Clinical and Molecular Evaluation of *SHOX*/PAR1 Duplications in Léri-Weill Dyschondrosteosis (LWD) and Idiopathic Short Stature (ISS)

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Context: Léri-Weill dyschondrosteosis (LWD) is a skeletal dysplasia characterized by disproportionate short stature and the Madelung deformity of the forearm. *SHOX* mutations and pseudoautosomal region 1 deletions encompassing *SHOX* or its enhancers have been identified in approximately 60% of LWD and approximately 15% of idiopathic short stature (ISS) individuals. Recently *SHOX* duplications have been described in LWD/ISS but also in individuals with other clinical manifestations, thus questioning their pathogenicity.

Objective: The objective of the study was to investigate the pathogenicity of *SHOX* duplications in LWD and ISS.

Design and Methods: Multiplex ligation-dependent probe amplification is routinely used in our unit to analyze for *SHOX*/pseudoautosomal region 1 copy number changes in LWD/ISS referrals. Quantitative PCR, microsatellite marker, and fluorescence *in situ* hybridization analysis were undertaken to confirm all identified duplications.

Results: During the routine analysis of 122 LWD and 613 ISS referrals, a total of four complete and 10 partial *SHOX* duplications or multiple copy number ($n > 3$) as well as one duplication of the *SHOX* 5′ flanking region were identified in nine LWD and six ISS cases. Partial *SHOX* duplications appeared to have a more deleterious effect on skeletal dysplasia and height gain than complete *SHOX* duplications. Importantly, no increase in *SHOX* copy number was identified in 340 individuals with normal stature or 104 overgrowth referrals.

Conclusion: MLPA analysis of *SHOX*/PAR1 led to the identification of partial and complete *SHOX* duplications or multiple copies associated with LWD or ISS, suggesting that they may represent an additional class of mutations implicated in the molecular etiology of these clinical entities. **(*J Clin Endocrinol Metab* 96: E404–E412, 2011)**

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Abbreviations: FISH, Fluorescence *in situ* hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; indel, insertions or deletions; ISS, idiopathic short stature; LWD, Léri-Weill dyschondrosteosis; MIM, Mendelian Inheritance in Man; MLPA, multiplex ligation-dependent probe amplification; PAR1, pseudoautosomal region 1; qPCR, quantitative real-time PCR; SDS, SD score; *SHOX*, short-stature homeobox-containing gene.

Léri-Weill dyschondrosteosis (LWD) [Mendelian Inheritance in Man (MIM) 127300] is a dominantly inherited skeletal dysplasia characterized by disproportionate short stature, mesomelic limb shortening, and the Madelung deformity of the forearm: the bowing of the radius and the distal dislocation of the ulna (1, 2). Idiopathic short stature (ISS; MIM 300582) is a condition defined as a height below −2 SD score (SDS) in the absence of known specific causative disorders (3). To date, heterozygous deletions of two distinct regions of the pseudoautosomal region 1 (PAR1), the short-stature homeobox-containing gene (*SHOX*; MIM 313865) (4, 5) and the downstream enhancer region (6–8) or point and small insertions or deletions (indel) mutations within *SHOX*, have been identified in up to 60% of LWD and approximately 15% ISS patients (9–11). Homozygous or compound heterozygous *SHOX* mutations as well as biallelic deletions of *SHOX* and/or the downstream PAR1 region result in a more severe phenotype, known as Langer mesomelic dysplasia (MIM 249700) (12–15). Conversely, *SHOX* overdosage, caused by either structural rearrangements or numerical abnormalities of the sex chromosomes, has been shown to be associated with normal to tall stature (16–18). Although the underlying pathological mechanism remains unknown, it has been proposed that the association of an extra copy of *SHOX*, especially with estrogen deficiency, results in tall stature through an abnormally prolonged growth period in the late teenage years and early adulthood (17). In contrast, *SHOX* duplications have been recently described in three LWD/ISS patients as well as in individuals with diverse clinical features, leading to uncertainty of their involvement in LWD/ISS etiology (19–22).

Until recently, the molecular diagnosis of LWD, Langer mesomelic dysplasia, and ISS patients was commonly undertaken by microsatellite and/or fluorescence *in situ* hybridization (FISH) of the *SHOX* encompassing PAR1 sequence (9, 10, 23). We and others (6, 9, 24–27) have shown the advantages of using multiplex ligation-dependent probe amplification (MLPA) for the identification of PAR1 deletions encompassing *SHOX* and/or the downstream enhancer region. This technique has permitted us now to identify 14 complete or partial *SHOX* duplications or multiple copies ($n > 3$) and one duplication upstream of *SHOX* in nine LWD and six ISS probands. We report our clinical and molecular findings on the largest number of *SHOX* duplications reported to date, with the aim to increase our knowledge of whether such alterations are involved in the etiology of LWD and ISS.

Patients and Methods

Clinical patients

The study was approved by the local ethical committees and all participants provided informed consent. The LWD and ISS patient samples were referred from endocrinology and genetic clinics. LWD patients were ascertained using the inclusion criteria of the presence of the Madelung deformity and mesomelic shortening of the limbs in the proband or a direct family member. Stature was recorded and SDS were determined according to the population standards for age and gender (28) (http://www.aepap.org/crecorbegozo.ht; http://www.magicfoundation.org/). ISS patients with stature less than −2 SDS were ascertained using the current consensus criteria (3).

Two control cohorts were also obtained and analyzed. A cohort of 340 normal controls, obtained from the Spanish DNA bank (University of Salamanca, Salamanca, Spain), with heights within the normal range for the Spanish population for age and gender ($-2 < SDS < +2$). The second control cohort consisted of 104 overgrowth referrals with heights greater than 3 SDS.

Peripheral blood was drawn from probands and, when possible, from relevant family members. All samples were reported to have a normal G-banding karyotype. Genomic DNA was isolated from whole blood using the salt precipitation method (blood kit; QIAGEN, Valencia, CA).

MLPA assay

MLPA analysis was carried out using the commercial *SHOX*/PAR1 MLPA kit (Salsas P018B-D1; MRC Holland, Amsterdam, The Netherlands) in accordance to the manufacturer's recommendations. In particular cases, *SHOX* duplications were further delimited using an additional MLPA assay including six novel probes, located between the Xp telomere and *SHOX* (kindly donated by Dr. Simon Thomas, Wessex Regional Genetics Service, Wiltshire, UK). The MLPA assays were analyzed with help of GeneMapper software (Applied Biosystems, Foster City, CA). Subsequently the ratios of the proband' peak areas *vs.* controls' samples were determined using an Excel data sheet (Microsoft Corp., Richmond, CA). Normal peaks were classified as showing a ratio of 0.65–1.35, whereas deletions and duplications were classified as having a ratio less than 0.65 or greater than 1.35, respectively. Each alteration was confirmed in an independent MLPA replicate.

Validation of SHOX copy number

Microsatellite analysis

Samples were analyzed for the presence of a triallelic pattern with the help of a panel of previously described PAR1 microsatellite markers: DXYS10137, DXYS10138, DXYS201, DXYS10092, DYS290, DXYS10093, and DXYS10083 (6, 24).

Quantitative real-time PCR (qPCR)

Three qPCR assays targeting sequences within *SHOX* and flanking regions were designed to confirm the *SHOX* copy number. Primer sequences and probes are described in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. The qPCR was performed in a final volume of 10 μl. The reactions, in duplicate, contained 1× LightCycler 480 probes master mix (Roche, Mannheim, Germany), 10 μM of each primer, 10 μM of both

TABLE 1. Clinical features of the 15 LWD/ISS probands with complete or partial *SHOX* or *SHOX* 5′ flanking duplications or multiple copies

Proband	Gender	LWD/ISS	Duplication type (extents)	Height SDS (adult or age in years)	MD (Y/N)	Other clinical characteristics
1	F	ISS	Complete	−2.57 (13.0)	N	Slightly shortened neck
2	F	ISS	Complete	−2.19 (adult)	N	
3	F	ISS	Complete	−2.29 (13.8)	N	Postponderal delay, dermatitis atopica
4	F	LWD	Complete	0.30 (adult)	Y	Shortening of the ulnas, cubitus valgus, articular enlarging of hands, articular pain in wrists, hips, and knees
5	F	ISS	Partial (5′-Ex3)	−2.01 (6.7)	N	
6	M	LWD	Partial (5′-Ex3)	−2.25 (13.0)	Y	
7	M	LWD	Partial (Ex2-6b)	−1.80 (10.7)	Y	
8	M	LWD	Partial (Ex2-6a)	−3.88 (15.5)	Y	
9	F	LWD	Partial (Ex2-6a)	−4.13 (5.0)	Y	Piloric stenosis, vesicle uretheral reflux
10	F	LWD	Partial (Ex2-6a)	−2.24 (13.0)	Y	Minimal cubitus valgus
11	M	LWD	Partial (Ex2-6a)	−2.06 (10.0)	Y	
12	F	LWD	Partial (Ex2-6a)	−3.95 (13.5)	Y	Menarche at 8 yr
13	F	LWD	Partial (Ex2-6a)	−1.88 (7.5)	N	Small for gestional age, premature adrenarche
14	F	ISS	Partial (Ex4-3′)	−2.40 (14.0)	N	Mental retardation, dysmorphic facies
15	F	ISS	5′ region	−2.10 (adult)	N	

F, Female; M, male; MD, Madelung deformity present (Y) or absent (N).

target (labeled with FAM) and calibrator [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), labeled with HEX] probes, and 10 ng of genomic DNA. The cycling conditions were as follows: a prerun at 95 C for 10 min, 45 cycles of 10 sec at 95 C, 30 sec at 60 C and 1 sec at 72 C, and finally a 40 C cooling step for 30 sec. A no-template negative control was included in each assay. The samples were amplified in a Lightcycler 480 system (Roche). Test samples and normal controls were first normalized to the GAPDH control and subsequently to the mean Ct value determined for five normal controls. Normal peaks were classified as showing a ratio of 0.65–1.35, whereas duplications were classified as having a ratio greater than 1.35.

FISH analysis

To determine if the detected extra copy or copies were the consequence of chromosomal translocations, we undertook FISH analysis on metaphase chromosomes prepared from peripheral blood lymphocytes by standard techniques (29). FISH was performed with cosmids from the distal PAR1, including two spanning the entire *SHOX*, LLNOYCO3′M′15D10 and LLNOYCO3′M′34F5 (30). Centromeric probes DXZ2 and DYZ3 were used as controls.

Exclusion of point mutations, small deletions, or insertions in SHOX

All LWD and ISS probands were excluded for *SHOX* mutations by denaturing HPLC, high resolution melting analysis, and/or DNA sequencing, as previously reported (9).

Results

Characterization of SHOX duplications

Four complete and 10 partial *SHOX* duplications or multiple copies ($n > 3$) as well as one duplication of the *SHOX 5′* flanking region were identified in nine LWD and six ISS probands (Table 1 and Fig. 1). Eight of nine LWD probands presented with partial *SHOX* duplications or multiple copies; one LWD proband had a complete *SHOX* duplication, whereas three of six ISS referrals presented with whole *SHOX* duplications; two presented with two different partial *SHOX* duplications, and one ISS proband presented with a duplication of the *SHOX 5′* flanking region (Fig. 2). No pathogenic PAR1 deletions or point or indel *SHOX* mutations were identified in the 15 probands. Proband heights and clinical details are shown in Fig. 1 and Table 1. No increases in *SHOX* copy number were detected in 340 normal height controls or 104 overgrowth referrals.

The complete *SHOX* duplications ranged in size from 38 to 346 kb, extending from the *SHOX 5′* flanking region through at least exon 6b in three ISS cases, and from the *SHOX 5′* flanking region through to approximately 100 kb downstream of *SHOX* in the single LWD case presenting with a complete gene duplication (Fig. 2). Partial *SHOX* duplications or multiple copies ranged from 13 to 294 kb, extending from the *SHOX 5′* flanking region

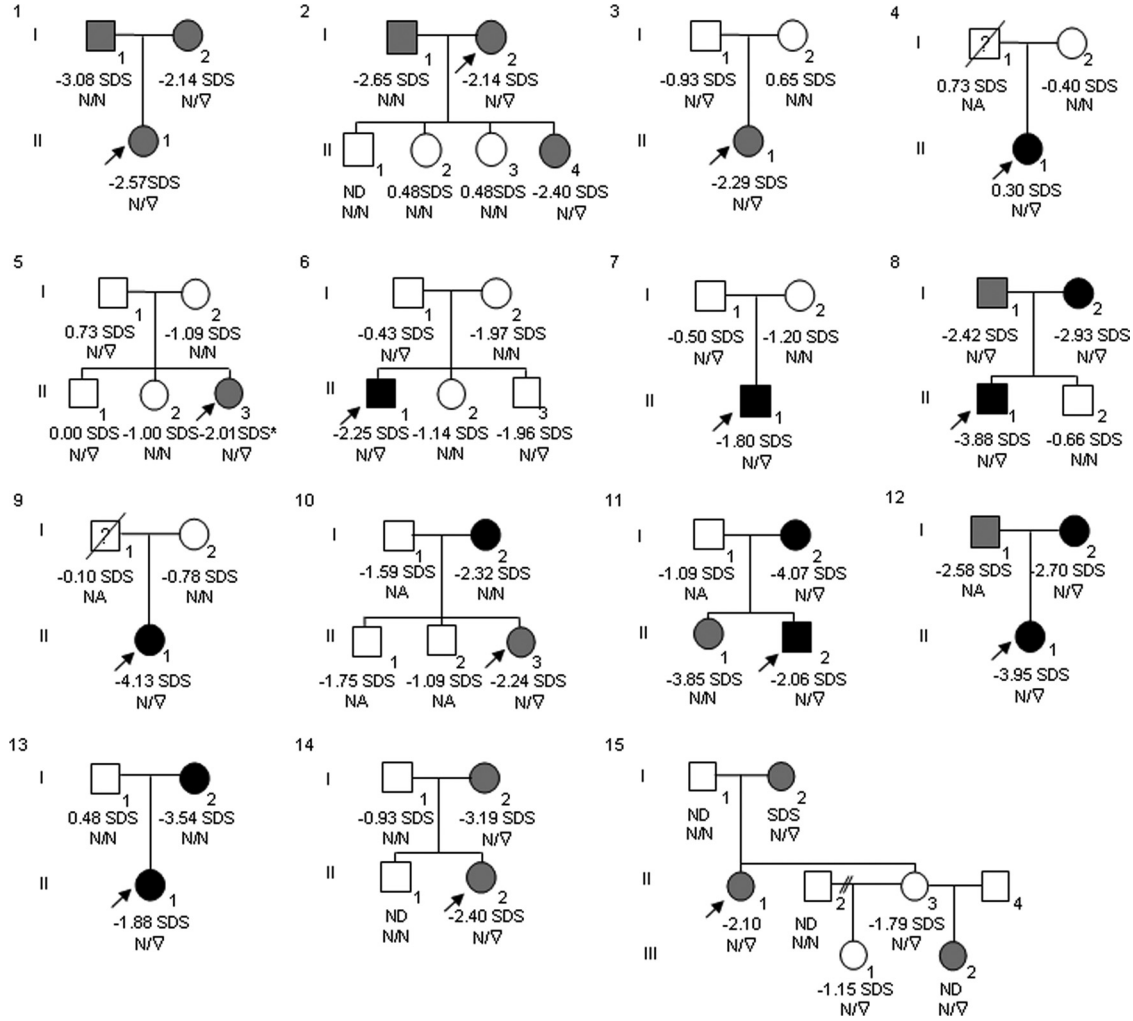

FIG. 1. Family pedigrees of the 15 LWD/ISS probands with duplications or multiple copy number of *SHOX* or the 5′ flanking region. The probands are indicated by the *arrow*. *Black-filled symbols* indicate the clinical diagnosis of LWD, whereas *gray-filled symbols* indicate the clinical diagnosis of ISS, *unfilled symbols* indicate normal stature and no dysplasia. The height SDS and *SHOX* copy number are shown below each individual. ND, Height not documented. Copy number status is indicated with *N* (normal, n = 2) and *inverted triangles* (increased *SHOX* copy number, n > 2). NA, No DNA available but heights were obtained from spouse or by telephone. In 11 cases the alterations were shown to be inherited (probands 1–3, 5–8, 11, 12, 14, 15). Paternity was confirmed by specific testing in proband family 13. Somatic mosaicism is suspected in I.2 of family 13. The inheritance of the duplication could not be ascertained in the remaining three cases due to lack of parental DNA samples (probands 4, 9, and 10). In family 15, II.3 has an inversion of chromosome 14q22-q24, and III.1 has mental retardation and premature adrenarche.

through to exon 3, in two cases (one LWD and one ISS); from exon 2 to exon 6a in six LWD cases; from exon 2 through to exon 6b in one LWD case; and from exon 4 to approximately150 kb downstream of *SHOX* in one ISS referral (Fig. 2). Proband 15 carried a duplication located upstream of *SHOX*, which did not include any *SHOX* coding sequence (Fig. 2). The duplication was at least 320 kb in size, extending from the most telomerically analyzed probe, P1 (~250 kb from telomere), to marker DYS201, 15 kb upstream of *SHOX*.

The height SDS of the eight LWD probands with partial *SHOX* duplications or multiple copies ranged between −4.13 and −1.80, whereas the single LWD who had a complete *SHOX* duplication (proband 4) had a height within the normal range (0.3 SDS). She presented with shortening of the ulnas, Madelung deformity, curving of the lower limbs, and cubitus valgus and complained of articular pain in the wrists, hips, and knees.

Six LWD referrals (probands 8–13) carried a similar duplication spanning *SHOX* exons 2–6a (Fig. 2). No *SHOX* haplotype was shared between these six probands. Interestingly, in one case (proband 8), both affected parents presented with a similar alteration as the LWD diagnosed proband (Fig. 1), although no common *SHOX* haplotype was shared between them (data not shown). PAR1 haplotype analysis confirmed that proband 8 had inherited the allele with the extra copies from his mother and shared the same nonduplicated paternal allele with his healthy brother, who had inherited the nonduplicated alleles form both parents. Probands 5 (ISS) and 6 (LWD) appear to share the same duplication extents, spanning from probe P2 in the *SHOX* 5′ flanking region to exon 3,

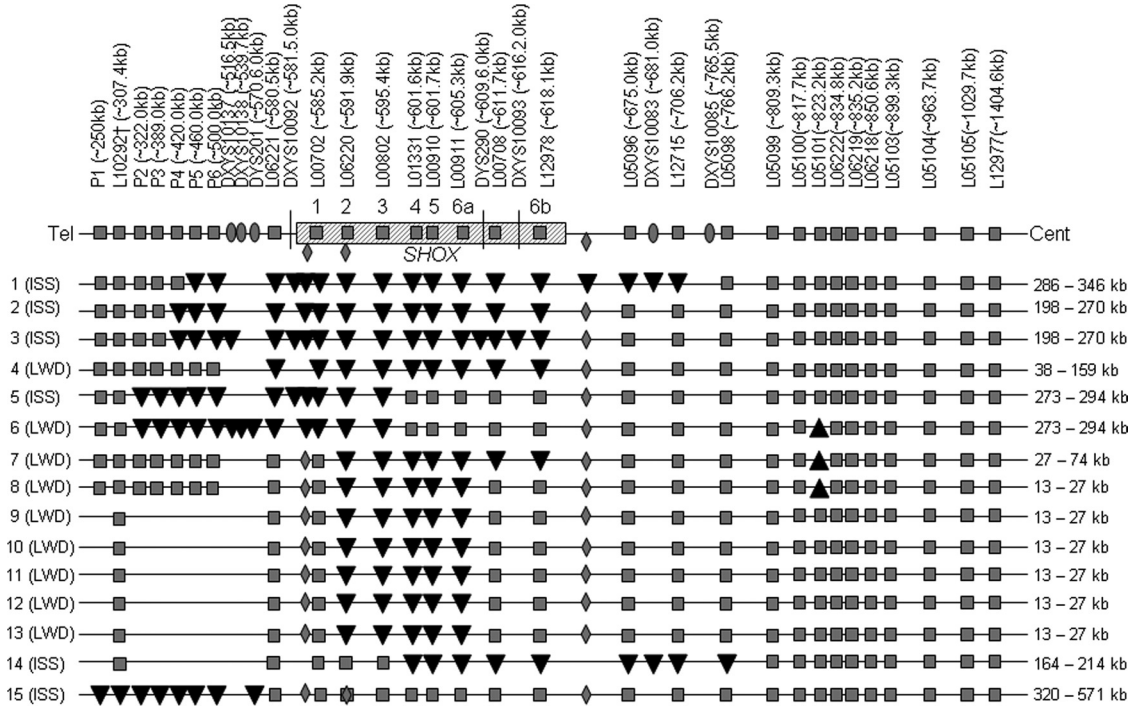

FIG. 2. Schematic representation of the genomic location and approximate extensions of the observed *SHOX* duplications in the 15 LWD/ISS probands, estimated from data obtained from MLPA, qPCR, and microsatellite marker analysis (diagram not drawn to scale). The approximate coordinates are according to chromosome X, National Center for Biotechnology Information (Bethesda, MD) assembly GRCh37. The MLPA probes are named according to kit P018D1 and are indicated by *gray boxes*, microsatellite markers are indicated by *gray ovals*, and qPCR amplicons are indicated by *gray diamonds*. ▼, An increased *SHOX* copy number ($n > 2$), determined by either MLPA or qPCR or by the observation of three alleles by microsatellite marker analysis. The duplication size range is indicated adjacent to each proband. ▲, The presence of a deletion in heterozygosity corresponding to MLPA probe L5101 observed in probands 6 and 8 and in homozygosity in proband 9. The deletion was characterized by fine-tiling Y-chromosome aCGH (Nimblegen Y chromosome specific array), and the breakpoint was subsequently characterized to have occurred between 820736 and 825695 on the X chromosome, National Center for Biotechnology Information assembly GRCh37/hg19. Part of the *AluY* sequence from the 3′ deletion end of the 4959-bp deletion is present (61 bp), which aligned with the *AluSc* sequence from the 5′ deletion end resulting in homologous recombination. The frequency of this 4959-bp deletion was not significantly different in ISS (11.6%, n = 613), normal-height controls (12.1%, n = 340), and overgrowth controls (4.8%, n = 104), indicating that this deletion represents a polymorphic copy number variant.

corresponding to a size of 273–294 kb (Fig. 2). Two unrelated ISS probands (probands 2 and 3), harboring a complete gene duplication, also appear to share the same 5′ and 3′ break points (Fig. 2).

Confirmation of duplications

SHOX copy number was confirmed by at least one alternative method, qPCR (probands 1–13, Fig. 2) and/or the observation of three alleles for one or more PAR1 microsatellite markers (probands 1, 3, 5, 6, 14, and 15, Fig. 2). The MLPA and qPCR data were concordant in 10 of 13 analyzed cases (Table 2) and differing in only one copy in two further cases (probands 8 and 9). Interestingly, three probands were observed, by MLPA and qPCR, to have multiple copies of the affected region (probands 8, 10, and 13; Table 2). Despite extensive optimization of the various techniques and multiple attempts and due to limitations of the quantification assays, the exact number of copies could not be determined for proband 13, which appeared to have the largest number of copies (5–18).

Fluorescence *in situ* hybridization

To investigate whether the extra copy/copies of *SHOX* were translocated to another chromosome, we undertook FISH analysis on metaphase chromosomes from probands 1, 4, 5, 8 (Supplemental Fig. 1), 12, and 13 and from both parents of proband 8 (Supplemental Fig. 1). No translocation was detected in any of the analyzed samples.

Discussion

We report on the largest collection of LWD or ISS cases associated with *SHOX* duplications or multiple copy number detected to date. For aiding the flow of reading, from this point on, we use the term *SHOX* duplications to include not only the 12 duplications but also the three cases that have multiple *SHOX* copies ($n > 3$). Four complete and 10 partial *SHOX* duplications as well as one duplication upstream of *SHOX* were detected in nine LWD and six ISS probands by MLPA and confirmed by

TABLE 2. *SHOX* copy number determination showing the comparison of qPCR and MLPA data

Proband	qPCR (5′ *SHOX*)	qPCR (*SHOX* ex2)	qPCR (3′ *SHOX*)	Average of duplicated qPCR	Average of duplicated MLPA probes	Number of copies
1	**1.62**	**2.05**	**1.70**	1.71	1.38	3
2	**(1.33)**	**1.90**	1.01	1.62	1.75	3
3	**1.57**	**1.45**	0.85	1.51	1.48	3
4	**1.34**	**1.55**	1.26	1.45	1.39	3
5	**1.45**	**1.67**	1.12	1.56	1.48	3
6	**1.39**	**(1.32)**	1.25	1.36	1.36	3
7	1.01	**1.77**	1.27	1.77	1.38	3
8	0.97	**3.54**	1.16	3.54	2.90	6–7
9	0.93	**2.12**	1.25	2.12	1.60	3–4
10	1.06	**2.18**	1.12	2.18	2.20	4
11	0.96	**1.49**	1.04	1.49	1.50	3
12	1.06	**1.37**	1.04	1.37	1.80	3
13	0.87	**8.06** (8.93, 7.19)	1.19	8.06	3.71 (2.51, 3.57, 5.07)[b]	5–18[a]

The qPCR amplicons with values above normal (*i.e.* more than two copies) are indicated in *bold*. Each qPCR was performed in duplicate and each assay was repeated twice. The test samples were normalized to the two copy GAPDH control gene. Each MLPA was repeated twice except for proband 13, which was repeated three times.

[a] The number of copies cannot be finely determined due to the large variability and the sensitivity limits of the assays. No qPCR was undertaken in proband 14 due to lack of DNA and in proband 15 because the duplicated region did not encompass any of the three amplicons. No qPCR was undertaken for proband 15 because the duplication did not include any of the analyzed regions.

[b] The average values of the qPCR and MLPA assays are shown for proband 13.

alternative methods. Importantly, no duplication was observed in 340 controls with normal height or 104 overgrowth referrals suggesting that they associate specifically with LWD or ISS.

SHOX duplications were identified in nine of 122 (7.3%) LWD and six of 613 (1.0%) ISS probands. No precise statistical analysis could be undertaken to compare frequencies between the LWD and normal height cohorts due to ascertainment bias and incomplete clinical data. Despite this and assuming the frequency of duplications to be equal in the normal population as the LWD/ISS cohort, we should have observed at least seven cases in the normal-height control cohort, if they would represent nonpathogenic copy number variants, and two in the overgrowth referrals, if they were associated with overgrowth.

When we compared the total number of duplications detected with the number of PAR1 deletions, encompassing *SHOX* or its enhancers, or *SHOX* mutations detected in our LWD/ISS cohort, the incidence of *SHOX* or *5′* flanking duplications compared with PAR1 deletions was significantly lower (15 *vs.* 49), a finding that is consistent with recent studies regarding the incidence of segmental duplications in other disorders (31, 32, 34, 35). In our laboratory, the frequency of PAR1 duplications is very similar to that of *SHOX* point mutations and indels (15 *vs.* 18).

Genotype/phenotype correlations

Partial *SHOX* duplications were more frequently associated with LWD (n = 8 probands) than ISS (n = 2). The heights of the LWD probands presenting with partial *SHOX* duplications varied between −4.13 and −1.80 SDS, whereas the single LWD proband who presented with a complete *SHOX* duplication had normal stature (0.30 SDS). This variability is not unexpected because it is well documented that SHOX haploinsufficiency due to *SHOX* deletion/mutations exerts a widely variable effect on LWD patient's height (−5.6 to 0.3 SDS) (30–32, 33–35). Interestingly, all six unrelated probands presenting with the overlapping partial duplication of *SHOX* exons 2–6a presented with similar, LWD characteristic dysplasic features, and height SDS varied between −4.13 and −1.88. Although no definitive conclusion can be drawn from our present data, partial *SHOX* duplications appear to be more deleterious than complete *SHOX* duplications, resulting more often in the fully penetrant LWD rather than ISS, suggesting an underlying molecular mechanism also resulting in SHOX haploinsufficiency.

The first intrachromosomal *SHOX* duplication was described in a LWD proband (31, 33). Recently complete *SHOX* duplications were identified in five further individuals: two with LWD (19, 21), one with ISS (22), and two with Asperger syndrome (19) or cleft lip palate, respectively (19). A large duplication was also recently reported in a patient referred for mild mental retardation, congenital abnormalities, and dysmorphic features (20). In our cohort, mental retardation and dysmorphic facies were also reported in the clinical records of ISS proband 14.

Recent articles have reported the detection of *SHOX* duplications by subtelomeric MLPA assay in clinical re-

ferrals unrelated with LWD or ISS (19, 20, 22). We reviewed the 745 clinical subtelomeric MLPA referrals that our diagnostic unit had carried out during the last 2 yr. Only two complete *SHOX* duplications were detected (0.27%). The first case was referred for behavioral disorders and psychomotor retardation. Both the proband and his unaffected carrier mother had normal heights, but no detailed auxological examination was undertaken. The second case identified was a prenatal referral due to the mother having a history of spontaneous abortions with cytogenetic aberrations.

Cosegregation analysis

Cosegregation of the duplication with the presence of short stature and Madelung deformity was apparent in three LWD families (probands 8, 11, and 12). LWD proband 8 inherited the allele with multiple partial *SHOX* copies from his mother, who shared classic clinical LWD features with her affected son, whereas the proband's brother, carrier of two normal *SHOX* alleles, has normal height, with an actual height SDS above the target height SDS (−0.66 *vs.* −2.83 SDS). We were unable to definitely determine cosegregation in six more LWD proband families (4, 6, 7, 9, 10, 11).

The increased copy number of *SHOX* exons 2–6a detected in proband 13 was not detected in genomic DNA extracted from his parents' lymphocytes suggesting that the multiple copies had either arisen *de novo* or germline mosaicism had occurred. Subsequently qPCR analysis was carried out on DNA extracted from a skin biopsy puncture sample from the affected mother. An increase in *SHOX* copy number was detected in the skin fibroblast DNA sample but absent in lymphocyte DNA, suggesting that somatic mosaicism had occurred. Although both mother and daughter presented with LWD, they show different degrees of affectation, the mother is shorter and has a milder form of Madelung deformity compared with her daughter. Analysis of a bone marrow sample from the mother would allow us to confirm this hypothesis.

Regarding the additional six ISS probands, although cosegregation is apparent in the family of probands 2 and 14, it could not be clearly demonstrated nor excluded in the families of probands 1, 3, and 15.

The assessment of cosegregation with the phenotype is often hampered in the absence of large families with a high number of affected and unaffected members. Furthermore, the large intra- and interfamiliar phenotypic variability observed in LWD (9, 36) is an additional hurdle for the cosegregation analysis of these patients.

Pathogenic mechanism

We propose that the clinical manifestation associated with partial and complete *SHOX* duplications is dependent on the physical localization of the duplicated sequence.

With regard to the apparent association between *SHOX* partial or complete duplications and LWD/ISS, we hypothesize that *SHOX* expression from the allele harboring the duplication is reduced or ablated, resulting in SHOX haploinsufficiency and thus clinically manifesting as LWD or ISS. This may occur through at least three possible mechanisms: 1) the extra whole or partial copy/copies disrupt the normal copy of *SHOX* and the inserted copy/copies is/are not functional because they lack the necessary regulatory sequences; 2) the extra copy/copies is/are present in tandem with the normal copy, either affecting *SHOX* regulatory sequences or modifying the distance between the gene and the enhancers or regulatory sequences; and 3) the extra copy/copies is/are localized further upstream or downstream from the normal copy, which would increase the distance between the normal copy and the regulatory regions. This model is partially supported by our own observations, as illustrated by case 15 findings: although no *SHOX* coding sequence was affected by the 5′ flanking duplication identified in proband 15, the duplication extended up to within 10–15 kb upstream of the *SHOX* promoters and may therefore have disrupted the interaction between the upstream enhancers and the *SHOX* promoters (37).

To be able to test these hypotheses, we should ideally analyze *SHOX* expression at the RNA and protein level in the bone marrow fibroblasts of these duplication carriers; however, none of the examined referrals have so far required surgical intervention.

In summary, the *SHOX*/PAR1 MLPA assay has proven to be an essential tool in the detection of copy number changes, including both deletions and now also duplications of *SHOX* or its flanking sequences in LWD/ISS referrals. Our results suggest that *SHOX* duplications may have an effect on human skeletal growth. We propose that an international registry should be initiated to further investigate the pathogenicity of PAR1 duplications encompassing *SHOX*. For this purpose, it would be essential to collect and document clinical and molecular data from not only the clinically affected but also their unaffected family members. This would help us to determine if *SHOX* duplications or increased *SHOX* copy number represent a novel class of mutations implicated in the molecular etiology of LWD and short stature.

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