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Holt–Oram Syndrome With Intermediate Atrioventricular Canal Defect, and Aortic Coarctation: Functional Characterization of a De Novo *TBX5* Mutation

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Holt–Oram syndrome (HOS) is a rare autosomal dominant disorder characterized by upper limb defects and congenital heart defects (CHD), which are often simple septal and conduction defects, less frequently complex CHDs. We report on a 9 year-old boy with clinical and radiologic features of HOS consisting of bilateral asymmetric hypoplastic thumbs, generalized brachydactyly, limited supination due to radioulnar synostosis, and sloping shoulders, and intermediate atrioventricular canal defect (AVCD) with aortic coarctation. A de novo, previously described mutation, (Arg279ter) was identified in the *TBX5* gene. Molecular characterization of this mutation was carried out due to the atypical CHD. In order to investigate whether the mutated transcript of *TBX5* was able to escape the post-transcriptional surveillance mechanism and to produce a truncated TBX5 protein, we analyzed the TBX5 transcript, and protein pattern in HOS, and WT cardiac tissues. Our results demonstrate that the mutant TBX5 transcript is cleared by the cellular mechanism of surveillance. This data provides some support for the hypothesis that a dominant negative mutation, which strongly impairs the WT allele, might be too hazardous to be maintained. The literature suggests that HOS is relatively common among syndromes associated with AVCD. © 2014 Wiley Periodicals, Inc.

Key words: Holt–Oram syndrome (HOS); atrioventricular canal defect (AVCD); aortic coarctation (COA); *TBX5*

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INTRODUCTION

Holt–Oram syndrome (HOS) (OMIM 142900) is an autosomal dominant disorder which affects 1/100,000 live births, and is characterized by well-described anterior pre-axial limb and congenital heart defects (CHD) [Basson et al., 1997; Li et al., 1997]. Although HOS is a highly penetrant disorder, inter- and intrafamilial variability is frequently described. Upper limb defects, usually bilateral and asymmetric, range from subtle carpal bone abnormalities to overt proximal defects such as phocomelia [Newbury-Ecob et al., 1996; Patel et al., 2012]. Simple septal CHDs and/or conduction system defects are common, but multiple and complex CHD can occur [Basson et al., 1994, 1999; Fan et al., 2003; McDermott et al., 2005; Boogerd et al., 2010]. No obvious correlation exists between the severity of the cardiac and skeletal abnormalities in HOS patients [Newbury-Ecob et al., 1996].

Atrioventricular canal defect (AVCD) (OMIM 606215) represents 7.4% of all CHDs [Perry et al., 1993], has a birth prevalence of 0.83 per 10,000 and can be associated with extracardiac defects and syndromes [Hartman et al., 2011]. Also known as atrioventricular septal defects or endocardial cushion defects, AVCD is characterized by a spectrum of anomalies of the atrioventricular valves, atrial and ventricular septa ranging from partial to complete AVCD;

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intermediate AVCD includes a well-formed two atrioventricular valves, clefting of the mitral valve, atrial septal defect (ASD) ostium primum and a small ventricular septal defect (VSD).

HOS is caused by mutations in *TBX5* gene [Basson et al., 1997; Li et al., 1997], which include nonsense to missense mutations [Cross et al., 2000; Ghosh et al., 2001]. Although HOS-associated mutations are distributed across *TBX5* exons, the majority are found within the T-box DNA-binding domain [Mori and Bruneau, 2004]. Initially it was hypothesized, that missense mutations might disrupt the T-box region and lead to severe organ-specific defects [Basson et al., 1999], but, phenotype–genotype correlation has not been supported [Brassington et al., 2003a; Boogerd et al., 2010]. Animal models suggest that non-coding mutations affecting the TBX5 expression level might also contribute to the number and complexity of CHD [Bruneau et al., 2001; Mori et al., 2006; Smemo et al., 2012].

In vitro analysis using *TBX5* expressing constructs containing nucleotide changes that correspond to HOS mutations, indicate the binding activity and interaction with other protein partners. At least for truncated proteins, it is still not clear whether such a protein would be biologically active or whether their transcripts would be subjected to nonsense mediated decay and therefore function as null allele.

In this report we characterize a null function allele in a *TBX5* mutation in a patient with HOS and AVCD and aortic coarctation (COA), which may provide support to the studies of genotype and HOS phenotype. We also review the occurrence of AVCD in HOS, an association probably underestimated in patients with subtle upper limb deformities.

CLINICAL REPORT

The male proband is the first child of healthy 25-year-old non-consanguineous parents. Gestational diabetes mellitus was diagnosed during the third trimester of pregnancy. There was no exposure to drugs, alcohol, smoking, or infections. Routine ultrasound scanning showed a normally growing fetus that was delivered at full term by cesarean section for cephalopelvic disproportion. His birth weight was 3.350 kg (75th centile).

Echocardiography at birth showed intermediate AVCD and COA, with significant both mitral and tricuspid valve incompetence. Neonatal coarctation repair and subsequent AVCD repair at the age of 2 months were performed. Due to significant mitral valve stenosis with tricuspid regurgitation at 8 months, the patient had mitral valve replacement and tricuspid valve commissuroplasty. At the age of 9 years the mitral prosthesis was replaced, but the postoperative course was complicated by severe congestive heart failure, leading to heart transplantation and eventually to death due to septicemia.

Genetics consultation at the age of 9 years was requested to evaluate the association of CHD and bilateral upper limb malformation. The patient's weight, height, and OFC were 20.8 kg (<3rd centile), 120 cm (<3rd centile), and 51.5 cm (10–25 centile), respectively. Physical examination showed bilateral asymmetric hypoplastic thumbs, generalized brachydactyly, limited supination due to radioulnar synostosis, and sloping shoulders, which were retrospectively confirmed by upper limb X-ray investigation performed early in infancy. Other systemic and lower limb examinations were normal. Facial appearance, hair, nail and teeth growth were normal. Psychomotor development was normal for age. Renal ultrasound was normal. Chromosome analysis on peripheral blood leukocytes revealed normal male karyotyping (46,XY). We performed *TBX5* gene screening (see results below). His parents had neither clinical evidence of HOS by upper limb and heart examination (including ECG and echocardiography), nor the disease-causing mutation.

MOLECULAR GENETIC RESULTS

TBX5 sequences of exons 1–9, including the 3′ and 5′ untranslated regions of this gene, were ascertained in the patient and both parents. A de novo mutation (Arg279ter) was identified in the HOS proband (Fig. 1A). The Arg279ter mutation, already described [Basson et al., 1997; Li et al., 1997], generates a stop codon 33 aa after the end of the T-box domain, therefore leaving intact this domain and deleting the most part of the C-terminal portion of this transcription factor. To investigate whether this mutated *TBX5* gene is transcribed and able to produce a truncated TBX5 protein, total RNA and proteins were extracted from the left ventricle of the HOS patient and quantified. For comparison left ventricle tissues were extracted from young cardiac patients with a WT TBX5. Q-RT-PCR analysis showed that the level of TBX5 transcript was strongly decreased compared with the WT transcript level (Fig. 1B). In accord with this data, Western blot analysis performed with two different antibodies against both the amino-terminal and the carboxyl end of TBX5, revealed the complete absence of the shorter isoform of TBX5 protein (Fig. 1C). These data indicate that this mutant behaves as haploinsufficient allele (Table I).

MATERIALS AND METHODS

Mutational Sequencing Analysis

A mutational screening was performed by direct gene sequencing of all coding exons including adjacent intronic as well as 5′ and 3′ untranslated sequences of *TBX5* gene. Genomic DNA was extracted from peripheral blood cells by using the QIAGEN (S.r.l., 20151, Milan, Italy) BioRobot® EZ1 System. Exons and the flanking intronic sequences were amplified by polymerase chain reaction (PCR) using specific primers as described previously [*] and using Primer3 (v. 0.4.0) software (http://frodo.wi.mit.edu/primer3/) based on the cDNA sequences available in GenBank.

PCR amplifications were performed with a volume of 50 μl mixture containing 1.5 mM $MgCl_2$, 10 mM dNTP, 50 ng genomic DNA, 20 μmol of each primer and 2.5 U Taq DNA polymerase. PCR product was used for PCR sequencing reaction with the CEQ DTCS Quick Start Kit. The sequencing reaction products were purified by precipitation with ethanol, resuspended in a sample loading solution, and analyzed with a CEQ 8800 capillary sequence (Beckman Coulter, Germany) according to the manufacturer's protocol. Resulting sequences were analyzed CEQ 8800 software packages and aligned against a reference sequence obtained from GenBank [Granados-Riveron et al., 2012].

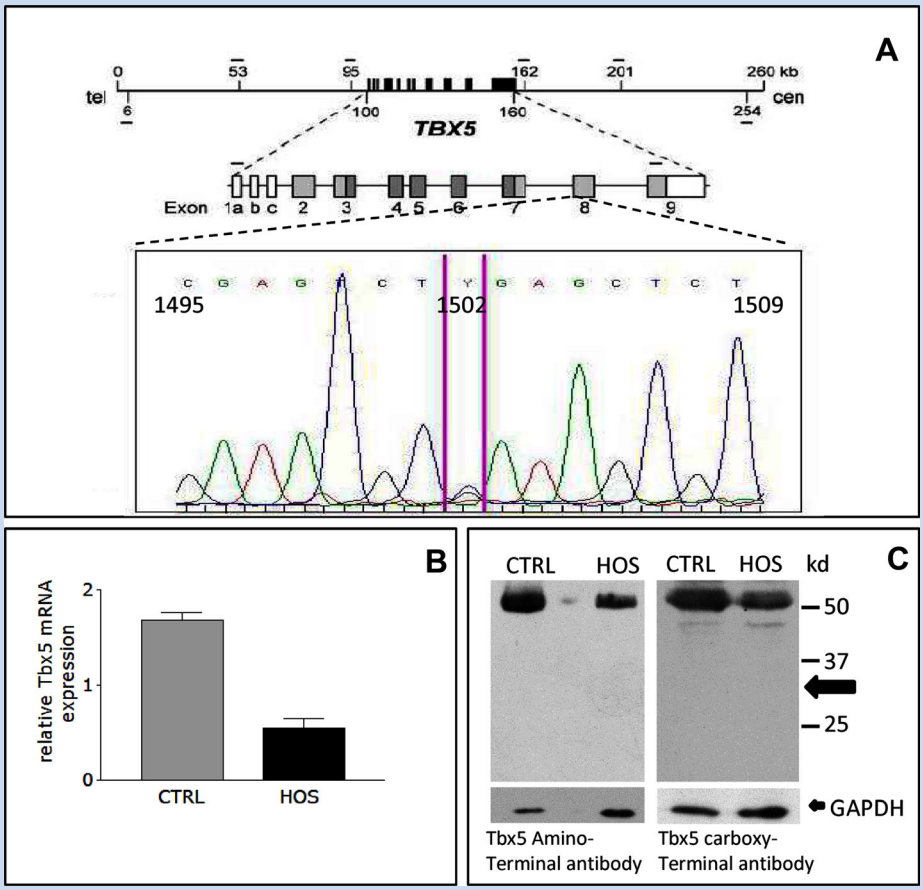

FIG. 1. Molecular characterization of the HOS patient mutation. A: Schematic representation of *TBX5* genomic structure, top, and the electropherogram of the mutation, bottom. B: Q-RT PCR quantification of TBX5 transcript in cardiac tissues from the HOS, and control patients. HPRT and HMBS were used for normalization. C: Western blot analysis of *TBX5* protein. Total proteins were extracted from HOS and a control patient as described in B. Two examples of Western blot performed using two different antibodies against *TBX5* are presented. The arrow indicates the expected position of the hypothetical truncated Arg279ter protein.

Western Blot Analysis

Cardiac tissues (~100 mg) were grounded in liquid nitrogen and homogenized in lysis buffer (20 mM Tris–HCl pH 8.0, 20 mM NaCl, 10% glycerol, 1% nonidet P-40, 10 mM EDTA, 2 mM PMSF, 2.5 μg/ml leupeptin, 2.5 μg/ml pepstatin). Proteins (20 μg/lane) were separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblots were probed with the following primary antibodies: anti-TBX5 N-terminal region (Aviva Systems Biology Corp., San Diego, CA; 92121), anti-TBX5 C-terminal region (Abnova), and anti-GAPDH (Cell Signaling Technology, Danvers, MA; 01923). Signals were revealed after incubation with recommended secondary antibody coupled to peroxidase using Enhanced chemiluminescence (ECL) from (GE Healthcare Europe GmbH, Italian branch, 20126, Milano, Italy).

Absolute Quantification by Real-Time RT-PCR

To perform an absolute quantification of TBX5 transcript, a standard curve was generated. Total RNA was extracted from ~50 mg of cardiac tissue using the RNeasy mini kit. After DNase treatment, 0.5–1 μg of total RNA was retro-transcribed using Quantitec Reverse Transcription kit (QIAGEN S.r.l., 20151, Milan, Italy) following the manufacturer's instruction. Primers used for mRNA analysis were as follows: for TBX5, F(5′-CCAGGAGCA-TAGCCAAATTTAC-3′) and R(5′-AGGGCTTCTTATA GGGAT-GGTC3′); for HPRT, F(5′-CCTGGCGTCGTGATTAGTG-3′) and R(5′-TGA GGAATAAACACCCTTTCCA-3′); for HMBS, F(5′-CTGAAAGGGCCTTCCTGAG-3′) and R(5′-CAGACTCCTCCAG TCAGGTACA-3′). To generate the standard curve, the TBX5, HPRT, and HMBS amplicons were cloned into p-GEM T-easy plasmid and the new constructs were purified and accurately quantified. Real-time PCR (qRT-PCR) was carried out using QuantiFast SYBR Green kit with Rotor gene (QIAGEN S.r.l., 20151, Milan, Italy) following the manufacturer's instructions.

DISCUSSION

The spectrum of CHDs in HOS has been well described [Sletten and Pierpont, 1996; Mori and Bruneau, 2004; McDermott et al., 2005]. Bruneau et al. [1999] reported a schematic representation of the

TABLE I. Summary of the Previously Reported Patients With Clinically and/or Molecularly Confirmed Holt–Oram Syndrome and AVCD

	Congenital heart defects	HOS diagnosis	Inheritance	References
Patient 1	AVCD unspecified + ASD	Clinical	Familial	Holmes [1965]
Patient 2	AVCD unspecified	Clinical	?	Smith et al. [1979]
Patient 3	AVCD partial + first degree AV block	Clinical	Familial	Marcus et al. [1985]
Patient 4	AVCD unspecified	Clinical	Familial	Boehme and Shotar [1989]
Patient 5	AVCD complete	Clinical	Sporadic	Del Corso et al. [1991]
Patient 6	AVCD unspecified	Clinical	?	Terrett et al. [1994]
Patient 7	AVCD complete	+Linkage analysis to 12q	Familial	Basson et al., 1994
Patient 8	AVCD complete + subaortic stenosis + aortic arch hypoplasia + COA	Clinical	Familial	Kumar et al. [1994]
Patient 9	AVCD complete + left ventricular hypoplasia + hypoplastic MV + aortic narrowing	Clinical	Familial	Sletten and Pierpont [1996]
Patient 10	AVCD complete + complete AV block	Clinical	Sporadic	Newbury-Ecob et al. [1996]
Patient 11	AVCD complete, multiple muscular VSD	100delG	Familial	Brassington et al. [2003a]
Patient 12	AVCD + membranous VSD	Arg237Trp	Sporadic	Brassington et al. [2003b]
Patient 13	AVCD + double outlet right ventricle	Ser261Cys	Familial	Brassington et al. [2003a]
Patient 14	AVCD unspecified	Glu69ter	Familial	He et al. [2004]
Patient 15	AVCD complete + common atrium	R279X	Sporadic	Heinritz et al. [2005]
Patient 16	AVCD + hypoplastic right ventricle + AV insufficiency + pulmonary valve stenosis	His220del	Sporadic	Boogerd et al. [2010]
Patient 17	AVCD + pulmonary stenosis	Large intragenic duplication	Familial	Patel et al. [2012]
Our patient	AVCD intermediate + COA + significant both MV and TV incompetence	Arg279ter	Sporadic	Present report

AVCD, atrioventricular canal defect; AV block, atrioventricular block; Ao, aortic; COA, aortic coarctation; MV, mitral valve; TV, tricuspid valve; VSD, ventricular septal defect.

mature heart indicating regions of *TBX5* expression and the location of HOS cardiac defects in 240 patients with aortic abnormalities (AA), AVCD, and mitral valve disease (MVD), mainly prolapse, rarely described in 4 (1.6%), 7 (2.9%), and 10 (4.1%) patients, respectively. Simple septal defects, or PDA were reported in 125 of 189 patients (66%), whereas 33 (17.5%) had complex CHD (hypoplastic left heart, total anomalous pulmonary venous return, truncus arteriosus). Complete AVCD in HOS can occur as an isolated CHD [He et al., 2004], or in association with other CHDs, including COA [Sletten and Pierpont, 1996], complete heart block [Newbury-Ecob et al., 1996], left-sided obstructions [Kumar et al., 1994; Sletten and Pierpont, 1996], multiple muscular VSDs or membranous VSD [Brassington et al., 2003b], and double outlet right ventricle [Brassington et al., 2003a]. The association of AVCD and HOS remains uncommon, with 17 reported patients after the era of *TBX5* identification carrying a disease causing mutation. An additional HOS case, genotyping status unknown, was reported in a descriptive epidemiology analysis (0.15% total AVCD) [Hartman et al., 2011]. AVCD displays great anatomic variability, likely reflecting genetic heterogeneity, and association with distinct genetic disorders [Marino and Digilio, 2000]. Additional CHDs are more common in patients with syndromic non-Down AVCD, although left-sided obstructive lesions are frequent in syndromic and non-syndromic patients [Digilio et al., 1999]. HOS should be added to the syndromes possibly associated with AVCD and left-sided obstructive heart defects.

A more difficult question is why severe CHD are not frequently observed in HOS. Bruneau et al. [1999] suggested that particularly severe malformations might be lethal to embryos. Alternatively, since most of these autosomal dominant mutations are predicted to cause *TBX5* haploinsufficiency, robust atrial expression may allow partial compensation by the non-mutated allele.

A heterogeneous spectrum of *TBX5* mutations were reported in HOS patients showing AVCD. These mutations included: 100delG, Arg237Trp, Ser261Cys [Brassington et al., 2003b], and Glu69ter [He et al., 2004], R279X [Heinritz et al., 2005], His220del [Boogerd et al., 2010], and large intragenic duplication [Patel et al., 2012]. No specific clinical or molecular detail is available regarding the 7AVCD patients among 240 HOS series reported by Bruneau et al. [1999]. The mutation we describe, the Arg279ter mutation [Basson et al., 1997; Li et al., 1997; Brassington et al., 2003a], introduces a stop codon after the T-box domain, putatively generating a truncated protein which should retain DNA-binding activity and loose the possibility to activate transcription and to interact with other protein partners. Indeed EMSA in vitro studies demonstrated that the mutant R279terTBX5 protein binds oligonucleotides containing the canonical *TBX5* consensus sequence even with higher affinity than the full-length protein [Ghosh et al., 2001]. However R279terTBX5 protein is unable to activate the ANF promoter or to interact with TAZ transcription factor to activate Fgf10 expression [Murakami et al., 2005]. Therefore, if this truncated protein is synthesized in HOS cardiac tissues, it might act as a strong dominant negative mutant. Indeed, carboxy-terminal truncations have been described in the T-box protein branchyury with dominant negative effect [Herrmann, 1991]. A cellular mechanism that guarantees RNA quality control is nonsense-mediated mRNA decay (NMD), which degrades transcripts harboring a premature termination codon. To investigate whether the mutated transcript

of TBX5 was able to escape the post-transcriptional surveillance mechanism, and to produce a truncated TBX5 protein we analyzed the TBX5 transcript, and protein pattern in HOS, and WT cardiac tissues (Fig. 1). Our result demonstrates that the mutant TBX5 transcript is cleared by the cellular mechanism of surveillance. In contrast to our observations, in zebrafish, where NMD is a crucial mechanism for embryonic development and survival [Wittkopp et al., 2009], the transcript for the TBX5 mutant heartstrings (hst), which is similar to the human *TBX5* truncation mutant TBX5-R279ter, is reported to be expressed at WT levels [Garrity et al., 2002]. However, in this model system, the expression level of the TBX5 mutated protein was not investigated. Moreover, there were no genetic evidence for dominant effects of the *hts* mutation, and the hts heterozygotes were phenotypically indistinguishable from WT embryos [Garrity et al., 2002].

In conclusion, our report provides some support for the hypothesis that a dominant negative mutation, which strongly impairs the WT allele, might be too hazardous to be maintained. We agree with previous reports, which emphasized that the proportion of unusual non-septal CHD in HOS patients is underestimated and should be considered in counseling families with HOS mainly due to the wide intrafamilial variability. We suggest that HOS should be added to the list of syndromes associated with AVCD, and left-sided obstructive heart lesions.

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