ONLINE MUTATION REPORT

A novel point mutation A170P in the SHOX gene defines impaired nuclear translocation as a molecular cause for Lérid-Weill dyschondrosteosis and Langer dysplasia

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The human gene (SHOX) resides within the pseudoautosomal region of the X and Y chromosomes and encodes a paired related homeodomain transcription factor. Nominal levels of the SHOX protein have been implicated in bone development and longitudinal body growth, as heterozygous and homozygous loss of SHOX functions result in Lérid-Weill dyschondrosteosis (LWD) and Langer dysplasia (LD), respectively. Apart from mesomelic short stature and a characteristic deformity of the forearm leading to a limited mobility of the wrist (Madelung deformity), some individuals with LWD and LD present with a subset of clinical stigmata frequently observed in females with Turner syndrome, including high arched palate, curvature of radius/ulna/tibia, and short fourth metacarpals. These clinical features are variable, leading to a significant phenotypic heterogeneity particularly among persons with LWD. In addition, SHOX mutations are a major cause of isolated short stature conditions, with an estimated incidence of 2–3% in children presenting with idiopathic growth retardation. This is higher than the incidence of achondroplasia and growth hormone deficiency together.

The SHOX gene product has been shown to reside within the nucleus of several cell types, and acts as a transcriptional activator of target genes that have yet to be identified. This activity is likely to be regulated at different levels, including control of temporal and spatial expression patterns, nuclear translocation, and modification of its transactivating activities. In fact, regulation of SHOX expression was recently shown to involve sequential transcriptional and translational checkpoints, emphasising the importance of fine tuning the amount of functional SHOX protein.

Here we report a novel missense mutation, 508G>C (A170P), affecting the SHOX homeodomain. This mutation cosegregates with the LWD phenotype in a highly consanguineous Spanish gypsy family. Genotype-phenotype and pedigree analysis revealed that individuals homozygously carrying the identified mutation displayed the severe short stature condition of LD. In contrast to the wild type SHOX gene product, the protein carrying this mutation exhibits an exclusively cytoplasmic localisation, defining the impairment of SHOX nuclear localisation as a molecular mechanism underlying the aetiology of LWD and LD.

MATERIALS AND METHODS

Clinical evaluation of the participants

Data on height were available for the following individuals: III-2 (140.8 cm [-3.6 SDS], f, 59y, LWD); III-6 (148 cm [-4.5 SDS], m, 35y, LWD); IV-4 (109 cm [-8.5 SDS], f, 38y, LD); and V-4 (116.6 cm [-5.1 SDS], m, 12y, LWD). Further anthropometric measurements were available for III-2, with a sitting height of 80.1 cm (-1.9 SDS), an arm span of 133.5 cm (-4.5 SDS) and leg length of 72.5 cm. This individual presented with shortening and bowing of the forearms, and lucency of the distal ulnar border of the radius, but no obvious Madelung deformity. Patient V-4 presented with LWD and growth hormone deficiency as confirmed after propanolol exercise (peak GH: 7.4 μg/l) and clonidine stimulation (peak GH: 6.7 μg/l). Serum IGF-1 was 41.7 μg/l, which was well below the 0.1st percentile of the reference range for gender and age.

Patient material and mutation analysis

Blood samples were obtained after informed consent was given by each individual or his/her parents. Controls comprised 100 ethnically related healthy individuals. Genomic DNA was isolated from blood samples by standard protocols.

Key points

- The short stature homeobox gene SHOX resides within the pseudoautosomal region of the sex chromosomes, and encodes two isoforms of a paired related homeodomain protein. The SHOX gene product is found within the nucleus of all cell types analysed so far, and has been demonstrated to act as a transcriptional activator of target genes yet to be identified.
- SHOX haploinsufficiency leads to phenotypically heterogeneous short stature conditions, including idiopathic growth failure and Lérid-Weill dyschondrosteosis, and is involved in growth retardation and skeletal abnormalities in Turner syndrome. The homozygous loss of SHOX functions causes mesomelic dysplasia of the Langer type.
- The current study of a highly consanguineous family with a recorded history of Lérid-Weill dyschondrosteosis and Langer dysplasia provides compelling evidence that a missense mutation A170P (508G>C) causes disease by impairment of the nuclear translocation of the SHOX protein.
- Insertion of a seven amino acid fragment predicted to represent the nuclear localisation signal adjacent to the mutated site fully restores nuclear localisation of the SHOX protein.
- Our data explain Lérid-Weill and Langer syndrome conditions on a molecular and cellular level, and strengthen the growing awareness that regulation of subcellular localisation contributes to clinically relevant phenotypes.

Abbreviations: LWD, Lérid-Weill dyschondrosteosis; LD, Langer dysplasia
procedures. PCR amplification and direct sequencing were performed using SHOX and FGFR3 specific primers described elsewhere. Sequencing reactions were performed on both strands following the standard protocol with the Dye terminator kit (Applied Biosystems, Foster City, CA, USA) on a MegaBACE 1000/DNA Analysis System (Molecular Dynamics and Amersham Life Science, Amersham, UK). The sequences obtained were compared with the published gene sequences (Y11536 for SHOX and AF243114 for FGFR3) using the BLASTN program.

Plasmid constructs

The G508C mutation was introduced into the full length SHOX cDNA by site directed mutagenesis using the primer 5’-CTTGGTTGCGGCACTTGAGCTCCGTTCTGGAACC-3’ with the Quick-Change Multi Site-directed Mutagenesis Kit (Stratagene, www.stratagene.com) according to the manufacturer’s recommendations.

Insertion of the predicted nuclear localisation signal into the mutant SHOX G508C was achieved by replacing an XhoI/NsiI restriction fragment of this cDNA with a PCR generated fragment containing both the mutation and the wild type sequence (primer sequences are available upon request). All cDNAs were cloned into the eukaryotic expression vector pcDNA4/TO (Invitrogen, Carlsbad, CA, USA) and used for transient transfection of U2Os osteosarcoma cells.

Cell culture and transient transfections

U2Os Tet-on osteosarcoma cells (BD biosciences, Clontech, www.clontech.com) were grown in DMEM/10% FCS. Transient expression of different plasmid constructs was achieved by transfecting these cells using FuGENE reagent (Roche, Mannheim, Germany) following the manufacturer’s instructions.

Immunofluorescence and Western blot analysis

For immunofluorescence analysis, cells grown on cover slips were stained using a rabbit anti-SHOX antiserum and FITC conjugated goat anti-rabbit antibodies following the standard protocol published elsewhere. Slides were evaluated by conventional fluorescence microscopy and all results were confirmed by confocal analysis.

For subcellular fractionations, cells were harvested by centrifugation (3000 rpm/five minutes/4°C). The pellet was washed twice with ice cold PBS, resuspended in 300 μl of hypotonic buffer A (10 mM Tris-HCl, pH 8.0; 5 mM MgCl2; 0.05% NP40; 1 mM EDTA; and 0.1 mM DTT, protease inhibitors from Roche) and incubated on ice for 20 minutes. Lysed cells were centrifuged (5000 rpm/five minutes/4°C) and the supernatant was taken as cytoplasmic extract. The remaining pellet was washed with ice cold PBS, resuspended in 300 μl of hypotonic buffer B (hypotonic buffer A with 0.4 M KCl), and incubated on ice for 40 minutes followed by centrifugation at 5000 rpm. The supernatant contained the nuclear extract.

Western blot analysis was carried out following the standard procedure using rabbit anti-SHOX primary antibody at a dilution of 1:3000. As cytoplasmic and nuclear controls, mouse anti- M2PK (DF4) (pyruvate kinase isozyme M2) (Schebo, Giessen, Germany) and mouse anti-C23/ Nucleolin (Santa Cruz, Heidelberg, Germany) were used at dilutions of 1:1000 and 1:300, respectively.

RESULTS

We analysed samples from 17 members of a highly consanguineous Spanish gypsy family for clinical manifestations of LWD and LD. Of these, 11 individuals (II-2, II-4, III-1, III-2, III-6, IV-5, IV-6, V-1, V-2, V-3, and V-4) presented with clinical and radiological features of dyschondrosteosis, such as mesomelic shortening and bowing of the forearms, triangularisation of the distal radial epiphysis, or lucency of distal ulnar border of radius (fig 1A). Four family members (IV-2, IV-4, IV-7, and V-6) exhibited clear symptoms of LD (fig 1B). Besides LD, patient V-4 also was found to have growth hormone deficiency. With an adult height of 109 cm, IV-4 was the shortest member of this family. Individual IV-1 was diagnosed with achondroplasia, and a 1138G>A mutation in the FGFR3 gene was confirmed.

Mutation analysis revealed that family members III-2, III-6, IV-5, IV-6, V-1, V-2, V-3, and V-4 carried a missense

![Figure 1](http://jmg.bmj.com/firstpublishedas10.1136/jmg.2003.016402onlinemutationreport)
Figure 2. (A) SHOX genomic region and cDNA structure of SHOXA. Red bar, homeodomain; blue bar, RRAKCRK. (B) Sequence analysis in III-2. Wild type on left and the G to C mutant on right. (C) Sequence alignment of third helix of homeodomain of SHOX, its fish orthologue (Ol-Prx3/MOG-12), its human parologue SHOX2, and the mouse orthologue of SHOX2 (OG-12). Bar above third helix, NLS; black arrows, conserved amino acids within paired related homeodomain proteins; red arrows, conserved amino acids in all homeodomain proteins. (D) Wild type SHOX (1); mutant SHOX constructs (2); insertion construct SHOX/A170P+RRAKCRK (3) in the vector pcDNA4/TO; Nu, nuclear; Cy, cytoplasmic. (E) Immunofluorescence of transfected U2O (pcDNA constructs above) stained with anti-SHOX antibodies. (F) Western blot of nuclear and cytoplasmic extracts from same cells.
mutation 508G>C within exon 4 of the SHOX gene, leading to the amino acid exchange A170P (fig 2B). Blood samples for genetic analysis were not available from II-2 and II-4. Members IV-2 and V-6 were found to be homozygous for the identical mutation. None of the healthy control samples showed the same nucleotide exchange. The identified mutation has been reported to the human SHOX mutation database ([http://www.shox.uni-hd.de; 17] The mutation resides at the C-terminal end of the homeodomain within a stretch of basic amino acid residues which represent a conserved feature among nuclear localisation signals. 18–21 We therefore screened a nuclear localisation signal data base ([http://cubic.bioc.columbia.edu/db/NLSdb/; 22] using the SHOX amino acid sequence as a query. This analysis predicted a seven amino acid motif RRxKxRK as a non-classical type nuclear localisation signal (fig 2A). Since the identified novel missense mutation resided within this motif, we predicted its interference with the correct subcellular localisation of the SHOX protein. To corroborate this hypothesis, we introduced the identified missense mutation into the full length SHOX cDNA by site directed mutagenesis, and cloned the resulting mutant into the eukaryotic expression vector pcDNA4/TO (fig 2D). This plasmid was transiently transfected into U2Os osteosarcoma cells, and the subcellular localisation of the encoded protein was analysed by immunofluorescence using a SHOX specific antibody. Unlike the wild type SHOX gene product, the mutant protein localised exclusively to the cytoplasm of all transfected cells (fig 2E), demonstrating that the nuclear translocation capacity is affected by the mutation. These results were confirmed by subcellular fractionation of transfected cells and subsequent Western blot analysis (fig 2F). As controls for proper cytoplasmic and nuclear separation, we used pyruvate kinase isozyme M2 (M2PK) as a cytoplasmic marker and C23/nucleolin as a nuclear marker. 22–24 Complementing the immunofluorescence results, we detected wild type SHOX mainly in the nuclear fraction, whereas the protein carrying the A170P mutation was detected only in the cytoplasmic fraction of transiently transfected cells (fig 2F).

We were also interested to find out whether nuclear localisation could be restored by inserting a wild type sequence encoding the predicted nuclear localisation signal next to the mutated site. We therefore transiently transfected U2Os cells with the appropriate plasmid constructs (fig 2D). Our results clearly demonstrate distinct nuclear localisation of the encoded fusion proteins (fig 2E). These immunofluorescence results were confirmed by Western blot analysis with subcellular fractions of transfected cells (fig 2F).

In summary, these data clearly define subcellular mislocalisation of mutated SHOX protein as the molecular mechanism underlying the LWD and LD phenotypes in the investigated family.

DISCUSSION
Mutations affecting the pseudoautosomal homeobox gene SHOX cause several short stature conditions, including LWD and LD. In the present study, we identified a novel missense mutation (508G>C) within the homeobox of the SHOX gene in a highly consanguineous family, and analysed the molecular mechanism underlying the LWD and LD phenotypes of individual family members. In agreement with previous reports, subjects carrying a heterozygous mutation displayed LWD, and homozygously affected individuals exhibited the severe short stature condition of LD. 4–6,25 Because of repeated consanguineous marriage, the analysed family featured a remarkable prevalence of LWD and LD cases. A father to son transmission (III-1 to IV-2) of an initially X chromosomal mutation was seen, due to a pseudoautosomal crossover during male meiosis. As a result of consanguineous marriage, this event led to homozygosity of the 508G>C mutation on the X and Y chromosomes of IV-2 and therefore to an LD phenotype. Since this mutation could be traced to both grandmothers of IV-2, the founder mutation was likely to have occurred before or within the first generation of the available genealogical tree.

The identified missense mutation resided within a seven amino acid motif predicted to represent a nuclear localisation signal. We were able to show that the A170P mutation interfered with proper subcellular localisation by inhibiting SHOX nuclear translocation. This mislocalisation could be reverted by introducing the predicted seven amino acid motif (RRxKxRK) adjacent to the site of mutation, suggesting that it represented the nuclear localisation signal of the SHOX encoded protein. This nuclear localisation signal was within the recognition helix of the homeodomain which exhibits extraordinary sequence conservation among all homeodomain proteins. In fact, four amino acids within the motif (R168, R169, A170, and R173) are invariably conserved throughout the entire family of paired related homeodomain proteins with amino acid R169 conserved in all the homeodomain proteins, 26 suggesting that similar mutations in other homeodomain proteins may also lead to an impairment of nuclear translocation and abolish their intrinsic function as transcriptional regulators. Interestingly, the fish orthologue of SHOX (Medaka OG-12/MOG-12/Ol-Prx3, NCBI ref. no. AF001393), the human parologue of SHOX (SHOX2/SHOT, NCBI ref. no. AF022654), and the mouse orthologue of SHOX2 (OG-12/Prx3, NCBI ref. no. AK032007) show exact similarity with predicted NLS for SHOX in their respective third helices, indicating a common mechanism for the nuclear import of all these proteins (fig 2C).

The mutation identified in this study led to an exchange between two uncharged polar amino acids (P replaced A) and, therefore, should not be expected to interfere with the charge dependency of the nuclear localisation signal recognition by the nuclear transport machinery. The A170P amino acid substitution is, rather, predicted to destroy the structure of the third homeodomain helix (recognition helix), since proline is known to evoke bending of the polypeptide backbone. The observed impairment of SHOX nuclear localisation was therefore likely to occur because of structural changes in the protein.

In summary, the data presented here define impairment of nuclear localisation of the SHOX encoded transcription factor as a molecular cause of LWD and LD, most likely due to structural changes in the protein. We would predict that similar mutations affecting the corresponding motif of other paired related homeobox genes may cause clinical conditions based on the same cellular principle: interference with nuclear translocation.

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Conflicts of interest: none declared
PHOG, a candidate gene for involvement in the short stature of Turner syndrome.


