Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)
ISSN: 2349-2759
Available online at www.jipbs.com

Research article

Prediction of the functional consequences of a novel homozygous TBX5 variant in isolated AVSD patient

Alaaeldin. G. Fayez[1*], Nora N. Esmaiel[1], Arwa A. El Darsh[2], Engy A. Ashaat[2], Alaa K. Kamel[3], Ghada M. Shehata[1], Azza M. Ahmed[4], Mona O. El Ruby[2]

[1]*Molecular Genetics and Enzymology Department, Human Genetics and Genome Research Division, National Research Centre, P.O. Box 12622, Dokki, Giza, Egypt.*
[2]*Clinical Genetics Department, Human Genetics and Genome Research Division, National Research Centre, P.O. Box 12622, Dokki, Giza, Egypt.*
[3]*Human Cytogenetics Department, Human Genetics and Genome Research Division, National Research Centre, P.O. Box 12622, Dokki, Giza, Egypt.*
[4]*Pediatrics Department, Medical Research Division, National Research Centre, P.O. Box 12622, Dokki, Giza, Egypt.*

Key words: Isolated cardiac septal defects; TBX5 gene; Novel homozygous variant.

***Corresponding Author: Alaaeldin Gamal Fayez,** Molecular Genetics and Enzymology Department, Human Genetics and Genome Research Division, National Research Centre, 33 El Bohouth Street (Former El Tahrir Street), P.O. Box 12622, Dokki, Giza.

Abstract

Objectives: The T-box transcription factor TBX5 gene is an important factor in mammalian cardiac development for both cardiac septation and morphogenesis. Autosomal dominant variants in the human TBX5 gene cause the known Holt-Oram syndrome (HOS); a disorder characterized by heart and upper limb deformities. Because there are rare studies for detection of TBX5 variants in isolated congenital cardiac septal defects, the current study aimed to identify the putative function of novel detected TBX5 gene variant in non-HOS patient with congenital cardiac septal defects (CSD). **Methods:** This is a case study of a one year old female congenital heart disease (CHD) patient with atrioventricular septal defect (AVSD). Using Direct Sanger sequencing, screening of T-box DNA binding domain corresponding exons for the patient who had normal Nkx2-5 and GATA4 genotyping was performed, as well as, a group of twenty eight non-cardiac children were screened as controls. **Results:** A novel homozygous missense (p.L135R) variant in the N- terminal of the T- box domain was detected with a predictive disease causing effect. This variant was absent in the variants registration databases and in 28 healthy controls, indicating its possible role in causing (CHD). **Conclusions:** To our knowledge, we show for the first time homozygous TBX5 variant in non-HOS associated cardiac malformations in an Egyptian patient. Absence of definite congenital heart defects symptoms in the unaffected carrier parents for the p.L135R variant supporting dosage-sensitivity hypothesis and a probable new mode of Tbx5 variants inheritance.

Introduction

Congenital heart defects (CHD) can occur as an isolated defect or as part of a syndrome. Holt–Oram Syndrome (HOS) is one of CHDs syndromes, characterized by limb deformities as well as heart malformations and genetically inherited in an autosomal dominant fashion [1]. HOS is clinically heterogeneous, as the clinical manifestations of the disease are considerably variable, even among affected family members who share identical TBX5 variant [2].

The strict clinical diagnostic criteria for HOS are representing the presence of preaxial radial ray malformation of at least one upper limb along with a personal or a family history of septation defects and/or atrioventricular conduction defects. Around 70% of the HOS patients show a variant in the TBX5 gene [3, 4]. Recently, TBX5 variants are also found to be associated with nonsyndromic diseases such as atrial fibrillation [5-8] or dilated cardiomyopathy [9].

Basson *et al.,* (1997) and Li *et al.*, (1997) identified that haplo-insufficiency causing variants of TBX5 cause HOS with structural cardiac defects, mainly atrial septal defect (ASD) and limb abnormalities [10, 11]. Variants in other T-box genes have been associated with malformations such as septal defects and dilated cardiomyopathy (TBX20), cleft palate (TBX22), and syndromes such as DiGeorge syndrome (TBX1) and Ulnar– Mammary syndrome (TBX3) [12].

Basson *et al.* (1999) proposed that the missense variants which disturb different regions of the T box might be associated with organ-specific defects depending on studies of several kindred with HOS. Nevertheless, the presence of two main regions inside the T-box domain; one binds to the minor targeted DNA groove and another binds to the major groove, assists the prediction of the role of the amino acid substitution present in the N-terminal end of the T box (i.e., Gly80Arg) which disturbs the TBX5 binding to the major groove of target DNA causing severe heart malformations but relatively mild limb defects. Alternatively, amino acid substitutions in the C-terminal end of the T box such as (Arg237Trp) were predicted to perturb TBX5 binding to the minor

groove of target DNA causing mild cardiac abnormalities but severe limb defects [13].

However, when a larger number of variants and individuals were analyzed, this relation has been questioned, only two out of 20 individuals showed a clinical HOS phenotype consistent with the Basson *et al.* (1999) hypothesis as reported by Brassington *et al.* (2003), Dreßen *et al.,* (2016) [14, 15].

In the current study, we aimed to further explore the function of the detected novel variant, located in the N terminal of the T-box DNA binding domain, in a case of isolated congenital heart defects without skeletal manifestations of HOS. Screening of all possible variants within the T-box DNA binding domain of the TBX5 gene, and coding regions of Nkx2-5 and GATA4 genes were performed. A novel TBX5 variant (p. L135R) located in the N terminal of T-box DNA binding domain with the disease causing prediction score was identified. Significance of this study due to that the first time homozygous TBX5 variant, with positive predictive function value, was found in non-HOS associated cardiac malformations in an Egyptian patient pointing a new mode of Tbx5 variant inheritance.

Experimental

Subjects and methods

Subjects

This study included one family with an affected girl proband diagnosed as isolated atrioventricular septal defect (AVSD) and her normal healthy parents. The study also included 28 healthy as controls.

Methods

Clinical evaluation

The following parameters were conducted for the proband

History:

- Three-generation pedigree construction including consanguinity, similar conditions in the family and parental consanguinity.
- Complete history including parents' occupation, pregnancy and delivery histories, exposure to drugs, fever, trauma, irradiation or any maternal chronic illness as Diabetes, PKU and history of low folate intake. Parental ages at birth of the child, family history and developmental milestones were also recorded.
- Detailed history of cardiac symptoms such as; cyanosis, dyspnea, palpitations, arrhythmias, syncope, etc.
- Detailed history of extra-cardiac symptoms such as; developmental delay, learning disabilities.
- Family history was obtained, including detailed history of cardiac disease for all affected family members to provide valuable clues about possible causation and inheritance.

Examination:

- Detailed clinical examination with special emphasis on cardiac examination, dysmorphic features and examination of different body systems to clinically rule out syndromic cases.
- Anthropometric measurements including height (Ht), weight (Wt) and head circumference (HC). Measurements were compared to Egyptian growth charts [16].
- Echocardiography: 2D, M- mode and Doppler echocardiography were performed for all patients. Using echocardiogram HP 5500 in CUCH echocardiographic Lab.

Molecular analysis

Genomic DNA was extracted using the QIAamp DNA Blood Mini kit according to the manufacturer's recommendation (QIAGEN, Hilden, Germany) [17].

Variant analysis

TBX5 genotype analysis of the family was carried out using direct Sanger sequencing, the whole coding sequences of exons 3, 4, 5 and 6 and exon-intron boundaries corresponding to T-box DNA binding domain were sequenced by specific previously reported primers [14]. Also, the coding regions of Nkx2-5 and GATA4 genes were sequenced using specific PCR primers as reported by Goldmuntz *et al.* (2001) and Okubo *et al.* (2004)[18, 19]. A full list of the used TBX5 primers is illustrated in table (1). Uniform PCR conditions comprised an ammonium buffer (50 mM Tris HCl pH 9.2, 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 0.5% Tween 20, 10% dimethylsulfoxide), 0.5U recombinant Taq DNA polymerase (2U/ul) (Thermo Fisher Scientific Inc., USA), 0.2 mmol/l each dNTP and 0.8 pmol of each primer for 1ug of genomic DNA under 53-65^{o}C annealing temperatures. PCR amplification was then purified by using The MEGAquick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology, Inc., Kyeonggi do, Korea) [20]. Sequencing was performed using the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer) on the ABI3730XL sequencer in Macrogen Inc., South Korea [21]. The newly detected variant has been submitted to the ClinVar database under Variation ID 375289 [22], dbSNP database under rs1057519050 [23] and 1000 Genomes Browser at NCBI, phase 3 [24].

In Silico Analysis

The sequencing chromatograms were checked by manual inspection of characteristic double peaks and analyzed by DNA Baser Sequence Assembler [25]. Prediction of the disease causing variants and putative effect of the variants were identified using the MutationTaster [26], SNPnexus [27] tools and Variant Effect Predictor

(VEP), Ensemble genome browser v83.38, assembly GRCh38.p5 (Genome Reference Consortium Human Build 38) [28]. Modeling and exploration of the wild and mutant type of TBX5 protein were done by Protein Homology/ analogy Recognition Engine V 2.0 (Phyre^2) tool [29] and Swiss-PdbViewer [30] to recognize and build the structure models for wild and mutant proteins. Effect of the variant on mRNA folding was performed by GeneQuest program (Lasergene 6.0) [31], and on premature RNA splicing was performed by MutationTaster, NNSPLICE 0.9 [32] and RegRNA 1.0 [33]. The stability of the mutant protein was assessed using iStable [34].

Table 1. PCR primer sequences of corresponding exons to T-box DNA binding domain of TBX5 gene

Exon	Forward (5'---3')	Reverse (5'---3')	Size
3	AGTTTGGGGAAG GAATGCCCACTA C	TTCTCCTCGTCC CTCTCTCTACAC A	200
4	AACGGGGCTAGT TTCCGCTTCCACG	CTTTTCAACTTT TTGGGAGAAGG TTCCACTTTTC	315
5	AGATACCTAAGG GAGACGGGA	AGAGAGGACAA GAGGGAGACAA GGC	348
6	GCGGGGAGCAGG GTTTTA	GTCGAAGTTGG TGACTGCTGC	348

Cytogenetics analysis: Chromosomal analysis was performed for the studied case using whole blood samples for conventional and high resolution techniques [35]. Karyotype description followed the international system of human cytogenetic nomenclature [36].

Results and Discussion

Results

Clinical results

Clinical evaluation: The proband is a female patient aged 1year when first referred to the CHD clinic of the NRC for genetic counseling. Her main complaint was failure to thrive, cyanosis at age 3months with recurrent chest infections that urged hospitalization. The parents are 2nd degree consangouse. The father was 36, the mother was 32 years old at birth of the proband. The mother was suffering from rheumatic heart disease. She had 6 siblings: 3 of them died of CHD (2males and 1 female) with no skeletal manifestations. Her elder sister has Rheumatic activity (Figure 1A). The patient had delayed milestones of development, slight dysmorphic features (hypertelorism, epicanthic folds), normal chest, abdominal and neurological systems. No upper limb defects were found, and no thumb hypoplasia or aplasia detected (Figure 1B). Anthropometric evaluation showed that the patient was short, with small head and low weight. When evaluated at age 3 years she was under weight (-2.9 SD), short for her age (-2.7SD) and tendency to microcephaly (-2.5SD). Abdominal ultrasound reported atrophic left kidney in the patient. X ray upper limbs revealed normal hands, forearm, and upper arm of both upper limbs. No thumb defects or hypoplasia could be detected. Both clavicles and shoulder bones were normal for age (Figure 1B).

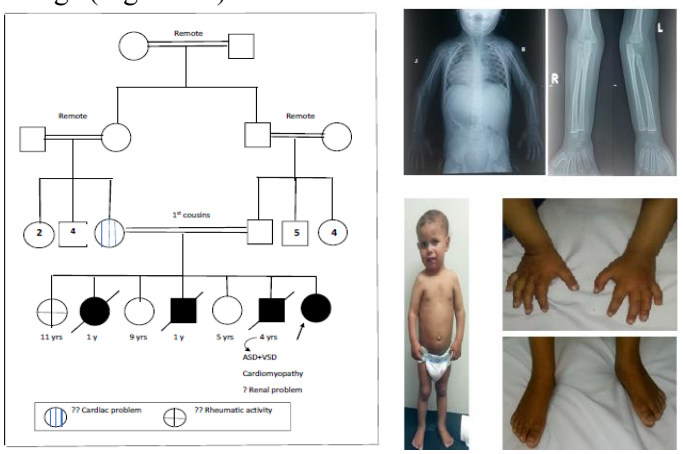


Figure 1. (A) represents 3 generation Pedigree of the affected proband, and (B) represents radiographical findings of the affected proband showing normal upper and lower limb with no bilateral bony defects of thumbs, hands, upper and lower parts of limbs. Intact clavicles with normal shoulders joints are also evident.

Cardiac examination and Echocardiographic finding: revealed pan-systolic murmur heard all over the pericardium, two small muscular VSDS with left to right shunt, large sized sinus venous type ASD with left to right shunt, small sized PDA with left to right shunt (PSG=41mmHg) and dilated right side of the heart (RA - RV) with moderate tricuspid regurgitation.

Chromosomal analysis: 46,XX. The parents were normal.

Variant analysis

Sequencing results: Using Direct Sanger sequencing of whole exonic and exon-intron boundary regions of T-box DNA binding domain of TBX5 gene, we identified an identical novel homozygous missense variant, p.Leu135Arg, in exon 5 (ENSE00003508688) located in the N-terminal of T-box DNA-binding domain and led to substituting leucine (L) by arganine (R) amino acids. p. Leu135Arg variant was found in a heterozygote state in the parents (Figure 2). This variant was not found in publicly available SNP databases of the National Center for Biotechnology Information (NCBI), Ensembl, Human Gene Variant Database (public HGMD), the 1000 Genomes Project (TGB), ClinVar, the Exome Aggregation Consortium (ExAC) browser, NHLBI Exome Sequencing Project (ESP) server databases and in 28 healthy controls included in the current study.

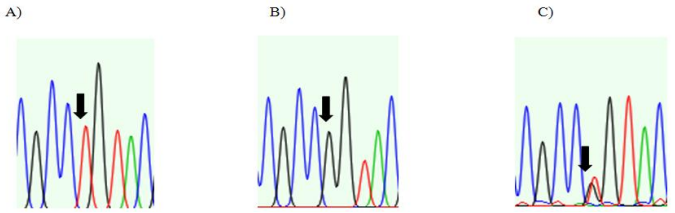


Figure 2. Partial sequence chromatogram displaying the TBX5 gene for a normal case [TT] (A), our patient presented homozygous Leu135Arg [GG] (B) and her father presented heterozygous Leu135Arg [TG].

In silico analysis: Detected p.L135R variant located within the DNA-binding domain of TBX5 and it is predicted to result in substitution of an amino acid residue of TBX5 that is highly conserved among species (Figure 3).

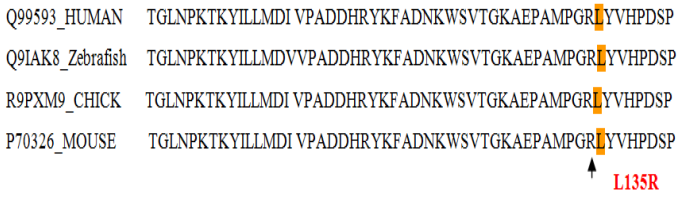


Figure 3. Deduced amino acid sequence of partial TBX5 protein in the human, zebrafish, chicken and mouse with uniprot IDs. The conserved L135 was shaded by brown. Missense variant was indicated in red bold writing type.

Effect on the TBX5 protein properties; using MutationTaster (MT), SNPnexus and Ensemble variant effect predictor, disclosed that prediction scores of p. Leu135Arg in SIFT (Sorting Intolerant from Tolerant substitutions based on sequence homology and the physical properties of amino acids), PolyPhen-2 (Polymorphism Phenotyping using straightforward physical and comparative considerations), fathmm-MKL (predicting the functional effects of protein missense variants by combining sequence conservation within hidden Markov models (HMMs)) and CONsensus DELeteriousness score of SNVs or Condel (computing a weighted average of the scores of five of these tools SIFT, PolyPhen2, VariantAssessor, LogRE and MAPP) algorithms showed high damaging effect of p.Leu135Arg on TBX5 protein (Table 2).

Table 2. predictive scores of p.L135R

HGVS Names/ Codons	Ensemble Transcript ID	Polyphen	SIFT	MT	FATHMM	Condel
p.Leu135Arg CTG/CGG	ENST00000310346	0.979	0	102 D	D	0.990

Predictive pathogenecity of p.Leu135Arg due to definite difference in the chemical and physical properties between leucine (L), non-polar hydrophobic amino acid, and arganine (R), polar positive charge hydrophilic amino acid. Therefore the charged mutant R135 has electrostatic constraints and non-bonded energies more significantly than uncharged wild L135. Electrostatic potential map, protein molecular surface properties and the different types of calculated energies were showed in (Figure 4 and Table 3).

Table 3. Computed energy force scores of wild L135 and mutant R135.

AA	Energy force scores (KJ/mol)					
	Bonds	Angles	Torsion	Non-bonded	Electrostatic constraints	Total Energy
wL135	0.381	3.966	1.076	-24.42	7.20	-9.800
mR135	1.608	5.395	3.000	-7.090	946.14	950.97

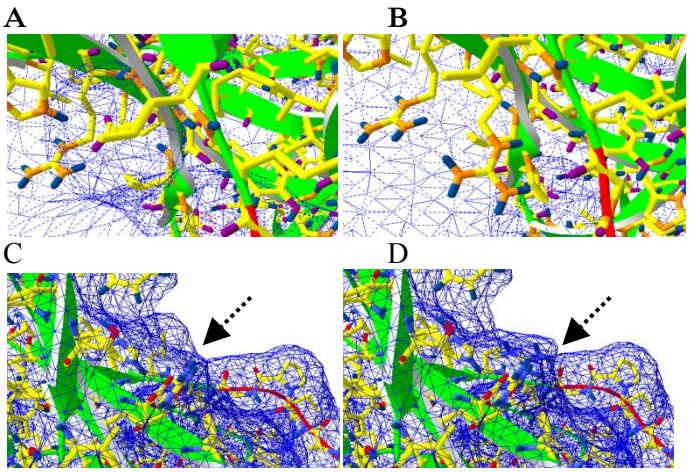


Figure 4. (A/B) Molecular model of human free TBX5 protein based on PDB file 2X6v showing change in electrostatic potential and in (C/D) TBX5 molecular surface shape based on electrostatic potential from (A) wild type to (B) mutant type.

To gain further insight into the effect of the p.L135R variant on the function of TBX5 protein, we compared the predicted structure of TBX5 mutant to wild-type TBX5 by creating a model of the DNA-binding domain of TBX5 based on PDB file 2X6U. The core of the T-box domain consists of a seven-stranded β barrel (from strand A to G), which is closed by a lid structure comprising a two-stranded β sheet (Figure 5).

Using Protein Homology/analogY Recognition Engine V 2.0 (Phyre [2]), we found that L135 located in β-Strand C' inside the hydrophobic core close to the DNA binding active site (Figure 6), so changing the non-polar hydrophilic L135 to the polar hydrophilic R135 may decrease the stability of the hydrophobic core and hence DNA binding active site conformation especially that guanidium group of mutant R135 forms extra two hydrogen bonds with wild R134 and N105 (Figure 7). Consistent with this, the iStable software package

predicted a decrease in protein stability as a consequence of this specific variant as shown in table (4). The p.L135R is predicted to disrupt the stability and structural conformation of the N-terminal of the T-box domain, thereby affecting binding of the DNA binding site to the minor groove of the DNA target.

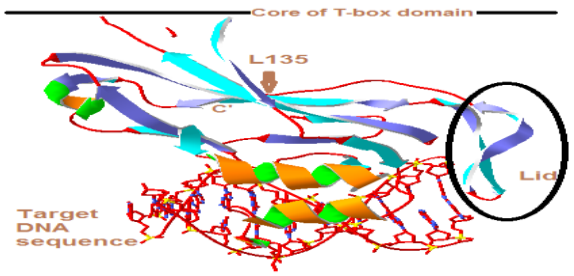


Figure 5. Ribbon diagram of T-box DNA binding domain of TBX5 protein showing the TBX5–DNA complex. Core of T-box domain interacted with red double helix DNA strand, based on PDB file 2X6U. The L135 located in C' β strand is labeled with brown capital letters. The lid is depicted as black circle. This figure was generated with Swiss Prot deep view program in the 3D display form

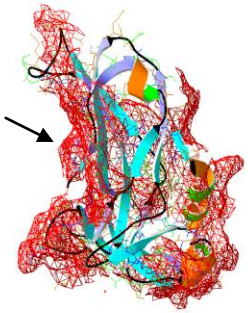


Figure 6. Hydrophobic patches map for T-box DNA-binding domain, based on PDB file 2X6v, shows incorporated blue arrowed L135 in the hydrophobic core. This figure was generated by Swiss Prot deep view program in the 3D display form.

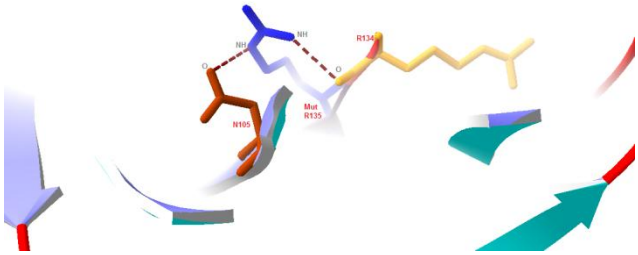


Figure 7. Slab view of human TBX5 protein free from nucleic acid based on PDB file 2X6v showing the hydrogen bonds between the mutant R135 with R134 and N105 residues. This figure generated with Swiss Prot deep view program in the 3D display form.

Effect on TBX5 splicing: Using MutationTaster and NNSPLICE 0.9 (The Berkeley Drosophila Genome Project (BDGP), we found that changing codon CTG to CGG in context wild CGCCTGT sequence inside the constitutive exon 5 led to create new donor splicing site (gccgccggtacgtgc) with score 0.99 higher than the nearest consensus donor with score 0.40 at mean. A new donor splicing site gain may cause alternative splicing producing abnormal transcript isoform with partial residual deletion from L135 till H170 (Table 5 and Figure 8A-C).

mRNA folding analysis; Using the program GeneQuest (Lasergene 6.0), we determined the effect of change of CTG to CGG codon on RNA folding. GeneQuest uses the Vienna RNA folding procedure, taken from Zuker's optimal RNA folding algorithm, to predict variant dependent mRNA folding when compared to the reference sequence. We found that mutant CGG codon at position 135 might leads to significant faulty RNA folding without effects on its stability (minimum free energy [MFE]) (Figure 9A, B).

Table 4. Meta results of mutant TBX5 protein stability using iStable package

		Reference			Meta result
Predictor	***i-Mutant 2.0 PDB***	***i-Mutant 2.0 SEQ***	***MUpro***	***CUPSAT***	***iStable****
Result	Decrease	null	Decrease	Decrease	Decrease
Conf.**			1		0.540953
ΔΔG*** (Kcal/mol)	-1.52	-1.78		-2.68	-0.831585

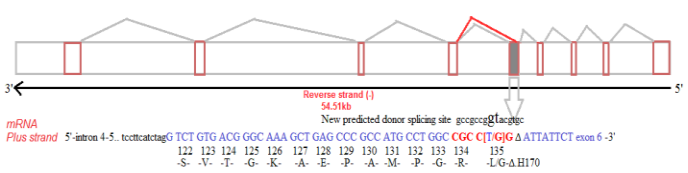
*** *iStable*; Integrated predictor for protein **stability** change upon single variant.

** **Conf.**; confidence score and ranges between 0-1.

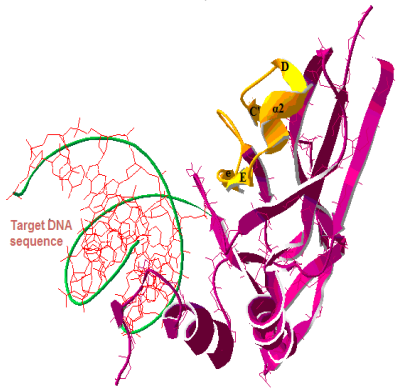
*** **ΔΔG**; free energy change value; ΔΔG<0 means decrease stability; ΔΔG>0 means increase stability.

Table 5. Effect of T/G substitution on splicing processing

Tools	Effect	Score	Wt. detection sequence	Exon-intron border
MT	Donor increased	wt: 0.35 mu: 0.99	wt:GCCGCCTGTACGTGC mu:GCCGCCGGTACGTGC	CGCC|tgta
NNSPLICE 0.9	Donor create	mu: 0.99	Mu:gccgccggtacgtgc	



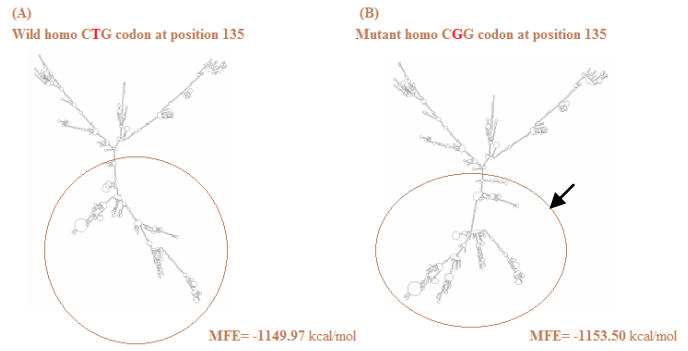
A)



B)

C)

Figure 8. (A) structure features of normal and alternative splicing resulting of p.L135R . L135R is predicted to create a new donor splicing site led to truncated protein from L135 to H170. (B and C) Molecular model of human TBX5 protein free from nucleic acid based on PDB file 2X6v shows the deleted strands and helix indicated with yellow color including C', D, E and e β-Strands, and α2 helix with bold black letters. This figure was generated by Swiss Prot deep view program in the 3D display form.



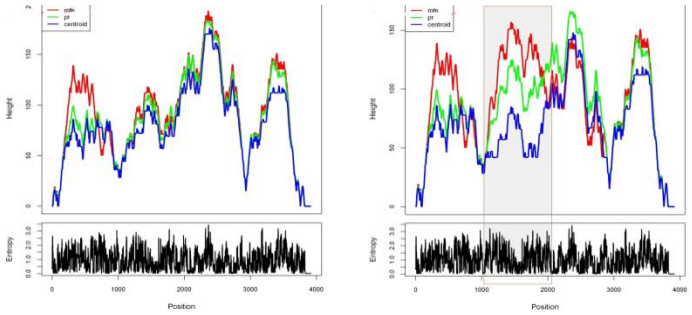


Figure 9. Prediction of TBX5 mRNA folding: Prediction of mRNA secondary structure, as determined by the Vienna RNA folding procedure, showing differences in folding, as arrowed, between wild (A) and mutant (B). The difference between minimum free energy (MFE) of wild and mutant mRNA was margin, but the mountain plot representation of the MFE, plotted by red color, and entropy for each position showed significant positional difference in MFE due to the detected variant, as squared by gray shadow color. Transcript variant 1, mRNA_ NCBI Reference Sequence: NM_000192.3 was used for the prediction.

Discussion

TBX5 is an important gene expressed in the central conduction system of the heart including the atrioventricular node, atrioventricular bundle and ventricular bundle branches. TBX5 is required for normal patterning and function of the proximal ventricular conduction system, in morphogenesis and maturation of atrioventricular canal components, and for patterning and functioning of the left and right bundle branch [38].

Approximately 85% of TBX5 gene variants are novel, and of the published 102 HOS-related variants, up to 44 were either missense or nonsense variants located mostly at the T-box domain of the coding protein [39]. In the present study, a novel homozygous missense variant in TBX5; the p.L135R variant located at the T-box domain, was identified in the patient who had congenital AVSD without skeletal manifestations of upper limbs characteristic of classic HOS. Nkx2-5 and GATA4 genotypes for the same patient were normal.

A three-dimensional structural model of the human TBX5 protein (PDB: 2X6U) has been made available [40]. This model allows more precise predictions of the consequences of the variant p.L135R. The structural model and the iStable software package predicted a decrease in protein stability and changed the structural conformation of the T-box domain core resulting in aberrant conformation of the DNA binding site or addition an extra donor splicing site leading to translation of truncated TBX5 protein.

The location of the L135R variant in the tertiary structure of the T-box DNA binding domain supports a decrease in DNA binding activity. Leucine as non-polar hydrophobic residue-to-arginine as positive charge hydrophilic residue

substitution at this position could generate extra favorable charge–charge interaction with the surrounded residues of T-box hydrophobic core and creates extra hydrogen bonds with R134 and N105. Hence, leucine-to-arginine substitution could lead to change the conformation of the DNA binding site and decrease its binding with the targeted downstream gene promoters.

TBX5 variant detection rate in HOS patients ranges from 54% to 74% [3, 41], the vast majority of TBX5 variants lead to loss-of-function, either by lack of protein or diminished DNA binding, or gain-of-function [42, 5, 6]. According to our *in silico* functional analysis of the p.L135R variant, this variant might lead to loss-of-function either by lack of TBX5 protein stability or change in DNA binding conformation.

Basson *et al.* (1999) pointed that the association between the location of missense variants and the HOS clinical phenotypes; where an amino acid substitution near the amino-terminal end of the T-box domain, which should affect interactions with the major groove of the target DNA promotor sequence , produces very significant cardiac malformations with mild or no limb deformations. In contrast, amino acid substitution at the carboxyl end of the T-box domain, which should affect interactions between TBX5 and the minor groove of the target promoter sequence, produces severe limb abnormalities with minor CHDs [13].

On the other hand, Brassington *et al.* (2003) reported that neither the type of variant nor the location of a variant in the T-box domain is predictive of the expressivity in individuals with HOS [14]. In agreement with Eker *et al.* (2016) and Dreßen *et al.* (2016) who detected novel p. The161Pro and p. Pro85The variants respectively; despite the presence of two variants; one located at the carboxyl end of the T-box domain and the other located at the amino end of the T-box domain, yet the two variants were found in cases with severe heart and upper limb defects [39, 15]. While Smemo *et al.* (2012) speculated that a variant in TBX5 enhancer elements can cause isolated CHD [43], Baban *et al.* (2014) reported that gain-of-function variant might lead to deleterious effects on heart development, but not on the upper limb development [6]. Al-Qattan and Abou Al-Shaar (2015) agreed with Basson *et al.* (1999) findings that location of a missense variant regarding T-box domain determines the type of phenotypes either CHD or HOS. In addition, they pointed out that the extended protein variants are more inclined to cause severe bilateral skeletal malformations and more severe cardiac anomalies, while the intragenic duplications inclined to cause more severe cardiac anomalies rather than severe skeletal anomalies. Our a novel missense substitution variant was detected in amino-terminal end of the T-box domain and was presented in isolated AVSD patient, a finding supporting the association between the amino-terminal missense variant of residue contributing in interactions with the major groove of the target promotor sequence and isolated CHD phenotype [44].

In our study, the parents of the proband detected to be carriers (heterozygous) for TBX5 variant without the distinct CHD phenotype. This finding could be explained according to the TBX5 dosage-sensitivity hypothesis which was postulated by Smemo *et al.* (2012) [43], and has been confirmed in multiple animal models [45-47], or to the effect of other modifier genes such as KLF13 gene which was postulated by Darwich *et al.* (2017) who reported that loss of a KLF13 allele in Tbx5 heterozygote mice significantly increases the penetrance of TBX5-dependent cardiac abnormalities including atrial, atrial-ventricular and ventricular septal defects [48].

Postma *et al.* (2008) and Baban *et al.* (2014) detected p.G125R and p.S372L respectively in two patients with CHDs phenotype and not resemble the classical HOS. This could be attributed to the concept that TBX5 target genes are highly sensitive for TBX5 dosage, and differences in dosage might lead to differences in phenotypes [5, 6]. Darwich et al. (2017) reported that KLF13 act as a genetic modifier gene of the TBX5, because it co-expressed with TBX5 in several cardiac cells including atrial cardiomyocytes and cells of the interatrial septum to synergistically activate transcription of cardiac genes [48]. Further studies for detection of other modifier genes are required.

Holt-Oram syndrome is a well-known autosomal dominant genetic disorder linked to TBX5 variants. Due to rare studies about TBX5 variants in isolated CHD, the mode of inheritance is still undefined and needs more investigations. In the current study, finding homozygous TBX5 novel variant in non-HOS isolated CHD phenotype proband could point to an autosomal recessive inheritance pattern. Reamon-Buettner and Borlak (2004) found nine heterozygous variants in diseased cardiac tissues of non-HOS patients with complex cardiac malformations, two out of nine variants were found in one patient with AVSD as compound heterozygous for p.Pro96Leu and p.Leu102Pro [49]. Smemo *et al.* (2012) scanned ~700 kb of the enhancer elements of TBX5 gene in a cohort of non-syndromic patients with isolated atrial and/or ventricular septal defects, and identified a patient with a homozygous variant in an enhancer ~90 kb downstream of TBX5 gene [43]. Baban *et al.* (2014) identified a novel heterozygous p.S372L variant in non-skeletal defects TOF patient [6].

Conclusion

In our study, as the patient's parents appear to be unaffected carriers of the p.L135R variant, we can suggest that a single affected allele of this variant is not sufficient to significantly cause skeletal deformations and/or congenital heart defects supporting dosage-

sensitivity hypothesis and a probable new mode of Tbx5 variants inheritance. We conclude that these data warrant the inclusion of the TBX5 L135R variant in future case-control studies for isolated CHD, as this variant could confer susceptibility to the disease and hence more effective genetic counseling and treatment for non-HOS CHD patients. More studies are to be conducted and recommended to support our findings.

Acknowledgements

The authors are very grateful to patient and her family for their participation and cooperation during this study. Regarding the funding; the reagents and consumables were funded by National Research Centre under project ID; 10010610. Results interpretation and writing were funded by authors.

References

1. Holt M, and Oram S: Familial heart disease with skeletal malformations. Br Heart J. 1960; 22: 236-242.
2. Cross J, Ching H, Li L, Armstrong-Buisseret L, Spranger S, Munnich A, Bonnet D, Pentinnen M, Jonveaux P, Mortier G, Van Ravenswaaij C and Gardiner CA: The mutation spectrum in Holt-Oram syndrome. J Med Genet. 2000; 37:785-787.
3. McDermott D, Bressan M, He J, Lee JS, Aftimos S, Brueckner M, Gilbert F, Graham GE, Hannibal MC, Innis JW, Pierpont ME, Raas-Rothschild A, Shanske AL, Smith WE, Spencer RH, St John-Sutton MG, van Maldergem L, Waggoner DJ, Weber M and Basson CT: TBX5 genetic testing validates strict clinical criteria for Holt-Oram syndrome. Pediatr Res. 2005; 58:981– 986.
4. Lichiardopol C, Militaru C, Popescu B, Hila G and Mixich F: Holt-Oram syndrome. Rom. J. Morphol. Embryol. 2007; 48:67–70.
5. Postma A, van de Meerakker J, Mathijssen I, Barnett P, Christoffels V, Ilgun A, Lam J, Wilde A, Deprez R and Moorman A: A Gain-of-Function TBX5 Mutation Is Associated With Atypical Holt–Oram Syndrome and Paroxysmal Atrial Fibrillation. Circ Res. 2008; 102:1433-1442.
6. Baban A, Pitto L, Pulignani S, Cresci M, Mariani L, Gambacciani C, Digilio MC, Pongiglione G and Albanese S: Holt-Oram syndrome with intermediate atrioventricular canal defect, and aortic coarctation: functional characterization of a de novo TBX5 mutation. Am J Med Genet A. 2014;164A:1419–1424
7. Guo D, Li R, Yuan F, Shi H, Hou X, Qu X, Xu Y, Zhang M, Liu X, Jiang J, Yang Y and Qiu X: TBX5 loss-of-function mutation contributes to atrial fibrillation and atypical Holt-Oram syndrome. Molecular Medicine Reports. 2016; 13:4349-4356. doi.org/10.3892/mmr.2016.5043
8. Ma J, Yang F, Mahida S, Zhao L, Chen X, Zhang M, Sun Z, Yao Y, Zhang Y, Zheng G, Dong J, Feng MJ, Zhang R, Sun J, Li S, Wang QS, Cao H, Benjamin EJ, Ellinor PT, Li YG and Tian XL: TBX5 mutations contribute to earlyonset atrial fibrillation in Chinese and Caucasians. Cardiovasc. Res. 2016; 109:442–450.
9. Zhang X., Qiu X., Yuan, F., Wang J., Zhao C., Li R., et al. TBX5 loss-of-function mutation contributes to familial dilated cardiomyopathy. Biochem Biophys Res Commun. 2015; 459:166–171.
10. Basson C, Bachinsky D, Lin R, Levi T, Elkins J, Soults J, Grayzel D, Kroumpouzou E, Traill TA, Leblanc-Straceski J, Renault B, Kucherlapati R, Seidman JG and Seidman CE: Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram syndrome. Nat Genet. 1997; 15:30-35.
11. Li Q, Newbury-Ecob R, Terrett J, Wilson D, Curtis A, Yi C, Gebuhr T, Bullen PJ, Robson SC, Strachan T, Bonnet D, Lyonnet S, Young ID, Raeburn JA, Buckler AJ, Law DJ and Brook JD: Holt-Oram syndrome is caused by mutations in TBX5 a member of the Brachyury T gene family. Nat Genet. 1997; 15: 21-29.
12. Greulich F, Rudat C and Kispert A: Mechanisms of T-box gene function in the developing heart. Cardiovasc Res. 2011; 91:212–22.
13. Basson C, Huang T, Lin R, Bachinsky D, Weremowicz S, Vaglio A, Bruzzone R, Quadrelli R, Lerone M, Romeo G, Silengo M, Pereira A, Krieger J, Mesquita SF, Kamisago M, Morton CC, Pierpont ME, Müller CW, Seidman JG and Seidman CE: Different TBX5 interactions in heart and limb defined by Holt-Oram syndrome mutations. Proc Natl Acad Sci USA. 1999; 96:2919–2924.
14. Brassington A, Sung S, Toydemir R, Le T, Roeder A, Rutherford A, Whitby FG, Jorde LB and Bamshad MJ: Expressivity of Holt-Oram syndrome is not predicted by TBX5 genotype. Am J Hum Genet. 2003; 73:74-85.
15. Dreßen M, Lahm H, Lahm A, Wolf K, Doppler S, Deutsch MA, Cleuziou J, Pabst von Ohain J, Schön P, Ewert P, Malcic I, Lange R and Krane M: A novel de novo TBX5 mutation in a patient with Holt–Oram syndrome leading to a dramatically reduced biological function. Mol Genet Genomic Med. 2016; 5:557–567. doi: 10.1002/mgg3.234
16. Ghalli I, Salah N, Hussien F, Erfan M, El-Ruby M and Mazen I: Egyptian growth curves for infants, children and adolescents. In: Satorio A., Buckler J.M.H., Marazzi N., editors. Crecere nel mondo. Ferring Publisher; Italy: 2008.
17. https://www.qiagen.com/eg
18. Goldmuntz E, Geiger E and Benson D: NKX2.5 mutations in patients with tetralogy of Fallot. Circulation 2001; 104:2565-2568.
19. Okubo A, Miyoshi O, Baba K, Takagi M, Tsukamoto K, Kinoshita A, Yoshiura K, Kishino T, Ohta T, Niikawa N and Matsumoto N: A novel GATA4 mutation completely segregated with atrial septal defect in a large Japanese family. J Med Genet. 2004; 41: 97.
20. http://eng.intronbio.com. Accessed 23 Sept 2015.
21. http://dna.macrogen.com. Accessed 11 Nov 2015.
22. https://www.ncbi.nlm.nih.gov/clinvar/variation/375289. Accessed 1 Jun 2016.
23. https://www.ncbi.nlm.nih.gov/snp/1057519050. Accessed 1 Jun 2016.
24. https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/?chr=12&from=114836484&to=114836484&gts=rs1057519050&mk=114836484:114836484|rs1057519050. Accessed 1 Jun 2016.
25. Heracle BioSoft (2013): DNA Baser Sequence Assembler v4.7.0., http://www.DnaBaser.com. Accessed 1 Dec 2015.
26. Schwarz J., Cooper D., Schuelke M., Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods 2014; 11: 361-362.
27. http://snp-nexus.org Accessed 3 Dec 2015.
28. Flicek M, Amode R, Barrell D, Beal K, Billis K, Brent S, Carvalho-Silva D, Clapham P, Coates G, Fitzgerald S, Gil L, García Girón C, Gordon L, Hourlier T, Hunt S, Johnson N, Juettemann T, Kähäri A, Keenan S, Kulesha E, Martin F, Maurel T, McLaren W, Murphy D, Nag R, Overduin B, Pignatelli M, Pritchard B, Pritchard E, Riat H, Ruffier M, Sheppard D, Taylor K, Thormann A, Trevanion S, Vullo A, Wilder S, Wilson M, Zadissa A, Aken B, Birney E, Cunningham F, Harrow J, Herrero J, Hubbard T, Kinsella R, Muffato M, Parker A, Spudich G, Yates A, Zerbino D and Searle S: Ensembl 2014. Nucleic Acids Res 2014; 42: 749-755.
29. Kelley L, Mezulis S, Yates C, Wass M and Sternberg M: The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 2015; 10:845-858.
30. http://www.expasy.org/spdbv. Accessed 12 Dec 2015.
31. Zuker's RNA folding algorithm. http://www.bioinfo.rpi.edu/~zukerm/seqanal. Accessed 12 Dec 2015.
32. http://www.fruitfly.org/seq_tools/splice.html. Accessed 12 Dec 2015.
33. Huang H, Chien C, Jen K and Huang H: RegRNA: an integrated web server for identifying regulatory RNA motifs and elements. Nucleic Acids Research. 2006; 34:W429–W434. doi: 10.1093/nar/gkl333.
34. http://predictor.nchu.edu.tw/ istable. Accessed 5 Jun 2017.
35. Verma R and Babu A: Human chromosomes: manual of basic Tissue culture techniques and chromosome preparation. New York: Pergamon Press. 1989; 44-52.
36. ISCN 2009: an international System for human cytogenetic nomenclature (2009). In: Shaffer LG, Slovak ML, Campbell LJ, Eds. Basel: S. Karger, 2009.
37. https://www.ncbi.nlm.nih.gov/nuccore/31652233. Accessed 24 Nov 2015.
38. Moskowitz I, Pizard A, Patel V, Bruneau B, Kim J, Kupershmidt S, Roden D, Berul C, Seidman C and Seidman J: The T-Box transcription factor Tbx5 is required for the patterning and maturation of the murine cardiac conduction system. Development 2004; 131:4107-4116.
39. Eker H, Altunoglu U, Toksoy G and Kayserili H: Holt-Oram syndrome because of the novel TBX5 mutation c.481A>C. Clin Dysmorphol. 2016; 4:192-194. doi: 10.1097/MCD.0000000000000121.
40. Stirnimann C, Ptchelkine D, Grimm C and Muller C: Structural basis of TBX5-DNA recognition: theT-boxdomaininitsDNA-boundand-unbound form. J Mol Biol. 2010; 400:71–81.
41. Debeer P, Race V, Gewillig M, Devriendt K and Frijns J: Novel TBX5 mutations in patients with Holt-Oram syndrome. Clin Orthop Relat Res. 2007; 4:20–26.

42. Heinritz W, Shou L, Moschik A and Froster U: The human TBX5 gene mutation database. Hum Mutat 2005; 26:397.
43. Smemo S, Campos L, Moskowitz I, Krieger J, Pereira A and Nobrega M: Regulatory variation in a TBX5 enhancer leads to isolated congenital heart disease. Human Molecular Genetics 2012; 21:3255–3263.
44. Al-Qattan M and Abou Al-Shaar H: A novel missense mutation in the TBX5 gene in a Saudi infant with Holt-Oram syndrome. Saudi Med. J. 2015; 36:980-982.
45. Takeuchi J, Koshiba-Takeuchi K, Suzuki T, Kamimura M, Ogura K and Ogura T: Tbx5 and Tbx4 trigger limb initiation through activation of the Wnt/Fgf signaling cascade. Development 2003; 130:2729–2739.
46. Mori A, Zhu Y, Vahora I, Nieman B, Koshiba-Takeuchi K, Davidson L, Pizard A, Seidman JG, Seidman CE, Chen XJ, Henkelman RM and Bruneau BG: Tbx5-dependent rheostatic control of cardiac gene expression and morphogenesis. Dev. Biol., 2006; 297:566–586.
47. Albalat R, Baquero M and Minguillon C: Identification and characterisation of the developmental expression pattern of tbx5b, a novel tbx5 gene in zebrafish. Gene. Expr. Patterns. 2010; 10:24–30.
48. Darwich R, Li W, Yamak A, Komati H, Andelfinger G, Sun K and Nemer M: KLF13 is a genetic modifier of the Holt-Oram syndrome gene TBX5. Hum Mol Genet. 2017; 26:942-954. doi: 10.1093/hmg/ddx009.
49. Reamon-Buettner S and Borlak J: TBX5 Mutations in Non-Holt-Oram Syndrome (HOS) Malformed Hearts. Hum Mutat. 2004; 24:104.