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Association between TBX20 gene polymorphism and congenital heart disease among Egyptian subjects

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Abstract

Background: T-box-transcription-factor-20 (TBX20) is a major type of T-box protein family that plays a plausible function in the optimum functional and developmental of cardiac tissues. This work aimed to assess the contribution of TBX20 gene polymorphisms with elevated risk of congenital heart disease (CHD) among Egyptian subjects. **Methods:** This case-controlled study consisted of 175 participants [75 patients diagnosed with CHD along with 100 unrelated healthy controls], matched with age and gender. The genetic variant for TBX20* was genotyped and characterized with the aid of the polymerase chain reaction-restriction fragment-length-polymorphism (PCR-RFLP) technique.

Results: Our findings revealed a higher frequency of TBX20*(T/T) genotype among CHD patients compared with healthy controls. Additionally, testing genetic association models revealed a significant association with protection against CHD in the recessive model. This might imply this rare genotype is a predisposing factor to protect against congenital heart disease. Moreover, the TBX20(C/C) genotype exhibited higher prevalence among males than females among CHD compared to unrelated healthy controls. On the contrary, the frequency of TBX20*(T/T) genotype was higher among both males and females than healthy controls. The frequency of TBX20*(C/C) genotype within TOF cases was higher in CHD cases compared to healthy controls. However, the frequency of TBX20*(T/T) genotype was significantly associated with VSD cases compared to healthy controls.

Conclusions: In conclusions, our findings revealed the protective function of the TBX20*rs3999950 gene variant against the development of congenital heart diseases among Egyptian subjects.

Keywords: *TBX20 transcription factor; rs3999950*; *Genetic variants*; *congenital heart disease*.

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1. Introduction

Congenital heart defect (CHD) is a type of defect in the structure and/or function of the heart tissues that is attributed to abnormal heart development before birth (1,2). It is the most frequent congenital anomaly that ranges from five per one hundred live births worldwide (3). Recently, CHD has been considered the crucial reason for childhood mortality and morbidity globally (4). After precise literature screening, numerous studies have been conducted investigating the existence of congenital heart diseases among children due to various genetic factors along with epigenetic modifications [5,6].

T-box transcription factor (TBX20) is considered a crucial member of the T-box family that contributed to many biological processes and shares a definite conserved deoxyribonucleic acid-binding domain [7]. In addition, it plays a potential role in the optimum functional and developmental of cardiac tissues including the formation of cardiac septum, and hence prevents the advancement of congenital heart diseases (8). Moreover, several articles observed that many family members were exposed to normal cardiac function throughout initial embryogenesis including TBX5 and TBX20 (9). The TBX20 gene is situated on chromosome number 7p14.2 and consists of two main splice variants with eight exons and seven introns along the minus strand (10).

Recently, various mutations were identified in the TBX20 gene due to the acquisition or lack of the function of this transcription factor and are thought to be associated with a diverse of heart malformations involving cardiac septal defects, congestive heart failure, and dilated cardiomyopathy (11,12). Based on the fact that screening the single nucleotide variants (SNV) of certain genes is the key method to identify the hereditary predispositions related to specific issues, thus studying the cellular mechanisms by which the pathogenesis of these diseases could be raised (13-15). Other reports investigated the potential function of the TBX20 gene with other important genes and illustrated the signaling pathways by which this transcription factor maintained the proper function of cardiac tissues (12,16). Taken together, this work was designed to explore the association of the *TBX20*(rs3999950; c.774T>C; Ala265Ala)* variant with the susceptibility and development of congenital heart disease among Egyptian children.

2. Subjects and Methods

2.1. Study participants

This retrospective case-controlled study is a hospital-based design including 175 participants (75 patients diagnosed with congenital heart diseases that subdivided into three categories involving 21 patients with septal defects, 43 patients with ventricular septal defects, and 11 patients with tetralogy of Fallot, along with 100 unrelated healthy controls that matching with gender and age and from the same geographical region. All the subjects enrolled in this work were recruited from the Department of Cardiology, Internal Medicine Specialized Hospital, Mansoura University, Egypt. The study was approved by the Ethical Committee of Research, Mansoura University Hospitals (Code number: R.20.11.1071). Furthermore, the study was carried out in accordance with the regulations and recommendations of the Declaration of Helsinki. The collection of blood samples from all the subjects was performed and manipulated as previously described (17). Briefly, peripheral blood samples were extracted under aseptic conditions with the aid of sterilized vacutainer tubes and subcategorized into two main compartments, the first aliquot was drawn with the presence of EDTA-anticoagulant for purposes of hematological measurements and hereditary assessment, while the other aliquot was separated with gel-separator tubes and subjected for continuous centrifugation to extract serum for biochemical evaluation. All samples were identified and given matched numbers that corresponded to all investigations.

2.2. Isolation and purification of genomic DNA

Generally, the extraction of genomic DNA was executed from all participants in this work using the phenol-chloroform method in the presence of proteinase K digestion. The purification of extracted DNA was evaluated with the aid of Nanodrop™ ND-1000 Spectrophotometer (18).

*2.3. Genotyping and amplification of TBX20*rs3999950 variant*

The amplification and characterization of the *TBX20*rs3999950* variant were accomplished with the aid of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, as formerly described [12]. The two primers used in the amplification process were [Forward primer (F1): 5'-CCT CAC TGT AAT TTG GCC TG-3', while the reverse primer (R1): 5'-GCC CTG AAA CTC AAT AGC TC-3']. The PCR reaction was adjusted in a total volume of 25 µL containing 3 µL purified template DNA, 2 µL of forward primer, 2 µL of reverse primer, 13 µL of PCR Master Mix (2×), and 5 µl nuclease-free sterile water (ddH_2O). The PCR conditions were manipulated with an initial denaturation step for 7 minutes at 94º C, followed by 28 cycles of 94º C for 30 seconds, 50º C for 60 seconds, and 72º C for 30 seconds with a final extension step for 5 minutes at 72º C. The visualization of PCR products was performed with the aid of 3% agarose gel using 15 µL PCR product, and 5 µL 10X buffer followed by electrophoresis at 60 mV for 25 minutes, and the imaging process was taken with ethidium bromide.

2.4. Statistical analysis

All tabulated data obtained from this work was analyzed using IBM SPSS software version 22.0 (SPSS Inc, Chicago, USA). The equilibrium of the Hardy-Weinberg equation was performed using Fisher's exact chi-square test. The allelic/genotypic analysis of the *TBX20*rs3999950* variant was applied using the online SNPstats tool for genomic analysis (https://www.snpstats.net/start.htm, accessed on 20 May 2023). The odd ratios (OR) and 95% confidence intervals (CI) were evaluated among patients compared to unrelated healthy controls. The significance of interests regarding the statistical analysis was adjusted with a *p-value* less than 0.05.

3. Results

3.1. Genetic and allelic frequencies of TBX20(rs3999950; c.774T>C) variant among studied subjects*

The distribution of allelic/genotypic for *TBX20*(rs3999950; c.774T>C)* variant among CHD patients compared to healthy controls was displayed in **Table 1**. The higher genotype frequency among CHD patients was C/C genotype, also the higher frequency among healthy controls was C/C genotype. The exact test for Hardy-Weinberg equilibrium (HWE) among studied subjects for the *TBX20*(rs3999950; c.774T>C)* variant was displayed in **Table 1**. HWE showed equilibrium among all subjects and CHD patients (*p-value* = 0.59 and 0.33, respectively). On the contrary, HWE failed to achieve a suitable equilibrium between the control group (p-value = 0.031), and this was probably due to the lower frequency of the T/T genotype that should be tested later among larger control samples.

Table (1): Testing for Hardy-Weinberg equilibrium (HWE) for *TBX20*(rs3999950; c.774T>C)* among the studied subjects.

TBX20*(rs3999950; c.774T>C) exact test for Hardy-Weinberg equilibrium (n=175)						
	C/C	**C/T**	**T/T**	**C**	**T**	**P-value**
All subjects	84	77	14	245	105	0.59
Cases	32	31	12	95	55	0.33
Controls	52	46	2	150	50	**0.031***
***p-value* < 0.05 indicates statistically significant.**						

3.2. Results of agarose gel electrophoresis of the PCR products

The interpretation of agarose gel electrophoresis results was applied with the aid of illumination under UV light for the *TBX20*(rs3999950; c.774T>C) variant*. The product size of this variant was visualized at 350 bp which corresponds to the designed primers. The digested fragments of the TBX20*(rs3999950; c.774T>C) variant were processed by applying an enzymatic endonuclease digestion system using EcoRI and XbaI.

3.3. Association of the TBX20(rs3999950; c.774T>C) variant with increased risk of CHD*

The genetic association models for *TBX20*(rs3999950; c.774T>C)* variant with an elevated risk of CHD compared to healthy controls were illustrated in **Table 2**. As depicted, the frequency of the *TBX20*(T/T) genotype* was higher among CHD patients compared to healthy controls (16% vs. 2% respectively). This might shed light on the potential role of this genotype as a predisposing factor to the occurrence of congenital heart disease. Our findings showed a significant association with CHD patients under the codominant model compared to healthy controls (C/C vs. C/T vs. T/T; OR= 0.1, 95% CI= 0.02-0.49, *p-value* = 0.0023). Similarly, upon testing the recessive model, the *TBX20*(rs3999950; c.774T>C)* variant represented statistical difference among CHD patients compared to control groups (T/T vs. C/C + C+T; OR=0.11; 95% CI=0.02-0.49, *p-value* = 0.0005).

Table (2): The genetic association models for *TBX20*(rs3999950; c.774T>C)* variant with an elevated risk of CHD.

Model	**Genotype**	**Cases**	**Controls**	**OR (95% CI)**	**P-value**
Codominant	C/C	32 (42.7%)	52 (52%)	1.00	**0.0023***
	C/T	31 (41.3%)	46 (46%)	0.91 (0.48-1.72)	
	T/T	12 (16%)	2 (2%)	**0.10 (0.02-0.49)**	
Dominant	C/C	32 (42.7%)	52 (52%)	1.00	0.22
	C/T-T/T	43 (57.3%)	48 (48%)	0.68 (0.37-1.25)	
Recessive	C/C-C/T	63 (84%)	98 (98%)	1.00	**0.0005****
	T/T	12 (16%)	2 (2%)	**0.11 (0.02-0.49)**	
Overdominant	C/C-T/T	44 (58.7%)	54 (54%)	1.00	0.54
	C/T	31 (41.3%)	46 (46%)	1.21 (0.66-2.21)	
Genetic testing of association of TBX20*(rs3999950; c.774T>C) variant in CHD PATIENTS compared to Controls (n=175, adjusted by SEX). * p-value < 0.05 indicates statistically significant.					
Abbreviations: OR; odd ratios, CI; confidence intervals, P; probability					

3.4. Association of the TBX20*(rs3999950; c.774T>C) variant with increased risk of CHD according to gender compared to healthy controls.

The distribution of *TBX20*(rs3999950; c.774T>C)* variant among CHD patients compared to healthy controls stratified by gender was demonstrated in **Table 3**. Our results indicated that the frequency of *TBX20*(C/C) genotype* was higher in male patients compared to healthy controls (OR=0.33, 95% CI=0.13-0.82). On the other hand, the frequency of *TBX20*(T/T) genotype* was higher among both male and female patients compared to healthy controls (OR=0.06, 95% CI =0.01-0.53).

3.5. Association of the TBX20*(rs3999950; c.774T>C) variant stratified by different types of CHD compared to healthy controls.

The correlation between *TBX20*(rs3999950; c.774T>C) variant* stratified by different types of CHD compared to healthy controls was displayed in **Table 4**. Interestingly, the frequency of *TBX20*(C/C)* genotype among TOF cases was higher in CHD patients compared to healthy controls (72.7% vs. 52%). On the other hand, the frequency of *TBX20*(T/T) genotype* among VSD cases is higher in CHD patients compared to healthy controls (20.9% vs. 2.0%) with a significant statistical difference *p-value* = 0.004, (**Table 4**).

Table (3): Association of *TBX20*(rs3999950; c.774T>C) variant* among CHD patients compared to healthy controls stratified with gender.

	Female			**Male**		
	Cases	**Controls**	**OR (95% CI)**	**Cases**	**Controls**	**OR (95% CI)**
C/C	11	32	1.00	21	20	**0.33 (0.13-0.82)**
C/T	18	22	0.42 (0.17-1.06)	13	24	0.63 (0.24-1.66)
T/T	6	1	**0.06 (0.01-0.53)**	6	1	**0.06 (0.01-0.53)**
Genetic testing of association of *TBX20*(rs3999950; c.774T>C) variant* in CHD patients compared to Controls (n=175, stratified by SEX). * p-value < 0.05 indicates statistically significant. **Abbreviations: OR; odd ratios, CI; confidence intervals, P; probability**						

Table (4): The correlation between *TBX20*(rs3999950; c.774T>C) variant* stratified by different types of CHD.

TBX20*rs3999950	**Controls**	**VSD**	**ASD**	**TOF**	**Total**
C/C	52	15	9	8	84
	52.0%	34.9%	42.9%	72.7%	48.0%
T/T	2	9	2	1	14
	2.0%	20.9%	9.5%	9.1%	8.0%
C/T	46	19	10	2	77
	46.0%	44.2%	47.6%	18.2%	44.0%
Genetic testing of association of *TBX20*(rs3999950; c.774T>C) variant* in different types of CHD patients compared to Controls (n=175). * p-value < 0.05 indicates statistically significant.					

4. Discussion

Congenital heart disease (CHD) is a group of functional anomalies that occur during embryogenesis (19). They are classified according to various criteria including pathological and morphological issues with asymptomatic till adulthood (20). The mortality rate for CHD decreased slowly, with higher incidence among adults with CHD compared to children with CHD within the United States and various developed countries (21). After screening literature, various reports studied the contribution of different genetic factors to the elevated risk of CHD among different ethnic subjects (8-10).

The TBX20 gene is situated on chromosome number 7q14.3 and is represented by eight exons. Recently, seven members of the T-Box gene family were expressed and translated during the embryonic level and within cardiac vertebrates including TBX1, TBX2, TBX3, TBX4, TBX5, TBX18, AND TBX20 (12,22). The transcription activity for the TBX20 gene shared different domains that interact with various transcription factors involving GATA4, and TBX5 to adjust the degree of synthesis related to embryonic hearts (23). Numerous genetic risk factors contributed to the event of congenital heart diseases within homo sapiens, and these variables include the posttranslational modifications and genetic mutations within the coding region related to the TBX20 gene and their correlations with congenital cardiac disorders (24,25). Our findings indicated the association of *TBX20*(rs3999950; c.774T>C) genetic variant* with congenital heart diseases among Egyptian subjects. In a study done by Kirk et al. that reported the complete map of full coding regions within the TBX20 gene in 353 CHD patients, they indicated the contribution of two novel heterozygous mutations *TBX20*(p.I152M and p.Q195X)* among two index familial CHD patients (11). Interestingly, they observed that family one carried the missense mutation of *TBX20*p.I152M* with atrial congenital heart and ventricular septal defects, while family two was found to carry the other mutation of *TBX20*p.Q152X* with atrial defect, left atrioventricular valve, dilated cardiomyopathy, and coarctation of the aorta (11). Another study screened the potential role of the TBX20 gene within 192 unrelated CHD children and implicated the involvement of two mutations in four CHD children including two children with *TBX20*p.H186D* mutations accompanied by two children with *TBX20* p.L197P* mutations (1).

In addition, a report performed among 203 unrelated CHD patients using sequence analysis technique for the TBX20 gene from exon two to exon six revealed three other nonsynonymous mutations in three CHD patients with atrial septal defects including *TBX20*(p.A63T, p.I121F, and p.T262M)* (26). Another record studied the total set of coding regions within the TBX20 gene among 170 unrelated subjects suffering from atrial septal defect using sequencing analysis, they observed a novel mutation within one patient with a positive family history and this mutation was referred to as *TBX20*p.I121M* (27). Moreover, a three-generation family diagnosed with atrial septal defects was screened without functional analysis and identified a rare mutation related to this gene called *TBX20*(p.D176N)* (28). Furthermore, another group team was subjected to perform sequencing technique for flanking introns and their corresponding coding exons for the TBX20 gene among 146 CHD patients and identified the contribution of various TBX20 mutations in diminishing the transcriptional level including *TBX20*(p.R143W)* (29).

Another report examined 38 patients with atrial/ventricular septal defect and identified the contribution of various mutations within the TBX20 gene in the pathogenesis of the disease but without functional characterizations for these target mutations including *TBX20*(p.Y309D, p.T370O, and p.M395R)* (30). Additionally, another article

revealed the association of genetic variants within the TBX20 gene with an elevated risk of congenital septal heart defects (31). Moreover, Yn et al. identified lower activity for the TBX20 gene and its function in diminishing CHD risk among Chinese subjects (32). Another report studied the potential impact of the TBX20*rs3999950 variant and identified the role of the *TBX20*(T/C)* genotype in the development of congenital heart diseases among Chinese children (12). Interestingly, this work confirmed the involvement of *TBX20*(rs399950; T/T genotype)* against the development of congenital heart diseases among Egyptian subjects. This finding implied that this genetic variant could contribute as a potential factor in decreasing the adverse effects of CHD among Egyptian populations. On the contrary, Lin et al. observed the role of the TBX20 gene in the progression of congenital heart diseases (16). Fewer limitations were identified through the establishment of this work in the form of a relatively small pilot sample size; therefore, our team recommends performing another large-scale multicenter survey to illustrate the functional mutations within the TBX20 gene and their roles in the susceptibility for CHD among Egyptian subjects.

Conclusion

This study confirmed that the *TBX20*rs3999950 variant* showed a protective effect against the development of congenital heart defects among Egyptian populations.

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