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ORIGINAL ARTICLE

Association of *TBX20* Gene Polymorphism with Congenital Heart Disease in Han Chinese Neonates

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Abstract As a transcription factor mainly expressed in cardiovascular system, T-box 20 (TBX20) plays an important role in embryonic cardiovascular system development and adult heart function. Previous studies have identified associations of two SNPs in the T-box DNA-binding domain of TBX20 with congenital heart disease (CHD) in two Caucasian families, but the associations of TBX20 mutations underlying the more common populations with CHD remain to be uncovered. In this study, 25 unrelated Chinese Han neonates with CHD and 25 healthy children as controls were investigated for TBX20 mutations. SNP genotyping was performed by PCR-DNA sequencing. The selected SNPs were well genotyped and SNP rs3999941 was found to be strongly associated with CHD ($p = 0.007$). The minor allele of rs3999941 showed a high-risk factor for CHD (OR 4.24; 95 % CI 1.41–12.71). Besides, we found a new SNP site located at the 657th nucleotide of the exon 5 of *TBX20* gene which may also be associated with CHD, c.657A>C. The frequency was significantly different between two groups ($p = 0.011$), the minor allele of SNP c.657A>C also showed a risk factor for CHD (OR 2.56; 95 % CI 1.02–6.46). These findings suggested that the TC genotype of SNP rs3999941 and AC genotype of the new SNP c.657A>C in the *TBX20* gene may be risk factors for CHD and thus screening of these SNPs may have some implications in the prevention and treatment of CHD in Han Chinese children.

Keywords Congenital · Heart disease · T-box20 · Single nucleotide polymorphisms · Genotype · Allelic gene

Introduction

Congenital heart disease (CHD) refers to a group of structural and functional heart abnormalities, including septal defects, valve defects, and lesions affecting the outflow tract. It is the most common defect with a postnatal incidence of 0.8 % [32] and an approximately much higher prenatal incidence [17, 18, 33, 34]. It has been increasingly recognized that CHD is a complex disease which may be caused by both environmental and genetic factors [36]. Approximately, 5–8 % of CHD is due to chromosomal abnormalities, and 3 % to classical genetic defects with a high recurrence risk in first-degree family members [8–10, 16, 20].

T-box (TBX) factors belong to an evolutionary conserved family of transcriptional regulators with diverse roles in development [25]. Their regulatory functions are integral in the patterning, recruitment, specification, differentiation, and growth processes underlying heart formation [2, 11, 14]. Mutations in T-box genes are associated with congenital heart defects. TBX20 is an ancient member of the T-box superfamily related to TBX1. Previous studies

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revealed mutations of *TBX20* gene in familial secundum atrial septal defects ASDs [15]. Kirk et al. found that TBX20 mutations were associated with diverse cardiac pathologies, including defects of septation and valvulogenesis and cardiomyopathy [22]. Posch et al. described a novel mutation in TBX20 associated with familial ASD spanning three generations of kindred [27].

Nevertheless, the vast majority of studies on the relationship between TBX20 and CHD were conducted on white subjects in the Western [12, 13, 28, 29], little is known in this regard on the Chinese populations. Therefore, this study was conducted to investigate the mutations of *TBX20* gene in Han Chinese neonates with CHD. The work may lead to a better understanding of the genetic susceptibility to CHD in the Han Chinese population, which may assist in the prenatal diagnosis and facilitate the prevention and treatment of CHD.

Materials and Methods

Ethical Statement

This study was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University and conformed to the Declaration of Helsinki. Written informed consent was obtained from the parents of every participant.

Study Subjects

During the period from September 2011 to February 2012, 25 unrelated Chinese neonates with CHD (13 boys and 12 girls; age range, 4 h to 28 days; median age, 14.5 days) were recruited as case subjects from the Second Hospital of Tianjin Medical University. 25 unrelated healthy neonates (14 boys, 11 girls; age range, 12 h to 28 days; median age, 14.1 days) were recruited randomly from the neonatology department of the Second Hospital of Tianjin Medical University as control subjects. The diagnosis of CHD in all subjects was performed based on clinical symptoms and examination results from echocardiography and cardiac catheterization. Neonates who had a family history of CHD or whose patients had any other documented abnormalities/syndromes such as Noonan, DiGeorge, HolteOram, Marfan, Alagille, and Char syndrome were excluded from this study. All patients and control subjects were from the Chinese Han population. The basic demographics of CHD patients were presented in Table S1.

SNP Selection

SNP genotype data for TBX20 gene were obtained by retrieving the dbSNP database, and 16 SNPs were found located in the coding regions of TBX20 gene. These SNPs were reconfirmed and analyzed using online SNPper software and 7 SNPs from both databases were obtained: 2 in the exon 1, 2 in the exon 2, 1 in the exon 3, and 2 in the exon 5 (Table S2). Considering the exons 2–6 contribute to the T-box DNA-binding domain, the 2 SNPs in exon 1 were excluded. After comparing the left 5 SNPs with relevant reports in the literature, finally two SNPs: rs6950175 and rs3999941 in exon 5 of *TBX20* were selected as targets.

Mutation Detection

Genomic DNA was isolated from peripheral blood leukocytes using ReadyAmp™ Genomic DNA Purification System (Promega, USA). The exon 5 region of the *TBX20* gene containing the DNA-binding domain and the flanking intron sequence was amplified by PCR (Forward primer, 5′-CCTCACTGTAATTTGGCCTG-3′; Reverse primer, 5′-GCCCTGAAACTCAATAGCTC-3′; product length, 365 bp). The PCR product was purified using the AxyPrep DNA Gel Extraction kit (Axygen, CA) and sequenced on a PRISM 3730 sequencer (ABI, Japan). DNA sequences were recorded and analyzed with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems, USA).Whenever a sequence variant was found, the sample was sequenced again from the opposite direction to confirm the nucleotide change.

Statistical Analysis

Gene and allele frequencies were analyzed by Chi-Square test, and genotype data on SNPs were subjected to the association analysis. Odds ratio (OR) and 95 % confidence intervals (CI) for the relative risk for single locus genotypes were computed using the online version of SNPStats (http://bioinfo.iconcologia.net/snpstats/start.htm). All the analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p < 0.05$.

Results

In the present study, 25 CHD patients (male: 13, female: 12, median age 14.5 days) and 25 controls (male 14, female 11,

Table 1 The basic characteristics of the cases and controls

Variable	Case ($N = 25$)		Control ($N = 25$)		p values from χ^2
	No.	(%)	No.	(%)	
Sex					
Female	12	48.0	11	44.0	0.777
Male	13	52.0	14	56.0	
Median age (days)	14.5		14.1		

median age 14.1 days) were recruited. Chi-square tests were used to assess whether the cases and controls were similar. As shown in Table 1, the case and control populations were well matched in terms of sex and age distributions.

By retrieving SNP data from dbSNP database and reconfirmed with SNPper, two SNPs, rs6950175: c (765C→A, Ile255Ile) and rs3999941: c (766T→C, Phe256Leu) were selected. They are both in exon 5 of *TBX 20* gene, and the exon is 159 bp in length (from nucleotide 655 to 813), and the corresponding DNA and amino acid sequences are shown in Figure S1.

Detection of Amplification Products

PCR was performed and the amplicons were analyzed by 2 % agarose electrophoresis. As shown in Fig. 1, each amplicon was unique and appeared at the expected size of 365 bp, indicating that the primers and PCR conditions were adequate, and the products were qualified for DNA sequencing.

Genotype Assignment

Sequence alignment was performed between the results we obtained and the sequence posted in the NCBI database. The sequences of exon 5 in *TBX20* gene of each subjects sequenced were perfectly matched with the sequence posted in the NCBI database.

The studied SNPs were successfully genotyped. The association of both the SNP genotypes and allelotypes with the risk of CHD was analyzed. For SNP rs6950175, a homozygous C/C genotype was detected in both the patients and control subjects as shown in Fig. 2. No mutations were detected in any of the participants.

SNP rs3999941 was detected in both groups of subjects, which presented as homozygous T/T and heterozygous T/C (doublet position) genotypes (Fig. 3). The frequency of T/C genotype of rs3999941 was significantly higher in CHD patients compared with healthy control subjects ($\chi^2 = 9.93$, $p = 0.002$), and the odds ratio (OR) for the TC genotype was 7.11 (Table 2). The minor allele (C) of rs3999941 was present at a much higher frequency in the case group than in the control group ($\chi^2 = 7.29$, $p = 0.007$), and the odds ratio (OR) for the C allelotype was 4.24 (Table 3).

A new SNP, c.657A>C was detected in the subjects and presented as homozygous A/A and heterozygous A/C genotypes (Fig. 4). The frequency of A/C genotype of c.657A>C was significantly higher in CHD patients compared with healthy control subjects ($\chi^2 = 6.52$, $p = 0.011$), and the odds ratio (OR) for the AC genotype was 4.57 (Table 2). The minor allele (C) of c.657A>C showed a significantly higher frequency in newborns with CHD compared with control subjects ($\chi^2 = 4.11$, $p = 0.043$), and the OR for the C allelotype was 2.56 (Table 3).

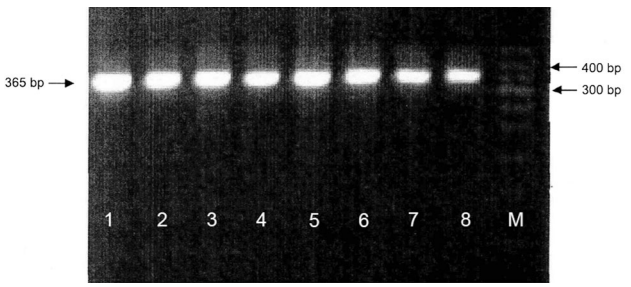

Fig. 1 Analysis of amplicons by agarose electrophoresis. *M*, DM500 DNA ladder marker (Kangwei biotech); *1–4* case group; *5–8* control group

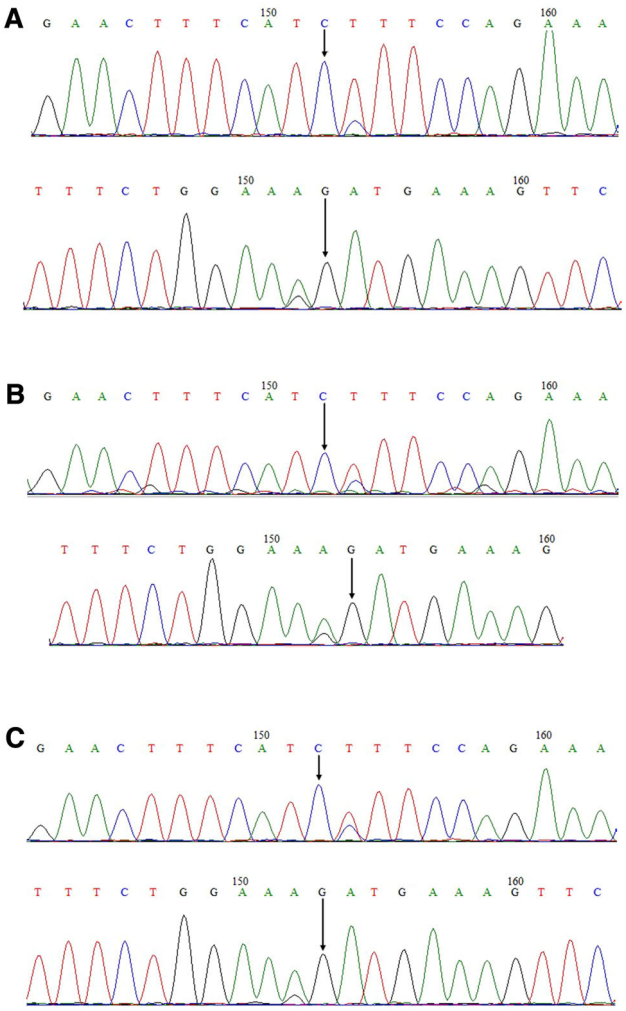

Fig. 2 Sequencing results of rs6950175 (c.765C>A) sites of coding region SNPs in TBX20 genes. Subjects in cases and controls all showed C/C-type homozygous genes. **a** Positive and negative sequencing results of DNA sample from subject 2 of case group with VSD + ASD + PDA. **b** Subject 6 of case group with VSD. **c** Subject D6 of control group. *Upper* positive sequencing result; *below* negative sequencing result

Discussion

Previous animal studies have identified and characterized a number of T-box genes involved in embryonic cardiogenesis [4, 5, 35]. TBX20 is an ancient member of the T-box superfamily related to TBX1, whose gene located on chromosome 7q14.2 or 7p14-15 and has 8 exons, encoding a 289–888 amino acid protein with a T-box DNA-binding domain. As a transcription factor, it plays pivotal roles in cardiac orphogenesis and maintenance of normal heart function in human adults [1, 3, 6, 30]. TBX20 was also regarded as a disease gene of human atrial septal defects (ASDs) [15, 26]. The expression of TBX20 has been demonstrated to be up-regulated during the development of Tetralogy of Fallot in humans [13]. Previous studies on the association of the *TBX20* gene with CHD have mostly focused on Europeans. Due to the different genetic backgrounds and epidemic trends, those results have limited significance on CHD prediction and prevention among the Chinese Han population. From the present study, we have obtained a better understanding about the TBX20 gene polymorphism and genetic background among the Chinese Han population.

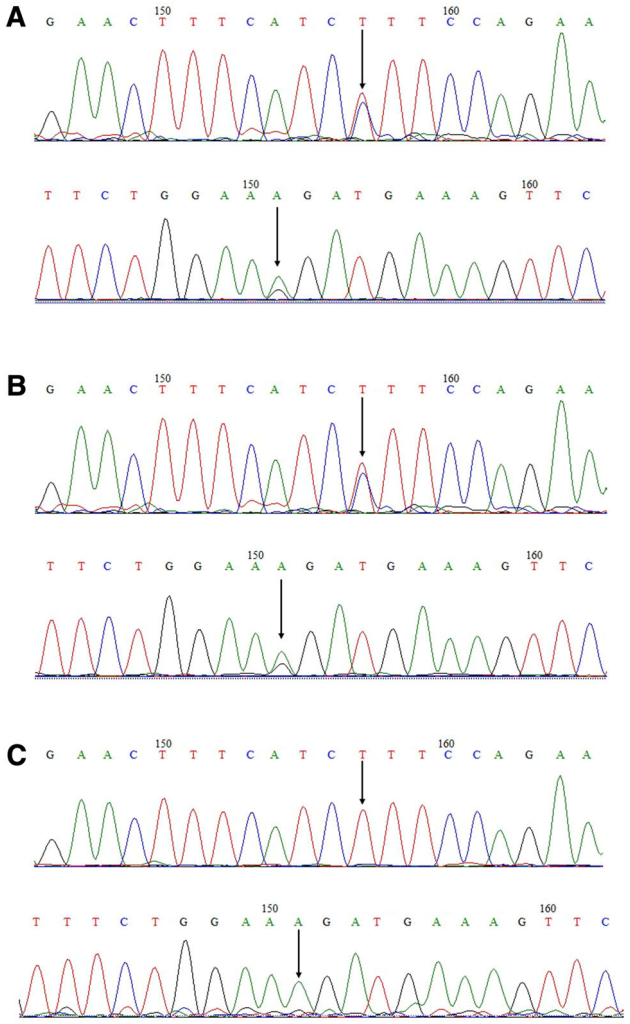

Fig. 3 Sequencing results of rs3999941(c.766T>C) sites of coding region SNPs in TBX20 genes. **a** Positive and negative sequencing results of DNA sample from subject 4 of case group with TOF. **b** Subject 23 of case group with ASD + VSD. **c** Subject 6 of control group. *Upper* positive sequencing result; *below* negative sequencing result

In the present study, we identified two known SNPs, rs6950175 and rs3999941, and a new SNP, c.657A>C, in exon 5 of *TBX20* in a population of Han Chinese neonates with a variety of neonatal CHD including atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA), tetralogy of fallot (TOF), and transposition of great arteries (TGA). SNP rs6950175:c.765C>A (Ile255Ile) is a synonymous mutation. The mutation causes changes of nucleotide 765 of the *TBX20* gene from C to A, which does not lead to change in protein sequence. No polymorphism of rs6950175 was detected in our study. However, in case of rs3999941:c.766T>C, nucleotide 766 changes from T→C, which leads to changes in mRNA codon (UUU→CUU) and in amino acid 256 (Phe → Leu), the changed protein sequence may lead to changes of secondary structures and tertiary structures of TBX20 protein and result in heart malformations of neonates.

The new SNP, c.657A>C (Ile219Ile), is also a synonymous mutation which leads to changes of nucleotide 657 from A to C, but amino acid 219 remains isoleucine (Ile). It was believed that synonymous mutations would have no effect on the fitness of an organism initially. But now increasing evidences suggest that synonymous mutations could have functional consequences. This might be achieved

Table 2 Association between SNP genotypes and the risk of CHD

SNP	Genotype	Frequency		Odds ratio (95 % CI)	χ^2	*p* values from χ^2
		Case (%)	Control (%)			
rs3999941	TT	9 (36)	20 (80)	0.14 (0.04–0.50)	9.93	0.002*
	TC	16 (64)	5 (20)	7.11 (1.99–25.47)		
c.657A>C	AA	7 (28)	16 (64)	0.22 (0.07–0.72)	6.52	0.011*
	AC	18 (72)	9 (36)	4.57 (1.38–15.11)		

Note that asterisks mean differences between groups were statistically significant

Table 3 Association between SNP allelotypes and the risk of CHD

SNP	Allelotype	Frequency		Odds ratio (95 % CI)	χ^2	p values from χ^2
		Case (%)	Control (%)			
rs3999941	T	34 (68)	45 (90)	0.24 (0.08–0.71)	7.29	0.007*
	C	16 (32)	5 (10)	4.24 (1.41–12.71)		
c.657A>C	A	32 (64)	41 (82)	0.39 (0.16–0.98)	4.11	0.043*
	C	18 (36)	9 (18)	2.56 (1.02–6.46)		

Note that asterisks mean differences between groups were statistically significant

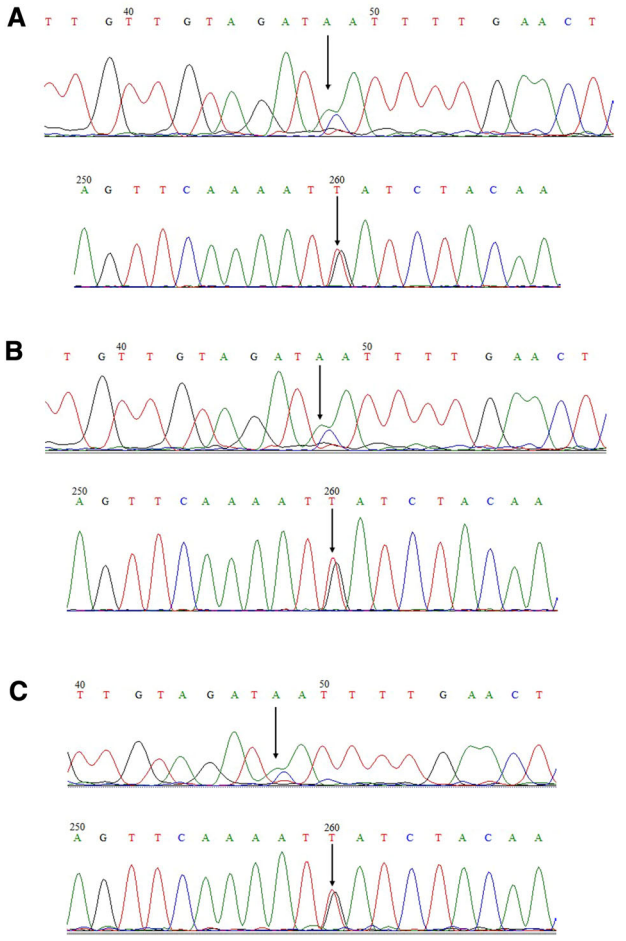

Fig. 4 Sequencing results of c.657A>C sites of coding region SNPs in TBX20 genes. **a** Positive and negative sequencing results of DNA sample from subject 1 of case group with VSD. **b** Subject 5 of case group with ASD + VSD. **c** Subject 5 of control group. *Upper* positive sequencing result; *below* negative sequencing result

by alterative mRNA splicing [7], affecting mRNA stability [24] and thus protein expression and enzymatic activity, alteration of methylation pattern [31], or by affecting tertiary protein structure [21]. Here, we do not know the detailed mechanism about how the c.657A>C mutation affects the pathogenesis of CHD, further research needs to be performed.

According to our results, the variant of SNP rs6950175 in both CHD patients and control subjects showed homozygous C/C genotype, which suggested a limited association between rs6950175 and CHD in Han Chinese neonates. For rs3999941, ORs for TC genotype and C allele were greater than 1, suggesting the TC genotype and C allele might increase the risk of CHD in newborn Han Chinese. In contrast, ORs for the TT genotype and T allele were less than 1, suggesting that the TT genotype might be protective against CHD development. The newly found SNP c.657A>C also showed high frequency in CHD subjects. ORs for AC genotype and C allele suggested that the AC genotype and C allele might be the risk factors of CHD in newborn Han Chinese. In contrast, ORs for the AA genotype and A allele were less than 1, suggesting the AA genotype and A allele protective factors against CHD in Han Chinese population.

As approximately 5–8 % of CHD is caused by genetic variations, understanding the contributions of different susceptibility alleles to CHD might highlight new diagnostic and management recommendations in the prediction and prevention of CHD. Once a mother is identified as a susceptible individual for carrying a CHD baby, preventive care may be given to minimize the chance of the child to develop the disease [19, 23]. Overall, our findings provided new evidences for the association between SNPs of TBX20 gene and CHD in newborn Chinese Han population. Our results, combined with those of previous reports, may assist in the prenatal diagnosis and facilitate the prevention and treatment of CHD.

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Conflict of interest The authors declare no competing interests.

References

1. Ahn D-G, Ruvinsky I, Oates AC, Silver LM, Ho RK (2000) *tbx20*, a new vertebrate T-box gene expressed in the cranial motor neurons and developing cardiovascular structures in zebrafish. Mech Dev 95:253–258

2. Andersen TA, Troelsen Kde L, Larsen LA (2014) Of mice and men: molecular genetics of congenital heart disease. Cell Mol Life Sci 71:1327–1352
3. Brown D, Binder O, Pagratis M, Parr BA, Conlon FL (2003) Developmental expression of the *Xenopus laevis* Tbx20 orthologue. Dev Genes Evol 212:604–607
4. Brown DD, Martz SN, Binder O, Goetz SC, Price BM, Smith JC et al (2005) Tbx5 and Tbx20 act synergistically to control vertebrate heart morphogenesis. Development 132:553–563
5. Cai X, Zhang W, Hu J, Zhang L, Sultana N, Wu B et al (2013) Tbx20 acts upstream of Wnt signaling to regulate endocardial cushion formation and valve remodeling during mouse cardiogenesis. Development 140:3176–3187
6. Carson CT, Kinzler ER, Parr BA (2000) *Tbx12*, a novel T-box gene, is expressed during early stages of heart and retinal development. Mech Dev 96:137–140
7. Cartegni L, Chew SL, Krainer AR (2002) Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet 3:285–298
8. Digilio MC, Marino B, Giannotti A, Toscano A, Dallapiccola B (1997) Recurrence risk figures for isolated tetralogy of Fallot after screening for 22q11 microdeletion. J Med Genet 34:188–190
9. Ferencz C, Neill CA, Boughman JA, Rubin JD, Brenner JI, Perry LW (1989) Congenital cardiovascular malformations associated with chromosome abnormalities: an epidemiologic study. J Pediatr 114:79–86
10. Grech V, Gatt M (1999) Syndromes and malformations associated with congenital heart disease in a population-based study. Int J Cardiol 68:151–156
11. Greulich F, Rudat C, Kispert A (2011) Mechanisms of T-box gene function in the developing heart. Cardiovasc Res 91:212–222
12. Hammer S, Toenjes M, Lange M, Fischer JJ, Dunkel I, Mebus S et al (2008) Characterization of TBX20 in human hearts and its regulation by TFAP2. J Cell Biochem 104:1022–1033
13. Hammer S, Toenjes M, Lange M, Fischer JJ, Dunkel I, Mebus S et al (2008) Characterization of TBX20 in human hearts and its regulation by TFAP2. J Cell Biochem 104:1022–1033
14. Hariri F, Nemer M, Nemer G (2012) T-box factors: insights into the evolutionary emergence of the complex heart. Ann Med 44:680–693
15. Hirayama-Yamada K, Kamisago M, Akimoto K, Aotsuka H, Nakamura Y, Tomita H et al (2005) Phenotypes with GATA4 or NKX2.5 mutations in familial atrial septal defect. Am J Med Genet Part A 135:47–52
16. Hoffman JI (1990) Congenital heart disease: incidence and inheritance. Pediatr Clin North Am 37:25–43
17. Hoffman J (1995) Incidence of congenital heart disease: II. Prenatal incidence. Pediatr Cardiol 16:155–165
18. Hoffman JI, Kaplan S (2002) The incidence of congenital heart disease. J Am Coll Cardiol 39:1890–1900
19. Kenny D, Hijazi ZM (2012) To inhale or to nebulize: treating the pulmonary vascular bed post-operatively in children with congenital heart disease. Cardiol J 19:335–336
20. Kidd SA (1995) Congenital heart disease: a 10 year cohort. J Paediatr Child Health 31:362
21. Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV et al (2007) A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 315:525–528
22. Kirk EP, Sunde M, Costa MW, Rankin SA, Wolstein O, Castro ML et al (2007) Mutations in cardiac T-box factor gene *TBX20* are associated with diverse cardiac pathologies, including defects of septation and valvulogenesis and cardiomyopathy. Am J Hum Genet 81:280–291
23. Liu C-X, Shen A-D, Li X-F, Jiao W-W, Bai S, Yuan F et al (2009) Association of TBX5 gene polymorphism with ventricular septal defect in the Chinese Han population. Chin Med J 122:30
24. Nackley AG, Shabalina SA, Tchivileva IE, Satterfield K, Korchynskyi O, Makarov SS et al (2006) Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. Science 314:1930–1933
25. Naiche LA, Harrelson Z, Kelly RG, Papaioannou VE (2005) T-box genes in vertebrate development. Annu Rev Genet 39:219–239
26. Plageman TF, Yutzey KE (2004) Differential expression and function of Tbx5 and Tbx20 in cardiac development. J Biol Chem 279:19026–19034
27. Posch MG, Gramlich M, Sunde M, Schmitt KR, Lee SH, Richter S et al (2010) A gain-of-function TBX20 mutation causes congenital atrial septal defects, patent foramen ovale and cardiac valve defects. J Med Genet 47:230–235
28. Posch MG, Gramlich M, Sunde M, Schmitt KR, Lee SH, Richter S et al (2010) A gain-of-function TBX20 mutation causes congenital atrial septal defects, patent foramen ovale and cardiac valve defects. J Med Genet 47:230–235
29. Qian L, Mohapatra B, Akasaka T, Liu J, Ocorr K, Towbin JA et al (2008) Transcription factor neuromancer/TBX20 is required for cardiac function in Drosophila with implications for human heart disease. Proc Natl Acad Sci 105:19833–19838
30. Qiao Y, Wanyan H, Xing Q, Xie W, Pang S, Shan J et al (2012) Genetic analysis of the TBX20 gene promoter region in patients with ventricular septal defects. Gene 500:28–31
31. Ramser J, Ahearn ME, Lenski C, Yariz KO, Hellebrand H, von Rhein M et al (2008) Rare missense and synonymous variants in UBE1 are associated with X-linked infantile spinal muscular atrophy. Am J Hum Genet 82:188–193
32. Reller MD, Strickland MJ, Riehle-Colarusso T, Mahle WT, Correa A (2008) Prevalence of congenital heart defects in metropolitan Atlanta, 1998–2005. J Pediatr 153:807–813
33. Reller MD, Strickland MJ, Riehle-Colarusso T, Mahle WT, Correa A (2008) Prevalence of congenital heart defects in metropolitan Atlanta, 1998–2005. J Pediatr 153:807–813
34. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB et al (2012) Heart disease and stroke statistics—2012 update a report from the American heart association. Circulation 125:e2–e220
35. Ryan K, Chin AJ (2003) T-box genes and cardiac development. Birth Defects Res Part C, Embryo Today: Rev 69:25–37
36. van der Bom T, Zomer AC, Zwinderman AH, Meijboom FJ, Bouma BJ, Mulder BJ (2011) The changing epidemiology of congenital heart disease. Nat Rev Cardiol 8:50–60