Exome Sequencing Identifies a c.148-1G>C Mutation of TBX5 in a Holt-Oram Family with Unusual Genotype–Phenotype Correlations

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Key Words
Holt-Oram syndrome • Mutations • TBX5 • Exome sequencing

Abstract
Background/Aims: Congenital heart defects (CHD) can occur with upper limbs deformities. Holt-Oram syndrome is the main type of heart-hand syndromes, characterized by upper limb radial ray malformations, CHD and/or conduction abnormalities. Mutations of the TBX5 gene, most of which are found within the T-box domain, are one cause of the disease. We aimed to find the cause of the disease in a family with two children exhibiting symptoms of Holt-Oram syndrome while the parents tend to be normal.

Methods: Chromosomal microarray analysis and exome sequencing were applied in the proband segments bearing the specific mutation and single nucleotide variants (SNVs) suspected of being involved in the disease were analyzed by polymerase chain reaction and direct sequencing.

Results: A splice acceptor site mutation c.148-1G>C of TBX5 was detected in both the father and the proband. The mutation may result in an aberrant transcript which will most probably undergo nonsense-mediated decay (NMD) system resulting in haploinsufficiency of TBX5 protein. In the meantime, 3 candidated SNVs were detected.

Conclusions: c.148-1G>C of TBX5 should be the pathogenic cause of the disease in this family. Works have been done to find a possible explanation of the unusual genotype–phenotype correlations in this family and further studies are still needed.

Introduction

Congenital heart defects (CHD) is one of the most common birth defects and the main cause of death in infancy, affecting 1 in 100 live births [1]. On some occasion, upper limbs deformities can occur with CHD and a broad category of disease named heart-hand syndromes...
was identified. Heart-hand syndromes include Holt-Oram syndrome (HOS) (OMIM 142900), Tabatznik's syndrome and heart-hand syndrome type III [2]. Meanwhile, patients with other diseases such as Ellis-van Creveld syndrome (OMIM 225500) and McKusick-Kaufman syndrome (OMIM 236700) can also exhibit both limbs and cardiac abnormalities.

Most of these heart-hand defects are thought to have a genetic basis, one of which is the mutation of the \( \text{TBX5} \) gene [3, 4]. \( \text{TBX5} \) is a member of T-box family transcription factors with a highly conserved DNA binding domain named T-box [5]. A direct role of \( \text{TBX5} \) in animal models indicates that the gene is required for cardiogenesis and limb development [6]. The \( \text{TBX5} \) heterozygous knock-out mutant mouse represents a phenocopy of HOS [7].

Mutations in \( \text{TBX5} \) gene have been classically associated with HOS, the principle type of heart-hand syndromes. HOS is a rare autosomal dominant syndrome characterized by upper limb radial ray malformations, and/or conduction abnormalities [8-10]. Upper limb malformations can range from subtle carpal bone abnormalities to severe reduction defect or phocomelia. Cardiac compositions are similarly varied, patients can present with atrial septal defect (ASD), or ventricular septal defect (VSD), or multiple and complex structural heart abnormalities, or conduction defects [11, 12]. More than 90 \( \text{TBX5} \) mutations have been identified and high inter- and intra-familial variability of phenotypic expressivity has been revealed by genotype-phenotype analysis [13, 14].

Here, we report a family with an acceptor splice site mutation of \( \text{TBX5} \) (TBX5:NM_000192:exon3:c.148-1G>C). Between the two family members who share this mutation, the proband displayed typical symptoms of HOS while his father tends to be normal. Genotype-phenotype relations were discussed in this unusual case, as well as the possible reason of these atypical findings.

**Materials and Methods**

**Patients and samples**

We studied three individuals from a family with two children exhibiting symptoms of HOS (Fig. 1a). Echocardiography, electrocardiography, radiographs, complete blood count, hearing test and physical examinations were given to the proband (patient II/4). Echocardiography, electrocardiography, radiographs and physical examinations were carried out for the parents.

**Chromosomal microarray analysis (CMA)**

Genomic DNA was isolated from peripheral leukocytes of the family members and the control group using the QIAamp DNA Blood Midi Kit (Qiagen, Duesseldorf, Germany) following the manufacturer’s instructions.

DNA of the proband was amplified, labeled and hybridized to the CytoScan HD array platform (Affymetrix, USA) following the manufacturer's protocol. The array offered 2,696,550 probes including 743,304 single nucleotide polymorphisms (SNPs) and 1,953,246 nonpolymorphic probes. CEL files obtained by scanning the CytoScan arrays were analyzed using the Chromosome Analysis Suite software (Affymetrix, USA) and the annotations of the genome version GRCh37 (hg19). Gains and losses that affected a minimum of 50 markers in a 100 kb length were initially considered.

**Exome sequencing and analysis**

Targeted enrichment was performed using Agilent Technologies (Santa Clara, CA). Capture libraries of the proband were constructed using SureSelect® Human All Exon V5. An average requirement of a 100-fold enrichment was achieved for all of the libraries prepared. The purified amplicons were sequenced bi-directionally (paired-end 125 base pair) on an Illumina HiSeq 2500 Next-Generation Sequencing system using v3.0 SBS chemistry. Paired-end sequences were first aligned to the NCBI human reference genome (hg19), and the reads were mapped by Burrows-Wheeler Alignment (BWA) version 0.5.9 [15]. To identify potential mutations, we performed local realignments using the Genome Analysis Toolkit (GATK) [16].
Mutation Detection
Candidate variants through exome sequencing were confirmed using Sanger sequencing. Primers were designed for polymerase chain reaction (PCR) amplification. The sequence of the primers and corresponding annealing temperatures can be seen in Table 1. DNA sequencing (of both strands) was performed by an ABI 3730 genetic analyzer (Applied Biosystems, USA).

Function predictions
Human Splicing Finder (HSF) Version 2.4.1 was applied to predict whether splicing errors would be caused by the intronic mutation [17]. Then HSF and GENSCAN were both used to see changes of the transcript caused by the splicing mutation [18]. SIFT was used for in silico predictions [19].
Results

Clinical findings

Individual I/1: A 31-year-old man, with a height of 157 cm and a weight of 65 kg. Physical examination and radiography of the limbs revealed no sign of abnormalities (Fig. 1e/f/g). Cardiac physical examination showed a regular heart rate of 63 bpm, normal heart sounds and no murmurs with the confirmation of echocardiography. A normal sinus rhythm with normal PR interval and no dysrhythmias was displayed by ECG. ST segment elevation was found in V2-V3 with little clinicopathological significance (Fig. 1h).

Patient II/1: The first child of the family with birth weight of 3650 g (G1P1, 37 weeks) died on the second day of his birth. According to the medical report left, the boy presented with thumb hypoplasia and syndactyly of the index finger and middle finger. But there was no image of echocardiography for the patient.

Patient II/2 (proband): A 6-month-old boy with birth weight of 3595 g (G2P2, 40 weeks), who was born by vaginal delivery after an uneventful pregnancy with a normal neuropsychomotor development. He presented with a finger-like thumb on left hand and the result of radiography confirmed this (Fig. 1b/c). Auscultation displayed a regular heart rate of 160 bpm, a loud second heart sound and a systolic heart murmur III. Echocardiography revealed a ventricular septal defect and isolated dextrocardia (Fig. 1d). ECG showed normal sinus rhythm with a normal PR interval and no dysrhythmias. Neither chondrodysplastic changes in the lower limbs and clavicles, nor any extracardiac malformations (ears, nails, hair, lips, teeth and gums) were found. The kidneys and genital system were normal. Hematological and biochemical abnormalities were excluded. At the time of writing, the patient was lucky enough to have spontaneous closure of VSD without operation.

CMA and exome analysis

CytoScan HD array revealed no significant deletions or duplications. The whole-exome sequencing experiment produced 207,882,012 reads. The number of unique mapped reads after excluding possible repeated PCR was 171,054,838 (87.19%, out of total reads).

Fig. 2. (a/b) Show c.148-1G>C mutation and wild type of TBX5. (c/d) Show c.4102T>C and wild type of PCSK5; (e/f) Show c.613C>T and wild type of CAPN2; (g/h) Show c.1886C>G and wild type of FRAS1.
When measured at 10x and 100x coverage, 99.65% and 81.58% of the intended target was covered, respectively. After filtering the data by a minimum genotype quality of 20, a frequency lower than 5% and a SIFT score of less than 0.05, 626 variants (26 splicing SNVs, 600 nonsynonymous SNVs, 4 stopgain SNVs and 4 stoploss SNVs) were found in 534 genes. The splicing mutation of \( \text{TBX5} \) (c.148-1G>C) was identified. Exon 3 of \( \text{TBX5} \) was amplified, and results of direct DNA sequencing confirmed that the mutation was found in both the father and the proband but not in the mother (Fig. 2). One hundred normal DNA samples were sequenced to testify it not a polymorphism.

To own an informative analysis of the filtered exome sequencing data, a list of genes mostly seen in heart-hand defects (\( SALL1, SALL4, \text{TBX4}, \text{GLI3}, \text{ROR2}, \text{EVC}, \text{EVC2}, \text{TFAP2B}, \text{HRAS}, \text{HAND2}, \text{HOXD13}, \text{PAPA2}, \text{PAPA3}, \text{SPRY4}, \text{WNT7A} \) and \( \text{ZRS} \) of \( \text{SHH} \)) was checked and no pathogenic mutation was found among the other 625 variants. In the meantime, none of the 625 variants were involved in CHD and limb deformities in genome-wide association study (GWAS). Then DAVID Bioinformatics Resources (version 6.7) was used to annotate the list with 534 genes included [20, 21]. Genes involved in heart and limb development (GO:0035136: forelimb morphogenesis; GO:0007507: heart development; GO:0003007: heart morphogenesis; GO:0048729: tissue morphogenesis; GO:0051146: striated muscle cell differentiation; etc) were selected and segments bearing the specific SNVs were sequenced. Among them, 3 candidates (\( \text{PCSK5} \) c.4102T>C, \( \text{CAPN2} \) c.613C>T, \( \text{FRAS1} \) c.1886G>G) were confirmed. Results of direct DNA sequencing were listed in Table 2.

**Importance of the mutation**

Variation (%) of HSF Matrices is WT site broken (-30.48) indicating splicing errors due to mutation c.148-1G>C. GENSCAN output suggests that it results in an aberrant transcript.

<table>
<thead>
<tr>
<th>Gene</th>
<th>dbSNP137</th>
<th>Amino Acid Change</th>
<th>SIFT</th>
<th>P</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPN2</td>
<td>rs149096348</td>
<td>NM_001146068:exon7:c.C613T:p.R205C</td>
<td>0</td>
<td>C/T</td>
<td>C/T</td>
<td>C/C</td>
</tr>
<tr>
<td>FRAS1</td>
<td>rs187365033</td>
<td>NM_001166133:exon17:c.C1886G:p.F629R</td>
<td>0.01</td>
<td>C/G</td>
<td>C/C</td>
<td>C/G</td>
</tr>
</tbody>
</table>

**Table 2. Direct sequencing results of suspected SNVs in the family.** P: Proband; M: Mother; F: Father

**Fig. 3.** Schematic presentation of normal transcript and aberrant transcripts predicted by HSF and GENSCAN. The genomic locations of exons 2-4 and the cryptic exons are indicated by open and black boxes respectively. Locations of the cryptic exons are marked with arrowheads.
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Discussion

In this study, we discovered a healthy non-consanguineous couple with two children exhibiting symptoms of HOS. Exome sequencing helped us to identify the splice acceptor site mutation c.148-1G>C of TBX5 to be responsible for the pathology in this family. This splice mutation (c.148-1G>C) was already reported in a Holt-Oram syndrome family [22]. In contrast it is not present in the Exome Aggregation Consortium (ExAC) database [23] supporting its probable pathogenicity. But in the present family, the clinical evaluation of the father who shares the same mutation with the proband was normal. There is a lack of explanations to the unusual genotype–phenotype correlations in this Holt-Oram family.

Initially, the clinical evaluation of the family raised suspicions of Ellis-van Creveld syndrome and McKusick-Kaufman syndrome, two rare autosomal recessive diseases that patients can also have both limbs defects and congenital heart disease [24, 25]. However, considering that there is no evidence of other characteristics such as extra fingers/toes, ectodermal dysplasia and genital abnormalities (hypospadias, chordee, cryptorchidism) and the patient was too young to define as short-limbed dwarfism, it is hard to make a definite diagnosis. To make a full scan of all the genes responsible for diseases causing both hand and skeletal anomalies and own an informative analysis, exome sequencing was used in the study after a negative outcome of CMA analysis.

Exome analysis of the proband revealed the splice mutation (c.148-1G>C) of TBX5 with proofs showing that haploinsufficiency of TBX5 protein caused the symptoms of the patient. One thing should be noted is that the aberrant transcript would be degraded by the NMD system. Even if the new transcript can be translated into protein, the core T-box domain (amino acid 53-241 of TBX5 protein, NP_000183) would be damaged due to the location of the acceptor site.

by activation of cryptic splice sites while no cryptic splice site is predicted by HSF (Fig. 3). In either case, the mutation will result in haploinsufficiency of TBX5 protein and there is a high possibility that the aberrant transcript would be degraded by the NMD system. Even if the new transcript can be translated into protein, the core T-box domain (amino acid 53-241 of TBX5 protein, NP_000183) would be damaged due to the location of the acceptor site.

Table 3. The clinical evaluation of 12 splicing mutations of TBX5. ASD: Atrial Septal Defect; VSD: Ventricular Septal Defect; PDA: Patent Ductus Arteriosus; ND: No Data

<table>
<thead>
<tr>
<th>TBX5 mutation</th>
<th>Cardiac defects</th>
<th>Upper limbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.148-1G&gt;C</td>
<td>ASD, VSD, PDA, Isolated dextrocardia</td>
<td>+/-</td>
</tr>
<tr>
<td>c.148-3C&gt;A</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>c.243-2 A&gt;G</td>
<td>Conductive heart failure</td>
<td>+</td>
</tr>
<tr>
<td>c.242+1G&gt;A</td>
<td>VSD</td>
<td>+</td>
</tr>
<tr>
<td>c.362+1G&gt;T</td>
<td>Conductive abnormalities</td>
<td>+</td>
</tr>
<tr>
<td>c.363-5T&gt;C</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>c.510+1G&gt;T; 509A&gt;T</td>
<td>ASD, VSD, Aortic coarctation</td>
<td>+</td>
</tr>
<tr>
<td>c.664-1G&gt;A</td>
<td>ASD, an anomalous right coronary artery</td>
<td>+</td>
</tr>
<tr>
<td>c.756-1G&gt;A</td>
<td>VSD, Conductive abnormalities</td>
<td>+</td>
</tr>
<tr>
<td>c.756-3 C&gt;G</td>
<td>ASD, VSD</td>
<td>+</td>
</tr>
<tr>
<td>c.755+2T&gt;C</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>c.755+2T&gt;G</td>
<td>VSD</td>
<td>+</td>
</tr>
</tbody>
</table>
who shares the same mutation. Similar phenomenon has been reported in a family with atypical clinical findings (atrial septal defects associated with postaxial hexodactyly in all extremities) bearing a *TBX5* missense mutation (V263M) [26].

A speculation is that the described phenotypic findings could be the result of two (or even more) molecular alterations. In this scenario, the effect of a single mutation of c.148-1G>C of *TBX5* may not be enough to cause the abnormalities or may only explain the existence of cardiac anomalies (minor changes of the father’s EKG). Since no significant deletions or duplications were revealed by CytoScan HD array, several candidate variations in genes annotated to be involved in heart and/or forelimb development were sequenced. 3 of them (*PCSK5* c.4102T>C, *CAPN2* c.613C>T, *FRAS1* c.1886C>G) were confirmed. None of them presents as a "de novo" mutation and the *CAPN2* c.C613T was inherited from the mother. But whether or not these candidates play a role in the disease could only be elucidated by functional and/or interactional studies of *TBX5* protein.

Then we checked whether same thing happened in other cases of *TBX5* splicing mutations [27-31]. The clinical evaluation of 12 mutations was listed in Table 3 and no carrier of splicing mutation of *TBX5* was reported as in this case. The phenomenon seems not to correlate with the splice sites. However, it is possible that family members with no symptom were not detected in these families and more detailed information needs to be collected to investigate the clinical characteristics of *TBX5* splicing mutations.

In summary, genetic counseling could be made available for newborns in this family based on the present study. These findings lead to a perspective that even people with no clinical symptom can be detected with a pathological *TBX5* mutation. Also there maybe still mechanisms uncovered of HOS which raise interesting questions about the genotype-phenotype heterogeneity in Holt-Oram patients.

**Ethics Statement**

Approval was obtained from local ethics committees as per the revised Declaration of Helsinki (2004). Family individuals and members of the control group were recruited in Xin Hua Hospital. Informed consent was given to all the participants of this study.

**Abbreviations**

CHD (Congenital heart defects); SNVs (single nucleotide variants); PCR (polymerase chain reaction); HOS (Holt-Oram syndrome); ASD (atrial septal defect); VSD (ventricular septal defect); CMA (chromosomal microarray analysis); SNPs (single nucleotide polymorphisms); BWA (Burrows-Wheeler Alignment); GATK (Genome Analysis Toolkit); NMD (nonsense-mediated decay).

**Acknowledgements**

We are grateful to the family members for their collaboration in this study. The study is supported by Grants below. 1) Project supported by the Shanghai Committee of Science and Technology China (124119a3900). 2) Project supported by Shanghai Municipal Commission of Health and Family Planning (2013ZYJB0016). 3) National Natural Science Foundation of China (81270233).

**Disclosure Statement**

The authors declare that they have no conflict of interest.
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