SNPs	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)	Annealing temperature (°C)
N1	rs17052019 rs2277923	TCTCCTGCCCTTGTGCTCA	CGTAGGCCTCTGGCTTGAAGG	332	61
N2	rs3729938 rs3729753 rs3729754	TGGAGAAGACAGAGGCGGACAA	CGTAGGCGTTATAACCGTAGGGAT	396	63
N3	rs703752 rs11552707	ACAACAACCTCGTGAACCTCGG	ATCGTCATTCTTACAGCAATAGGT	542	58



**Figure 1.** Sequence diagram of novel heterozygous DSV (1433A>G) site. Arrows indicated the mutations. Genotype: Above, AA; below, AG. Green, black, red and blue represented A allele, G allele, T allele and C allele, respectively.

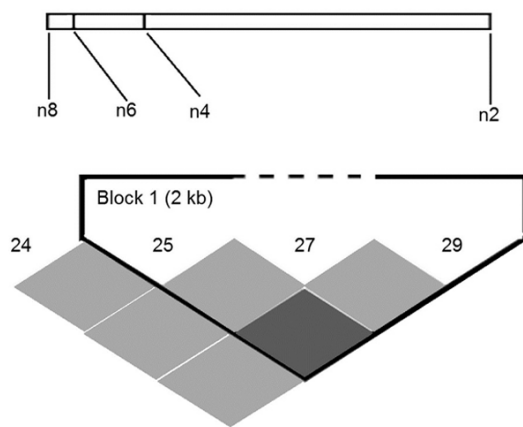
Tris-HCL, 500 mM KCL and 15 mM MgCl<sub>2</sub>. The amplification conditions were as follows: 95°C for 5 min; 36 cycles at 95°C for 30 s, X°C for 30 s, 72°C for 40 s; final extension at 72°C for 5 min (X°C: N1, 61°C; N2, 63°C; N3, 58°C). PCR kits were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China).

The genotypes of 7 SNPs of NKX2-5 gene were determined using DNA direct sequencing method (Beijing Liuhe Huada Gene Polytron Technologies Inc., Beijing, China). The DNASTAR software (DNASTAR Corp., WI, USA) was used to compare DNA sequencing diagrams and find the related polymorphism sites.

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**Table 4.** The genotype and allele frequency distribution of all SNPs sites between CHD and control groups

SNPs	Genotype/allele	Frequency		P	Logistic regression analysis	
		CHD N = 70	Con N = 136		OR, 95% CI	P
rs2277923	GG	13 (0.186)	47 (0.346)	0.032	0.432, [1.152-4.655]	0.018
	GA	37 (0.529)	65 (0.478)			
	AA	20 (0.286)	24 (0.176)			
	G	63 (0.450)	159 (0.585)	0.009	1.719, [1.140-2.594]	0.014
	A	77 (0.550)	113 (0.415)			
rs3729753	GG	68 (0.971)	131 (0.963)	0.759	0.771, [0.202-2.933]	0.702
	GC	2 (0.029)	5 (0.037)	0.759	1.298, [0.245-6.865]	0.759
	G	138 (0.986)	267 (0.982)	0.760	0.773, [0.148-4.041]	0.763
	C	2 (0.014)	5 (0.018)			
rs703752	GG	52 (0.743)	103 (0.757)	0.879	1.080, [0.556-2.099]	0.819
	GT	17 (0.243)	30 (0.221)			
	TT	1 (0.014)	3 (0.022)	0.879	1.556, [0.159-15.243]	0.704
	G	121 (0.864)	236 (0.868)	0.924	0.971, [0.534-1.765]	0.812
	T	19 (0.136)	36 (0.132)			
1433A>G	AA	68 (0.971)	136 (1.00)	0.048	1.616E9, [0.000-]	0.999
	AG	2 (0.029)	0 (0.000)	0.048	0.000, [0.000-]	0.999
	A	138 (0.986)	272 (1.000)	0.115	0.000, [0.000-]	0.999
	G	2 (0.014)	0 (0.000)			



**Figure 2.** Linkage disequilibrium map (n2: rs2277-923; n4: rs3729753; n6: rs703752; n8: 1433A>G).

### Statistical analysis

All 7 SNPs were assessed for Hardy-Weinberg equilibrium (HWE) by  $\chi^2$  statistics. The comparisons of genotype and allele frequencies were evaluated by  $\chi^2$  test. The association of NKX2-5 gene polymorphisms with CHD risk was estimated by computing odds ratio (OR) and 95% confidence interval (CI) from the multivariate logistic regression analysis. All statistical analysis was performed by SPSS 17.0 software (SPSS Inc., Chicago, USA). In addition, haplo-

type constructions and Mendel linkage disequilibrium were analyzed using online computer platform SHEsis and Haploview software (<http://analysis.bio-x.cn/myAnalysis.php>) [12]. Haplotypes with frequencies > 3% in the combined cases and controls were examined.  $P < 0.05$  was regarded as statistically significant.

### Results

#### Sequencing results

In 7 SNPs in detection, rs2277923, rs3729753 and rs703752 existed polymorphism. A novel heterozygous DSV (1433A>G) in 3'UTR of NKX2-5 gene was identified in one tetralogy of Fallot (TOF) patient and one persistent left superior vena cava (PLSVC) patient. Its 1433th base A mutated into G. The sequencing results were shown in **Figure 1**. The rest rs17052019, rs3729938, rs3729754 and rs11552707 sites found no polymorphism, and the genotypes were AA, CC, CC and TT, respectively.

#### Results of statistical analysis

rs2277923, rs3729753, rs703752 and novel DSV (1433A>G) SNPs of CHD patients and controls were in line with the genetic balance Hardy-Weinberg test. GG, GA and AA genotypes

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**Table 5.** Haplotype analysis results of rs2277923, rs3729753, rs703752 and 1433A>G SNPs site

Haplotype	CHD (frequency)	Con (frequency)	P	OR, 95% CI
GCGA	2.00 (0.014)	4.95 (0.018)	/	/
AGGA	58.01 (0.414)	77.00 (0.283)	0.005	1.835, [1.193-2.824]
GGGA	60.99 (0.436)	154.05 (0.566)	0.015	0.596, [0.393-0.904]
ACTA	0.00 (0.000)	0.05 (0.000)	/	/
AGTA	16.99 (0.121)	35.95 (0.132)	0.785	0.918, [0.495-1.703]
GGTA	0.01 (0.000)	0.00 (0.000)	/	/
AGTG	2.00 (0.014)	0.00 (0.000)	/	/

were detected in rs2277923 site, and the frequency distribution of 3 genotypes had significant difference between CHD and control groups ( $P = 0.032$ ). Logistic regression analysis revealed that, the GG genotype was negatively correlated with CHD ( $P = 0.018$ ; OR = 0.432; 95% CI: 1.152-4.655). The frequency distribution of two alleles had significant difference between two groups ( $P = 0.009$ ). Logistic regression analysis revealed that, A allele was positively correlated with CHD ( $P = 0.014$ ; OR = 1.719; 95% CI: 1.140-2.594). AA and AG genotypes were detected in novel DSV (1433A>G) site. The frequency distribution of two genotypes had significant difference between two groups ( $P = 0.048$ ), but the relative risk analysis showed no statistical significance ( $P = 0.999$ ). The differences of rs3729753 and rs703752 polymorphism distribution had no statistical significance between groups ( $P > 0.05$ ) (Table 4).

### Haplotype and linkage disequilibrium analysis

The linkage disequilibrium figure was constructed according to the distribution of rs2277923, rs3729753, rs703752 and novel DSV (1433A>G) SNPs sites (Figure 2). Only 2 SNPs (rs2277923 and rs703752) of this section were linked ( $D' = 1.0$ ;  $r^2 = 0.18$ ). There were 7 haplotypes according to haplotype analysis of 4 SNPs. One of the most common haplotypes, GGGA, showed obvious protective effects in CHD patients ( $P = 0.015$ ; OR = 0.596; 95% CI: 0.393-0.904). AGGA haplotype showed obvious risky effects in CHD occurrence ( $P = 0.005$ ; OR = 1.835; 95% CI: 1.193-2.824) (Table 5).

### Discussion

CHD is seriously harmful to the health of infants and young children. It arises from abnormal

heart development during embryogenesis [13, 14]. The regulation morphogenesis of CHD has been extensively studied, which confirms that some mutated genes such as GATA4 and NKX2-5 can cause CHD [15].

NKX2-5 protein is the important transcription factor of the heart. The human NKX2-5 gene locates at chromosome 5q35, and contains two exons encoding protein of 324 amino acids and 5'UTR as well as 3'UTR regions [7]. It is the fifth found NK-2 family gene, so named NKX2-5. NKX2-5 gene is a highly conserved homeobox protein gene and expressed in the developing heart, as well as in adult heart. In the embryonic heart, NKX2-5 plays essential roles in cardiac progenitor determination, cardiomyocyte differentiation, cardiac morphogenesis and conduction system development [16-20]. In adult heart, NKX2-5 is required for cardiomyocyte homeostasis and postnatal formation of ventricular conduction system [21, 22]. Approximately 38 mutations in NKX2-5 gene have been identified in diverse types of CHD, including atrial septal defect, ventricular septal defect and TOF. In comparison with other population, these mutations have not been found often in mongoloid CHD patients. Analysis of NKX2-5 gene in subjects from southern China with sporadic CHD shows that, there is no any nonsynonymous mutation in 224 CHD patients, excepting 3 reported SNPs in 128 patients. It is suggested that the variant of the NKX2-5 gene may differ between races and regions [23, 24].

In this study, 4 SNPs of the NKX2-5 gene in 70 Chinese Bai patients with CHD are screened for genetic mutations. One SNP (rs2277923) is found to be significantly associated with the risk of CHD, and A allele of rs2277923 is likely a risk factor for CHD. When further exploring the genotype, it is found that people with GG genotype of CHD are at lower risk than people with AA or AG genotype, but AA genotype is not associated with the occurrence of CHD independently after the logistic regression analysis. It is believed that the allele frequency and genotype frequency are not consistently attributed to the existence of heterozygous. In addition, a

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novel mutation (1433A>G) in 3'UTR region of NKX2-5 gene may be associated with the incidence of TOF and PLSVC in Chinese Bai people. Analysis of the linkage disequilibrium shows that, there is no high-degree linkage at this region, and only 2 SNPs (rs2277923 and rs703752) have a linkage ( $D' = 1.0$ ;  $r^2 = 0.18$ ). Moreover, analysis of haplotype has provided evidence that the GGGA haplotype of NKX2-5 gene has a protective effect in the incidence of CHD, and the AGGA has a risky effect.

The rs2277923 SNPs of NKX2-5 gene is the 63th base G mutates into A in the coding chain, causing 21st codon GAG converting into GAA. As the third codon is degenerate codon, it still codes Glu after base substitution. So it is synonymous mutation (Glu21Glu). Results of this study suggest that, A allele of rs2277923 in NKX2-5 gene is likely a risk factor for CHD, which is similar to previous study [25] which investigates the genetic variations of NKX2-5 associated with sporadic CHD in 125 cases and 105 controls of Chinese Han people. In that study, the A allele frequency distribution of rs2277923 has significant difference between CHD and control group ( $P = 0.04$ ;  $OR = 1.48$ ;  $95\% CI: 1.01-2.14$ ), which indicates that A allele of rs2277923 in NKX2-5 gene contributes to the risk of CHD. That study also finds that, the transcription activity of rs2277923 SNPs encoding protein is lower than the wild type by 20%, which may account for the contributor to CHD. Besides, a meta-analysis regarding the associations between two genetic variants in NKX2-5 and risk of CHD reveals that, the rs2277923 variant in NKX2-5 gene may contribute to CHD risk for Chinese [26]. However, there are some differences from our study. Above study [26] does not provide evidence that the rs2277923 SNPs in NKX2-5 gene is associated with sporadic CHD in Chinese Han people. The different research conclusions may be related to the racial and geographic differences. The objects of this study are the Bai minority people. They have no migration history or history of marriage with other nationalities within three generations, and settle in Dali Bai minority autonomous district. Additionally, the sample size of this study is small, and we have not respectively divided subtypes of CHD for analysis. It is necessary to enlarge sample for further research as to the association of NKX2-5 gene mutations and sporadic CHD in Chinese Bai people.

As the mutations in the coding region can affect the protein function or make the protein cannot express, most of relative researches are focused on this genetic regions. In recent years, it is found that the 3'UTR, 5'UTR, promoter region and enhancer region also play an important role in the occurrence of CHD. Mutations in these regions can lower the protein physiological levels of gene encoding by affecting the efficiency of protein transcription, changing the methylation status near the site, affecting RNA folding, or interfering the combination of transcription factors and cofactors [27-31]. Our study finds a novel DSV in the 3'UTR of NKX2-5 gene in one TOF patient and one PLSVC, but none in controls. TOF is considered to be a neural crest cell and/or second heart field related conotruncal heart defects that occur during embryonic development [32]. In the embryonic period, the right and left anterior cardinal veins constitute the main venous drainage of the cephalic portion. As a result of the development of left innominate vein that bridges the anterior cardinal veins at 8 weeks gestation, the proximal part of the left cardinal vein regresses, and only a small portion of it remains as the left superior intercostal vein (LSVC). A defect in the regression of this vein is thought to be the cause of persistence of LSVC [33]. TOF and PLSVC have the similar formation time in the embryonic period. Dinesh et al. [34] find that, SNP 1212G>T in 3'UTR of NKX2-5 is observed in 40% of CHD cases in Mysore, South India. Similarly, a research on the role of related gene variants in the development of hypoplastic right heart syndrome (HRHS) shows that, 4 HRHS cases present a low frequency variation in the 3'UTR of NKX2-5 gene [35]. Notably, recent research demonstrates that, the influence of low frequency variation is significantly greater than the common variants in CHD [36]. Therefore, the noncoding regions need to be further studied in the future.

In conclusion, the Chinese Bai people with CHD have NKX2-5 gene mutations. This study may provide a foundation for a more integrated understanding of the molecular basis of human CHD. Further studies are required to confirm the risk factors of SNP rs2277923 in NKX2-5 gene in relation to CHD occurrence, and the function of novel DSV in 3'UTR should be verified.

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### Disclosure of conflict of interest

None.

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