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MUTATION IN BRIEF

Expanding the Spectrum of *TBX5* Mutations in Holt-Oram Syndrome: Detection of Two Intragenic Deletions by Quantitative Real Time PCR, and Report of Eight Novel Point Mutations

Wiktor Borozdin[1], Ana M. Bravo Ferrer Acosta[1], Michael J. Bamshad[2], Elke M. Botzenhart[1], Ursula G. Froster[3], Johannes Lemke[4], Albert Schinzel[4,5], Stephanie Spranger[6], Julie McGaughran[7], Dorothea Wand[8], Krystyna H. Chrzanowska[9], and Jürgen Kohlhase [1*]

[1]Institut für Humangenetik und Anthropologie, Universität Freiburg, Freiburg, Germany; [2]Dept. of Pediatrics, Division of Genetics & Developmental Medicine, University of Washington School of Medicine, Seattle, Washington; [3]Institut für Humangenetik, Universität Leipzig, Leipzig, Germany; [4]Institute of Medical Genetics and [5]Department of Pediatrics, University of Zurich, Zurich, Switzerland; [6]Praxis für Humangenetik, St.-Jürgen-Strasse, Bremen, Germany; [7]Queensland Clinical Genetics Service, Royal Children's Hospital and Health District, Brisbane, Queensland, Australia; [8]Institut für Humangenetik und Medizinische Biologie, Universität Halle-Wittenberg, Halle/ Saale, Germany; [9]Department of Medical Genetics, Children's Memorial Health Institute, Warsaw, Poland

*Correspondence to: PD Dr. J. Kohlhase, Institut für Humangenetik und Anthropologie, Universität Freiburg, Breisacher Str. 33, 79106 Freiburg, Germany; Tel.: +49 761 2707050; Fax: +49 761 2707041; E-mail: juergen.kohlhase@uniklinik-freiburg.de

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Mutations in the gene *TBX5* cause Holt-Oram syndrome (HOS), an autosomal dominant disorder characterized by anterior (i.e., radial ray) upper limb malformations and congenital heart defects and/or cardiac conduction anomalies. The detection rate for *TBX5* mutations in HOS patients has been given as 30-35% in most reports. However, a detection rate of 74% was reported when strict clinical inclusion criteria for HOS were applied prior to *TBX5* analysis. Still, in a significant proportion of typical HOS cases no mutation can be found within the *TBX5* coding region and flanking intronic sequences. One explanation could be that large but submicroscopic deletions of *TBX5* could cause HOS, yet only one such *TBX5* deletion has been reported to date. We developed a quantitative Real Time PCR strategy to detect large, submicroscopic deletions in *TBX5*. Using this assay, we screened a total of 102 *TBX5* mutation negative patients and discovered two novel intragenic deletions. One deletion of 7756 bp removes exon 6 and a considerable part of the neighboring intronic sequences, and the other of 3695 bp removes exon 9 with the stop codon and the 3'UTR completely as well as a part of the preceding intron 8. We conclude that quantitative Real Time PCR is a reliable method to detect submicroscopic deletions within *TBX5*. However, such deletions explain only ~2% of of the *TBX5* mutational spectrum in HOS cases. In addition, we also present eight novel *TBX5* mutations (three nonsense, one splice mutation, four short deletions) as detected by direct sequencing in 21 families not previously analyzed for mutations. © 2006 Wiley-Liss, Inc.

KEY WORDS: TBX5; Holt-Oram syndrome

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INTRODUCTION

Holt-Oram syndrome (HOS; MIM# 142900) is an autosomal dominant malformation syndrome characterized by cardiac malformations and defects of the anterior elements of the upper limb (Newbury-Ecob et al. 1996). Cardiac phenotypes include a wide spectrum of congenital heart defects (Newbury-Ecob et al. 1996; Sletten and Pierpont 1996; Basson et al. 1999), with secundum-type atrial septal defect (ASD) and ventricular septal defect (VSD) being the most common. Conduction disturbances are frequent. The upper limb anomalies in HOS vary widely and range from absence of the thumb to triphalangeal thumb although hypoplasia of thenar eminences or limited supination of the forearm are the mildest defects observed (Newbury-Ecob et al. 1996). HOS is caused by mutations in *TBX5*, a transcriptional activator of the T-Box family located on chromosome 12q24 (Basson et al. 1994; Bonnet et al. 1994; Basson et al. 1997; Li et al. 1997; Bruneau et al. 2001).

Mutations in the *TBX5* (MIM# 601620) gene have been found in 30-35% of familial and sporadic cases with HOS (Cross et al. 2000; Brassington et al. 2003). Recently, a detection rate of 74% was reported following application of more stringent clinical criteria for HOS. For example, patients with malformations of the kidneys, suggesting an alternative diagnosis such as Okihiro or Fanconi syndrome, were excluded (McDermott et al. 2005). Neither the type nor the location of a mutation in *TBX5* appeared to predict type and/ or severity of malformations in individuals with HOS (Brassington et al. 2003).

Another possible reason for the low detection rate of *TBX5* mutations is that HOS is genetically heterogeneous. However, with the exception of a single HOS pedigree in which linkage to chromosome12q24 was excluded (Terrett et al. 1994), there is little evidence that genetic heterogeneity among HOS cases is common. Alternatively, the low detection rate could also reflect that most cases of HOS have not been screened for large deletions or mutations in *TBX5* regulatory regions (McDermott et al. 2005). To our knowledge, only one study to date has screened *TBX5* for deletions in HOS cases without *TBX5* sequence variants (Akrami et al. 2001). This study applied *multiplex amplifiable probe hybridization* (MAPH) to 20 patient samples and detected one deletion that spanned exons 3 to 9. However the exact size of the deletion could not be determined. In this study, we analyzed a cohort of HOS patients for *TBX5* mutations and used quantitative Real Time PCR with SYBR-Green I to detect and map deletions in the *TBX5* mutation negative cases.

MATERIALS AND METHODS

Patients

Patients from 21 unrelated families with the clinical diagnosis of Holt-Oram syndrome were analyzed for *TBX5* mutations and - if negative - for *SALL4* mutations, because *SALL4* mutations may in some cases lead to a phenotype indistinguishable from Holt-Oram syndrome (Kohlhase et al. 2003). The 12 families of those who were negative for mutations in either gene together with patients from additional 90 unrelated families (a total of 102 patients) with the clinical diagnosis of Holt-Oram syndrome but without detectable *TBX5* and *SALL4* mutations (as analyzed previously) were investigated in this study for the presence of a *TBX5* deletion. Inclusion criteria for mutation analysis were presence of typical anterior upper limb malformations with or without congenital heart defects or cardiac conduction anomalies, and no other evident clinical diagnosis. Inclusion criteria for deletion analysis were (1) presence of typical anterior upper limb malformations with or without congenital heart defects or cardiac conduction anomalies, (2) no other evident clinical diagnosis, and (3) previously negative *TBX5* and *SALL4* mutation analysis by direct sequencing. Venous blood was collected from patients and unaffected relatives after obtaining informed consent.

Mutation analysis

Mutation analyses of *TBX5* and *SALL4* were performed by PCR and direct sequencing as described (Kohlhase et al. 2002; Brassington et al. 2003). Mutations were numbered according to the *TBX5* cDNA sequence with the A of the ATG start codon being +1.

Quantitative PCR

TBX5 deletion detection and fine mapping of the deletions was performed using a quantitative PCR approach with SYBR-Green I detection (Boehm et al. 2004; Borozdin et al. 2004; Laccone et al. 2004). Using the PRIME program (Genetic Computer Group, Wisconsin, USA) we designed 8 primer pairs for amplicons positioned in exons 1, 2, 6, 8, 9 and in introns 3, 6, and 7 of *TBX5*. Six additional primer pairs were designed in order to map

larger deletions (Fig. 1C; primer sequences available on request). Three amplicons mapping to subtelomeric regions (2q24.2, 3p26.3, 4p15.2) were used as internal reference controls (Boehm et al. 2004; Borozdin et al. 2004). Genomic DNA from an unrelated individual without malformations was used as a deletion-negative control. *TBX5* genomic sequence including 100 kb upstream and downstream of the coding region was retrieved from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/) and masked for repeats (http://www.woody.embl-heidelberg.de). Amplicons were mapped relative to the *TBX5* coding region (Fig. 1C). The investigated genomic region comprises about 260 kb, approximately 100 kb on the 5' and on the 3' side of the *TBX5* gene (101-154 kb). The physical location of this region is 113150000 - 113409999 bp for the complete sequence (exactly 260 kb), and 113254456 – 113308967 bp for the *TBX5* coding region, calculated from the telomere of the p-arm of chromosome 12. Since *TBX5* is transcribed from telomere to centromere on the q-arm, the startpoint of the analyzed sequence (bp 0 = bp 113409999) is more telomeric, and the end (bp 260,000 = bp 113150000) more centromeric. For real time PCR we used the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Darmstadt, Germany) and white-colored 384-well plates (ABgene, Hamburg, Germany). Reactions contained 0.25 mM each primer and 5 µl QuantiTect˙ SYBR® Green PCR Master Mix (Qiagen, Hilden, Germany) in a total volume of 10 µl. Assays included DNA standards in a final concentration of 5.0, 2.5, 1.25, or 0.625 ng/µl, a no-template control, or 2.5 ng/µl of the patient DNA in replicates (n=6). Cycling conditions were 50°C for 2 min, 95°C for 15 min, and 40 cycles of 94°C (15 sec), 60°C (15 sec), and 72°C (1 min). For all amplicons the same conditions were applied. In order to avoid the generation of unspecific products, a melting curve analysis and agarose gel electrophoresis of products was performed routinely following the amplification. A standard curve was constructed for each amplicon by plotting the cycle number (ct), at which the amount of target in standard dilutions reaches a fixed threshold, against the log of the amount of starting target. Absolute quantification of target amplicons in the patients was performed by interpolation of the threshold cycle number (Ct) against the corresponding standard curve. Quantitative data were further normalized against a normal diploid reference genome by calculating the ratio relatively to the average amount of reference amplicons for each amplicon. In this manner ratio-values of 1.0 indicate a diploid situation, values of 0.5 or 1.5 indicate partial haploidy or partial triploidy, respectively (Table 1).

Breakpoint cloning

In order to determine the exact size of the deletion, long range PCR was performed with forward and reverse primers of the amplicons located in the non-deleted regions neighboring the deletions. For cloning of the breakpoints in families 1 and 2, two additional primer pairs (sequences available on request) were designed for amplification of the breakpoint-spanning regions with the TaKaRa LA Taq™ (TaKaRa Bio Inc. Otsu, Shiga, Japan). The reaction contained 0.5 µg of genomic DNA, 5 µl LA PCR Buffer, 5 µl $MgCl_2$ (25 mM), 8µl dNTPs (2.5 mM each), 20 pmol of each primer and 2.5 units of TaKaRa LA Taq™ enzyme to a total volume of 50 µl. An initial denaturation step at 94°C for 1 min was followed by 30 cycles of 94°C for 30 sec and 68°C for 5 min. The reaction terminated with a final elongation step at 72°C for 10 min. The PCR products were subcloned into pGEM-T Easy vector (Promega, Heidelberg, Germany) for further analysis. Plasmids were isolated from bacterial colonies by routine methods and sequenced using T7 and SP6 primers, respectively.

Electronic database information

Accession numbers and URLs for data in this article are as follows: GenBank, http://www.ncbi.nlm.nih.gov (for *TBX5* mRNA sequence [NM_000192.3], genomic contig including *TBX5* [NT_009775.15]). Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/OMIM (for Holt-Oram syndrome [OMIM 142900], Repeat-masker at EMBL, http://www.woody.embl-heidelberg.de. *TBX5* mutation database (Heinritz et al. 2005), http://www.uni-leipzig.de/<genetik/TBX5.

RESULTS AND DISCUSSION

TBX5 mutation analysis in 21 index patients revealed 9 *TBX5* mutations, 8 of which have not been previously described to our best knowledge (Table 1, original data not shown but presented for review). The mutation c.100dupG found in one additional family has been reported before as c.100_101insG (Brassington et al. 2003). Seven mutations are truncating (3 nonsense mutations, 4 small deletions), and one splice donor site mutation is expected to result in exon skipping or activation of a cryptic splice site, and as such all mutations are predicted to cause the HOS phenotype via *TBX5* haploinsufficiency. The phenotype of the patients with point mutations is also given in Table 1. Interestingly, patient 667-1 had unilateral anophthalmia in addition to HOS, which is unlikely a

consequence of the *TBX5* mutation and might indicate a concurrent mutation of another gene. Of the remaining 12 *TBX5* mutation negative index patients, none was found to carry a *SALL4* mutation.

These 12 cases and 90 additional patients were investigated for *TBX5* deletions. Two deletions were detected. In family 1 (419-1,3; phenotypic features in Table 1), quantitative Real Time PCR revealed a heterozygous deletion (Fig. 1C; Table 2) of the exon 6 amplicon but not of the amplicons in intron 3 and 3' in intron 6. The deletion was present in the DNA of the index patient, but not in her mother and her brother, both unaffected. Two further amplicons 2 kb up- (intron 5) and downstream (5' in intron 6) of exon 6 were also found to be deleted. The minimal deletion size was estimated as 4.5 kb. Interestingly, in the father we consistently detected normalized ratios (*TBX5* amplicon/ reference amplicon) of 0.7 to 0.8. He would have been expected to carry a full mutation since he meets the diagnostic criteria for HOS, but the fact that a reduction by only 20 to 30 % was found suggests that he is very likely mosaic for the deletion with a much higher rate of cells carrying the deletion in the affected tissues, i.e. in the upper limbs and the heart. In order to determine the exact size of the deletion, long range PCR was performed, and we amplified a deletion-spanning fragment of approximately 4 kb (wild type 12 kb) from the index patient. Sequencing of the subcloned PCR fragment (Fig. 1D) with flanking primers revealed a deletion size of 7756 bp, containing 2440 bp of intron 5, the entire exon 6 (153 bp) and 5163 bp of intron 6 (c.511-2440_c.663+5163del7756). The 3' breakpoint is situated within an AluYa5 repeat but the 5' breakpoint does not lie within or next to a predicted repetitive element.

In order to prove mosaicism in the father of the index patient, we designed a new amplicon spanning the breakpoints of the deletion. By Real Time PCR, the index patient, her father and her mother were tested for this amplicon as well as for the exon 6 amplicon within the deletion. Since an unaffected person would not show amplification of the deletion-spanning product, the index patient's DNA was set as reference. As expected, the index patient showed ratios of 0.99/1.03 for the deletion-spanning amplicon and of 0.96/1.00 for the exon 6 amplicon. Her father showed ratios of 1.51/1.59 for the exon 6 amplicon consistent with the previous experiments. However, he also showed ratios of 0.37/0.41 for the deletion-spanning amplicon, confirming that he indeed carries the deletion in a mosaic state. The mother showed ratios of 2.06/2.08 for the exon 6 amplicon as expected, and 0.02/0.01 for the deletion-spanning amplicon (background).

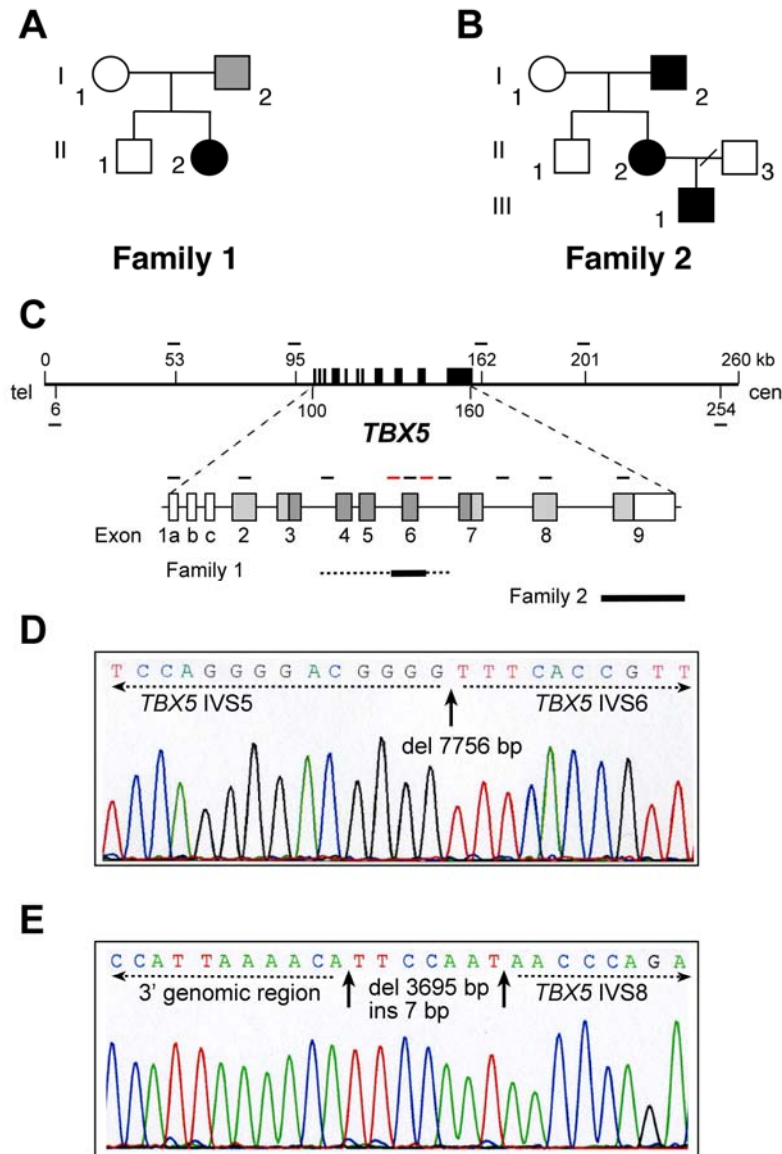

Figure 1. Pedigrees of the families, in whom deletions were detected (**A**,**B**). In family 1, the daughter (A; II.2) is clearly affected with Holt-Oram syndrome. The father (A; I.2) is marked with a grey symbol because he is clinically affected, but Real Time PCR results indicated that he is mosaic for the deletion. In family 2 (B), the male index patient (B; III.1), his mother (B; II.1) and her father (B; I.2) are affected with typical Holt-Oram syndrome (cardiac and radial ray malformations). Schematic diagram (**C**) of the *TBX5* genomic region on chromosome 12q24.1 with positions of the detected deletions. 260 kb of genomic DNA sequence (line) consisting of the *TBX5* gene plus 100 kb flanking sequence on each side were analyzed by quantitative Real Time PCR for deletions. The little horizontal bars indicate the positions of amplicons. 6 kb in the drawing corresponds to 113,40 Mb in the genomic contig, 53 kb to 113,35 Mb, 95 kb to 113,31 Mb, 162 kb to 113,24 Mb, 201 kb to 113,20 Mb, 254 kb to 112,46 Mb. The boxes on top of the line indicate the *TBX5* exons enlarged below with grey indicating coding and white UTR regions (darker grey indicates the T-Box). In family 1, the deletion is positioned between the preserved amplicons of exon 2 and intron 7. Two further amplicons, 2 kb up- (intron 5, red) and downstream (5' in intron 6, red) of exon 6 were also found to be deleted, whereas the two neighboring amplicons located 6 kb up- (5' of exon 4) and downstream (3' in intron 6) were preserved. The deletion in family 1 is 7756 bp in size and removes *TBX5* exon 6 as well as flanking sequences of IVS 5 and IVS 6 (**D**). In family 2, the 3695 bp deletion removes the entire exon 9 as well as 1.3 kb of IVS 8 and 1.6 kb the 3' sequence. Reverse complementary (in relation to *TBX5* sense strand) sequence (**E**) of the breakpoints in family 2 with the 5' breakpoint residing in IVS8 and the 3' breakpoint located 3' of the *TBX5* gene. *TBX5* mRNA sequence [NM_000192.3], genomic contig including *TBX5* [NT_009775.15]).

Table 1. Phenotypic features of patients with *TBX5* point mutations and larger deletions

Patient	Description	Base change	Protein	Heart defect	Hands	Arms	Other
667-1	Index patient, female	c.100dupG	p.G33*fs*	AV dissociation	HPL thumb (B)	ND	Anophthalmia (L)
667-2	Brother	c.100dupG	p.G33*fs*	ASD, MVI	Prolonged MC I	ND	ND
667-3	Father	c.100dupG	p.G33*fs*	ASD	Anomalies, NS	ND	ND
603-1	Index patient, male	c.451C>T	p.Q151X	ASD II, MVI II, AVB I	TDT (L) with UD distal phalanx	normal	none
619-1	Index patient, male	c.504delT	p.F168*fs*	ASD II VSD, PDA	TT with UD distal phalanx (R) HPL TDT (L)	normal	ASG with limited range of motion
619-2	Mother	c.504delT	p.F168*fs*	MVP+TVP+ regurgitation	AT (R) HPL thumb (L)	Shortened forearms LPS	ASG with limited range of motion
666-1	Index patient, female	c.641delG	p.V214*fs*	normal	HPL thumb (B) RD (B)	ND	ND
666-2	Son	c.641delG	p.V214*fs*	AVSD	HPL thumb (B) RD (B)	ND	ND
666-3	Fetus (abortion)	c.641delG	p.V214*fs*	ND	ND	ND	ND
668-1	Index patient, male	c.755+2T>C	unclear	ND	HPL thumb (B) Stiff thumb (B)	Shortened forearms LPS	ND
668-2	Paternal uncle	c.755+2T>C	unclear	VCC	Short thumb (B)	Shortened forearms	
668-3	Son of 668-2	c.755+2T>C	unclear	ND	ND	ND	ND
668-4	Paternal grandmother	c.755+2T>C	unclear	ND	ND	ND	ND
620-1	Index patient, female	c.873C>A	p.Y291X	VSD	HPL thumb (R), TDT (L)	HPL radius (B) LPS	none
620-2	Mother	c.873C>A	p.Y291X	ASD	BD II-V (B)	LPS	none
370-1	Index patient, male	c.939delG	p.I313*fs*	ASD	DT (B)	LPS	HPL chest wall
370-2	Daughter	c.939delG	p.I313*fs*	ASD, VSD	UD of distal phalanx (I, L)	LPS	ND
649-1	Index patient, female	c.1024delT	p.P341*fs*	ASD, VSD	AT (B), RD (B)	Humerus HPL, radius DPL (L), APL (R)	none
706-1	Index patient, male	c.1366C>T	p.Q456X*s*	PDA	TT (L), HPL TT(R)	normal	none
419/1	Index patient, female	IVS5_IVS6del	unclear	ASD, VSD	HPL TT (R) HPL Thumb (L)	Clavic. hook (R) HPL radius L>R	none
419/3	Father	IVS5_IVS6del	unclear	none	Thenar HPL (B)	normal	Sinus bradycardia
UT1	Index patient, male	IVS8_EX9del	unclear	Multiple ASDs	AT (B)	Radius APL (L), HPL (R), narrow chest, sloping shoulders	None
UT2	Mother	IVS8_EX9del	unclear	Large ASD	AT (L), TT (R)	Humerus HPL (B), radius HPL (B), narrow chest, sloping shoulders	None

Abbreviations: APL: Aplastic; ASD: Atrial septal defect; ASG: abnormal shoulder girdle; AT: Absent thumb; AVSD: atrial-ventricular septal defect; B: bilateral; BD: Brachydactyly; DPL: dysplastic; DT: digitalized thumb; HPL: hypoplastic; L: left; LPS: Limited pro- and supination of forearms; MC: metacarpal; MVI: Mitral valve insufficiency; MVP: Mitral valve prolapse; ND: No data; NS: not specified; PDA: patent ductus arteriosus at birth; R: right; RD: Radial deviation; SD: Syndactyly; TDT: Triphalangeal digitalized thumb; TT: Triphalangeal thumb; TVP: Tricuspid valve prolapse; UD: Ulnar deviation; VCC: ventricular coronary communication; VSD: Ventricular septal defect; Digits are numbered from I (anterior i.e., thumb) to V (posterior). Mutation numbering according to *TBX5* cDNA sequence [NM_000192.3] with the A of the ATG start codon being +1.

In family 2 (UT1,2; phenotypic features in Table 1) both the affected proband and his affected mother were found heterozygous for a deletion of the exon 9 amplicon but not of the neighboring amplicons by quantitative Real Time PCR. The deletion size could therefore be determined as between 2 and 15 kb (Fig. 1C). By long range PCR, we amplified a deletion-spanning fragment of approximately 1.4 kb (wild type 5.1 kb) from the index patient. Sequencing of the subcloned PCR fragment (Fig. 1D) with flanking primers revealed a deletion size of 3695 bp, containing 1296 bp of intron 8, the entire exon 9 (805 bp) and further 1594 bp 3’ genomic sequence. This

deletion was found in combination with a 7 bp insertion (c.985-1296_c.1557*1826del3695ins7; Fig. 1D). 5 bp upstream of the 5' breakpoint a mammalian-wide interspersed repeat (MIR) was found, but the 3' breakpoint does not lie within or next to a predicted repeat.

None of the deletions found have yet been reported, and they are considered to be pathogenic because they remove larger parts of the coding region (family 1) and/ or the complete 3' UTR (family 2). To date, reports on larger deletions of *TBX5* have been relatively sparse, and to our knowledge only one previous report described a strategy to look for *TBX5* copy number changes in a cohort of HOS patients (Akrami et al. 2001). In this report, one deletion including exons 3-9 was found using MAPH among 20 patients negative for *TBX5* point mutations, but the exact breakpoints were not determined. Our results show that quantitative Real Time PCR is another reliable method to detect deletions within or including the *TBX5* gene. In our study, the detection rate seems lower, but a comparison is difficult because of (1) the low number of patients in the study by Akrami et al. and (2) the possible phenotypic and genetic heterogeneity of the patients investigated in our study. Nevertheless, both studies suggest that *TBX5* deletions are contributing only to a small extent (1.9% in our study and 5% in the previous study) to the mutational spectrum of *TBX5* in unselected patients with the diagnosis HOS. Further studies with a selected cohort of HOS patients diagnosed according to the suggested strict clinical criteria (McDermott et al. 2005) would be helpful in order to determine a more exact number on the contribution of deletions to the mutational spectrum of *TBX5*. A major problem with all deletion tests is that the likelihood to detect a deletion depends on the density and size of probes. Since we detected only deletions within *TBX5* but no deletions of the entire gene, it seems that our strategy could have missed some deletions involving only exons 3-5 or introns not covered by the selected amplicons. This problem, however, is common to all strategies developed to date for screening *TBX5*.

Table 2. Real Time PCR results

	Amplicon position							
Family	113,31 Mb	Ex 1 *TBX5*	Ex 2 *TBX5*	Ex 6 *TBX5*	Intr 7 *TBX5*	Ex 8 *TBX5*	Ex 9 *TBX5*	113,24 Mb
1	-	0,99	1,05	**0,49**	0,99	1,02	1,00	0,98
2	-	1,04	1,01	1,05	1,00	0,98	**0,49**	0,97
C	1,01	0,99	0,97	1,03	0,98	1,00	1,03	0,99

Results of the *TBX5* Real Time PCR applied to index patient DNA samples of two families diagnosed with Holt-Oram syndrome (families 1 and 2) as compared to an unaffected control person (C). The normalized ratios (designed amplicon/ reference amplicon) are presented. Values interpreted as a haploid situation (deletion) range from 0.44 to 0.53 whereas a diploid situation was assumed for values from 0.93 to 1.11. Bold lettering indicates a deletion. 113,31 Mb corresponds to 95 kb in Fig. 1C, 113,24 Mb to 162 kb.

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