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COMMENT

The role of *TBX18* in congenital heart defects in humans not confirmed

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INTRODUCTION

The gene *TBX18* (T-Box Transcription Factor 18 MIM*604613) is mapped to chromosome 6q14.3. *TBX18* is well known to be related to kidney and urinary tract abnormalities [1] and was recently also associated with skeletal abnormalities [2]. *TBX18* is part of the T-Box transcription factor family, and T-Box genes such as *TBX1* and *TBX5* are known to have a role in heart development [3]. However, not much is known about the role of *TBX18* in congenital heart defects (CHDs) in humans.

Tbx18 is a well-conserved gene in mice, zebrafish and chickens. In mice, *Tbx18* expression is associated with the recruitment and differentiation of cells at the cardiac venous pole [4]. Homozygous Tbx18-/- mice die at birth, and their hearts show defects of the systemic venous return, delayed myocardial differentiation and severely reduced growth of the head of the sinoatrial node [5, 6]. Heterozygous Tbx18+/− mice do not die at birth or show obvious morphological defects [7]. Mice with misexpression of Tbx18 induced by the *Cre/loxP* principle showed decreased Tbx18 expression in atrial and ventricular myocardial cells of the chamber myocardium and died at embryonic and postnatal stages [8]. Ventricular septal defects (VSDs) were seen in 6 out of the 16 mice on embryonic day 18.5. On postnatal day 6, the four mice still alive had an atrial septal defect (ASD) and one also had a VSD. The authors thus concluded that correct Tbx18 expression is important for normal chamber development [8].

In 2013, Ma et al. reported variants in the promoter region of *TBX18* in patients with a VSD [9]. In our previous work on individuals with proximal 6q deletions (6q11q15), we found 15 individuals with a deletion including *TBX18*, including 9 with a CHD [10]. The CHDs of the deletion patients included patent ductus arteriosus, ASD, common atrium, tetralogy of Fallot (TOF), anomalous pulmonary venous return, and right bundle branch block (Supplementary Table S1). Since these cardiac abnormalities show some overlap with the defects reported in mouse studies, *TBX18* was a gene of interest for further study.

To provide more evidence on the potential role of *TBX18*, we studied *TBX18* in a cohort of 253 CHD patients without a molecular diagnosis by screening for variants likely to disrupt the function of *TBX18*.

METHODS

Patient selection and Sanger sequencing

We included CHD patients referred to the University Medical Center Groningen (UMCG) for genetic counselling from 2006 to 2021 in whom no molecular diagnosis was made. All the patients in this clinical cohort consented to use of residual material and data.

First, we performed Sanger sequencing, including the promotor region, for 40 patients with CHD. We selected 31 patients with CHD comparable to the CHD seen in our *TBX18* deletion patients and added 9 patients with random CHD (see Supplementary Table S1 for details on specific CHDs) [10]. Supplementary Table S2 presents the CHD types of the selected patients (mainly ASD ($n = 13$) and TOF ($n = 14$)). As some individuals had additional features that were also seen in *TBX18* deletion patients [10], we included these individuals based on these additional features, which included scoliosis/kyphosis ($n = 4$), abnormal vertebrae ($n = 3$) and an abnormality of the outer ear ($n = 3$).

Sanger sequencing was performed on residual anonymised DNA. *TBX18* (NM_001080508.2) was bi-directionally sequenced in the UMCG using the BigDye™ Terminator Sequencing Kit and ABI DNA Sequencer (Applied Biosystems). Primers (Supplementary Table S3) were designed using Clone Manager Software (Sci Ed Software LLC) based on Hg19 (NC_000006.11). For the promotor region, we used the primers designed by Ma et al. [9].

Exome sequencing

After Sanger sequencing found no pathogenic variants, we expanded the study to search for variants in *TBX18* by reanalysing exome sequencing data for another 213 patients. The exome sequencing-based CHD gene panel was performed in the UMCG genomic diagnostic laboratory for all 213 patients (unselected cohort), as described previously [11]. The sequencing data were anonymised. A cohort analysis was performed for sequence variants in *TBX18* (NM_001080508.3) using Alissa Interpret software (Agilent Technologies). The promotor region of *TBX18* could not be analysed using exome sequencing data because it lies outside the regions captured by the exome kit. We used Alamut Visual v2.15 software (SophiaGenetics) for in silico analysis of variants using embedded splicing and missense prediction tools (Supplementary Methods).

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Table 1. Molecular variants found in *TBX18*.

	V1	V2	V3	V4		V5	V6	V7	V8	V9
Variant	−1075C>A	−978C>G	−972C>T	c.142G>A [p.Gly48Arg]		IVS4-20G>C	c.652G>T [p.Val218Leu]	c.946C>T [p.Arg316Cys]	c.1103A>G [p.Asn368Ser]	c.1470G>A [p.Ser490Ser]
Genomic position			g.85474871G>A	g.85473758C>T			g.85466535C>A	g.85454037G>A	g.85447124T>C	g.85446757C>T
Variant type				Missense			Missense	Missense	Missense	Synonymous
Exon	Promotor region	Promotor region	Promotor region	Exon 1		Exon 4	Exon 4	Exon 6	Exon 8	Exon 8
Zygosity	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Homozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Variant found in number of Sanger patients (%)	13/40 (32.5%)	5/40 (12.5%)	3/40 (7.5%)	11/40 (27.5%)	18/40 (45.0%)	3/40 (7.5%)	0/40	0/40	1/40 (2.5%)	1/40 (2.5%)
WES patients (%)	Not tested	Not tested	Not tested	105/213 49.3%	59/213 27.7%	0/213	1/213 0.47%	1/213 0.47%	0/213	0/213
Allele frequency GnomAD (%)	3360/31,386 10.07%	2172/31,404 6.92%	1304/31,404 4.15%	90,169/186,006 48.48%	23,550[a]	3313/280,862 1.18%	1/251,304[b] 0.0003979%	1/250,064[c] 0.0003999%	134/206,668 0.06484%	239/282,446 0.08462%
CADD score				18.93			26.6	32	14.18	5.931
Conservation				Not highly conserved (dog)			Highly conserved (zebrafish)	Highly conserved (zebrafish)	Not highly conserved (dog)	Highly conserved (frog)
Predicted pathogenicity				Tolerated			Deleterious	Deleterious	Tolerated	n/a
Predicted splice effect				No effect on splice site		Variant found in the intron-exon boundary before exon 4, no effect on splice site	No effect on splice site	No effect on splice site	No effect on splice site	No effect on splice site

Frequency above the threshold used in diagnostic settings (>0.05%).
CADD Combined Annotation Dependent Depletion [15].
[a]Number of homozygotes in gnomAD.
[b]$N = 1$ European non-Finnish 0.0008798%.
[c]$N = 1$ East Asian 0.005461%.

Variant interpretation and classification

Since an anonymous cohort analysis was performed, identified variants could not be linked to an individual. For all variants, we checked their allele frequency in the general population in gnomAD and their missense tolerance Z scores [12, 13]. Variants with an allele frequency <0.05% were considered potentially relevant. The GAVIN [14] variant prioritisation tool was used to interpret Combined Annotation Dependent Depletion (CADD) [15] scores, with scores >35.8 predicted to be more likely pathogenic and scores <20.11 more likely to be benign.

RESULTS

We identified nine molecular variants (Table 1). Variants V1–V3 were part of the promotor region. We classified variants V1–V5, V8 and V9 as benign because their allele frequency in the general population was >0.05% or their CADD score was <20.11 (if known). The missense variants V6 (c.652G>T) and V7 (c.946C>T) were classified as variants of unknown significance (VUS). Both have a low allele frequency in the general population (reported only once in gnomAD), are highly conserved and are tagged as deleterious by missense prediction tools. V6 has a CADD score of 26.6. V7 has a CADD score of 32. However, missense Z scores reported in gnomAD suggest that *TBX18* is tolerant of missense variations [12, 13].

In addition, 15 intronic variants were identified in the exome sequencing data, but all were predicted to have no effect on the splice site (data not shown).

DISCUSSION

We looked for evidence of the potential role of *TBX18* in CHD in a clinical cohort of 253 patients with unexplained CHD. We did not find any pathogenic *TBX18* variants. Neither did we find a pathogenic variant in the promotor region of *TBX18*, which we could study in 40 of the 253 patients. We did detect two missense VUS, V6 and V7, but we are not convinced that these are disease-causing as *TBX18* is suggested to be tolerant of missense variants.

In 2013, Ma et al. reported three VUS and one polymorphism in the promotor region of *TBX18* in a cohort of 326 VSD patients. The authors suggested that these four variants might play a role in VSD aetiology and hypothesised that, in particular, downregulation of *TBX18* expression would cause CHD. They therefore only sequenced the promoter region of *TBX18*. The four variants showed decreased transcriptional activities of the *TBX18* promotor in functional studies [9], suggesting that the variants could be disease-relevant. While we also found the same polymorphism, V3 (–972C>T) in our data, it has an allele frequency of 4.15% in the gnomAD control database, making the conclusions drawn from the earlier functional testing unclear. The other three Ma et al. variants are not reported in gnomAD, leaving the promotor region a potential region of interest for further studies.

Since *TBX18* is a translational regulator, one would expect other genes to be up- or downregulated in cases of diminished *TBX18* expression. Although the expression patterns are well studied, it remains unknown which molecular circuits act downstream of *TBX18* [3, 4]. Studies in mice did not show co-expression of *Tbx18* with genes essential for heart development like *Gata4* or *Nkx2-5* [4]. *Gata4* is expressed in the precardiac mesoderm and expands to the endocardium and myocardium. *Nkx2-5* is expressed in cardiac progenitors within the mesoderm and in myocardial cells. *Gata4* and *Nkx2-5* do form complex regulatory loops, but these interactions have not yet been found for *Tbx18* [16].

Limitations

While no pathogenic *TBX18* variants have been related to CHD in humans thus far, extremely rare variants might still exist. Thus, our sample size of 253 CHD patients might be too small to elucidate pathogenic variants in *TBX18*. We also could not study the promotor region in 213 patients because this region was not captured by the exome kit.

Concluding remark

Based on our data, we conclude that variants in the coding sequence of *TBX18* do not play a major role in CHD. Nonetheless, *TBX18* is not yet fully excluded as a gene of interest for CHD in humans. Comprehensive analysis of the TBX18 gene, including its promotor region, in a larger cohort of CHD patients is needed to confirm whether *TBX18* plays a role in CHD.

DATA AVAILABILITY

Data generated as part of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualisation: AE, WSK-F. Data curation: AE, KMA. Formal analysis: AE, KMA. Funding acquisition: AE, CMAR-A. Investigation: AE. Methodology: AE, WSK-F. Project administration: AE. Resources: AE, KMA, WSK-F. Supervision: WSK-F. Writing—original draft: AE. Writing—review and editing: AE, KMA, MMH, CMAR-A, WSK-F.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study design for Sanger sequencing was discussed with the accredited Medical Ethics Review Committee of the University Medical Centre Groningen. The committee waived full ethical evaluation. All the patients in this clinical cohort consented to use of residual material and data.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41431-022-01242-3.

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