

Microduplication of 7q36.3 encompassing the SHH long-range regulator (ZRS) in a patient with triphalangeal thumb-polysyndactyly syndrome and congenital heart disease

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Abstract. Triphalangeal thumb-polysyndactyly syndrome (TPT-PS) is an autosomal dominant disorder with complete penetrance and a variable expression consisting of opposable triphalangeal thumbs, duplication of the distal thumb phalanx, pre-axial polydactyly and duplication of the big toes (hallux). The causative gene of TPT-PS has been mapped to 7q36.3. Sonic hedgehog (SHH) expressed in the zone of polarizing activity (ZPA) has an important role in defining the anterior-posterior axis and numbers of digits in limb bud development. Point mutation or duplication in the ZPA regulatory sequence (ZRS), a *cis*-regulator of SHH, will lead to TPT-PS. The present study describes a 1-year-old female congenital heart disease (CHD) patient with TPT-PS phenotype. In this Han Chinese family with TPT-PS, high resolution single nucleotide polymorphism array technology identified a novel 0.29 Mb duplication comprising ZRS at 7q36.3 where *LMBR1* is located. Additionally, a novel deletion of 22q11.21 was detected in the proband with Tetralogy of Fallot. However, the parents and other relatives of the patient did not harbor this genomic lesion nor CHD. The findings supported the hypothesis that an increased copy number variation of ZRS is the genetic mechanism underlying the phenotype of TPT-PS, and corroborated that 22q11.21 deletion is a genetic cause of CHD.

Introduction

Triphalangeal thumb-polysyndactyly syndrome (TPT-PS; OMIM #174500), also called pre-axial polydactyly type II (PPD2; OMIM #174500), is a well-defined autosomal dominant

disorder with complete penetrance and variable expression. The phenotype consists of opposable triphalangeal thumbs, duplication of distal thumb phalanx, pre-axial polydactyly, and duplication of the big toes (hallux).

Using linkage analysis, a malformation linked, highly polymorphic locus was identified within a large Dutch family with TPT-PS and, for the first time, the disease locus was located at the 7q36 region (1). Variable expression of TPT-PS was demonstrated by the asymmetrical limb deformities of affected individuals and the differences observed between monozygotic twins in that family.

The Sonic hedgehog (SHH) protein secreted by the mesenchymal cells is restricted to a region termed the zone of polarizing activity (ZPA) in the limb bud as a key regulator in defining the anterior-posterior axis and numbers of digits in early limb development and morphogenesis (2,3). In 2002, a translocation breakpoint was first identified in a pre-axial polydactyly patient (4). In 2003, it was reported that point mutations segregated with polydactyly in three unrelated families with PPD2 and in the Hemimelic extra toes mouse mutant. A further study identified a *cis*-regulator, designated ZPA regulatory sequence (ZRS), driving normal SHH expression in ZPA, which lies at 1 Mb upstream of the target gene *SHH* within intron 5 of the Limb region 1 homolog (*LMBR1*; OMIM #605522) (5). The ZRS is highly conserved from mammals to fish and can drive the expression of a reporter gene in ZPA (5,6). Subsequently, several different point mutations that are clustered within a highly conserved region of ZRS in humans have been reported to be associated with TPT-PS (7-9).

In 2008, for the first time in humans, a 588,819 bp duplication of 7q36 comprising ZRS was reported in a large family with TPT-PS (10). This previous study demonstrated that a duplication of ZRS results in a similar phenotype, that is, TPT-PS as single nucleotide mutations within ZRS. In the same year, six duplications of at least 131, 291, 158, 246, 235 and 398 kb were identified in six families with TPT-PS (11). These duplications shared a 32,757 bp common overlapping segment containing the ZRS enhancer.

A previous study (12) suggested that copy number variations (CNVs) can be associated with syndromic congenital

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heart disease (CHD), including limb anomalies. The present study reported the identification of a novel 0.29 Mb duplication in 7q36.3 encompassing ZRS in *LMBR1* in a 1-year-old female patient with the TPT-PS phenotype and CHD associated with a *de novo* 22q11.21 deletion.

Materials and methods

Ethical approval and patient consent. The present study was approved by the Review Board of The Second Xiangya Hospital of the Central South University (Changsha, China). All individuals provided informed written consent. Blood samples were collected following informed written consent from the patient's parent on behalf of the child enrolled.

Clinical presentation. The present study investigated a four-generation family from Central-South China (Hunan) with a phenotype comprising triphalangeal thumbs and pre-axial polysyndactyly leading to the diagnosis of TPT-PS, and the phenotype varied among affected individuals. The proband, a 1-year-old female patient, was the second child of non-consanguineous parents. Central cyanosis, cardiac murmur and a malformation of both thumbs were observed at birth.

At 1 year of age, the patient was referred to the in-patient department of The Second Xiangya Hospital of the Central South University (Changsha, Hunan, China) for surgical treatment of cardiac defects. Tetralogy of Fallot (TOF) was diagnosed by two-dimensional color Doppler echocardiography. Physical examination highlighted the limb anomalies of the proband, which comprised triphalangeal thumbs of both hands and polysyndactyly in the right thumb, and so typical TPT-PS was diagnosed (Fig. 1). Next, the patient's father was also ascertained to be expressing triphalangeal thumbs bilaterally but without polysyndactyly, and CHD was excluded using color Doppler echocardiography. A pedigree analysis revealed that the proband's grandmother, one of the grandmother's brothers and the grandmother's mother manifested similar thumb phenotypes, but had all died a number of years previously. This indicated that the phenotype was transmitted in an autosomal dominant manner throughout four generations in the family (Fig. 2). The pedigree had no history of CHD, with the exception of the female patient with TOF.

Cytogenetic analysis. Chromosome analysis was performed on the peripheral blood samples of the patient and the patient's parents by conventional G-Banded techniques (550 bands resolution). A 2 ml sample of peripheral blood was collected from each individual. All samples were subjected to lymphocyte culture according to standard cytogenetic protocol.

DNA extraction. Genomic DNA was prepared from peripheral blood using a DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, CA, USA) on the QIAcube automated DNA extraction robot (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol.

Sequence analysis. The entire coding regions, including the flanking intronic sequences of *LMBR1* (RefSeq: NM_022458) and *TBX1* (RefSeq: NM_080647) were amplified with polymerase chain reaction (PCR; primer sequences are presented

in Table I and the supplier was Sangon Biotech Co., Ltd., Shanghai, China). Sequences of the PCR products were determined using the ABI 3100 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), as previously described (13).

Single nucleotide polymorphism (SNP) array analysis. Genomic DNA samples were adjusted to a final concentration of 50 ng/ml. The Human Omni1-Quad Chip (Illumina, Inc., San Diego, CA, USA) and the Illumina BeadScan genotyping system (Beadstation Scanner) were employed to obtain the signal intensities of SNP probes. The Genome Studio software (version 2011; Illumina, Inc.) was used to analyze the genotypes [human genome build 37 (Hg19)] and to evaluate the experimental quality. The call rates of the samples were >99.0%.

Results

The karyotypes of the patient and the patient's parents were normal. Sequence analysis did not identify any missense or nonsense mutation in *LMBR1* and *TBX1*. To explore the presence of genomic imbalances, an SNP-array system was employed to analyze the whole genome for CNVs (Human Omni1-Quad Chip, Beadstation Scanner and GenomeStudio V2011 software). A total of 343 CNVs were identified. By comparing these CNVs with the Database of Genomic Variants (<http://dgv.tcag.ca>), the present study identified a novel 0.29 Mb duplication of chromosome 7q36.3 (Chr7: 156,484,201-156,772,643), which contains two genes, *LMBR1* and *NOM1* (Fig. 3). The patient's TPT-PS affected father also harbored the same duplication, however, the patient's TPT-PS unaffected mother did not.

In addition, the present study also identified a *de novo* 2.6 Mb deletion of chromosome 22q11.21 (chr22: 18,874,965-21,464,479) in the proband. This 2.6 Mb deletion region includes *TBX1*, *HIRA*, *RTN4R* and numerous other genes (Fig. 4). However, the proband's parents did not harbor the 22q11.21 deletion.

Discussion

The present study reported a complication that multiple pathogenic CNVs co-exist in one patient, and consequently resulted in congenital multi-system diseases, complicating limb anomalies with congenital heart defects.

The duplicated region of chromosome 7q36.3 in the patient reported in the present study spans 288,443 bp containing the genes *LMBR1* and, in part, *NOM1*. To the best of our knowledge, this is a novel 7q36.3 duplication. The duplication was identified to co-segregate with the TPT-PS phenotype in the patient and the patient's father, but was not present in the family members unaffected by TPT-PS. This result notably suggested that the duplication involving ZRS is the disease-causing mutation underlying human TPT-PS.

Intron 5 of *LMBR1* contains a *cis*-acting regulator of limb-specific SHH, which is designated as ZRS (2,5). The role of ZRS in limb development has been interpreted as an enhancer driving SHH expression in the posterior limb bud, as well as a repressor that silences the anterior expression. The direct link between ZRS and the PPD phenotypes was

Table I. Primer sequences used to amplify ZRS (on *LMBR1*) and *TBX1*.

Gene	Exon	Forward primer (5'-3')	Reverse primer (5'-3')
<i>LMBR1</i>	ZRS-1	TTTCAAATGCTCACTTTACATGG	TTTTATGACCAGATGACTTTTTTCC
	ZRS-2	AGGCTGGACTTCCTACTCACTCT	GAATAAAAATGTCAGGAGGAAAAA
<i>TBX1</i>	2	AGCCAGTGC GTTCAGCATCGCCTC	AAGAGCTGCCTCCACCTACTTTG
	3-1	TGATCTCCGCCGTGTCCAGC	GGCCACCTTCGCGTTCTTCTT
	3-2	GGTGAAGAAGAACGCGAAGGTGG	GACGGCGAACAGCGAAGGAG
	4	TGCCTTCCACCAGCTAGG	AGACGACCCTTGGAGTTGG
	5	CTCTGGGTTCACCTCCACAT	CAGGCCTCTTAGGGACAGG
	6	CTCCCACCCAGATCCTC	AATCCGCTCAGGTCCTCC
	7	TTGGTGC GTCTTCTCCTAACACTC	AAGGCGCTCATGAGCGGCAGTG
	8	GGGACTGTGACCCTGAGGACTG	GGGTAGAGCGCGCACAGG
	9A-1	TCAGACACTGGACATTTGTGC	TCAGCATTCAACAAAGACGG
	9A-2	CAGAGTGCCAACCCTTCAAT	ACTGGGAGTGTGACTCTATGGA
	9B	AACACTTTGACCTTCCTCCACCC	TGGAGGATTGCTTCCATCACAG
	9C-1	TCGCATGGGGCGTCGGAGCT	CCCGAGATAGTGGTCGTAGGC
	9C-2	GGTGCTAAGCCCCTCGCTG	GCCGACGAGTACATGTTGGC
	9C-3	ACGGCTACCACCCGCACG	TATTCCTTGCTTGCCCTTGG

ZRS, ZPA regulatory sequence; *LMBR1*, limb region 1 homolog; *TBX1*, T-box-1.

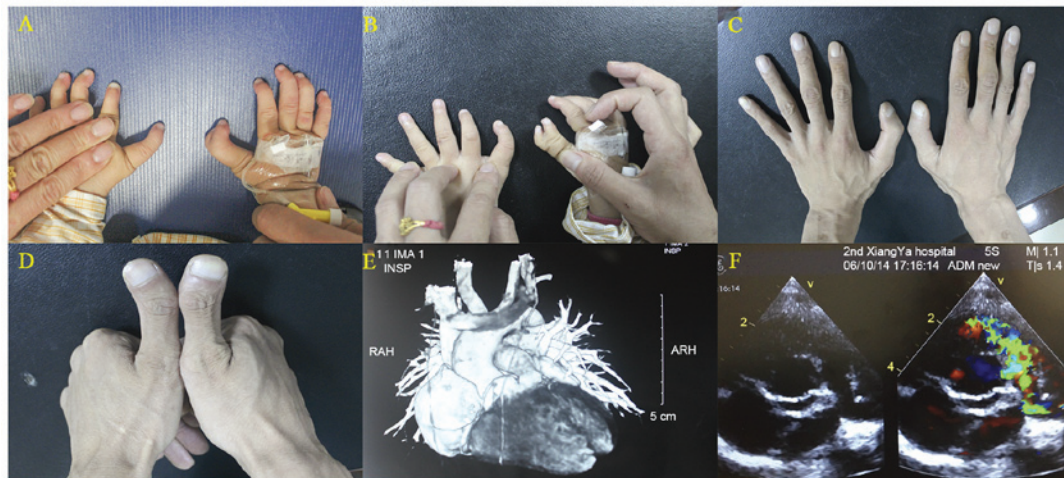


Figure 1. Clinical features. (A, B) The patient has triphalangeal thumbs on both hands and polysyndactyly in right thumb. (C, D) Her father has triphalangeal thumbs of both hands. (E) Computed tomography angiography and (F) echocardiogram of the patient indicate the Tetralogy of Fallot.

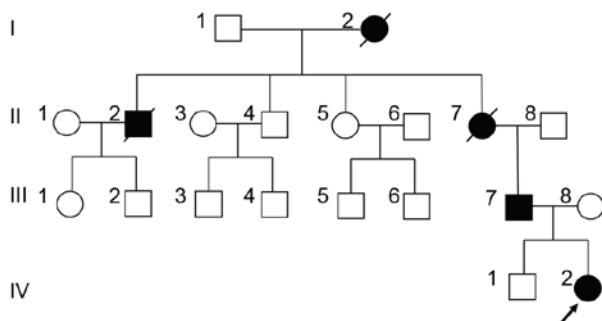
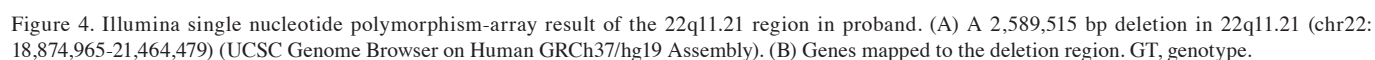
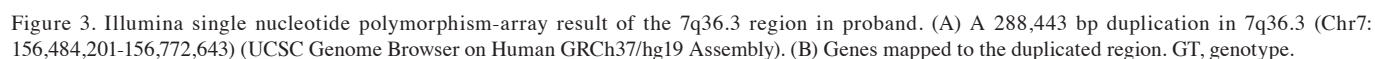


Figure 2. Pedigree of the triphalangeal thumb-polysyndactyly syndrome family. Squares indicate male members, circles, female; a symbol with a slash, the deceased members; closed symbols, the affected members; open symbols, unaffected members. The arrow indicates the proband, who presents with Tetralogy of Fallot as well.

established by the identification of a number of the different point mutations within ZRS, including in polydactylous cats, in mouse models of PPD, and in human PPD families (5-7,14). Duplication has been shown to be a major type of CNV in numerous human genetic diseases (15). One possible explanation is a gain of function mechanism leading to an augmented SHH expression in the limb bud. The pathogenicity of the duplication involving ZRS may be further supported by a previous study (16) in the Sasquatch mutant mouse whose phenotype is an isolated pre-axial polydactylosis. This mutant was generated by the transgenic insertion of a reporter gene in intron 5 of *Lmb1* and has been shown to have a 24 kb intronic duplication containing the mouse ZRS (5). In 2008, Klopocki *et al* (10) first reported the identification of a genomic



duplication containing ZRS in a large German family with TPT-PS. In the same year, Sun *et al* (11) identified six duplications in six families with TPT-PS. These independent findings and the results of the present study consistently implied that the duplication of ZRS long range enhancer is the molecular pathogenesis of TPT-PS. It is hypothesized that disruption of *cis*-regulator via ZRS duplication results in the dysregulation of SHH, leading to TPT-PS phenotypes.

The deletion of chromosome 22q11.21 in the patient reported in the present study spans 2,589,515 bp, containing *TBX1*, *HIRA*, *RTN4R* and numerous other genes. Chromosome 22q11.2 deletion syndrome (DiGeorge syndrome; OMIM #188400) is one of the most common genetic syndromes, with a prevalence of 1:4,000 to 1:6,000 (17,18). At least 30 genes have been mapped to the deleted region, among them *TBX1*, a major genetic determinant of the 22q11.2 deletion syndrome (19). The phenotypic spectrum is varied, including CHD, velopharyngeal insufficiency and cleft palate facial anomalies, immune disorders, and hypocalcemia secondary to hypoparathyroidism (20). The most common cardiac defects are conotruncal anomalies, including TOF, TOF with pulmonary atresia, truncus arteriosus and interrupted aortic arch (21). In the present study, the patient was diagnosed as TOF, but presented neither obvious facial anomalies nor immune deficiencies. Her parents did not harbor the 22q11.21 deletion and did not have CHD. It is inferred that it was the patient's deletion of 22q11.21 that caused the CHD and TOF. This is the first report, to the best of our knowledge, on a co-existence of 7q36.3 duplication with 22q11.21 deletion in a syndromic CHD.

In conclusion, the present study described a duplication of 7q36.3 containing the ZRS long range enhancer of SHH in a family with distinctive phenotypic features of TPT-PS. Additionally, a *de novo* 22q11.21 deletion containing *TBX1* was detected in the proband who is the unique member of the family with TOF, a type of CHD. The findings of the present study indicated that 7q36.3 duplication encompassing ZRS region is the underlying genetic cause of TPT-PS, and in addition, corroborate that 22q11.21 deletion is a genetic cause of CHD.

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