Two novel splicing mutations in *TBX5* cause Holt-Oram syndrome

Benyanan Phimphakan[1], Piyobol Kornrat[2], Thawornchai Limjindaporn[1], Manop Pithukpakorn[2,3], Chanin Limwongse[2,3], Natthida Petchampai[4], Wanna Thongnoppakhun[2] and Chairat Turbpaiboon[1]*

[1]Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
[2]Division of Molecular Genetics, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
[3]Division of Medical Genetics, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
[4]Department of Tropical Medicine, School of Public Health and Tropical Medicine, Tulane University, New Orleans, Louisiana 70112, USA.

**Corresponding author: cturbpaiboon@hotmail.com*

ABSTRACT

Holt-Oram syndrome (HOS), deformities owing to developmental defects of heart and upper limb, is caused by mutations in the *TBX5* gene encoding a transcription factor in T-box gene family. Its 1.5-kb coding sequence spans eight exons and encodes for the 518 amino acid TBX5 protein. Most *TBX5* mutations are located in the coding region and cluster either in the DNA-binding or in the transactivating domains of TBX5 protein. In this study, three Thai (Mongoloid) HOS patients were investigated for their causative variants by screening the *TBX5* coding sequence. Denaturing high performance liquid chromatography under partial denaturation mode was used to screen for the entire coding region before direct sequencing for specific mutation identification. We report two novel *TBX5* mutations (c.983-2A>C, IVS8 as-2 A>C and c.510+5G>A, IVS5 ds+5 G>A as a *de novo* change) which would affect the splicing process of *TBX5* transcript and another recently discovered missense mutation (c.241A>T, p.Arg81Trp) as the first report in Mongoloid HOS patients. Our findings could help extend the understanding of genotype-phenotype correlation in HOS patients which would be beneficial for clinical practice and counseling.

KEYWORDS

TBX5; Holt-Oram syndrome; heart-hand syndrome; congenital heart disease; congenital upper limb anomalies

INTRODUCTION

Holt-Oram syndrome (HOS, OMIM 142900) is a congenital anomaly characterized by variably defective development of heart and upper limb (radial ray longitudinal deficiency) (Holt and Oram, 1960). Its incidence is estimated to be 1:100,000 live births (Csaba *et al*., 1991). This deformity was unveiled for its causative mutations in the *TBX5* gene (localized at 12q24), a member of T-box gene family with an autosomal-dominant mode of transmission, high penetrance and variable expressivity (Newbury-Ecob *et al*., 1996). This gene comprises 9 exons with 1.5-kb coding sequence spanning exon 2 to 9 for 518 amino acid residues. As a transcription factor regulating normal heart and upper limb development, T-box protein 5 (TBX5) carries the conserved DNA-binding domain (T-box) about 190 residues near its N-terminus together with the transactivating domain (about 40 residues) close to its C-terminus (Muller and Herrmann, 1997; Ghosh *et al*., 2001; Zaragoza *et al*., 2004). Specific interaction with other proteins under correct spatiotemporal condition is required during cardiac and limb development. A number of its interacting partners have been identified, for example, Nkx2–5 (Hiroi *et al*., 2001), GATA4 (Garg *et al*., 2003), Hdac3 (Lewandowski *et al*., 2014), CRM1 (Kulisz and Simon, 2008), TAZ (Murakami *et al*., 2005) and LMP-4 (Krause *et al*., 2004).

Various types of *TBX5* mutation have been uncovered. The most common category of mutations causes truncated protein leading to haplo-insufficiency with severe deformity phenotypes (Basson *et al*., 1999) while the non-truncated group, e.g. missense mutations, expresses the phenotypes variable in severity depending on the mutation positions on the protein (Al-Qattan and Abou Al-Shaar, 2015). A majority of missense mutations has been observed within the DNA-binding domain, which can differentially affect the interaction with both target

Received 17 October 2018; Accepted 8 March 2019

DNA sequence (T-box binding element) and protein partners (Al-Qattan and Abou Al-Shaar, 2015). In addition, mutations affecting protein dosage, e.g. gene duplication, can give rise to HOS phenotype (Vaughan and Basson, 2000). Certain positions on this gene can be regarded as mutation hotspots as they occur repeatedly among independent cases (Barnett and Postma, 2014) but neither position nor type of mutation can be inferred for disease severity (Brassington *et al*., 2003). Even though this syndrome is found to be hereditary among family members, about nearly half of all reported mutations are *de novo* sporadic cases with negative family history.

According to current *TBX5* mutation records in database (HGMD®, www.hgmd.cf.ac.uk), most of them are located in the coding region, thus mutation analysis for newly diagnosed patients with HOS should start with coding sequence screening. Up to eight coding exons with large intronic sequence intervening among them, screening for any specific exon carrying putative variant prior to sequence analysis provides another economical approach for routine investigative studies. Single-strand conformational polymorphism (SSCP) has ever been applied for coding sequence scanning but may not achieve high mutation detection rate (Cross *et al*., 2000); therefore, denaturing high performance liquid chromatography (DHPLC) can be another option for this purpose with more rapid and convenient technique than SSCP. In this study, three unrelated Thai patients with clinical diagnosis of HOS were included for specific mutation analysis in the *TBX5* gene. Proper DHPLC condition under partial denaturation mode was developed for screening *TBX5* coding sequence before being subject to Sanger sequencing.

MATERIALS AND METHODS

Clinical summary

Three unrelated Thai individuals with a clinical diagnosis of HOS from Siriraj Hospital, Bangkok, Thailand were investigated in this study. The first proband (HOS1) is a 41-year-old Thai male patient with the clinical finding of atrial septal defect and limb reduction defect. His elder child appeared to have no obvious congenital abnormality while the younger one had short right arm with only three fingers and left arm with an absent thumb. His pedigree was shown in figure 1A. The second proband (HOS2) is a 38-year-old Thai male patient with the clinical finding of asymmetrical upper limb defects. His congenital limb abnormalities predominated on the left side including short left arm and forearm with fused thumb and index finger, while the only abnormality of his right upper extremity is the abnormal right thumb. In addition, he suffered from cardiomegaly with the family history of congenital heart disease of his father and brother as shown in the pedigree in figure 1B. The other proband (HOS3) in this study is a 26-year-old Thai male patient visiting Siriraj Hospital with the history of palpitation and chest pain for 2 days (functional class I). Physical examination and investigations (echocardiography & electrocardiography) revealed large secundum atrial septal defect (22.9 X 18.7 mm). This congenital heart problem was later corrected by ASD closure device. He then developed one episode of atrial flutter which was corrected by cardioversion. His upper limb defects included small left hand, contracture/ankylosis of left fingers, left radial deviation, shortening of left forearm, small right preaxial finger, and bilateral thumb absence. No other family members were found to have clinical signs of HOS as shown in the pedigree in figure 1C.

Ethic statement and clinical samples

This study was approved by Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University (COA no. *Si*217/2016). Venous blood samples of three male patients, clinically diagnosed as HOS at Siriraj Hospital were collected for further molecular genetic analyses of causative mutations.

DNA extraction and amplification

Genomic DNA (gDNA) of all blood samples was isolated from peripheral leukocytes by Puregene® Blood Core Kit, QIAGEN® according to a manufacturer's protocol (QIAGEN Sciences, MD, USA). The concentrations of purified gDNA samples were measured and the gDNA was diluted for condition appropriate for polymerase chain reaction (PCR). The PCR mixture consisted of 50 ng of gDNA, 10 pmol of each primer, 0.2 mM deoxynucleoside triphosphate (dNTP), 3% vol/vol dimethyl sulfoxide (DMSO as additive) only for amplification reaction of exons 5 and 9 (1st fragment), 0.4 unit of Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, MA, USA) and Phusion HF buffer (Thermo Fisher Scientific, MA, USA) in a final volume of 20 µL. The amplification reactions were carried out with an initial denaturation step at 98ºC for 30 seconds followed by 30 cycles of denaturation at 98ºC for 7 seconds, annealing at 62ºC or 72ºC for 20 seconds and extension at 72ºC for 30 seconds, and a final extension step at 72ºC for 7 minutes. All coding exons of the *TBX5* gene

with at least 10-base-pair flanking noncoding sequence, i.e. conserved sequences for splicing regulation were amplified by PCR using specific primers (McDermott *et al*., 2005) and annealing temperatures as shown in table 1. Amplicons were checked by 1.5% (w/v) agarose gel electrophoresis.

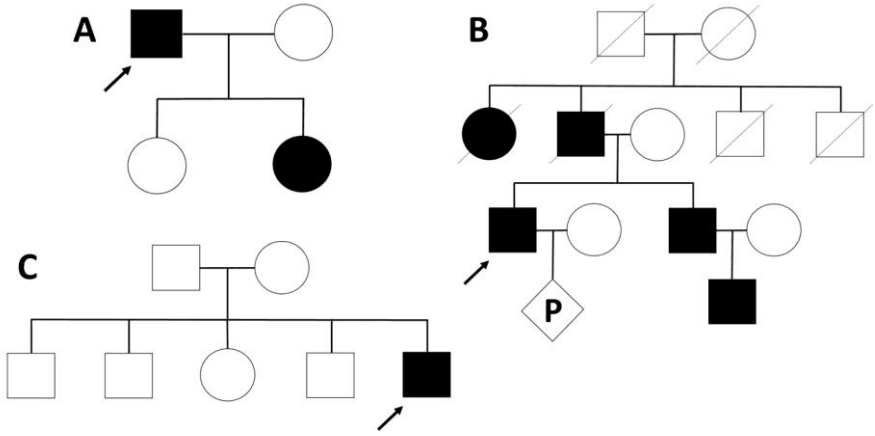

Figure 1: Pedigrees of 3 HOS patients in this study (figures 1A, 1B and 1C for patients HOS1, HOS2 and HOS3, respectively). The proband in each family was indicated by arrow.

Coding sequence screening by DHPLC

Amplicons from all patients were screened for heterozygosity using DHPLC by the differential retention of homoduplex and heteroduplex DNA on ion-pair reversed-phase liquid chromatography using the WAVE™ Nucleic Acid Fragment Analysis System 3500HT equipped with DNASep® HT cartridge technology (Transgenomic Inc., Omaha, Nebraska, US) under partial denaturation mode. First, nucleotide sequence of each amplicon was calculated to establish a melting profile suggesting proper partially denaturing temperatures which are able to distinguish heterozygosity as shown in table 1. Ten microlitres of PCR products were denatured at 95°C for 5 minutes and reannealed by gradual decreasing 1°C for every 22 seconds until reaching 25°C to increase heteroduplex formation prior to applying into DHPLC system. DHPLC eluting chromatograms of the cases of interest were compared with the one of normal control for distinguishing exon carrying putative variants.

Direct sequencing and *in silico* prediction of the *TBX5* variants

Excess primers and dNTPs were removed by ExoSAP-IT® (Affymetrix, Ohio, US) prior to Sanger sequencing reaction. Sequencing primers were the forward and reverse primers of the PCR amplification. Sequencing electropherogram was analyzed for variant existence by comparing with the reference sequence (RefSeq: NG_007373.1 and NM_000192.3). The suspected sequence variants were analysed by a DNA variant analysis software, Mutation Surveyor (demo) v.4.0.6. Nucleotide alteration was examined with the database on The Human Genome Mutation Database at the Institute of Medical Genetics in Cardiff (HGMD®, www.hgmd.cf.ac.uk) and NCBI dbSNP: Short Genetic Variations (www.ncbi.nlm.nih.gov/SNP/). Biological significance of the observed variants, for a missense change, was predicted by three of the most commonly used *in-silico* tools (Richards *et al*., 2015), i.e. SIFT (Ng and Henikoff, 2003; Kumar *et al*., 2009), PolyPhen-2 (Adzhubei *et al*., 2013), MutationTaster (Schwarz *et al*., 2010) as well as Condel web server (Gonzalez-Perez and Lopez-Bigas, 2011). Prediction of splice-site-disrupting variants was performed using two of the most popular tools, Human Splicing Finder (HSF, www.umd.be/HSF3) and Variant Effect Predictor (VEP) (McLaren *et al*., 2016). The latter incorporates two outperform methods: MaxEntScan (Yeo and Burge, 2004) and dbscSNV which includes all potential single nucleotide variants covering those within splicing consensus regions (scSNVs) and two ensemble prediction scores, adaptive boosting (ADA) and random forest (RF) scores (Jian *et al*., 2014). MaxEntScan was chosen as having the best predictive performance among individual tools (Jian *et al*., 2014). Both ADA and RF scores of dbscSNV were used in this study due to their superior performance than most of other splicing-prediction tools (Soens *et al*., 2017).

Table 1 Specific PCR primers for all coding exons of *TBX5* gene. Annealing temperatures for each PCR amplification and sizes of PCR amplicon were described. Proper partially denaturing temperatures used in DHPLC established from melting profile of each amplicon were also shown.

Exon	Primer	Annealing Temperature (°C)	Amplicon size (bp)	DHPLC Melting Temperature (°C)				
2	F- GCTTCTTGTCCTCAGAGCAGAACCT R- CAAGAGAAGCCGAGCAGGAAAGCCA	72	276	66.9	64.5	61.5	-	-
3	F- AGTTTGGGGAAGGAATGCCCACTAC R- TTCTCCTCGTCCCTCTCTCTACACA	72	200	60.5	60.1	-	-	-
4	F- AACGGGGCTAGTTTCCGCTTCCACG R- CTTTTTGGGAGAAGGTTCCACTTTT	62	307	60.5	60.0	59.3	-	-
5	F- CCTGGTGCGTGAACTGAAGCACGC R- GAGGGAGACAAGGCGGGGAATCCAG	72	275	66.5	63.5	62.8	-	-
6	F- CCGATATAACAAGGCGAATTTAGA R- ATTCAGAGGAGCAAAGTTCCAG	62	352	60.8	58.8	56.5	55.0	-
7	F- ATTAGCTCATGTCCTGAGGTGGTCT R- GTGGGGAGGAGAAAGTTGAGGAATC	72	203	60.3	59.7	59.3	-	-
8	F- CTTTTCTGGTGGATTCTCTCACACC R- GGGTAGGAACATGTCAAGGGAAC	62	515	61.5	58.5	58.0	52.7	-
9 (1^{st})	F- TACTTTGGCCAAATAACTGTCTCC R- AGGTCTGGTGCTGGAACATTC	72	478	63.7	61.2	59.8	58.1	56.5
9 (2^{nd})	F- GCCCATGGACAGGCTACCCTACC R- CGACCTTGAGTGCAGATGTGAAC	72	455					

RESULTS

All affected cases with clinical HOS included in this study suffered from both upper limb and cardiac abnormalities in a wide spectrum. These limb deformities were involved with radius, carpal bones and radial sided phalanx ranging from absent thumb to phocomelia, either unilaterally or bilaterally (symmetric or asymmetric), while their congenital heart defects included both structural and conduction abnormalities. All amplicons of *TBX5* coding sequences were screened for heterozygous variants using the established conditions of DHPLC prior to further identification of specific nucleotide alteration using direct sequencing. Appropriate partially denaturing temperatures of DHPLC screening for *TBX5* coding amplicon were established from the melting profiles as shown in table 1.

Among these amplicons, different heteroduplex patterns of DHPLC elution in different exons were found (figure 2). Nucleotide changes according to sequencing results (figure 3) of the amplicons with heteroduplex eluting chromatograms demonstrate two novel mutations and one recently discovered mutation of *TBX5* gene (Ersoy *et al*., 2016). For the proband HOS1, adenine to thymine alteration at the coding nucleotide 241 (c.241A>T) or the first nucleotide of codon 81 (AGG → TGG, p.Arg81Trp) was found (figure 3A). This disease-causing missense mutation (arginine to tryptophan) was recently discovered in members of a Caucasian family with HOS (Ersoy *et al*., 2016) for the first time but this study is the first report in Mongoloid family with HOS. Computational evaluation for the pathogenicity of this missense mutation, p.Arg81Trp, revealed a consensus positive prediction among all *in silico* tools tested, i.e. SIFT (score 0, deleterious), PolyPhen-2 (score 0.995, probably damaging), MutationTaster (score 1, disease causing) and Condel web server (score 0.902, deleterious).

Based on sequence analysis of the proband HOS2, the first novel mutation was observed at the upstream nucleotide of consensus acceptor splice site of intron 8 by altering from adenine to cytosine (AG → CG) as shown in figure 3B (c.983-2A>C, IVS8 as-2 A>C). The other novel mutation found in this study was observed in the proband HOS3. This guanine-to-adenine variation was revealed at the fifth nucleotide of intron 5 (c.510+5G>A, IVS5 ds+5 G>A) as shown in figure 3C.

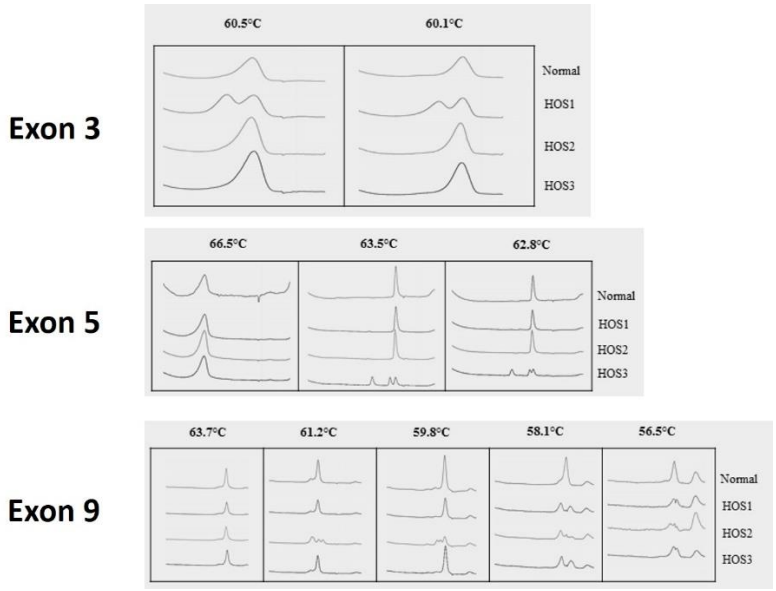

Figure 2: DHPLC elution chromatograms showing heteroduplex pattern of exons 3, 5 and 9 in patients HOS1, HOS3 and HOS2, respectively.

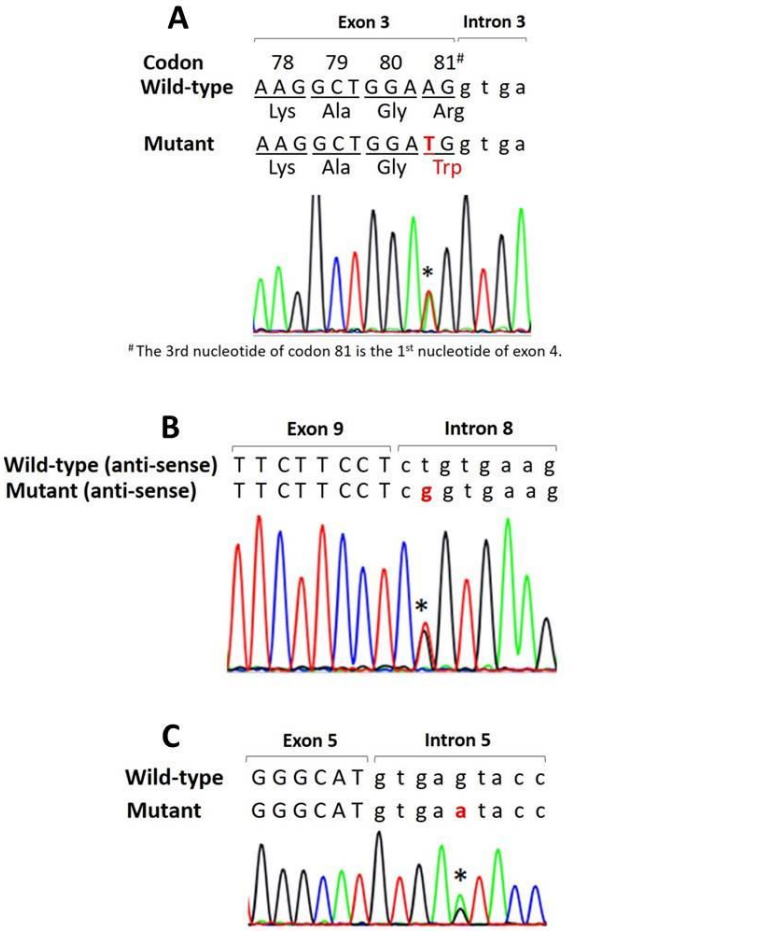

Figure 3: Sequence analysis showing heterozygous single-nucleotide substitution at the base indicated by asterisk (figures 3A, 3B and 3C for patients HOS1, HOS2 and HOS3, respectively). The mutated nucleotides were marked in red above each electropherogram.

Biological significance of these three *TBX5* variants was then predicted by various *in silico* splicing prediction tools mentioned above as shown in table 2. Albeit being a missense mutation (p.Arg81Trp), the nucleotide substitution itself at a penultimate position in exon 3, c.241A>T, can have an effect as an atypical splice-disrupting variant as predicted by VEP and HSF (table 2, HOS1 column). For the c.983-2A>C (IVS8 as-2 A>C) which is an undoubted acceptor splice-site mutation, the prediction tools revealed the consensus strongly-positive results (table 2, HOS2 column), implying the reliability of such computational confirmation for the variant pathogenicity. These prediction tools also indicated the obviously positive results for the c.510+5G>A (IVS5 ds+5 G>A) found *de novo* in the proband HOS3 by acting as a donor splice-site mutation (table 2, HOS3 column).

Table 2 *TBX5* gene variants identified in this study with the results of *in silico* splice-site disrupting prediction tools which support their pathogenicity.

HOS patients		**HOS1**	**HOS2**	**HOS3**
***TBX5* variants** (based on NM_000192.3)		**c.241A>T (p.Arg81Trp)**	**c.983-2A>C (IVS8 as-2 A>C)**	**c.510+5G>A (IVS5 ds+5 G>A)**
Results of prediction tools				
1. Variant Effect Predictor (VEP)				
• **Consequence**		Splice region variant: 50% Missense variant: 40%	Splice acceptor variant: 100%	Splice region variant: 47% Intron variant: 47%
• **Impact**		Moderate	High	Low
• **dbscSNV**	**ADA score**	0.9990	0.9999	0.9998
	RF score	0.9500	0.9280	1.0000
• **MaxEntScan**	**Alternate sequence score**	7.2267	2.2717	-0.3927
	Score difference	1.4533 (16.7%)	8.0423 (77.9%)	8.2186 (105.0%)
	Reference sequence score	8.6801	10.3141	7.8259
2. Human Splicing Finder (HSF)		Potential alteration of splicing – Creation of an exonic ESS site – Alteration of an exonic ESE site	Alteration of the WT acceptor site, most probably affecting splicing	Alteration of the WT donor site, most probably affecting splicing

Remarks: ADA and RF scores indicate splicing impact; the larger the score is (close to 1), the higher the probability of affecting splicing is
- MaxEntScan: Score difference = % change relative to wild-type sequence (RefSeq).
- ESS = exonic splicing silencer, ESE = exonic splicing enhancer, WT = wild-type.

DISCUSSION

Radial deficiencies are defined as abnormal development of radial-sided structures. It can be combined with other organ involvement as syndromic presentation, e.g. HOS (with heart defects), Okihiro syndrome (with impaired ocular motility). HOS is characterized by wide spectrum of mainly bilateral (mostly asymmetrical, more severe on the left (Smith *et al*., 1979)) upper limb malformation, especially in the preaxial or radial ray development and congenital heart defect (CHD), predominantly septation with/without conduction problem. There is no correlation in severity of the limb and heart anomalies (Brassington *et al*., 2003). It is widely described among diverse ethnicities with high variability of phenotype even within the family (Newbury-Ecob *et al*., 1996). In this study, all probands from three unrelated families demonstrated typical signs of HOS including both heart and upper limb defects with existence of inter- and intrafamilial clinical variability. Similar to previous reports of being both hereditary and sporadic, pedigrees of the first two probands represent the familial transmission of HOS among members while the proband HOS3 is the only affected member (*de novo* case) in the last family.

Phenotypic variability of HOS limb abnormalities ranges from mild deformity with partially limited forearm movement to severe form of limb reduction defect (ectromelia), which affects any bones (radial side) of forearm, wrist, palm and thumb (Basson *et al*., 1994; Cross *et al*., 2000). Thumb is more commonly affected than proximal structures and its deformities can be found in various forms, e.g. clinodactyly, triphalangism, fused thumb with index finger, hypoplasia or even aplasia (Debeer *et al*., 2007). However, carpal bone malformation is more specific in HOS than thumb deformities (Poznanski *et al*., 1970). Like other typical findings in previous reports of HOS patients, asymmetrical limb defects with varying degree of severity were described in each proband of

this study with most deformities involved with thumb and radial-sided structures of forearm or wrist. Humerus and ulna abnormalities are sometimes included in HOS limb deformities (Newbury-Ecob *et al*., 1996; Barisic *et al*., 2014) as found in the proband HOS1 (limb reduction defect).

Various single or multiple cardiac manifestations in HOS are usually observed with the most common lesion of septation defects, both atrial (mainly secundum) and ventricular (Newbury-Ecob *et al*., 1996). More severe form of structural heart development including atrioventricular canal, e.g. Tetralogy of Fallot, aortic coarctation may be involved (Li *et al*., 1997; Bohm *et al*., 2008). Conduction problems, particularly atrioventricular block including atrial fibrillation are also frequently observed in HOS patients either with or without cardiac structural defects (Newbury-Ecob *et al*., 1996). In this study, both structural defect (ASD) and arrhythmia (atrial flutter in HOS3) were noted in the probands. For the HOS2 case, only cardiomegaly was defined without evidence of any other structural or functional defects but family history of congenital heart disease was present among his 1st-degree relatives. According to previous report, CHD may not be observed in about 20% of diagnosed HOS cases (Barisic *et al*., 2014).

According to online database (HGMD®, www.hgmd.cf.ac.uk), over hundred causative mutations in *TBX5* have been reported in patients with HOS. From the sequencing result of the proband HOS1 in this study, an Arginine-to-Tryptophan mutation at codon 81 in exon 3 (c.241A>T) was identified. This disease-causing missense mutation was recently discovered in members of a Caucasian family with HOS for the first time (Ersoy *et al*., 2016) but our study is the first report in Mongoloid (Thai) family with HOS. Clinical variability observed among affected family members of the present study and with comparison to the previously reported Caucasian cases (Ersoy *et al*., 2016) revealed both inter- and intrafamilial variable expressivity of this mutation. Together with previous report, this mutation can cause the upper limb defects in a wide spectrum of phenotype affecting from thumb up to humerus with asymmetrical predomination. For cardiac phenotypic heterogeneity, septal defects of either atrium or ventricle can be observed with the involvement of conduction problem. Nonetheless, effects from other modifier genes and environment should be inevitably considered. Due to only a single amino acid substitution in the protein was resulted from this missense mutation, it is likely to manifest less severe clinical phenotypes (frequently asymmetrical) than other types of mutation (Basson *et al*., 1999). The causative amino acid residue was shown to be highly conserved among different species (Ersoy *et al*., 2016) as it is located in the DNA-binding domain (T-box) of TBX5 protein but not regarded as mutation hotspot (Muller and Herrmann, 1997; Ghosh *et al*., 2001). According to the crystallographic studies, the 81st residue of TBX5 protein was found to interact with DNA major groove via hydrogen bonding (Muller and Herrmann, 1997; Basson *et al*., 1999), thus affecting this target DNA sequence interaction if mutated. According to mutation database (HGMD®, www.hgmd.cf.ac.uk) and literature review on mutations in HOS (Barnett and Postma, 2014), missense and nonsense mutations are the most common cause of HOS and the majority of those mutations have been found in the DNA-binding domain (T-box) of TBX5 protein (Mori and Bruneau, 2004). However, there has been no evident correlation between clinical severity and either location or type of mutations in HOS patients (Brassington *et al*., 2003).

Splicing-related mutation is another common type found in *TBX5*-associated HOS patient (Barnett and Postma, 2014). For the other two novel mutations uncovered in this study, the first (c.983-2A>C, IVS8 as-2 A>C) was revealed in the proband HOS2 as a base substitution mutation at the universal acceptor splice site of intron 8 (AG → CG). This mutation definitely disrupts normal splicing process at the intron 8/exon 9 boundary, thereby aberrant splicing of intron 8 occurs. The ninth exon encodes for the C-terminal part (residues 328-518) of TBX5 protein harboring its transactivating domain (residues 339-379) (Muller and Herrmann, 1997; Stirnimann *et al*., 2010), thus perturbed interaction with other partner proteins via this domain can occur in this mutant protein.

Another novel as well as a *de novo* mutation unveiled in the proband HOS3 (c.510+5G>A, IVS5 ds+5 G>A) is also involved with the normal splicing process at the exon 5/intron 5 boundary. Since this mutated nucleotide is located in the short conserved sequences (5'-GURA*G*U-3'; R = purine nucleotide) at the 5' splice site of metazoan intron which is recognized by 5' end of U1 snRNA in the spliceosome (Will and Luhrmann, 2011; De Conti *et al*., 2013; Caminsky *et al*., 2014), this substituted nucleotide can derail normal splicing of intron 5 and lead to synthesis of dysfunctional TBX5 protein due to deranged structure of the DNA-binding (T-box) domain (partially coded by exons 5-6) (Muller and Herrmann, 1997; Stirnimann *et al*., 2010). Accordingly, pathogenicity of this variant is also supported by the

three independent *in silico* splicing-prediction tools, dbscSNV *(*ADA and RF scores), MaxEntScan and Human Splicing Finder (HSF) as a strong causative mutation affecting donor splice site of intron 5 (table 2, HOS3 column). Aberrantly spliced mRNAs caused by these 2 novel mutations can give rise to deleterious consequences for the proper regulation of normal upper limb and heart development due to either qualitative (truncated, elongated or dysfunctional protein) or quantitative (inadequate protein production owing to nonsense-mediated mRNA decay) defects of their translated proteins. Further RNA or functional study of these 2 mutations in developmental regulation might help verify their molecular consequences in HOS pathogenesis.

In conclusion, our study reports 2 novel mutations (c.983-2A>C, IVS8 as-2 A>C and a *de novo* c.510+5G>A, IVS5 ds+5 G>A) which would affect the splicing process of *TBX5* transcript during regulation of upper limb and heart development. Another recently discovered missense mutation (c.241A>T, p.Arg81Trp) is also reported for the first time in Mongoloid (Thai) HOS patient. Our findings could help elucidate our understanding of the genotype-phenotype correlation in HOS patients which would be beneficial for clinical practice and counseling. In addition, DHPLC is still a good screening method for batch analysis of HOS patients prior to direct *TBX5* sequencing.

ACKNOWLEDGEMENTS

TL, MP, CL, WT and CT are supported by the Siriraj Chalermphrakiat Grant, Faculty of Medicine Siriraj Hospital, Mahidol University. CT is also supported by the Talent Management Project, Mahidol University. This research project is supported by Siriraj Research Fund, Faculty of Medicine Siriraj Hospital, Mahidol University.

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