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Association between SNP rs59382073 in TBX2 3′ UTR and susceptibility to congenital heart diseases

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ABSTRACT

Background: The formation of the endocrinal cushion requires one of the members of the T-box transcription factor family, namely Tbx2. Yet, we have scarce information about the potential association of TBX2 3′ untranslated region (UTR) variations with congenital cardiac malformations. The aim of our study is the determination of the relationship between single-nucleotide polymorphism (SNP) *rs59382073* in TBX2 3′ UTR and CHD susceptibility.

Methods: Venous blood samples were collected from 120 patients with CHD (encompassing 28 neonates, 72 infants, and 20 children) and additional 120 apparently healthy subjects and of matched age and sex. Genotyping of *TBX2 3′UTR* was performed using Step OnePlus PCR system using Taqman predesigned SNP assay (rs59382073). The distribution of genotype and allele frequency in both the congenital heart diseases (CHD) and the control groups were analysed.

Results: rs59382073 minor allele T carriers in TBX2 3′ UTR (homozygous TT and heterozygous GT subjects) had a significantly higher risk of CHD compared to wild-type GG subjects (OR 5.7; 95% CI, 2.99–11.1; $P < 0.001$ and OR 9.6; 95% CI, 3.1–29.6; $P < 0.001$), with the most likely subtypes being septal defect and conotruncal defects ($P < 0.001$ each).

Conclusion: T allele carriers of rs59382073 in TBX2 3′UTR had a greater risk of CHD than wild-type GG, septal defect and conotruncal defects were more common in T allele carriers than wild-type GG.

1. Introduction

Among the different birth defects worldwide, Congenital heart defects (CHD) are the most common class. Approximately every 8–10 of 1000 newborns may have CHD, and 10 out of 100 aborted fetuses have heart anomalies (Triedman and Newburger, 2016). The etiology of CHD is not yet fully understood. It has been recorded that inherited and environmental factors cause CHD. Growing coding-region mutations of certain genes, especially transcription factors, were found in patients with CHD (Barua and Junaid, 2015). Thus, to reach a proper diagnosis and treatment and favorable prognosis for CHD, it is crucial to identify the molecular mechanism behind it (Zipkin et al., 2015).

A lot of the multiple genes that were identified by human genetic studies, and are responsible for inherited and sporadic congenital heart diseases, encode the transcription factors moderating certain events in the development of the heart, among which are ventricular septation or outflow tract morphogenesis. A gene, involved in Holt-Oram syndrome (HOS), was the first to be recorded as a single-gene mutation in the T-box transcription factor gene TBX5 causing inherited CHD (Li et al., 1997).

Playing an active role in cardiogenesis, Tbx2 is a known member of the T-box transcription factors (Christoffels et al., 2010). In a complex regulation net where it is finely regulated, including upstream transcription factors and signaling molecules such as Tbx20, retinoid acid, and Bmp2, (Cai et al., 2005; Ma et al., 2005; Sakabe et al., 2012; Singh et al., 2005) and downstream targeting genes like Nkx2-5, Tbx5, Msx1,

Abbreviations: CHD, congenital heart diseases; HC, head circumference; AVSD, atrial ventricle septal defect; LVOTO, left ventricle outflow tract obstruction; RVOTO, right ventricle outflow tract obstruction; APVR, anomalous pulmonary vein return; UTR, untranslated region; SNP, single nucleotide polymorphism.

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and Msx2 (Habets et al., 2002; Boogerd et al., 2008; Barron et al., 2005). OFT (Outflow tract) defects and small AVC (Atrioventricular canal) was presented by Tbx2-null mice embryos, while relatively low cell proliferation was generated by Tbx2 transgenic mice in OFT and AVC regions (Harrelson et al., 2004). Besides, mice fetuses with aberrantly upregulated Tbx2 expression presented large linear heart tubes with failure to form chambers (Christoffels et al., 2004). Interference with cardiogenesis and induction of CHD may occur by any factor that alters gene expression level, such as changes in its non-coding region like the 3′ untranslated region (3′UTR) or promoter region (Zhang et al., 2019). Up until now, the association between mutations in the TBX2 coding region and CHD susceptibility has been recognized by only a few studies, while variants of TBX2 in the promoter region were recorded to cause ventricular septal defect (Pang et al., 2013). Still, the potential role of the polymorphisms in TBX2 3′UTR regulating gene expression through the binding of microRNAs (miRNAs) remains vague. The aim of our study is assessing the possible associations between SNP rs5938207 in TBX2 3′UTR and the likelihood of CHD.

2. Subjects and methods

2.1. Study design and population

In our case-control study, 120 non-related patients (28 neonates, 72 infants, and 20 children) with non-syndromic isolated and non-isolated congenital cardiac defects (necessitated surgical intervention due to critical, life-threatening symptoms or failure of congestive cardiac dysfunction to respond to medications) were recruited from Benha University Hospital in the period from February 2019 to May 2021. The control group consisted of 120 healthy subjects with matched age and sex and with no family history of cardiac diseases. Patients were diagnosed according to ESC Guidelines for CHD (Baumgartner et al., 2010).

We excluded from our study the following cases: isolated patent ductus arteriosus, patent foramen ovale, small-size septal defects, congenitally corrected transposition of the great arteries, bicuspid aortic valve, coronary anomalies, and moreover CHDs that relate mainly to the vascular system. We excluded all patients having genetic syndromes (diagnosed by clinical examination and karyotyping) or family history of CHD. Furthermore, we did not recruit any cases combined with other non-cardiovascular malformations, tumors, or systematic diseases. As per the commonly used criteria (Botto et al., 2007), we classified the 120 CHD cases into nine wide categories inclusive of 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 Ethical Scientific Committee of Faculty of Medicine, Benha University, approved the study protocol conferring to the World Medical Association Declaration of Helsinki (Puri et al., 2009). We also collected informed consents from parents/guardians prior to enrollment in the study.

Three-generation pedigree constructions, thorough patient history collected from their parents or medical records, and extensive clinical examinations were undertaken for all participants. In all cases, we conducted echocardiography, electrocardiogram and plain chest X-rays.

2.2. Laboratory investigations

Genotyping for single–nucleotide variant (*rs59382073*) in TBX2 3′ UTR was conducted for all participants as follows:

1. Blood Samples Collection: one milliliter of venous blood samples was collected under complete aseptic conditions from each subject, placed in vacutainers which contain Na2EDTA as an anticoagulant, and immediately kept frozen at −80 °C for later genomic DNA extraction.

2. DNA Extraction: using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) as per the manufacturer's protocol. Subsequently, we determined the DNA concentration in every sample utilizing Nanodrop One spectrophotometer (Thermo Scientific, USA). For genotyping qPCR assay, we used a concentration of (1 μg) from each sample (Carnevale et al., 2007).

3. Genotyping by quantitative polymerase chain reaction (q PCR): genotyping of *3′ untranslated region of in T-Box Transcription Factor gene* (*TBX2 3′UTR*) rs59382073, was performed using TaqMan Predesigned SNP Genotyping Assay (Thermo Fisher Scientific, USA). According to the manufacturer's instructions a total volume of 25 μl was used for qPCR. The reaction setup started by adding nuclease free water to DNA volume (1000 ng) to reach volume of 11.25 μl in PCR tubes. Followed by adding 12. 5 μl of TaqMan Universal PCR Master Mix (2×), No AmpErase UNG and 1.25 μl of 20× working SNP Assay for *TBX2 3′UTR* (rs59382073).

Genotyping assays were performed by StepOnePlus real-time PCR system (Applied Biosystems, USA) under the following reaction conditions: heat activation at 95 °C for 10 min, followed by cycling for 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. No transcript controls (NTC) were used for each PCR run with *TBX2 3′UTR* rs59382073 SNP assy.

4. Allelic discrimination: We conducted the analysis using StepOnePlus software for calculation of normalized dye fluorescence (ΔRn) for Allele G (major allele) or Allele T (minor allele). An automatic call of either Allele G (homozygous G/G), or allele T [(homozygous T/T) or (heterozygous G/T)] is performed by the software. Suppl. Fig. 1.

2.3. Statistical analysis

Organization of the data was performed using SPSS version 16 software (SPSS Inc., Chicago). Quantitative data was designed in the form of mean and standard deviation. The significance of difference was tested using: -Student's *t*-test and Mann– Whitney Test (*U* test) to compare the mean of two groups of quantitative data. Categorical data were presented in the form of numbers and percentages. Odds ratios (ORs) and the corresponding 95% CI were calculated. Regression analysis with the adjusted Odds Ratios was used to detect the significant predictors of congenital heart disease. $P < 0.05$ was considered significant.

3. Results

We conducted our study on 120 cases with congenital heart disease (CHD) including 28 neonates, 72 infants, and 20 children. There were 64 males (53.7%) and 56 females (46.7%), as well as 120 healthy control subjects of matched age and gender.

As for the anthropometric measures and body surface area, the median values of weight centile and body surface area were significantly ($P < 0.05$) higher in the control group (50 and 0.3 respectively) than the patients' group (22.5 and 0.17 respectively). However, there was an insignificant statistical difference between the studied groups regarding HC and length centiles ($P > 0.05$ for both) (Table 1).

Among the 120 CHD cases, 26.7% had cyanosis, 76.7% had pulmonary congestion, 33.3% of the cases had systemic congestion, and 26.7% of them had low cardiac output. Septal defect was the most commonly observed CHD by echocardiography, being detected in 40 cases (33.3%), and conotruncal defects were identified in 28 cases (23.3%), followed by atrioventricular septal defect (AVSD) in 16 cases (13.3%). Abnormal ECG findings were found in 90% (108/120) of the cases; 33.3% had right ventricular strain, 23.3% had left ventricular strain, and another 23.3% had biventricular strain, 6.7% had complete heart block and 3.3% had sinus tachycardia. As for chamber enlargement, 30% had left ventricle enlargement, 36.7% had right ventricle enlargement while 23.3% suffered from biventricular enlargement and 10% had no chamber enlargement. Regarding fractional shortening, it ranged from 23 to 36 with an average value of 29.2 (Table 2).

CHD susceptibility significantly increased in our study population by T allele of the SNP rs59382073 in the TBX2 3′UTR. T allele carriers

Table 1
Anthropometric measurements and body surface area among studied groups.

Variables	Group				Z_{MWU}	*P value*
	CHD patients ($n = 120$)		Controls (n = 120)			
	Median	IQR	Median	IQR		
Weight (kg)	4.3	3–6	7.3	3–15	2.83	**0.005 (S)**
Weight centile	22.5	3–50	50	10–50	2.67	**0.008 (S)**
HC (cm)	36	34–44	37	35–45.0	1.51	0.22 (NS)
HC centile	17	3–25	20	4.5–31.3	1.42	0.15 (NS)
Length/ Height (cm)	51	48–70.0	51	48–95	1.15	0.25 (NS)
Centile	50	25–75	50	25–75	1.14	0.25 (NS)
Body surface area	0.17	0.04–0.29	0.30	0.04–0.50	2.49	**0.013 (S)**

Data represented as median and IQR.
CHD: congenital heart diseases, HC: head circumference.

Table 2
Clinical characteristics, echocardiography and ECG findings among studied cases.

Variables		CHD (N = 120)	% (100%)
Cyanosis	No	88	73.3
	Yes	32	26.7
Pulmonary congestion symptoms	No	28	23.3
	Yes	92	76.7
Systemic congestion symptoms	No	80	66.7
	Yes	40	33.3
Symptoms of low cardiac output	No	88	73.3
	Yes	32	26.7
Echocardiography	Septal defects	40	33.3
	AVSD	16	13.3
	Conotruncal defects	28	23.3
	LVOTO	12	10.0
	RVOTO	12	10.0
	Complex	4	3.3
	Isomerism	4	3.3
	APVR	4	3.3
ECG findings	Normal	12	10.0
	Sinus tachycardia	4	3.3
	Biventricular strain	28	23.3
	Complete heart block	8	6.7
	Left ventricular strain	28	23.3
	Right ventricular strain	40	33.3
Chamber enlargement	Non	12	10.0
	Left ventricle	36	30.0
	Right ventricle	44	36.7
	Biventricular	28	23.3
Fractional shortening	Mean ± SD	29.2 ± 4.1	
	Range	23–36	

Data represented as mean ± SD or number (percentage).
CHD: congenital heart diseases, AVSD: atrial ventricle septal defect, LVOTO: left ventricle outflow tract obstruction, RVOTO: right ventricle outflow tract obstruction, APVR: anomalous pulmonary vein return.

(homozygous TT and heterozygous GT individuals) had a significantly higher risk of CHD compared to wild-type GG subjects (OR 5.7; 95% CI, 2.99–11.1; $P < 0.001$) and (OR 9.6; 95% CI, 3.1–29.6; P < 0.001). Furthermore, individuals with T allele were 5.2 folds more likely to have CHD than G allele (OR 5.2; 95% CI, 3.2–8.5; $P < 0.001$) (Table 3).

Table 3
Genotypes and allele frequencies of TBX2 3′UTR SNP rs59382073 among studied groups.

TBX2 3′UTR SNP rs59382073 (G-T)		CHD ($n = 120$)		Controls (n = 120)		OR (95%CI)	P
		No.	%	No.	%		
Genotypes	GG	52	43.3	100	83.3	**Ref**	–
	GT	48	40.0	16	13.3	**5.7 (2.99–11.1)**	**<0.001 (HS)**
	TT	20	16.7	4	3.3	**9.6 (3.1–29.6)**	**<0.001 (HS)**
Allele	G	152	63.3	216	90.0	**5.2**	**<0.001 (HS)**
	T	88	36.7	24	10.0	**(3.2–8.5)**	

Data represented as number (percentage).
UTR: untranslated region, SNP: single nucleotide polymorphism, CHD: congenital heart diseases.

Carriers of T allele (homozygous TT and heterozygous GT individuals) were 13.5 times more susceptible to have septal defects than wild-type GG subjects (OR 13.5; 95% CI, 4.3–41.6; P < 0.001). Also, they were 33.5 folds more susceptible to have conotruncal defects than those with GG genotype (OR 33.5; 95% CI, 4.3–257.7; P < 0.001) (Table 4).

4. Discussion

Some key genes are essential for cardiac growth. For instance, *six of the TBX family* (*TBX1*, *TBX18*, *and TBX20 of the TBX 1 subfamily*, *and TBX2*, *TBX3*, *and TBX5 of the TBX2 subfamily*) may work in a complex manner to control a subgroup of genes necessary for cardiac morphogenesis (Greulich et al., 2011). Localized on the chromosome 17q23, is the human TBX2 gene which consists of seven exons (Campbell et al., 1995; Campbell et al., 1998; Law et al., 1995). Congenital disorders may be a result of both duplications and microdeletions of the chromosome fragments containing *TBX2* (located on chromosome 17q23), which includes heart defects (Ballif et al., 2010; Radio et al., 2010). Yet, previous studies did not identify any mutations in the coding region.

Variants in the promoter region of TBX2 (g.59477201C > T, g.59477347G > A, g.59477353delG, and g.59477371G > A) were associated with VSD by decreasing gene expression, as reported by Pang et al. (2013). Hence, we suggested that genetic variations in TBX2 3′UTR might have a role in CHD risk when *TBX2* expression levels are altered, thus, having an impact on CHD development. As a result of the discrepancy in the results between diverse populations, we aimed to study the association between the SNP rs59382073 within TBX2 3′UTR and the risk of CHD in an Egyptian case-control study including 120 cases and 120 controls.

In our study to assess possible association between the SNP rs59382073 within TBX2 3′UTR and the risk of CHD, we have showcased in our study that the polymorphism rs59382073 is significantly associated with CHD susceptibility and has revealed that T allele carriers (homozygous TT and heterozygous GT individuals) had a significantly higher risk of CHD compared to wild-type GG subjects (OR 5.7; 95% CI, 2.99–11.1; $P < 0.001$) and (OR 9.6; 95% CI, 3.1–29.6; P < 0.001). Furthermore, individuals with the T allele were 5.2 folds more susceptible to have CHD than individuals with G allele (OR = 5.2, 95% CI = 3.2–8.5, P < 0.001).

It was revealed by Wang et al., in compliance with our results, that carriers of the T allele (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 groups (OR 2.17; 95% CI, 1.54–3.05; $P = 1.00 \times 10 - 5$). A 1.89-fold increase in CHD risk was showed by the combination (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. Therefore, in two cohorts both separately and together, TBX2 3′UTR variant rs59382073 was a significant

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

Subtypes	Freq.	GG (Wild type) ($n = 52$)		GT/TT (T carrier) ($n = 68$)		OR (95%CI)	P
		No.	%	No.	%		
AVSD	16	8	50.0	8	50.0	0.73 (0.25–2.1)	0.56 (NS)
Septal defects	40	4	10.0	36	90.0	13.5 (4.3–41.6)	<0.001 (HS)
Conotruncal	28	1	0.0	27	100.0	33.5 (4.3–257.7)	<0.001 (HS)
LVOTO	12	4	33.3	8	66.7	1.6 (0.45–5.6)	0.46 (NS)
RVOTO	12	4	33.3	8	66.7	1.6 (0.45–5.6)	0.46 (NS)
Other CHD	12	4	33.3	8	66.7	1.6 (0.45–5.6)	0.46 (NS)

Data represented as number (percentage).
CHD: congenital heart diseases, AVSD: atrial ventricle septal defect, LVOTO: left ventricle outflow tract obstruction, RVOTO: right ventricle outflow tract obstruction.

contributor to CHD risk (Wang et al., 2019).

These results were consistent with the expression pattern of *Tbx2*, mostly restricted in the atrioventricular canal (AVC), outflow tract (OFT) region, inflow tract (IFT) region and inner curvature. Defective chamber formation was a result of overexpression and mutations of Tbx2 in mice. Tbx2 gene downregulation is crucial for right ventricle formation (Habets et al., 2002; Harrelson et al., 2004; Christoffels et al., 2004). It was reported in previous genetic studies that Tbx2 expressing cells partially contribute to the formation of ventricular septum (Aanhaanen et al., 2009).

Our study revealed that T allele carriers (homozygous TT and heterozygous GT individuals) were 13.5 times more likely to have septal defects than wild-type GG subjects (OR 13.5; 95% CI, 4.3–41.6; $P < 0.001$). Furthermore, they were 33.5 folds more susceptible to have conotruncal defects than those with GG genotype (OR 33.5; 95% CI, 4.3–257.7; $P < 0.001$).

Our results were in accordance with previous studies which revealed that Tbx5 and Tbx20 promote the chamber formation through activating chamber-specific myocardial genes, whereas Tbx2 and Tbx3 suppress the process by repressing the same set of genes (Habets et al., 2002; Harrelson et al., 2004; Christoffels et al., 2004).

In addition, it was found by Xie et al., that the variants of TBX2 and TBX3 induced the incidence of conotruncal defects (CTDs) and explored the interesting downstream genes of TBX2 and TBX3 to illuminate the mechanisms of CTD etiology (Xie et al., 2018).

Somatic mutations and dysregulations by microRNAs (miRNAs) may have a crucial role in Congenital Heart Defects (CHDs) (Sabina et al., 2013). Biogenesis of miRNA and the regulatory effect of miRNAs to their target genes may be affected by the genetic variations in miRNA processing genes and miRNA binding sites (Liang et al., 2010), therefore, MiRNAs can downregulate gene expression by interaction with 3′ UTR of the target mRNA, cleavage, or degradation of the mRNA and/or inhibition of its translation and transcription (Kiriakidou et al., 2007; Nikolova et al., 2013; Pillai et al., 2005). As per Wang et al., the binding affinity of miRNAs can be affected by genetic variations within 3′UTR can affect, leading to heart malformations (Wang et al., 2017) Hence, the increase in the risk of CHD in T allele carriers can be clarified by the reduction of TBX2 level through giving rise to biding sites for two miRNAs (miR-3940 and miR-708) and facilitating their negative regulation.

5. Conclusion

In the Egyptian population, the T allele of the SNP rs59382073 in the TBX2 3′ UTR is associated with increased CHD susceptibility, and carriers of the T allele had a considerably higher risk of CHD, particularly septal and conotruncal defects. The TBX2 3′ UTR SNP rs59382073 appears to play a role in the development of CHD. Our findings highlight the significance of further research into the multiple gene variants of CHD in Egyptian children.

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CRediT authorship contribution statement

Eman Rateb Abd Almonaem: Conceptualization, Methodology, Software. **Doaa Refaey Soliman:** Data curation, Writing – original draft. **Marwa Abdel Monaem El Sayed:** Visualization, Investigation. **Inas A. Ahmed:** Conceptualization, Methodology, Software. **Eman G. Abdelrahman:** Software, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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