BASIC SCIENCE ARTICLE

Susceptibility to congenital heart defects associated with a polymorphism in *TBX2* 3′ untranslated region in the Han Chinese population

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BACKGROUND: Tbx2 plays a critical role in determining fates of cardiomyocytes. Little is known about the contribution of *TBX2* 3′ untranslated region (UTR) variants to the risk of congenital heart defect (CHD). Thus, we aimed to determine the association of single-nucleotide polymorphisms (SNPs) in *TBX2* 3′ UTR with CHD susceptibility.
METHODS: We recruited 1285 controls and 1241 CHD children from China. SNPs identification and genotyping were detected using Sanger Sequencing and SNaPshot. Stratified analysis was conducted to explore the association between rs59382073 polymorphism and CHD subtypes. Functional analyses were performed by luciferase assays in HEK-293T and H9c2 cells.
RESULTS: Among five *TBX2* 3′UTR variants identified, rs59382073 minor allele T carriers had a 1.89-fold increased CHD risk compared to GG genotype (95% CI = 1.48–2.46, $P = 4.48 \times 10^{-7}$). The most probable subtypes were right ventricular outflow tract obstruction, conotruncal, and septal defect. G to T variation decreased luciferase activity in cells. This discrepancy was exaggerated by miR-3940 and miR-708, while their corresponding inhibitors eliminated it.
CONCLUSION: T allele of rs59382073 in *TBX2* 3′UTR contributed to greater CHD risk in the Han Chinese population. G to T variation created binding sites for miR-3940 and miR-708 to inhibit gene expression.

INTRODUCTION

Congenital heart defect (CHD) is the most common class of birth defects worldwide. It is estimated that every 8–10 of 1000 newborns can have CHD, and 10 out of 100 aborted fetus have heart anomalies.[1] The etiology of CHD is complicated, and both environmental and genetic causes can perturb heart development and lead to heart malformations.[2] The whole-exome sequencing has identified coding-region mutations in about 400 genes contributing to CHD risk, which only explains ~10% of sporadic CHD cases,[3] leaving a large group of sporadic CHD cases with no explicitly revealed causes.

Tbx2 is a member of T-box transcription factors, which plays an active role in cardiogenesis.[4] It is finely regulated in a complex regulation net, including upstream transcription factors and signaling molecules, e.g., Tbx20, retinoid acid, and Bmp2,[5–8] and downstream targeting genes such as Nkx2-5, Tbx5, Msx1, and Msx2.[9–11] Studies in mouse model showed homozygous mice fetus with Tbx2$^{-/-}$ null mutation had defects of the atrioventricular canal and septum of the outflow tract.[12]

Considering the dose-dependent function manner of Tbx2,[5] we presumed that any factor altering gene expression level, for example, changes in its noncoding region such as 3′ untranslated region (3′UTR) or promoter region, may interfere cardiogenesis and induce CHD. Our previous study demonstrated that noncoding variants in TBX5 3′UTR increased CHD risk by regulating gene expression levels.[13] Few studies have identified mutations in *TBX2* coding region to induce human CHD till now, while variants of

TBX2 in the promoter region[14] were indicated to cause ventricular septal defect. However, the contribution of polymorphisms in *TBX2* 3′UTR, which is critically important for microRNAs (miRNAs) to bind mRNA and regulate gene expression, remains unknown. In the present study, we analyzed the contribution of *TBX2* 3′UTR to CHD risk in a Han Chinese cohort composed of 1241 CHD patients and 1285 healthy controls, filtered out the most probable CHD subtypes, and further studied the mechanism involved primarily.

METHODS

Study subjects

Cases in the present study comprised complex CHD subjects that necessitated surgical intervention due to critical, life-threatening symptoms or failure of congestive cardiac dysfunction to respond to medications. Besides, the inclusion criteria for the simple CHD cases, mainly ventricular septal defect (VSD) and atrial septal defect (ASD), were clinical and/or echocardiographic evidence of a significant shunt through the lesions.[15] From January 2014 to May 2016, we recruited 1241 consecutive Han Chinese CHD children from two different cohorts and 1285 healthy controls who were matched to cases in terms of age and sex from the same geographical area over the same period. Among them, 648 controls and 693 CHD cases were enrolled from the Institute of Cardiovascular Research, Jinan Military General Hospital (Jinan, Shandong Province, China), as well as 637 controls and 548 cases from Children's Hospital of Fudan University (Shanghai, China).

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Cases of isolated patent ductus arteriosus, patent foramen ovale, small-size septal defects, congenitally corrected transposition of the great arteries, bicuspid aortic valve, coronary anomalies, as well as CHD that relate mainly to the vascular system were excluded from the present study. All patients with genetic syndromes (e.g., Down's syndrome, Holt–Oram syndrome, Alagille syndrome, DiGeorge syndrome, William syndrome, and Noonan syndrome) or family history of CHD were excluded. Also, cases combined with other non-cardiovascular malformations, tumor or systematic diseases were not recruited.

According to the commonly used criteria,[16] the 1241 CHD cases were classified into nine broad categories, including anomalous pulmonary venous return (APVR), atrioventricular septal defects (AVSD), complex CHD, conotruncal defects, heterotaxy (including isomerism and mirror-imagery), left ventricular outflow tract obstruction (LVOTO), right ventricular outflow tract obstruction (RVOTO), septal defects, and CHD with other associations.

The study protocol was approved by the medical ethics committee of Children's Hospital of Fudan University. Written informed consents were obtained from every participants or their legal guardians.

Variant identification and genotyping

Blood of CHD children was collected at the same time when they received other necessary blood sampling for disease diagnosis, while in the cohort of healthy control, we used the residual samples from their blood test. Genomic DNA was isolated from the peripheral venous blood using conventional reagents. The single-nucleotide polymorphisms (SNPs) in a cohort of 288 cases and 288 controls firstly collected were identified using Sanger Sequencing. Genotyping of rs59382073 in samples collected later was performed using SNaPshot assay (ABI, Waltham, MA) according to the manufacturer's instructions. Briefly, the TBX2 3′ UTR sequences were amplified through PCR using forward primer GAGGCGGCCAATGAACTGCA and reverse primer CTGTGCG CTCTGTGGGTC. After removing excessive primers and deoxynucleotides using ExoII and SAP (Takara bio, Tokyo, Japan), specific SNaPshot primer (tttttAACACAGTCACTTGGGAGCT) was annealed to the PCR products, separated by capillary electrophoresis (ABI, Waltham, MA), and analyzed by Peak Scanner.

Plasmid constructs

We constructed plasmids for validation of the function of either allele of the SNP rs59382073 using the psiCHECK™-2 vector (Promega, Madison, WI). A 1081-bp fragment in TBX2 3′UTR was cloned from genomic DNA which contains the G allele. Later we designed primers with point mutation (Forward: TGGGAGCTTCTGGGCTAGGTCCAGATCCGCTCCAG; Reverse: CTGGAGCGGATCTGGACCTAGCCCAGAAGCTCCCA) and used the KOD-Plus (Takara bio, Tokyo, Japan) DNA polymerase to construct vectors containing the corresponding T allele plasmid. Then PCR products were digested by XhoI and NotI (Takara bio, Tokyo, Japan), and then cloned into the 3′UTR of the Renilla luciferase gene of the psiCHECK™-2 vector, respectively. The recombined plasmids sequences were confirmed by Sanger sequencing.

Transfection and luciferase reporter assays

Human embryonic kidney 293 (HEK-293T) cells and rat cardiomyocyte H9c2 cells were grown using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), added 1% penicillin/streptomycin solution (10,000 U/mL and 10 mg/mL, respectively). Mycoplasma contamination was regularly checked. Cells were cultured in 24-well plates and transfected with 500 ng psiCHECK2-G, psiCHECK2-T, or psiCHECK2-basic vector using 1.5 μL Lipofectamine 2000 (Life Technologies, Waltham, MA) at 70% confluence. Cotransfection with miRNAs were performed with extra 15 pM miRNA mimics or inhibitors (GenePharm, Shanghai, China) mixing with the constructed vectors in both HEK-293T cells and H9c2

cells. After 24 h of transfection, the transfected cells were lysed in 100 μL per well passive lysis buffer (Promega, Madison, WI), gently rocked at room temperature for 15 min, centrifuged at 6000 rpm for 3 min, and transferred to black 96-well plate. The lysate was then assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) using GloMax 96 Microplate Lumiometer (Promega, Madison, WI). Transfection experiments were performed in three replicates, and each luciferase assay was carried out in triplicate.

Statistical analyses

Independent sample t-tests (two-tail) were used to compare continuous demographic and phenotypic variables between controls and cases. Allele frequencies were confirmed by gene counting. Hardy–Weinberg equilibrium, genotype and allele frequency comparisons between groups were performed using the Chi-square tests. To evaluate the associations between the genotypes and CHD risk, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by using unconditional logistic regression analysis with adjustment for age and sex. The statistical packages SPSS 20.0 (SPSS, Chicago, IL) and GraphPad Prism (San Diego, CA) were used for the statistical analyses and graphing. Statistical differences were considered significant if $P < 0.05$.

RESULTS

TBX2 3′UTR polymorphism rs59382073 significantly increased CHD susceptibility in the Han Chinese population

Five polymorphisms with minor allele frequency (MAF) over 0.05 were found in the 288 cases and 288 controls firstly collected, including rs59382073, rs1058004, rs729782, rs3198587, and rs729781. Among them the frequencies of rs59382073 alleles were significantly different between control and case cohort ($P = 0.01$) (Table 1). Therefore, the variant rs59382073 was further studied in two independent case–control cohorts from Shanghai and Shandong.

Analyses showed control population in both groups were in Hardy–Weinberg equilibrium, as well as in combination (HWE-$p > 0.05$). T allele frequencies in cases were significantly higher compared to controls in combination (7.86% vs 4.55%, $P = 1.00 \times 10^{-6}$), Shanghai (6.93% vs 4.87%, $P = 0.03$), and Shandong (8.59% vs 4.24%, $P = 5.00 \times 10^{-6}$) cohorts, respectively (Table 2).

In consideration of rare proportion of TT genotype, we applied the dominant model of inheritance in the association studies to enhance the statistical power. Logistic regression analyses revealed that T allele carriers (homozygous TT and heterozygous GT individuals) had a significantly higher risk of CHD compared to wild-type GG subjects in both Shanghai (OR = 1.61, 95% CI = 1.12–2.31, $P = 0.01$) and Shandong group (OR = 2.17, 95% CI = 1.54–3.05, $P = 1.00 \times 10^{-5}$). The combination showed a 1.89-fold increased CHD risk (OR = 1.89, 95% CI = 1.48–2.46, $P = 4.48 \times 10^{-7}$) for the GT and TT genotypes compared to GG subjects (Table 3). Hence, TBX2 3′UTR variant rs59382073 was a significant contributor to CHD risk in two cohorts both separately and together.

TBX2 rs59382073 T allele was significantly associated with susceptibility of RVOTO, conotruncal, and septal defect

To confirm which CHD subtype is closely associated with rs59382073 polymorphism, a stratified analysis was performed according to standard CHD classifications as previously described,[16] including APVR (34, 2.7%), AVSD (30, 2.4%), conotruncal defect (192, 15.5%), complex (49, 3.9%), heterotaxy (19, 1.5%), LVOTO (52, 4.2%), RVOTO (71, 5.7%), septal defect (769, 62.0%), and other CHDs (25, 2.0%). The results showed that T allele carriers had a significantly increased risk of RVOTO (OR = 2.57, 95% CI = 1.39–4.76, $P = 2.64 \times 10^{-3}$), conotruncal (OR = 2.25,

Table 1. Genotyping of TBX2 3'UTR SNPs identified in 288 CHD patients and 288 controls

SNPs	Genotype	Control	Case	P-value	HWE-p	%Genotyped
rs59382073	G/G	264 (93.29%)	248 (87.02%)	**0.01**	0.71	98.61%
	G/T	19 (6.71%)	37 (12.98%)			
	T/T	0 (0.00%)	0 (0.00%)			
rs1058004	C/C	110 (39.15%)	104 (37.82%)	0.84	0.28	96.52%
	C/T	128 (45.55%)	124 (45.09%)			
	T/T	43 (15.30%)	47 (17.09%)			
rs729782	G/G	149 (54.38%)	148 (53.43%)	0.94	0.93	95.66%
	G/C	100 (36.50%)	105 (37.91%)			
	C/C	25 (9.12%)	24 (8.66%)			
rs3198587	G/G	132 (46.48%)	142 (50.35%)	0.56	0.82	98.26%
	G/C	122 (42.96%)	116 (41.13%)			
	C/C	30 (10.56%)	24 (8.51%)			
rs729781	A/A	106 (37.06%)	125 (44.17%)	0.22	0.67	98.78%
	A/G	139 (48.60%)	121 (42.76%)			
	G/G	41 (14.34%)	37 (13.07%)			

In bold, $P < 0.05$
SNP single-nucleotide polymorphisms, UTR untranslated region, CHD congenital heart defect, HWE Hardy–Weinberg equilibrium

Table 2. TBX2 3'UTR SNP rs59382073 allele frequencies comparison

Cohort	Total	Shanghai	Shandong
Subjects	2526	1185	1341
Major allele in controls (%)	2453 (95.45%)	1212 (95.13%)	1241 (95.76%)
Major allele in cases (%)	2287 (92.14%)	1020 (93.07%)	1267 (91.41%)
Minor allele in controls (%)	117 (4.55%)	62 (4.87%)	55 (4.24%)
Minor allele in cases (%)	195 (7.86%)	76 (6.93%)	119 (8.59%)
Minor allele frequency	0.06	0.06	0.06
Statistical analysis (X^2, P-value)	**23.79, 1.00×10^{-6}**	**4.59, 0.03**	**20.81, 5.00×10^{-6}**

In bold, $P < 0.05$
UTR untranslated region, SNP single-nucleotide polymorphisms, HWE Hardy–Weinberg equilibrium

Table 3. TBX2 3'UTR SNP rs59382073 association with CHD risk in two independent cohorts

Group	Genotype	Control	Case	OR (95% CI)	P-value	HWE-p
Shanghai		637	548			0.07
	G	1212 (95.13%)	1020 (93.07%)			
	T	62 (4.87%)	76 (6.93%)	1.46 (1.03–2.06)	**0.03**	
	G/G	579 (90.89%)	472 (86.13%)			
	G/T-T/T	58 (9.11%)	76 (13.87%)	1.61 (1.12–2.31)	**0.01**	
Shandong		648	693			0.87
	G	1241 (95.76%)	1267 (91.41%)			
	T	55 (4.24%)	119 (8.59%)	2.12 (1.52–2.95)	**8.00×10^{-6}**	
	G/G	594 (91.67%)	579 (83.55%)			
	G/T-T/T	54 (8.33%)	114 (16.45%)	2.17 (1.54–3.05)	**1.00×10^{-5}**	
Combined		1285	1241			0.13
	G	2453 (95.45%)	2287 (92.14%)			
	T	117 (4.55%)	195 (7.86%)	1.79 (1.41–2.26)	**1.00×10^{-6}**	
	G/G	1173 (91.28%)	1051 (84.69%)			
	G/T-T/T	113 (8.72%)	190 (15.31%)	1.89 (1.48–2.46)	**4.48×10^{-7}**	

In bold, $P < 0.05$
UTR untranslated region, CHD congenital heart defect, HWE Hardy–Weinberg equilibrium

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95% CI = 1.48–3.42, $P = 1.40 \times 10^{-4}$), and septal defect (OR = 1.98, 95% CI = 1.50–2.60, $P = 1.00 \times 10^{-6}$) (Table 4).

MiR-3940 and miR-708 repressed luciferase expression in rs59382073 T allele construct

Luciferase assays were conducted in both HEK-293T and H9c2 cells to determine the functional effect of rs59382073 polymorphism. We first transfected cells with psiCHECK2-G, psiCHECK2-T and psiCHECK2-basic (as a negative control) vectors respectively. We found that relative Renilla luciferase activity decreased in psiCHECK2-T and psiCHECK2-G vector transfected cells compared to psiCHECK2-Basic cells in both cell lines and the T construct showed the lowest expression level (Fig. 1). This result indicated that rs59382073 T allele was associated with decreased gene expression compared to the G allele.

Further, to explore whether the rs59382073 polymorphism changes CHD risk by affecting miRNAs biding affinities, we used RegRNA (http://regrna2.mbc.nctu.edu.tw/) to predict potential miRNAs binding sites. This prediction showed that the G to T variation created additional biding sites for five miRNAs, namely miR-127-5p, miR-28-5p, miR-3139, miR-3940, and miR-708 (Fig. 2). We then cotransfeced HEK-293T and H9c2 cells with psiCHECK2-G, psiCHECK2-T, or psiCHECK2-basic construct and miRNA mimics or negative control oligos. MiR-3940 and miR-708 exaggerated the
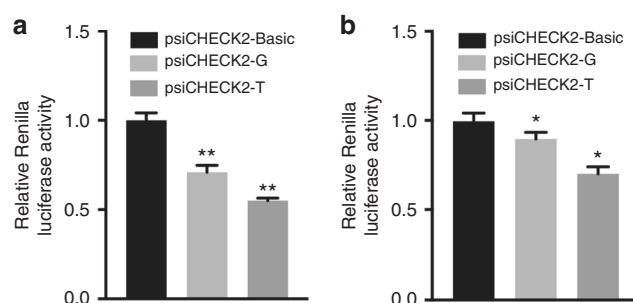
Fig. 1 Transfection of vector containing rs59382073 T allele decreased luciferase activities. **a** In HEK-293T cells, compared to psiCHECK2-Basic transfected cells, psiCHECK2-G luciferase activity decreased by 29.01% ($P = 4.8 \times 10^{-8}$) and psiCHECK2-T by 44.88% ($P = 2.8 \times 10^{-14}$). **b** In H9c2 cells, psiCHECK2-G luciferase activity decreased by 9.9% ($P = 0.02$) and psiCHECK2-T by 29.61% ($P = 1.2 \times 10^{-3}$). Bars, SE. $*P < 0.05$, $**P < 0.001$

discrepancy of luciferase activity level between psiCHECK2-G and psiCHECK2-T transfected HEK-293T cells (Fig. 3a), and so did miR-708 in H9c2 cells (Fig. 3b). Then we cotransfected cells with vector constructs and inhibitors of miR-3940, miR-708, miR-3139 or negative control oligos. Inhibitors of miR-3940 and miR-708 in HEK-293T cells and inhibitor of miR-3940 and miR-708 in H9c2 cells alleviated the discrepancy.

DISCUSSION

TBX2 gene localizes in chromosome 17q23, containing seven exons,[17–19] but no mutation in the coding region was clarified in previous studies. Previous studies reported genomic deletions and duplication at 17q23.1–q23.2, containing *TBX2* gene, in patients with multiple developmental abnormalities including CHDs. A patient carrying a 131 kb duplication, encompassing the *TBX2* gene, at 17q23.2 was manifested with mild mental retardation, prenatal onset growth retardation, cerebellar hypoplasia and complex heart defect.[20] Another series of seven patients having 2.2 or 2.8 Mb deletion at 17q23.1q23.2, flanked by segmental duplications, were manifested with mild to moderate developmental delay, hand and foot anomalies, heart defects, in most cases either patent ductus arteriosus or atrial septal defect, and other anomalies. Within the 2.2 Mb region of overlap, *TBX2* and *TBX4* were strong candidates contributing to microdeletion manifestations.[14] Variants in the promoter region of *TBX2* (g.59477201C>T, g.59477347G>A, g.59477353delG, and g.59477371G>A) were also reported to be associated with VSD, may acting by decreasing gene expression.[14] Taken together, alteration in *TBX2* levels contributed to both syndromic and isolated CHD. Thus, we presumed that genetic variations in *TBX2* 3′ UTR, which might also modulate gene expression levels, have impact on CHD development. In the present study, we examined the association between the SNPs within *TBX2* 3′UTR and CHD risk in a large sample set including 1241 cases and 1285 controls of Han Chinese from Shanghai and Shandong. Case–control studies showed that the polymorphism rs59382073 is significantly associated with the increased susceptibility of CHD (OR = 1.89, 95% CI = 1.48–2.46, $P = 4.48 \times 10^{-7}$) in the Han Chinese population, which has not been reported before.

During heart development, Tbx2 expression is restricted to the atrioventricular channel and outflow tract, and the downregulation of Tbx2 gene is essential for the right ventricle wall formation.[21] The lineage tracing studies demonstrated that the Tbx2 expressing cells finally become the ventricular septum.[22–24]

Table 4. Stratified analysis of CHD subtypes risk and rs59382073 variant

Classification	Freq.	Perc.	WT	T-carrier	OR (95% CI)	P-value
Subtypes						
APVR	34	2.7%	30	4	1.40 (0.48–4.04)	0.54
AVSD	30	2.4%	25	5	2.10 (0.79–5.58)	0.14
Complex	49	3.9%	44	5	1.19 (0.46–3.06)	0.71
Conotruncal	192	15.5%	158	34	2.25 (1.48–3.42)	**1.40×10^{-4}**
Heterotaxy	19	1.5%	19	0	0.00 (0.000-)	0.10
LVOTO	52	4.2%	49	3	0.64 (0.19–2.09)	0.46
RVOTO	71	5.7%	57	14	2.57 (1.39–4.76)	**2.64×10^{-3}**
Septal defects	769	62.0%	647	122	1.98 (1.50–2.60)	**1.00×10^{-6}**
Other CHD	25	2.0%	22	3	1.43 (0.42–4.85)	0.57
Total	1241	100.0%	1051	190	1.89 (1.48–2.46)	**4.48×10^{-7}**

In bold, $P < 0.05$

CHD congenital heart defect, *Freq* frequency, *Perc* percentage, *APVR* anomalous pulmonary vein return, *AVSD* atrial ventricle septal defect, *LVOTO* left ventricle outflow tract obstruction, *RVOTO* right ventricle outflow tract obstruction, *WT* wild-type genotype (G/G)

Tbx2 can inhibit Tbx5-induced chamber differentiation and endocardial cushion formation,[9,25] thus keeping myocytes in the primary phenotype, allowing these regions to develop another fate.[4] Our study detected that, compared to GG genotypes, GT and TT genotype had a significantly increased risk of RVOTO (OR = 2.57, 95% CI = 1.39–4.76, $P = 2.64 \times 10^{-3}$), conotruncal (OR = 2.25, 95% CI = 1.48–3.42, $P = 1.40 \times 10^{-4}$), and septal defect (OR
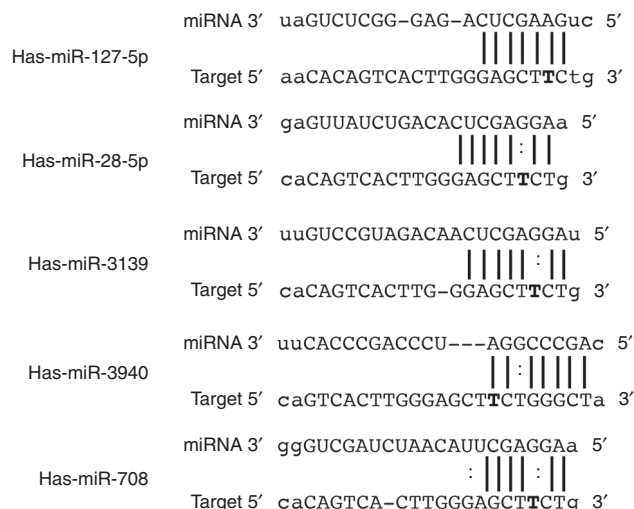

Fig. 2 Five additional miRNAs binding to rs59382073 T variant predicted by RegRNA

= 1.98, 95% CI = 1.50–2.60, $P = 1.00 \times 10^{-6}$). These affected subtypes were consistent with former molecular studies that the precise regulation of Tbx2 expression is essential to entire right ventricle and septum development.[22–24]

MiRNAs can negatively regulate gene expression by interacting with 3′ UTR of the target mRNA, cleaving or degrading the mRNA and/or inhibiting its translation and transcription.[26–28] Studies have shown that genetic variations within 3′UTR can affect the binding affinity of miRNAs, leading to heart malformations.[13,29–31] Using the dual-luciferase reporter assays, our study found rs59382073 G to T variation decreased reporter gene expression in both HEK-293T and H9c2 cells. Furthermore, we found five additional miRNA binding sites created by G to T variation predicted by RegRNA. Then we identified two miRNAs, miR-3940 and miR-708, that significantly exaggerated the reporter gene expression discrepancy, while their corresponding inhibitors alleviated it. Therefore, we presumed that T allele carriers have increased risks of CHD due to the reduction of *TBX2* level by giving rise to biding sites for the two miRNAs and facilitating their negative regulation.

Our study discovered the association between CHD risks and rs59382073 in *TBX2* 3′ UTR, and revealed that the G to T variation created miRNAs binding sites for miR-3940, miR-708 and miR-3139to downregulate gene expression. Due to the insufficient cases of CHD subtypes such as APVR, AVSD, heterotaxy, and other associations, future work is needed to expand the sample size of these lesions to guarantee enough statistical power for sub-analyses. Another limitation of our work is the lack of heart tissues to verify *TBX2* levels in different genotypes, due to the difficulties of getting prompt cardiac samples in CHD subjects. Future studies are needed to verify the in vivo role of miR-3940 and miR-708 in
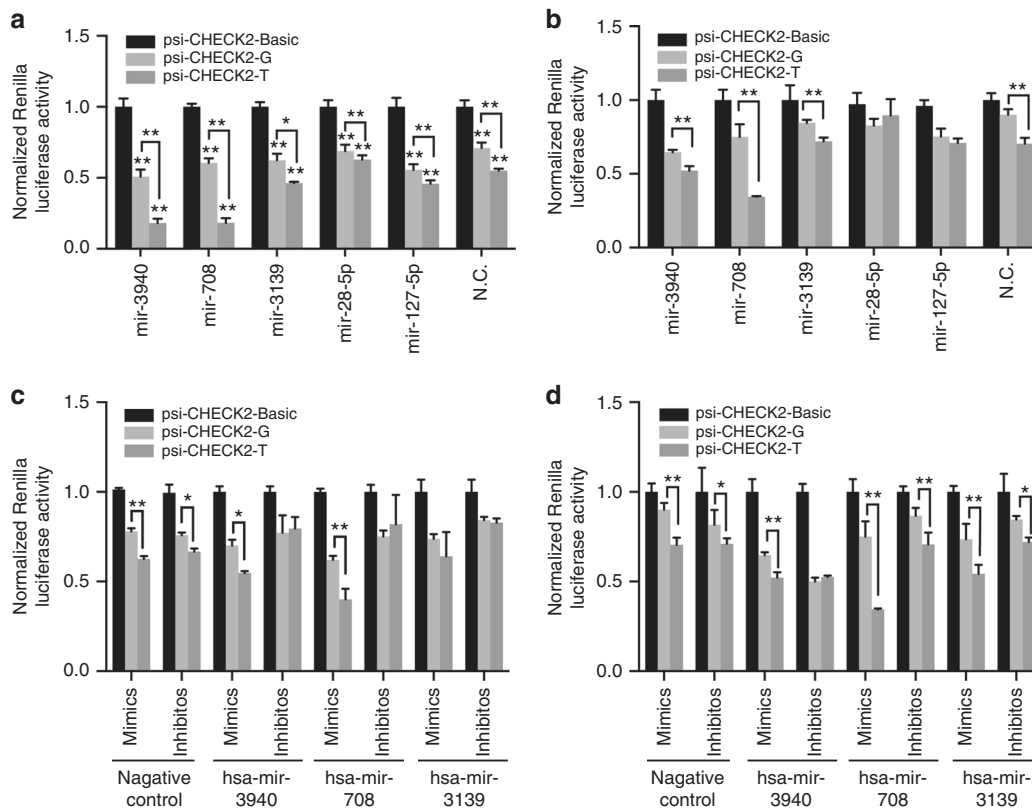

Fig. 3 Rs59382073 G to T variation created additional miRNAs binding sites and caused suppressed reporter gene expression. MiR-3940 and miR-708 in HEK-293T cells (**a**) and miR-708 in H9c2 cells (**b**) exaggerated the discrepancy of luciferase activity level between psiCHECK2-G and psiCHECK2-T transfected cells. **c** In HEK-293T cells, inhibitors of miR-3940 and miR-708 alleviated the discrepancy. **d** In H9c2 cells, inhibitors of miR-3940 and miR-708 alleviated the discrepancy. N.C. negative control miRNAs. Bars, SE. *$P < 0.05$. **$P < 0.001$

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decreasing *TBX2* level and to explore the possible value of the two miRNAs in early CHD diagnosis.

In conclusion, the T allele of the SNP rs59382073 in *TBX2* 3′ UTR is associated with increased CHD susceptibility in Han Chinese population, and carriers of T allele had significantly greater risk of CHD, especially of RVOTO, conotruncal defect, and septal defect. Functional studies demonstrated that the G to T alteration created additional binding sites for miR-3940 and miR-708 to repress gene expression. Our study sheds light on genetic variants in *TBX2* 3′ UTR in sporadic CHD, highlighting CHD risk assessment for the Han Chinese population.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

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REFERENCES
1. Triedman, J. K. & Newburger, J. W. Trends in congenital heart disease: the next decade. *Circulation* **133**, 2716–2733 (2016).
2. Pradat, P. et al. The epidemiology of cardiovascular defects, part I: a study based on data from three large registries of congenital malformations. *Pediatr. Cardiol.* **24**, 195–221 (2003).
3. Zaidi, S. et al. De novo mutations in histone-modifying genes in congenital heart disease. *Nature* **498**, 220–223 (2013).
4. Christoffels, V. M. et al. Development of the pacemaker tissues of the heart. *Circ. Res.* **106**, 240–254 (2010).
5. Cai, C. L. et al. T-box genes coordinate regional rates of proliferation and regional specification during cardiogenesis. *Development* **132**, 2475–2487 (2005).
6. Ma, L. et al. Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development* **132**, 5601–5611 (2005).
7. Sakabe, M. et al. Ectopic retinoic acid signaling affects outflow tract cushion development through suppression of the myocardial Tbx2-Tgfβ2 pathway. *Development* **139**, 385–395 (2012).
8. Singh, M. K. et al. Tbx20 is essential for cardiac chamber differentiation and repression of Tbx2. *Development* **132**, 2697–2707 (2005).
9. Habets, P. E. M. H. et al. Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev.* **16**, 1234–1246 (2002).
10. Boogerd, K.-J. et al. Msx1 and Msx2 are functional interacting partners of T-box factors in the regulation of Connexin43. *Cardiovasc. Res.* **78**, 485–493 (2008).
11. Barron, M. R. et al. Serum response factor, an enriched cardiac mesoderm obligatory factor, is a downstream gene target for *Tbx* genes. *J. Biol. Chem.* **280**, 11816–11828 (2005).
12. Harrelson, Z. et al. Tbx2 is essential for patterning the atrioventricular canal and for morphogenesis of the outflow tract during heart development. *Development* **131**, 5041–5052 (2004).
13. Wang, F. et al. A TBX5 3′UTR variant increases the risk of congenital heart disease in the Han Chinese population. *Cell Discov.* **3**, 17026 (2017).
14. Pang, S. et al. Novel and functional sequence variants within the *TBX2* gene promoter in ventricular septal defects. *Biochimie* **95**, 1807–1809 (2013).
15. Houyel, L. et al. Population-based evaluation of a suggested anatomic and clinical classification of congenital heart defects based on the International Paediatric and Congenital Cardiac Code. *Orphanet. J. Rare Dis.* **6**, 64 (2011).
16. Botto, L. D. et al. Seeking causes: classifying and evaluating congenital heart defects in etiologic studies. *Birth Defects Res. Part A Clin. Mol. Teratol.* **79**, 714–727 (2007).
17. Campbell, C. et al. Cloning and mapping of a human gene (TBX2) sharing a highly conserved protein motif with the Drosophila omb gene. *Genomics* **28**, 255–260 (1995).
18. Campbell, C. E., Casey, G. & Goodrich, K. Genomic structure of TBX2 indicates conservation with distantly related T-box genes. *Mamm. Genome.* **9**, 70–73 (1998).
19. Law, D. J. et al. Identification, characterization, and localization to Chromosome 17q21-22 of the human TBX2 homolog, member of a conserved developmental gene family. *Mamm. Genome.* **6**, 793–797 (1995).
20. Radio, F. C., et al., *TBX2* gene duplication associated with complex heart defect and skeletal malformations. *Am. J. Med. Genet. A* **152A**, 2061–2066 (2010).
21. Yamada, M. et al. Expression of chick *Tbx-2, Tbx-3,* and *Tbx-5* genes during early heart development: evidence for BMP2 induction of Tbx2. *Dev. Biol.* **228**, 95–105 (2000).
22. Sizarov, A. et al. Formation of the building plan of the human heart: morphogenesis, growth, and differentiation. *Circulation* **123**, 1125–1135 (2011).
23. Aanhaanen, W. T. J. et al. The Tbx2+ primary myocardium of the atrioventricular canal forms the atrioventricular node and the base of the left ventricle. *Circ. Res.* **104**, 1267–1274 (2009).
24. Moorman, A. F. M. et al. The heart-forming fields: one or multiple? *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **362**, 1257–1265 (2007).
25. Shirai, M., et al., T-box 2, a mediator of Bmp-Smad signaling, induced hyaluronan synthase 2 and Tgfbeta2 expression and endocardial cushion formation. *Proc. Natl Acad. Sci. USA* **106**, 18604–18609 (2009).
26. Kiriakidou, M. et al. An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell* **129**, 1141–1151 (2007).
27. Nikolova, E., Jordanov, I. & Vitanov, N. Dynamical analysis of the microRNA – mediated protein translation process. *BIOMATH* **2**, 1210071 (2013).
28. Pillai, R. S. et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309**, 1573–1576 (2005).
29. Pelletier, C. & Weidhaas, J. B. MicroRNA binding site polymorphisms as biomarkers of cancer risk. *Expert. Rev. Mol. Diagn.* **10**, 817–829 (2014).
30. Liang, D. et al. Genetic variants in MicroRNA biosynthesis pathways and binding sites modify ovarian cancer risk, survival, and treatment response. *Cancer Res.* **70**, 9765–9776 (2010).
31. Sabina, S. et al. Germline hereditary, somatic mutations and microRNAs targeting-SNPs in congenital heart defects. *J. Mol. Cell. Cardiol.* **60**, 84–89 (2013).