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ARTICLE

Identification of a Gypsy SHOX mutation (p.A170P) in Léri-Weill dyschondrosteosis and Langer mesomelic dysplasia

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We report the clinical and molecular characteristics of 12 Spanish families with multiple members affected with Léri-Weill dyschondrosteosis (LWD) or Langer mesomelic dysplasia (LMD), who present the *SHOX* (short stature homeobox gene) mutation p.A170P (c.508G>C) in heterozygosity or homozygosity, respectively. In all studied families, the A170P mutation co-segregated with the fully penetrant phenotype of mesomelic limb shortening and Madelung deformity. A shared haplotype around *SHOX* was observed by microsatellite analysis, confirming the presence of a common ancestor, probably of Gypsy origin, as 11 of the families were of this ethnic group. Mutation screening in 359 Eastern-European Gypsies failed to identify any carriers. For the first time, we have shown SHOX expression in the human growth plate of a 22-week LMD fetus, homozygous for the A170P mutation. Although the mutant SHOX protein was expressed in all zones of the growth plate, the chondrocyte columns in the proliferative zone were disorganized with the chondrocytes occurring in smaller columnal clusters. We have also identified a novel mutation at the same residue, c. 509C>A (p.A170D), in two unrelated Spanish LWD families, which similar to A170P mutation impedes nuclear localization of SHOX. In conclusion, we have identified A170P as the first frequent *SHOX* mutation in Gypsy LWD and LMD individuals.
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INTRODUCTION

Léri-Weill dyschondrosteosis (LWD; MIM 127300) is a dominantly inherited skeletal dysplasia characterized by disproportionate short stature, mesomelic limb shortening and the Madelung deformity: bowing of the radius and distal dislocation of the ulna.[1] Langer mesomelic dysplasia (LMD; MIM 249700) represents a more severe clinical form with disproportionate short stature due to shortening of the mesomelic and rhizomelic limb segments, abnormality of the humeral head, angulation of the radial shaft, carpal distortion, short femoral neck and hypoplastic or absent proximal half of the fibula.[2]

SHOX (short stature homeobox gene, MIM 312865), localized within the pseudoautosomal region 1 (PAR1) of the X and Y chromosomes[3,4] encodes a transcription factor implicated in skeletal growth.[5] SHOX haploinsufficiency results in LWD, whereas total deficiency results in LMD.[6–8] Mutations include deletions of *SHOX*[9,10] or the downstream enhancer region,[11–14] partial or complete *SHOX* duplications,[15] or point or insertion/deletion mutations within *SHOX*.

The *SHOX* missense mutation p.A170P was originally identified in a large consanguineous Spanish Gypsy LWD/LMD family,[16] and subsequently reported by us in a second Spanish Gypsy family.[17] Alanine 170 is localized within the five amino acids of the homeodomain, which form the SHOX nuclear localization signal.[16,18] Functional studies showed that the mutant protein was unable to enter the nucleus, thus impairing its function as a transcription factor.[16]

During routine analysis of LWD/LMD referrals, we have identified a total of 12 Spanish families presenting with the p.A170P mutation, including the two previously described,[16,17] and two Spanish families with a previously undescribed mutation at the same residue, p.A170D. Functional analysis of the A170D mutant showed that, similar to A170P, nuclear translocation was impaired. We subsequently

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undertook haplotype analysis around *SHOX* in these families to investigate the possibility of common ancestry for each mutation or whether alanine 170 represents a mutation hotspot. A common haplotype around *SHOX* was observed for the 12 families with the A170P mutation suggesting a common ancestor, whereas no haplotype was shared between the two A170D individuals.

We had the opportunity to evaluate SHOX expression in the human growth plate of a 22-week LMD fetus, confirmed to be an A170P homozygote. The chondrocytes in the reserve zone appeared enlarged and in pairs, whereas the columns in the proliferative zone were disorganized.

In summary, we report the first founder SHOX mutation, A170P, in the Gypsy LWD population and the presence of another mutation at the same residue, A170D, in two LWD families.

SUBJECTS AND METHODS

Subjects

The study was approved by the local ethics committees from the various institutions. Informed consent for the study was provided by all the participants or their legal representatives. Genomic DNA was isolated from whole blood, amniotic fluid or paraffin-embedded tissue using the Blood core B kit (Qiagen, Valencia, CA, USA), Master Pure DNA purification kit (Epicentre, Illumina, Madison, WI, USA) or QIAamp DNA FFPE tissue kit (Qiagen), respectively. The pedigrees of the 12 A170P families are shown in Figure 1, whereas the clinical data is listed in Table 1. The height range of LWD and LMD A170P affected individuals was −1.7 to −5.6 SD and −8.2 to −10.2 SD, respectively. The transabdominal ultrasonagraphical data and the post-termination radiographs of the LMD fetus (family 11, IV.9) are shown in Supplementary Figure 1.

Three control cohorts were analyzed for the mutation or for the associated haplotype: (1) 88 'healthy unrelated' Spanish Gypsies, (2) 359 Eastern-European Roma individuals: 233 of whom belonged to the Balkan and 126 to the Vlax migrational category and (3) 173 Spanish control subjects with heights within the normal range for the Spanish population for age and gender ($-2<$SDS$<+2$), obtained from the Spanish DNA bank (University of Salamanca, Spain).

Mutation detection

Deletion and duplication analysis was carried out using the commercial *SHOX*/PAR1 MLPA Kit (MRC Holland, Amsterdam, The Netherlands) and in accordance to the manufacturer's recommendations. *SHOX* mutations were screened using various techniques: high-resolution melting analysis (HRM) in a LightScanner HR96 system (Idaho Technologies, Salt Lake City, UT, USA), dHPLC (WAVE 4500HT, Transgenomic, Omaha, MO, USA) and/or DNA sequencing (ABI 3130, Applied Biosystems, Foster City, CA, USA), as previously described.[17]

The A170P mutation was screened in the Eastern-European Roma population using HRM on the Lightcycler 480 (Roche Applied Bioscience, Roche, Mannheim, Germany). The PCR conditions were 1× Roche Lightcycler master mix, 3.0 mM $MgCl_2$, 0.2 μM of each oligonucleotide, 5% DMSO and 10 ng DNA

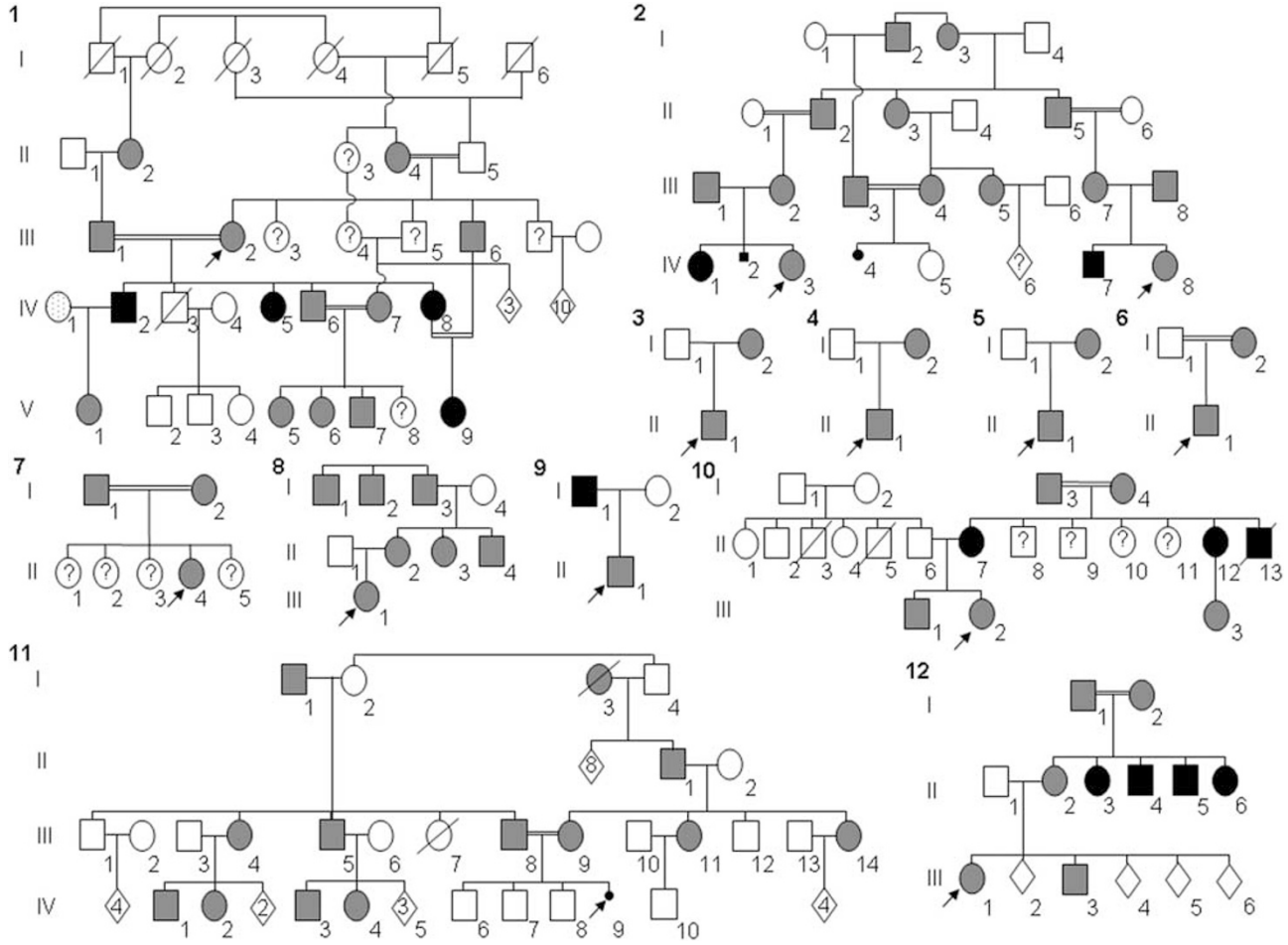

Figure 1 Pedigrees of the 12 LWD/LMD families carrying the A170P *SHOX* mutation. Individuals with LWD are shown as gray filled symbols, whereas individuals with LMD are shown as black filled symbols. Individual IV.1 of family 1 was clinically and genetically diagnosed with achondroplasia (*FGFR3* c.1138G>A, G380R). Unknown diagnosis is shown by a question marker.

Table 1 Clinical details of the documented LWD and LMD affected individuals and the prenatal diagnosis undertaken in the 12 Spanish families

Family	*Ethnic origin*	*Pedigree individual No.*	*Sex (F/M)*	*Status*	*Gen. Conf.*	*Age (years)*	*Height (cm)*	*Height SDS*	*MD*	*Other clinical and auxological details*
1	Gypsy	II.4	F	LWD	—	Adult	140.0	−3.7	MD	—
1	Gypsy	III.2	F	LWD	Y	Adult	140.8	−3.6	MD	—
1	Gypsy	III.6	M	LWD	—	Adult	148.0	−4.5	MD	—
1	Gypsy	IV.2	M	LMD	Y	Adult	SS	—	MD	—
1	Gypsy	IV.5	F	LMD	—	Adult	109.0	−9.1	—	—
1	Gypsy	IV.6	M	LWD	Y	Adult	SS	—	MD	—
1	Gypsy	IV.7	F	LWD	Y	Adult	SS	—	MD	—
1	Gypsy	IV.8	F	LMD	—	Adult	110.0	−9.0	—	—
1	Gypsy	V.1	F	LWD	Y	Adult	SS	—	MD	—
1	Gypsy	V.5	F	LWD	Y	Adult	SS	—	MD	—
1	Gypsy	V.6	F	LWD	Y	Adult	SS	—	MD	—
1	Gypsy	V.7	M	LWD	Y	12.0	116.6	−5.1	MD	—
1	Gypsy	V.9	F	LMD	—	7.0	89.0	−8.0	—	—
2	Gypsy	III.1	M	LWD	Y	Adult	SS	—	MD	—
2	Gypsy	III.2	F	LWD	—	Adult	SS	—	MD	—
2	Gypsy	III.3	M	LWD	Y	Adult	SS	—	MD	—
2	Gypsy	III.4	F	LWD	Y	Adult	SS	—	MD	—
2	Gypsy	III.5	F	LWD	Y	Adult	SS	—	MD	—
2	Gypsy	III.7	F	LWD	Y	Adult	SS	—	MD	—
2	Gypsy	III.8	M	LWD	Y	Adult	SS	—	MD	—
2	Gypsy	IV.1	F	LMD	—	Fetus	SS	—	—	—
2	Gypsy	IV.2	M	LMD	—	Fetus	NA	NA	NA	Electively aborted at 16 weeks gestation due to severe limb shortening with ultrasonography No genetic analysis undertaken
2[a]	Gypsy	IV.3	F	LWD	Y	Fetus	NA	NA	NA	Prenatal diagnosis at 19 weeks gestation
2[a]	Gypsy	IV.4	F	LMD	Y	Fetus	NA	NA	NA	Electively aborted after prenatal diagnosis at 18 weeks gestation
2	Gypsy	IV.5	F	N	Y	Fetus	NA	NA	NA	Prenatal diagnosis at 16 weeks gestation
2	Gypsy	IV.6	ND	ND	ND	Fetus	NA	NA	NA	No prenatal diagnosis undertaken as risk of LWD but not LMD
2	Gypsy	IV.7	M	LMD	—	Child	SS	—	—	
2[a]	Gypsy	IV.8	F	LWD	Y	Fetus	NA	NA	NA	Prenatal diagnosis at 19 weeks gestation
3	Gypsy	I.2	F	LWD	Y	Adult	SS	—	MD	—
3	Gypsy	II.1	M	LWD	Y	10.0	SS	—	MD	—
4	Spanish	I.2	F	LWD	Y	Adult	142.5	−3.3	MD	—
4	Spanish	II.1	M	LWD	Y	3.5	88.5	−2.7	MD	—
5	Gypsy	I.2	F	LWD	Y	Adult	143.0	−3.2	MD	—
5	Gypsy	II.1	M	LWD	Y	14.4	145.0	−2.4	MD	—
6	Gypsy	I.2	F	LWD	Y	Adult	146.1	−2.6	MD	Cervical scoliosis
6	Gypsy	II.1	M	LWD	Y	13.4	134.1	−2.8	MD	Cervical scoliosis
7	Gypsy	II.4	F	LWD	Y	11.8	113.0	−5.6	MD	Cervical scoliosis
8	Gypsy	II.2	F	LWD	Y	Adult	135.0	−4.6	MD	—
8	Gypsy	III.1	F	LWD	Y	6.5	99.5	−3.7	MD	—
9	Gypsy	I.1	M	LMD	—	Adult	120	−8.2	MD	—
9	Gypsy	II.1	F	LWD	Y	Child	SS	—	MD	—
10	Gypsy	II.7	F	LMD	Y	Adult	101.0	−10.2	MD	—
10	Gypsy	III.1	F	LWD	Y	8.5	SS	—	MD	—
11	Gypsy	I.1	M	LWD	—	Adult	SS	—	MD	—
11	Gypsy	I.3	F	LWD	—	Adult	SS	—	MD	—
11	Gypsy	I.9	M	LWD	—	Adult	143.0	−3.7	MD	—
11	Gypsy	III.4	F	LWD	—	Adult	145.0	−3.3	MD	—
11	Gypsy	III.5	M	LWD	—	Adult	155.0	−3.6	MD	—
11	Gypsy	III.8	M	LWD	Y	Adult	152.0	−4.1	MD	—
11	Gypsy	III.9	F	LWD	Y	Adult	144.0	−3.5	MD	—
11	Gypsy	III.11	F	LWD	—	Adult	140.0	−4.2	MD	—
11	Gypsy	III.14	F	LWD	—	Adult	147.0	−3.0	MD	—
11	Gypsy	IV.1	M	LWD	—	ND	SS	—	ND	—
11	Gypsy	IV.2	F	LWD	—	ND	SS	—	ND	—
11	Gypsy	IV.3	M	LWD	—	ND	SS	—	ND	—
11	Gypsy	IV.4	F	LWD	—	ND	SS	—	ND	—

Table 1 (Continued)

Family	Ethnic origin	Pedigree individual No.	Sex (F/M)	Status	Gen. Conf.	Age (years)	Height (cm)	Height SDS	MD	Other clinical and auxological details
11[b]	Gypsy	IV.9	M	LMD	Y	Fetus	SS	—[a]	MD	Electively aborted at 22 weeks gestation after prenatal diagnosis Body length 25 cm, humerus 30 mm, radius 19 mm, ulna 9 mm, femur 32 mm, tibia 25 mm, fibia 11 mm (Supplemetary Figure 1)
12	Gypsy	II.2	F	LWD	Y	Adult	142.0	−3.4	MD	—
12	Gypsy	III.1	F	LWD	Y	9.6	127.0	−1.7	MD	—

Abbreviations: LMD, Langer mesomelic dysplasia; LWD, Léri-Weill dyschondrosteosis; MD, Madelung deformity; N, normal; NA, no measurements could be undertaken as the individual was a fetus; ND; not determined; SS, short stature.
[a]Prenatal analysis undertaken with amniocentesis material.
[b]Analysis undertaken using DNA extracted from aborted tissue.
Status: LWD and LMD=A170P mutation in heterozygosity and homozygosity, respectively.

in a total volume of 15 μl. The sense and antisense oligos were 5′-CT TGGTTCAGCCTCATGGGAAG-3′ and 5′-TGTGGTGGTCCTGGGTGTAGC C-3′, respectively. The PCR cycling and HRM conditions were according to the manufacturer's recommendations (Roche Applied Bioscience).

Haplotype analysis

Haplotype analysis was undertaken to determine if a common ancestor was present for the two different mutations. Eight microsatellite markers located intragenically or in the 5′ and 3′ flanking regions of *SHOX* were analyzed as previously described.[11,17,19] The probability of observing the associated haplotype in the Spanish Gypsy and control populations was determined by analyzing the genotypes for each microsatellite marker in at least 100 chromosomes of the respective populations.

Subcellular localization studies

The A170D and A170P mutations were introduced into the vector pFLAG-CMV-1 (Sigma-Aldrich, St Louis, MO, USA) containing the full-length *SHOXa* cDNA using primers 5′-TCCAGAACCGGAGACCCAAGTGCCGCAAAC-3′ and 5′-GGTTCCAGAACCGGAGACCCAAGTGCCGCAAACAAG-3′, respectively, and the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's recommendations.

Immunocytochemistry: U2OS human osteosarcoma cells (Invitrogen, Life Technologies, Carlsbad, CA, USA) were grown in Dulbecco's modified Eagles medium supplemented with antibiotics and 10% fetal bovine serum (Invitrogen, Life Technologies) for 24 h on four-well chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA). Transient transfection of the different plasmid constructs was undertaken using FuGene (Roche Applied Bioscience) according to the manufacturer's instructions. At 24 h post transfection, the cells were washed and fixed with 4% paraformaldehyde (Sigma-Aldrich). Briefly, the cells were then washed with PBS and blocked with glycine buffer (1% BSA, 0.3 M glycine, Tween 0.2% diluted in PBS (PBS-T)). The preparations were incubated overnight at 4°C with a rabbit polyclonal SHOX antibody.[20] The cells were then washed with PBS-T and incubated with the secondary antibody, Alexa fluor 488 goat anti-rabbit IgG (Commercial Molecular Probes, Invitrogen). The cells were subsequently washed again with PBS-T, counterstained with DAPI (Sigma-Aldrich) and mounted with Mowiol 4–88 (Sigma-Aldrich). SHOX and DAPI localization were visualized using a confocal microscope SP5 (Leica, Leica Microsystems GmbH, Wetzlar, Germany).

Immunohistochemistry: Human radius and ulna growth plate sections were obtained from an electively aborted 22-week LMD fetus, confirmed to be an A170P homozygote by prenatal analysis (family 11, individual IV.9) and a spontaneously aborted 'healthy' 23-week fetus. Parents had provided informed consent for the use of their fetus for scientific research relevant to the clinical diagnosis. The LMD fetus was confirmed to carry two copies of the A170P mutation. Subsequently, histological analysis was undertaken as previously described.[20] Controls for specific antibody staining were performed by (1) using preimmune serum from the rabbit immunized against the SHOX peptide, at a dilution of 1:1000; (2) replacing the primary antibody with PBS; (3) using a rabbit polyclonal IgG isotype control (Ab27472, Abcam, Cambridge, UK) at a dilution of 1:50 and (4) staining sections of a normal adult colon with the SHOX antibody. Sections were examined using a DM5500B fluorescent microscope (Leica), and photos were taken using a DFC360FX digital camera (Leica) and visualized using the LA software (Leica).

RESULTS

Mutation detection and haplotype analysis

The *SHOX* mutation p. A170P (c.508G>C) was identified in 12 LWD/LMD probands (Figure 1). In the 12 families, a total of 34 LWD and four LMD individuals were subsequently genetically analyzed, all of whom were heterozygous or homozygous for the mutation, respectively.

The analysis of *SHOX* intragenic and flanking microsatellite markers revealed a common haplotype, present in one or two copies in all LWD and LMD affected family members, respectively. The haplotype block extended at least 77 kb, from microsatellite marker DXYS10038 to DXYS10093, to a maximum length of 165 kb (Table 2). Multiple haplotypes were observed with increasing physical distance from *SHOX*, suggesting the occurrence of multiple recombination events (Table 2). The frequency of this haplotype was determined in two control cohorts; 1 in 40 062 in Spanish Gypsies and 1 in 390 000 Spanish normal height controls.

As the A170P mutation was identified in 11 Spanish Gypsy families, we investigated its history. We looked into the genealogy of each family by last names and family interviews. Interestingly, the data obtained from one of the homozygous Gypsy probands (family 10) suggested that her family had migrated to Spain several generations ago from Eastern Europe. The common haplotype was observed in this family in the affected LWD and LMD individuals. Subsequently, we screened a panel of population controls from Bulgaria, representing Gypsy groups that remained in the Balkans during the early Gypsy diaspora across Europe. No A170P mutation was identified.

In two additional Spanish non-Gypsy families, we identified a novel missense mutation at the same residue, A170D (c.509C>A). No common *SHOX* haplotype was identified (data not shown).

Subcellular localization

We introduced the A170P and A170D missense mutations into the full-length *SHOX* cDNA by site-directed mutagenesis. The wild-type and mutant plasmids were transiently transfected into U2OS osteosarcoma cells, and the subcellular localization of the different SHOX proteins was analyzed by immunocytochemistry. Both A170D and A170P mutant proteins failed to localize to the nucleus, showing that nuclear translocation was impaired (Figure 2).

SHOX expression in a LMD human growth plate

We compared the expression of mutant SHOX in the human growth plate of a 22-week old fetus, molecularly confirmed to be homozygous

Table 2 Haplotypes determined from *SHOX* intragenic or flanking microsatellite markers in the 12 LWD/LMD families

Family	1	1	1	1	1	1	1	1	1	1	2	2	2
Member	III.2	III.6	IV.1	IV.2	IV6	IV.7	V.1	V.5	V.6	V.7	III.1	III.2	III.3
Status	LWD	LWD	ACH	LMD	LWD	LWD	LWD	LWD	LWD	LWD	LWD	LWD	LWD
DXYS10037	180–180	180–180	200–180	180–180	180–180	200–180	180–180	180–180	180–200	180–180	180–198	180–200	200–180
DXYS10038	217–**217**	217–**217**	209–213	**217–217**	217–**217**	217–**217**	213–**217**	217–**217**	**217**–217	217–**217**	**217**–217	**217**–213	209–**217**
DXYS201	135–**155**	135–**155**	145–155	**155–155**	135–**155**	157–**155**	155–**155**	135–**155**	**155**–157	135–**155**	**155**–135	**155**–147	141–**155**
DXYS10092	357–**359**	357–**359**	363–363	**359–359**	357–**359**	347–**359**	363–**359**	357–**359**	**359**–347	357–**359**	**359**–345	**359**–361	341–**359**
DYS290	292–**292**	292–**292**	306–296	**292–292**	292–**292**	296–**292**	296–**292**	292–**292**	**292**–296	292–**292**	**292**–292	**292**–292	294–**292**
DXYS10093	249–**235**	247–**235**	253–247	**235–235**	247–**235**	235–**235**	247–**235**	247–**235**	**235**–235	247–**235**	**235**–251	**235**–245	251–**235**
DXYS10083	156–160	156–160	170–160	160–160	156–160	154–160	160–160	156–160	160–154	156–160	160–172	160–154	160–162
DXYS10085	218–224	218–224	226–210	224–224	218–224	222–224	210–224	218–224	224–222	218–224	216–224	216–220	216–222

Family	2	2	2	2	2	2	2	2	2	3	3	4	4
Member	III.4	III.5	III.6	III.7	III.8	IV.3	IV.4	IV.5	IV.8	I.2	II.1	I.2	II.1
Status	LWD	LWD	N	LWD	LWD	LWD	LMD	N	LWD	LWD	LWD	LWD	LWD
DXYS10037	200–180	200–180	196∼200	200–180	180–200	198–180	180–180	200–200	180–200	200–180	202–180	202–180	202–180
DXYS10038	209–**217**	209–**217**	197∼197	**217**–197	209–**217**	217–**217**	**217–217**	209–209	197–**217**	209–**217**	213–**217**	209–**217**	209–**217**
DXYS201	143–**155**	143–**155**	149∼155	**155**–151	159–**155**	135–**155**	**155–155**	151–143	151∼**155**	143–**155**	151–**155**	143–**155**	143–**155**
DXYS10092	341–**359**	341–**359**	347∼355	**359**–343	349–**359**	345–**359**	**359–359**	341–341	343–**359**	359–**359**	343–**359**	347–**359**	347–**359**
DYS290	306–**292**	306–**292**	296∼306	**292**–306	292–**292**	292–**292**	**292–292**	294–306	306–**292**	296–**292**	296–**292**	296–**292**	296–**292**
DXYS10093	245–**235**	245–**235**	235∼255	**235**–245	249–**235**	251–**235**	**235–235**	251–245	245–**235**	243–**235**	235–**235**	261–**235**	261–**235**
DXYS10083	160–160	160–160	160∼162	160–160	170–160	172–160	162–160	162–160	160–160	158–160	154–160	158–160	154–160
DXYS10085	214–216	214–216	220∼222	216–214	216–212	224–216	222–216	222–214	214–212	218–214	224–214	218–214	224–214

Family	5	5	5	6	6	6	7	8	8	8	9	10	10
Member	I.1	I.2	II.1	I.1	I.2	II.1	II.4	II.1	II.2	III.1	II.1	II.6	II.7
Status	N	LWD	LWD	N	LWD	LWD	LWD	N	LWD	LWD	LWD	N	LMD
DXYS10037	200–192	200–180	192–180	194–200	198–180	200–180	180–180	198–200	180–190	200–180	180–198	198–192	180–180
DXYS10038	209–213	209–**217**	213–**217**	209–209	199–**217**	209–**217**	217–**217**	213–217	**217**–217	217–**217**	213–**217**	197–217	**217–217**
DXYS201	143–149	143–**155**	149–**155**	143–159	149–**155**	159–**155**	143–**155**	145–151	**155**–145	151–**155**	147–**155**	135–145	**155–155**
DXYS10092	355–343	355–**359**	343–**359**	347–349	349–**359**	349–**359**	339–**359**	361–353	**359**–361	353–**359**	361–**359**	317–361	**359–359**
DYS290	296–296	296–**292**	296–**292**	292–296	296–**292**	296–**292**	296–**292**	296–292	**292**–306	292–**292**	292–**292**	292–306	**292–292**
DXYS10093	235–235	235–**235**	235–**235**	233–233	247–**235**	233–**235**	253–**235**	235–231	**235**–257	231–**235**	245–**235**	235–257	**235–235**
DXYS10083	162–154	154–160	154–160	154–154	156–160	154–160	162–160	156–158	162–160	158–160	164–160	160–162	160–160
DXYS10085	216–224	216–214	224–214	220–224	222–214	224–214	216–214	224–228	216–212	228–216	224–216	222–212	224–224

Family	10	11	11	11	12	12	12
Member	III.2	III.8	IV.9	III.9	II.1	II.2	III.1
Status	LWD	LWD	LMD	LWD	N	LWD	LWD
DXYS10037	192–180	180–180	180–180	202–180	202–180	196–180	180–180
DXYS10038	217–**217**	209–**217**	**217–217**	209–**217**	209–217	213–**217**	217–**217**
DXYS201	145–**155**	145–**155**	**155–155**	143–**155**	151–137	145–**155**	137–**155**
DXYS10092	361–**359**	387–**359**	**359–359**	345–**359**	353–357	357–**359**	357–**359**
DYS290	306–**292**	296–**292**	**292–292**	308–**292**	292–296	292–**292**	296–**292**
DXYS10093	257–**235**	243–**235**	**235–235**	243–**235**	251–233	251–**235**	233–**235**
DXYS10083	162–160	164–154	154–160	154–160	158–158	160–160	158–160
DXYS10085	212–224	228–224	224–220	224–220	222–216	220–216	216–216

Abbreviations: LMD, Langer mesomelic dysplasia; LWD, Léri-Weill dyschondrosteosis; *SHOX*, short stature homeobox gene; ∼, haplotype could not be definitely defined but is predicted to be as stated.
The *SHOX* A170P mutation is located between markers DXYS10092 and DYS290. The common haplotype segregating with the mutation and phenotype is indicated in bold. The precise haplotype associated with the *SHOX* mutation was determined in 10 of the families, whereas a predicted haplotype was determined in families 7 and 10, as DNA was only available from the proband.

for the A170P mutation, with that of a 23-week normal fetal growth plate (Figure 3). SHOX was observed in the resting, proliferative and hypertrophic zones of both the control and the LMD growth plate. The chondrocytes appeared to be enlarged and in pairs in the reserve zone of the LMD growth plate. Their columnar stacking in the proliferative zone was also disorganized, with the chondrocytes appearing in less defined columns and in smaller clusters. The controls for antibody-specific staining are shown in Supplementary Figure 2.

DISCUSSION

A total of 12 families with multiple affected LWD and LMD individuals were found to carry the A170P *SHOX* mutation in heterozygosity and homozygosity, respectively. A common haplotype around *SHOX* was observed in all 12 families, suggesting the presence of a common ancestor. The probands originate from different Spanish provinces, 11 were of gypsy origin, thus suggesting that the common ancestor belonged to this ethnic group. The probability of this SHOX haplotype in the unaffected Spanish Gypsy population was low and even lower in the Spanish population. The haplotype block spanned between 77 and 165 kb. Multiple recombination events had occurred at increased distances. A study of linkage disequilibrium around *SHOX* showed that in Northern-European populations, disequilibrium decays rapidly with physical distance and that the recombination rate of the PAR1 is approximately 20-fold higher than the genome average.[21] Therefore, it is not unexpected to observe multiple recombinational events in these families.

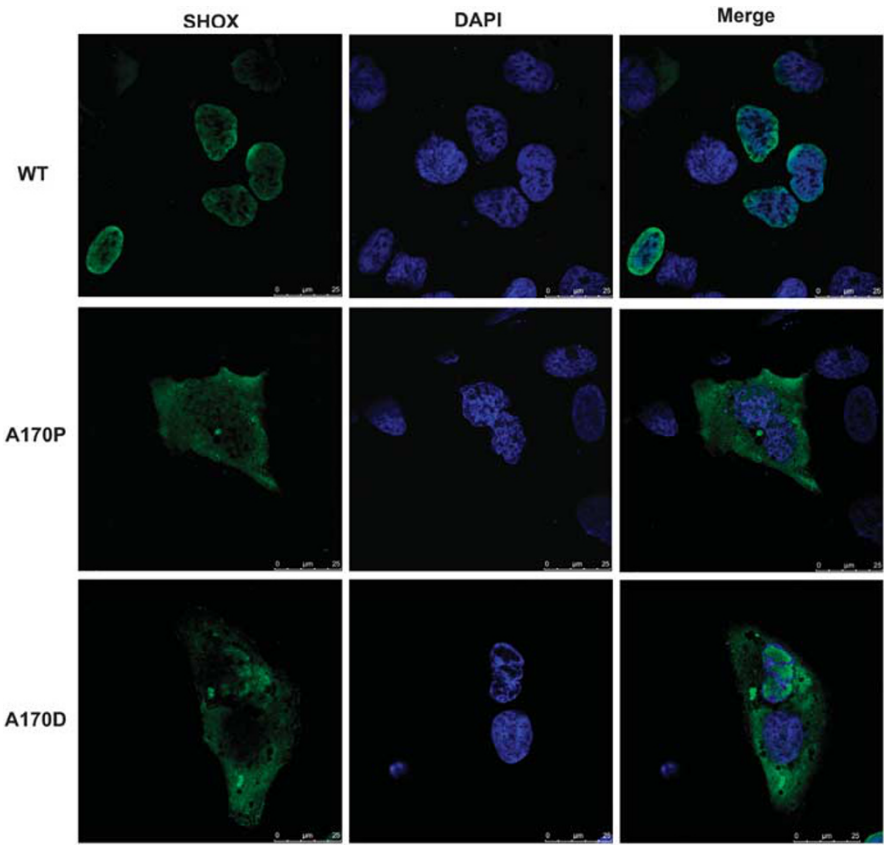

Figure 2 Subcellular localization studies of wild-type and mutant A170P and A170D SHOX proteins. U2OS cells were transiently transfected with the SHOX wild-type and mutant constructs. SHOX expression was detected using a rabbit polyclonal SHOX antibody and the nuclei were counterstained with DAPI. Images were observed using a confocal microscope at ×20 magnification.

As the A170P mutation was identified in 11 Spanish Gypsy families, we investigated its history and incidence of this mutation in the European Gypsy population. A panel of population controls from Bulgaria, representing Gypsy groups that remained in the Balkans during the early Gypsy diaspora across Europe, was screened for the A170P mutation. No mutation was identified, but failure to identify any mutation carrier does not rule out its presence in the Eastern Europe, where it could be confined to specific sub-isolate(s) not represented in the panel or could occur at very low frequency. The A170P SHOX mutation should therefore be the first choice in diagnostic analyses of LWD/LMD patients from this ethnic group. The mutation is more prevalent in the Spanish Gypsy population, in which six out of the seven prenatal diagnoses that we have undertaken in recent years belonged to high-risk Gypsy families.

Interestingly, the A170P mutation was highly penetrant, with all carriers presenting with mesomelic shortening of the limbs and the Madelung deformity, characteristics of LWD, in contrast to other *SHOX* mutations, which may present as either LWD or idiopathic short stature (ISS, MIM 300582). Only one individual did not fulfill the criteria of short stature (family 12, individual III.1). The high penetrance is likely to be due to the functional importance of alanine 170, which resides within a five non-classic basic amino acid nuclear localization signal (AKCRK), located in the homeodomain of SHOX.[16,18] Alanine 170 is one of the four amino acids which are invariably conserved in the homeodomain of all paired related homeodomain proteins. The A170P mutation has been shown to result in the incorrect subcellular localization of the SHOX protein, affecting the translocation from the cytosol to the nucleus, which would lead to the functional impairment of the transcription factor.[16] Indeed, these amino acids have been shown to not only be essential for SHOX nuclear localization, but may also participate in other processes such as DNA binding, dimerization[22] and interaction with its cofactors.[20]

We have investigated, for the first time, the effects of the A170P mutation on the growth plate of the radius and ulna of a LMD fetus. The chondrocytes in the reserve zone appeared enlarged and in pairs, whereas the proliferative zone was disorganized, with the columns appearing shortened and stacked side by side rather than in an orderly stacking. Despite this, the pattern of SHOX expression was unchanged between the normal growth plate and that of the LMD fetus. The histology of the growth plate in LMD fetuses has been previously described,[23] but at this time point, the implicated gene had not been discovered. Later, comparison of the histology and SHOX expression in the growth plate of normal and four LWD fetuses, two with *SHOX* deletions, one with a splice site mutation and one with a deletion of the *SHOX* enhancer region was studied.[24] In all cases of LWD and LMD, disorganization of the chondrocyte columnar stacking has been observed.

We have also identified two independent LWD probands with a previously unreported mutation (c.509C>A) affecting the same amino acid, p.A170D. We expressed the mutant protein in U2OS osteosarcoma cells and showed that the A170D mutant protein, as shown with the A170P mutation, failed to translocate to the nucleus, thus leading to the malfunction of the transcription factor.

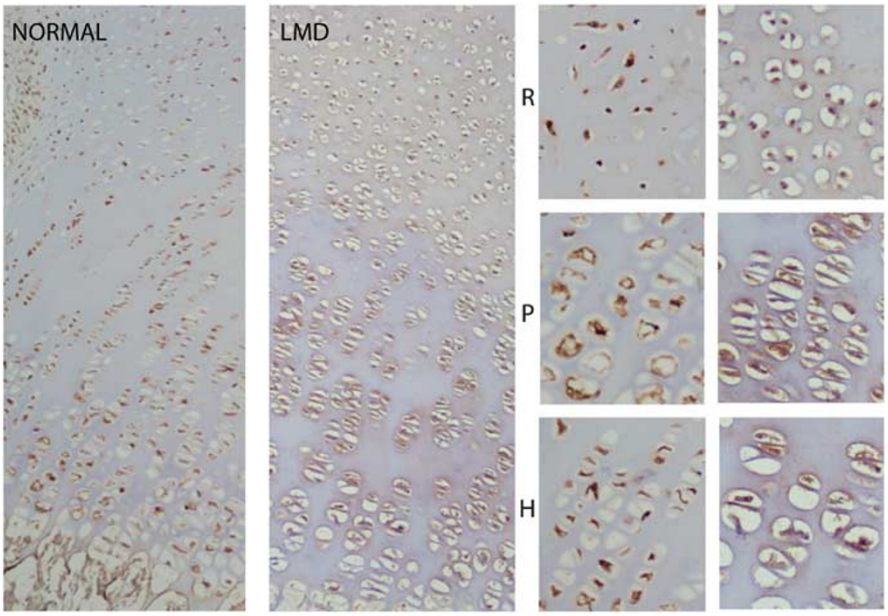

Figure 3 SHOX expression in the human fetal growth plate. Immunohistochemistry performed on the radius and ulna of a 22-week old LMD fetus homozygous for the A170P mutation (family 11, IV.9) and a 23-week old normal fetus. DAB immunostaining (×10 magnification) performed with anti-SHOX antibody in the LMD and normal fetal growth plate, showing the reserve (R), proliferative (P) and hypertrophic (H) zones. Specific staining can be observed in all zones of the LMD and control growth plates. The chondrocytes appear enlarged and in pairs in the reserve zone of the LMD growth plate. The chondrocyte columnar stacking in the proliferative zone of the LMD preparations is slightly disorganized compared to the normal growth plate. Controls for antibody specificity are shown in Supplementary Figure 2.

Including the cases reported in this study and identified by our group, six different mutations within the five amino acids of the SHOX nuclear localization signal have now been reported in 28 LWD/LMD/ISS probands (unpublished data and SHOX database: http://hyg-serv-01.hyg.uni-heidelberg.de/lovd/index.php?select_db=SHOX). Five additional mutations affecting the adjacent amino acids arginine 168 and 169 have been reported in 14 further LWD/ISS cases. Although it has been experimentally shown that these two amino acids are not included within the minimal nuclear localization signal, it has been postulated that they may support the minimal signal by their basicity.[18] Thus, the nuclear localization signal and adjacent basic amino acids appear to be a frequently mutated region associated with LWD and ISS. A note of caution must be mentioned in the detection of mutations in the nuclear localization signal, as both the A170P and A170D mutations result in a false positive deletion of *SHOX* exon 4 using the commercial MLPA assay.

In conclusion, we have identified A170P as the first common *SHOX* mutation in Spanish LWD individuals, which appears to have arisen from a common ancestor, most likely of Gypsy origin. Thus, it is advisable to screen the A170P mutation as a first step in the genetic screening cascade of *SHOX*/PAR1 alterations in Spanish gypsies with LWD or LMD. Moreover, we have also identified a novel mutation, A170D, altering the same conserved alanine 170 residue, which also impairs the nuclear localization of the protein.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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